



Evaluation of antimicrobial antibodies in patients with intestinal pathologies

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Dedication

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Abstract

Introduction: Inflammatory bowel disease (IBD) is a chronic immune-mediated condition characterized by dysregulated mucosal immune responses to intestinal antigens. Immunoglobulin A (IgA) and immunoglobulin G (IgG) play complementary roles in mucosal and systemic immunity, yet most studies examining IgA- and IgG-mediated responses to the gut microbiota—including analyses of antibody-coated fecal bacteria—have been conducted in North America, Europe, or East Asia. Immunologic data from South America remain limited.

Objective: The aim of this study was to characterize systemic and intestinal humoral immune responses in a Uruguayan cohort by evaluating total antibodies in plasma, and fecal soluble and bacteria-associated IgA and IgG across patients with IBD, irritable bowel syndrome (IBS), celiac disease, and healthy controls.

Methodology: Plasma IgA and IgG concentrations were quantified using direct enzyme-linked immunosorbent assays (ELISA). Soluble fecal IgA and IgG were measured by direct ELISA in available stool samples. Flow cytometry was employed to assess IgA- and IgG-associated bacterial signal, expressed as background-normalized fluorescence ratios. Within the IBD group, immunoglobulin (Ig) measures were additionally analyzed according to self-reported symptom presence status and C-reactive protein (CRP) levels.

Results: Plasma IgA and IgG concentrations did not differ significantly across diagnostic groups. Soluble fecal IgA and IgG similarly had no statistically significant differences observed at the group level. Flow cytometric measures of IgA- and IgG-associated bacterial signal showed no significant differences between diagnostic groups. Additionally, fecal Ig concentrations and Ig-associated bacterial signals did not differ significantly between symptomatic and asymptomatic subgroups within IBD.

An exploratory multivariable Spearman correlation analysis revealed coordinated patterns within compartments: plasma IgA and IgG concentrations were positively correlated, as were fecal IgA and fecal IgG concentrations. IgA- and IgG-associated bacterial measures were also positively correlated, and both soluble fecal IgA and IgG levels demonstrated modest but significant positive associations with IgG-coated bacterial fluorescence. No significant correlations were

observed between plasma Ig concentrations and mucosal or bacteria-associated antibody measures.

When CRP was analyzed as a categorical variable (elevated vs non-elevated), no statistically significant differences were observed in soluble fecal IgA, soluble fecal IgG, or in the proportion of IgG-coated bacteria within the IBD group. A statistically significant difference was identified in the proportion of IgA-coated bacteria, with higher values observed among IBD participants with non-elevated CRP; however, this finding was based on a small subgroup comparison (n = 8, n = 3) and should be interpreted cautiously.

Conclusion: In this Uruguayan cohort, systemic and intestinal Ig measures were characterized by substantial heterogeneity rather than clear diagnostic group separation, with no consistent differences in total plasma or fecal antibody levels or bacterial coating patterns between patient groups and healthy controls. However, multivariable analyses revealed coordinated IgA–IgG patterns within systemic and mucosal compartments and modest associations between fecal Ig levels and IgG-coated bacteria, indicating structured humoral organization despite limited group-level discrimination. These findings suggest that IgA- and IgG-based immune signatures may reflect compartment-specific and individual-level mucosal immune dynamics rather than disease classification alone.

Perspectives: This study provides population-specific immunologic data from South America and highlights the need for larger stool-based cohorts and stratified analyses to determine whether IgA- and IgG-based intestinal immune profiling can reliably detect and distinguish between inflammatory and non-inflammatory gastrointestinal disorders in this setting.

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List of Abbreviations

APC – Allophycocyanin
ASCA – Anti-Saccharomyces cerevisiae antibodies
Bas# – Absolute basophil count
Bas% – Basophil percentage
BMI – Body mass index
CBC – Complete blood count
CD – Crohn's disease
CRP – C-reactive protein
ELISA – Enzyme-linked immunosorbent assay
Eos# – Absolute eosinophil count
Eos% – Eosinophil percentage
Fc – Fragment crystallizable
FITC – Fluorescein isothiocyanate
FSC – Forward scatter
HCT – Hematocrit
HGB – Hemoglobin
IBD – Inflammatory bowel disease
IBS – Irritable bowel syndrome
Ig – Immunoglobulin
IgA – Immunoglobulin A
IgG – Immunoglobulin G
IQR – Interquartile range
Lym# – Absolute lymphocyte count
Lym% – Lymphocyte percentage
MCH – Mean corpuscular hemoglobin
MCHC – Mean corpuscular hemoglobin concentration
MCV – Mean corpuscular volume
Mon# – Absolute monocyte count
Mon% – Monocyte percentage
MPV – Mean platelet volume

Neu# – Absolute neutrophil count

Neu% – Neutrophil percentage

PBS – Phosphate-buffered saline

PBS-T – Phosphate-buffered saline with Tween-20

PCT – Plateletcrit

PDW – Platelet distribution width

PLT – Platelet count

PSC – Primary sclerosing cholangitis

RBC – Red blood cell count

RDW-CV – Red cell distribution width, coefficient of variation

RDW-SD – Red cell distribution width, standard deviation

SSC – Side scatter

UC – Ulcerative colitis

WBC – White blood cell count

1. Introduction

1.1 Overview of the Immune System: Innate and Adaptive Immunity

The innate immune system provides the first line of defense against pathogens through rapid, non-specific mechanisms. Key components include physical barriers like the skin and mucous membranes, which block entry of microbes, and physiological barriers such as acidic pH and antimicrobial enzymes. If invaders breach these surfaces, phagocytic cells (such as neutrophils and macrophages) ingest and destroy them. Additional innate immune populations also contribute to early defense, including granulocytes (such as eosinophils and basophils), which release antimicrobial and inflammatory mediators, innate lymphoid cells, which rapidly produce cytokines that shape local immune responses at barrier tissues, and dendritic cells, which act as tissue sentinels and initiate immune activation (Marshall et al., 2018).

Innate immune cells possess pattern recognition receptors (PRRs) that detect common pathogen-associated molecular patterns (PAMPs) and trigger inflammation. For example, toll-like receptors on macrophages recognize bacterial cell wall components, inducing release of cytokines that recruit more immune cells. This fast-acting system can stop infections within hours but does not confer long-lasting immunity (Marshall et al., 2018).

In contrast, the adaptive immune system is slower to respond but highly specific and capable of immunologic memory. Its main players are lymphocytes: B cells and T cells. B cells mature into plasma cells that secrete immunoglobulins (Ig), mediating humoral immunity. T cells include helper T cells ($CD4^+$) that coordinate responses and cytotoxic T cells ($CD8^+$) that kill infected cells. Adaptive responses require antigen presentation: specialized antigen-presenting cells (such as dendritic cells) process pathogens and display peptide fragments on major histocompatibility complex (MHC) molecules to T cell receptors. Upon recognizing a specific antigen-MHC complex, T cells proliferate and differentiate. Helper T cells release cytokines that activate macrophages and stimulate B cells to produce antibodies, while cytotoxic T cells directly lyse infected or abnormal cells. Memory B and T cells persist after an infection, enabling a faster and stronger response if the same pathogen reappears (Marshall et al., 2018), in a process known as acquired or adaptive immunity.

While these principles broadly define innate and adaptive immunity, they are particularly challenged at mucosal surfaces, where the immune system is continuously exposed to vast quantities of foreign antigens. Among these sites, the gastrointestinal tract represents the most immunologically complex environment, requiring constant integration of barrier defenses, innate sensing, and adaptive immune regulation to maintain tissue homeostasis.

1.2 The Gut Immune System

Nowhere is the interplay between innate and adaptive immunity more evident than in the gastrointestinal tract. The intestine is not merely a digestive organ but a highly specialized immune environment that must continuously balance tolerance toward dietary antigens and commensal microorganisms with rapid and effective defense against pathogens. This dual function is achieved through the coordinated activity of the intestinal epithelial barrier, gut-associated lymphoid tissue (GALT), diverse immune cell populations, and mucosal antibody responses (Nagler-Anderson, 2001).

1.2.1 Intestinal Barrier and Epithelial Immunity

The intestinal epithelium constitutes the first line of immune defense and functions as a dynamic, selectively permeable barrier rather than a passive physical wall. Tight junctions between epithelial cells regulate paracellular permeability, while a mucus layer produced primarily by goblet cells physically separates luminal microbes from the epithelial surface. Specialized epithelial cells further contribute to host defense: Paneth cells secrete antimicrobial peptides such as defensins, which directly limit microbial overgrowth (Nagler-Anderson, 2001).

Beyond barrier maintenance, intestinal epithelial cells actively participate in immune signaling. They express pattern recognition receptors and respond to microbial and dietary cues by producing cytokines, chemokines, and antimicrobial molecules that influence underlying immune cells. Through these signals, epithelial cells help orchestrate local immune responses, promoting either pro-inflammatory pathways during infection or regulatory programs that support tolerance under homeostatic conditions (Nagler-Anderson, 2001).

1.2.2 Gut-Associated Lymphoid Tissue (GALT)

Embedded within and beneath the epithelial layer is the gut-associated lymphoid tissue (GALT), a central component of mucosal immunity. GALT comprises organized inductive sites such as Peyer's patches in the small intestine and isolated lymphoid follicles throughout the colon, as well as diffuse immune populations in the lamina propria and mesenteric lymph nodes (Nagler-Anderson, 2001). The regional organization of these immune compartments and their associated cell populations along the intestinal tract is summarized in Figure 1 (reproduced from Mowat & Agace, 2014).

Within Peyer's patches and lymphoid follicles, antigens from the intestinal lumen are sampled by microfold (M) cells and dendritic cells and presented to naïve lymphocytes. These inductive sites are critical for initiating antigen-specific immune responses, including the differentiation of B cells into immunoglobulin A (IgA)-secreting plasma cells. As illustrated schematically in Figure 1, the lamina propria, located directly beneath the epithelial lining, serves as a major effector compartment and is densely populated with immune cells that execute mucosal immune functions (Mowat & Agace, 2014).

1.2.3 Immune Cell Populations in the Intestinal Mucosa

The intestine harbors a disproportionately large fraction of the body's immune cells. The lamina propria contains abundant plasma cells, T lymphocytes, macrophages, dendritic cells, and innate lymphoid cells (ILCs). Among these, regulatory T cells play a crucial role in maintaining tolerance to commensal microbes and dietary antigens, while ILCs contribute to epithelial repair and early immune responses (Mowat & Agace, 2014).

Intestinal dendritic cells continuously sample luminal contents and integrate microbial and environmental signals to direct T cell differentiation toward either regulatory or effector phenotypes. This capacity to induce tolerance or immunity depending on context is central to intestinal immune homeostasis. Disruption of these regulatory mechanisms can lead to inappropriate immune activation and chronic inflammation, as observed in inflammatory bowel disease (IBD) (Mowat & Agace, 2014).

1.3 Mucosal Antibodies and Antibody Specialization

Antibodies are central effectors of the adaptive immune system, providing highly specific recognition and targeted responses against a wide range of antigens. Produced by differentiated B cells (plasma cells), antibodies contribute to host defense through multiple mechanisms, including neutralization of pathogens and toxins, opsonization to enhance phagocytosis, and activation of the complement system. Beyond direct pathogen clearance, antibodies also play a critical role in immune regulation, shaping interactions between the host and commensal microbiota and limiting excessive inflammatory responses. The diversity of antibody isotypes and their compartmentalized distribution throughout the body reflect a high degree of functional specialization, allowing the immune system to tailor its responses to distinct anatomical and physiological environments.

1.3.1 IgA in Mucosal Immunity

A hallmark of mucosal immunity is the dominance of IgA production. The GALT generates more antibody-secreting cells—particularly IgA-producing plasma cells—than the spleen, lymph nodes, and bone marrow combined. In the gut, IgA is primarily secreted as dimeric secretory IgA, which is transported across the epithelium into the lumen (Nagler-Anderson, 2001).

IgA is the predominant antibody in mucosal secretions—such as intestinal fluids, saliva, and tears—and is key for protecting mucous membranes (Li et al., 2020). In fact, IgA is the second most abundant Ig class in humans behind immunoglobulin G (IgG). IgA in circulation is normally measured at approximately 2,000 µg/ml in adults, with a range from 700 to 3,400 µg/ml; however, reported serum reference ranges may vary depending on assay platform and calibration method. In the gut, IgA is usually produced as dimeric secretory IgA, which binds luminal microbes and toxins, preventing their attachment to epithelial surfaces and limiting penetration into host tissues. This process, termed immune exclusion, allows containment of potential threats without triggering strong inflammatory responses, thereby preserving epithelial integrity and microbial balance. IgA thus plays a central role in immune surveillance and maintenance of microbial homeostasis at mucosal surfaces. (Reinholdt & Husby, 2013).

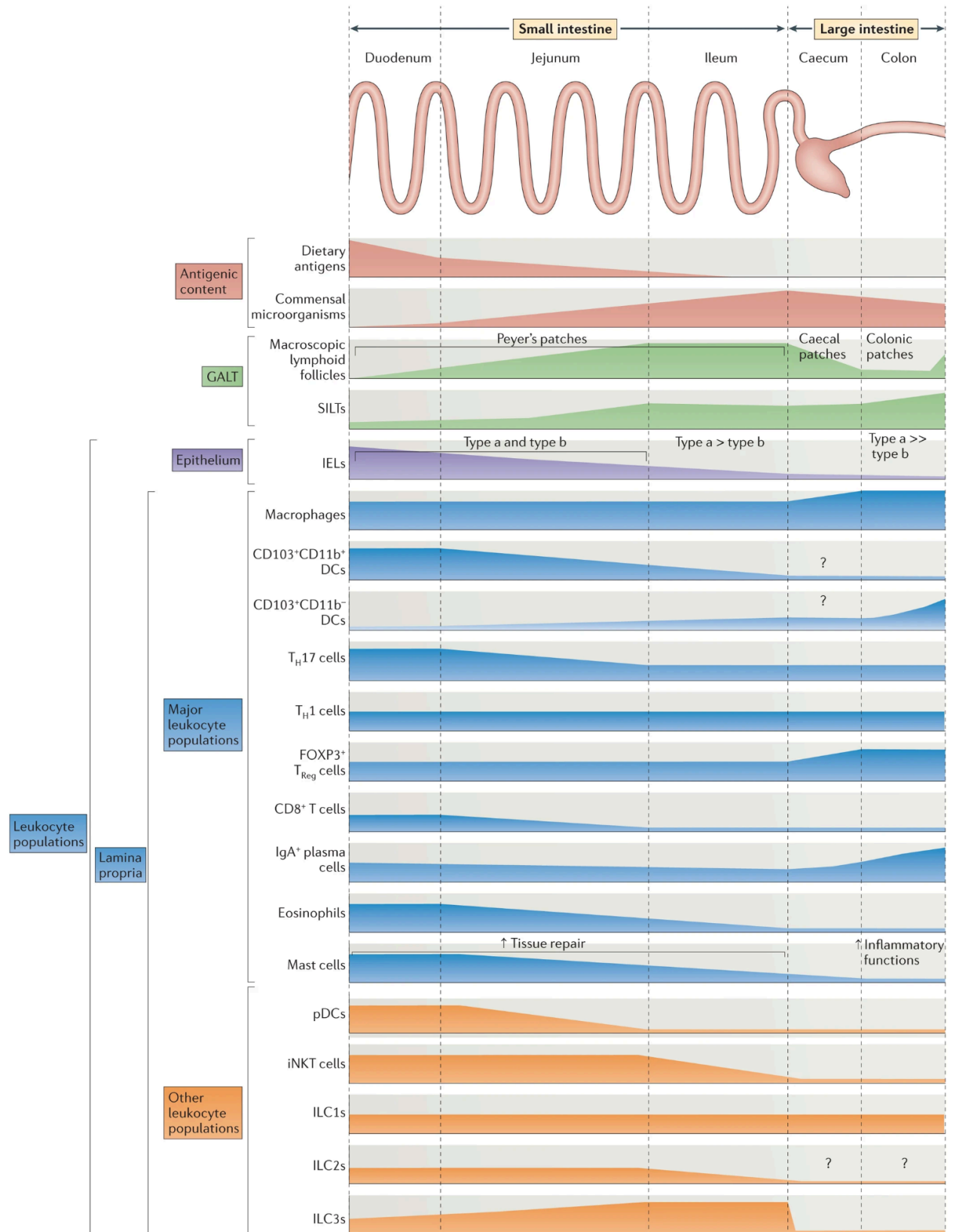


Figure 1. Immune organization and functional specialization along the intestinal tract

Schematic overview of immune organization along the intestinal tract, illustrating gradients in antigenic exposure, microbial density, and immune composition from the duodenum to the colon. Inductive immune sites, including Peyer's patches and isolated lymphoid follicles, are specialized for antigen sampling and initiation of adaptive immune responses. In contrast, the lamina propria functions predominantly as an effector compartment enriched in innate and adaptive immune cells that mediate mucosal defense, immune regulation, and maintenance of epithelial integrity. Major immune cell populations shown include antigen-sampling dendritic cells, regulatory and effector T-cell subsets, innate lymphoid cells involved in epithelial support and early immune responses, and IgA-secreting plasma cells that contribute to mucosal immune protection.

Note. Reproduced from "Regional specialization within the intestinal immune system", by A. M. Mowat and W. W. Agace, 2014, *Nature Reviews Immunology*, 14(10), 667–685. Copyright 2014 by Springer Nature.

1.3.2 IgG in Mucosal and Systemic Responses

In contrast, IgG operates predominantly in the systemic compartment. IgG is the most abundant antibody class in human serum and is essential for long-term systemic immunity (Vidarsson et al., 2014). IgG in circulation is normally measured at approximately 12,000 µg/ml (Reinholdt & Husby, 2013), though reported serum reference ranges vary depending on assay platform and calibration method. IgG antibodies promote pathogen clearance through opsonization, complement activation, and neutralization of circulating toxins and viruses (Vidarsson et al., 2014). While IgA dominates the intestinal lumen, IgG responses may arise against gut-derived antigens that breach the mucosal barrier or during systemic infections and vaccination. Together, IgA and IgG represent complementary arms of mucosal and systemic immune defense.

1.4 The Gut Microbiota and Host–Microbe Interactions

1.4.1 Composition and Function of the Gut Microbiota

The gut microbiota is a complex and diverse microbial community composed predominantly of bacteria, with members of the Firmicutes and Bacteroidetes phyla representing the dominant taxa. In addition to bacteria, the gut microbiota includes viruses (particularly bacteriophages), archaea, fungi, and protozoa, all of which contribute to the overall microbial ecosystem (Rinninella et al., 2019).

Gut microbial composition is dynamic across the lifespan and varies in response to multiple factors, including age, diet, geographic environment, antibiotic exposure, and host health status.

Microbial colonization begins at birth, undergoes rapid diversification during early childhood, and stabilizes into a relatively adult-like configuration by approximately three years of age, while remaining responsive to environmental and dietary changes throughout life (Rinninella et al., 2019).

Functionally, the gut microbiota plays a central role in the metabolism of dietary components that are otherwise indigestible by the host. Fermentation of complex carbohydrates results in the production of short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate, which serve as key mediators of host–microbe interactions. SCFAs provide energy to intestinal epithelial cells, contribute to the maintenance of epithelial barrier integrity, and regulate immune signaling pathways (Koh et al., 2016). Through these mechanisms, the gut microbiota influences both local mucosal immune responses and systemic immune function, supporting immune homeostasis.

1.4.2 Microbiota–Immune System Crosstalk

The intestinal immune system continuously senses microbial signals through pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which detect conserved microbial-associated molecular patterns (MAMPs). This innate immune sensing enables surveillance of microbial activity while limiting excessive immune activation under homeostatic conditions (Hooper & Macpherson, 2010).

A central feature of gut immunity is the ability to tolerate commensal microorganisms while remaining responsive to pathogenic threats. This balance is achieved through multiple regulatory mechanisms, including the induction of regulatory T cells (Tregs), the selective coating of commensal bacteria by secretory IgA, and the maintenance of controlled inflammatory responses that prevent tissue damage in a rapidly changing environment (Belkaid & Harrison, 2017).

Microbial metabolites further shape immune responses. For instance, butyrate, a microbiota-derived SCFA, promotes the differentiation and function of regulatory T cells and suppresses pro-inflammatory signaling pathways in homeostasis, thereby avoiding an exaggerated immune response under healthy conditions (Furusawa et al., 2013). In parallel, tryptophan-derived microbial metabolites activate aryl hydrocarbon receptor (AhR) signaling in

immune and epithelial cells, contributing to immune regulation and epithelial homeostasis (Zelante et al., 2013).

In inflammatory conditions such as IBD, dysbiosis is accompanied by reduced abundance of SCFA-producing bacteria and decreased microbial metabolic capacity, including butyrate biosynthesis, thereby weakening metabolite-mediated immune regulation (Lloyd-Price et al., 2019).

1.4.3 The Gut–Systemic Immunity Axis

Early-life exposure to the gut microbiota plays a critical role in immune education and the establishment of systemic immune tolerance. Microbial colonization during infancy influences immune maturation, shaping immune responses that persist into adulthood (Zhao & Elson, 2018).

Under conditions of altered barrier function or immune dysregulation, microbial products such as lipopolysaccharide (LPS) and other toxins can translocate from the intestinal lumen into the circulation. These microbial components modulate immune activity at distant sites by engaging innate immune receptors, thereby influencing systemic inflammatory responses (Charitos et al., 2025).

Through these mechanisms, gut immunity exerts effects beyond the gastrointestinal tract. Alterations in host-microbiota interactions have been linked to systemic inflammatory diseases, neuroimmune processes, and cancer immunity, including variability in responses to immunotherapy (Belkaid & Harrison, 2017; Yu et al., 2022; Matson et al., 2018).

Loss of the normal immune–microbiota balance in the gut is a unifying feature behind many intestinal disorders. Under healthy conditions, the mucosal immune system maintains a regulated coexistence with commensal microbes that supports barrier integrity and immune homeostasis (Bretto et al., 2025). Perturbations of this equilibrium can give rise to diverse immune responses, ranging from antigen-specific autoimmunity to low-grade or dysregulated immune activation that may or may not be accompanied by overt inflammation (Bretto et al., 2025, Pascual et al., 2014). The spectrum of immune involvement in gut disease is therefore broad, encompassing both inflammatory and functional disorders. The following sections introduce key examples across

this spectrum, with a particular focus on IBD as a well-characterized chronic immune-mediated intestinal condition.

1.5 IBD: Clinical, Immunological, and Population Context

1.5.1 Clinical and Pathological Classification of IBD

IBD refers to a group of chronic immune-mediated inflammatory conditions of the gastrointestinal tract driven by dysregulated immune pathways that lead to uncontrolled intestinal inflammation, principally comprising Crohn's disease (CD) and ulcerative colitis (UC) (Vuyyuru et al., 2022). Both CD and UC manifest as relapsing-remitting inflammation of the gut mucosa, resulting in symptoms like diarrhea, abdominal pain, and bleeding, and both require long-term immunomodulatory treatment, yet they differ in anatomical distribution, depth of tissue involvement, and clinical manifestations. The principal anatomical, histopathological, and immunological distinctions between CD and UC are summarized in Table 1.

Table 1. Comparison of CD and UC

This table summarizes the principal anatomical, histopathological, and immunological features distinguishing CD and UC, based on their patterns of gastrointestinal involvement, depth and distribution of inflammation, dominant immune pathways, cytokine profiles, and characteristic humoral immune markers.

*Abbreviations: ASCA, anti-Saccharomyces cerevisiae antibodies; pANCA, perinuclear anti-neutrophil cytoplasmic antibodies.

Domain	Crohn's Disease	Ulcerative Colitis
Anatomical distribution	Any part of the GI tract (most commonly ileum and proximal colon)	Colon and rectum only
Pattern of inflammation	Patchy, segmental ("skip lesions")	Continuous, starting in rectum and extending proximally
Depth of involvement	Transmural (full thickness)	Superficial (mucosa and submucosa)
Dominant immune profile	Th1 / Th17-driven cell-mediated immunity	Atypical Th2-like mucosal immunity
Key cytokines	TNF- α , IL-12, IL-23, IFN- γ , IL-17	IL-5, IL-13 (plus TNF- α , IL-6 to a lesser extent)
Histopathologic features	Transmural inflammation, granuloma formation	Mucosal ulceration, crypt abscesses
Barrier dysfunction	Defective innate immunity and impaired microbial containment	Impaired epithelial barrier, reduced mucus, altered tight junctions
Humoral immune features	Prominent IgG (and IgA) responses to microbial antigens	Increased IgG1 responses in colonic mucosa
Characteristic serologic markers	ASCA (IgG and IgA) frequently positive	pANCA (IgG) positive in ~60–70%
ASCA / pANCA pattern	ASCA ⁺ / pANCA ⁻	ASCA ⁻ / pANCA ⁺
Microbiota interaction	Loss of tolerance to gut microbes central to pathogenesis	Abnormal mucosal immune response to luminal antigens

1.5.2 Crohn's Disease

CD is a form of IBD characterized by chronic, relapsing inflammation that can affect any part of the gastrointestinal tract from mouth to anus, most commonly the ileum and proximal colon. A defining feature of CD is transmural inflammation, in which immune activation extends through the full thickness of the intestinal wall, often resulting in patchy, segmental involvement (“skip lesions”) with intervening areas of normal tissue (Table 1).

Mechanistically, CD is an immune-mediated condition with a complex etiology. Although its precise cause remains unclear, it is widely understood to arise from an inappropriate immune response to intestinal microbes in a genetically susceptible host. Numerous susceptibility genes have been identified, many of which are involved in immune regulation and microbial sensing, highlighting the central role of dysregulated host–microbiota interactions in disease pathogenesis (Tian et al., 2025).

1.5.3 Ulcerative Colitis

UC is the other principal form of IBD and is characterized by chronic inflammation limited to the colon and rectum. In contrast to CD, inflammation in UC is typically continuous and confined to the superficial layers of the intestinal wall, primarily affecting the mucosa and submucosa. Disease involvement usually begins in the rectum and extends proximally in a continuous fashion for a variable distance (Lynch & Hsu, 2025).

Like CD, UC follows a relapsing–remitting course. However, UC reflects sustained mucosal immune activation rather than transmural involvement. Despite these anatomical distinctions, UC shares key immunopathological features with CD, with both arising from complex interactions between genetic susceptibility, environmental exposures, the intestinal microbiota, and dysregulated mucosal immune responses (Lynch & Hsu, 2025). These shared features justify their classification under the umbrella of IBD and provide the framework for examining disease distribution and global incidence patterns.

1.5.4 Immune Pathogenesis of IBD

1.5.4.1 Immune Pathogenesis of CD

CD is fundamentally an immunologic disorder—an overactive immune reaction in the gut. Research indicates that CD involves a dysregulated T-helper 1 (Th1) and Th17 cell response in the intestinal mucosa. There is excessive production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-12 and -23 (which drive Th1/Th17 differentiation), interferon-gamma, and IL-17, among others (Table 1). Innate immune cells are also hyper-activated: macrophages and neutrophils in CD lesions release inflammatory mediators that contribute to tissue injury. The transmural granulomatous inflammation seen in CD suggests an inability of the immune system to adequately contain or down-regulate responses to intestinal microbes. One theory posits that CD patients have a defective mucosal barrier and innate immunity (for instance, impaired secretion of defensins by gut epithelial cells), leading to greater microbial penetration, which then triggers chronic IgG-dominated immune responses. IgG antibodies appear to play a key role: many CD patients develop antibodies against microbial antigens (e.g., *Saccharomyces cerevisiae* mannan, *E. coli* outer membrane porins)—these include both IgA and IgG isotypes known as ASCA (anti-*Saccharomyces cerevisiae* antibodies). The presence of ASCA (often IgG and IgA) is relatively specific for CD and reflects loss of tolerance to certain gut microbes. In contrast, these antibodies are usually absent in UC. The immune complex formation and complement activation by IgG in CD could exacerbate inflammation. Furthermore, CD pathology involves activated cytotoxic T cells and natural killer (NK) cells that contribute to tissue destruction. The chronic Th1/Th17 inflammation in CD leads to granuloma formation (clusters of activated macrophages attempting to wall off what they perceive as foreign material). In summary, CD can be seen as an overactive cell-mediated immune response in the gut, with prominent Th1/Th17 cytokine activity and IgG antibody production against intestinal microbiota (Tian et al., 2025).

1.5.4.2 Immune Pathogenesis of UC

UC has a somewhat different immune profile. The inflammation in UC is typically confined to the mucosa, and immune responses are often described as atypical Th2 in nature (Lynch & Hsu, 2025). Unlike a classic Th2 (allergic) response, UC's Th2-like response does not involve high

IgE or eosinophils, but it does include overproduction of cytokines like IL-5 and IL-13 by a subset of T cells (possibly NK-T cells in the colon). IL-13 in particular has been implicated in UC for its cytotoxic effects on epithelial cells, contributing to the hallmark ulceration. There is also evidence of TNF- α and other inflammatory cytokines (like IL-6) playing roles in UC, but to a lesser extent than in CD. Importantly, a majority (~60–70%) of UC patients have a unique autoantibody: pANCA (perinuclear anti-neutrophil cytoplasmic antibody). pANCA is an IgG antibody directed against certain neutrophil proteins and is thought to reflect an underlying abnormal immune activation in UC. Its presence (while not pathogenic itself) is a useful immunologic marker that distinguishes UC from CD, where pANCA is usually negative. On the other hand, UC patients generally do *not* have ASCA; that serologic division (ASCA-positive tends to be CD, pANCA-positive tends to be UC) underscores different immune targets in the two diseases. B cell responses in UC are also notable—UC patients have been found to have a disproportionate increase in IgG1 antibodies in the colon. This IgG1 subclass elevation may be involved in complement activation and mucosal damage. The mucosal immune system in UC seems to react abnormally to luminal contents, but instead of deep granulomas, it produces continuous superficial inflammation and crypt abscesses (collections of neutrophils in colonic crypts). The epithelial barrier is impaired, as evidenced by reduced mucus and altered tight junctions, increasing antigen uptake which fuels inflammation. Dendritic cells in UC are activated and express high levels of PRRs, likely driving the T cell responses. There is an imbalance of regulatory cells as well—regulatory T cell function may be reduced, failing to check effector T cells. In summary, UC involves a chronic immune reaction biased toward an atypical Th2 profile and humoral autoimmunity (pANCA), leading to continuous colonic mucosal inflammation. Key immune pathways include IL-13-mediated epithelial injury, recruitment of neutrophils (via IL-8/CXCL8 and other chemokines), and IgG1-complement interactions damaging the mucosa. These differences in immune pathways between UC and CD inform the selection of targeted treatments (for instance, therapies blocking TNF- α are effective in both, but blocking IL-12/23 is more tailored to CD Th1/Th17 biology. Newer therapies targeting integrin-mediated leukocyte trafficking are used in UC as well as CD) (Lynch & Hsu, 2025).

Although CD and UC differ in anatomical distribution, immune pathways, and characteristic antibody profiles (Table 1), the present study does not focus on direct comparisons between these

clinical subtypes. Instead, it examines shared immunologic features across IBD, with particular emphasis on IgA and IgG responses to intestinal antigens, independent of clinical classification.

1.5.5 Current Diagnostic Approaches in IBD

Accurate diagnosis of IBD requires a comprehensive and stepwise approach that combines clinical features with objective evidence of intestinal inflammation obtained through laboratory biomarkers, endoscopy with histopathology, and advanced imaging techniques.

1.5.5.1 Endoscopic Evaluations

The cornerstone of IBD diagnosis is endoscopy, particularly colonoscopy with ileoscopy and biopsy. Colonoscopy allows direct visualization of the colonic mucosa and terminal ileum, enabling the clinician to identify characteristic changes: in UC, a continuous inflamed mucosa starting in the rectum; in CD, scarring, stricture formation, patchy ulcers, skip lesions, and possibly fistula openings (Tian et al., 2025). Biopsies taken during endoscopy are key for diagnosis (UC, CD, or other) and aid in monitoring disease progression. Endoscopy is highly sensitive for detecting even mild inflammation and is currently essential for obtaining tissue to differentiate IBD from other causes of colitis (like infections or ischemia) (Lynch & Hsu, 2025). Its strengths lie in its diagnostic accuracy and ability to obtain tissue. However, it is an invasive procedure requiring bowel preparation, sedation, and carries certain risks. Colonoscopy complications include bleeding (especially if biopsies or polyp removals are done) and, more seriously, bowel perforation. Perforation is uncommon but not negligible—large studies report colonoscopy perforation rates on the order of 0.005% to 0.085% (roughly up to 1 in 1,200 to 1 in 20,000 procedures). Should a perforation occur, it often requires surgical repair (Kim et al., 2019). Therefore, while generally safe, endoscopy discomfort and anxiety are a barrier for some patients. It is worth noting that certain populations are underserved by current diagnostics. In parts of the world with limited endoscopic capacity, IBD may be significantly under-recognized (Watermeyer et al., 2023). For example, sub-Saharan Africa reports very low IBD incidence, but this likely reflects lack of diagnostic resources as much as true low occurrence (Hodges et al., 2025).

1.5.5.2 Imaging Techniques

Imaging studies complement endoscopy in the evaluation of IBD, particularly CD, which frequently involves segments of the small intestine beyond the reach of colonoscopy.

Cross-sectional imaging with CT enterography (CTE) and MR enterography (MRE) provides detailed visualization of bowel wall thickening, strictures, fistulas, and abscesses, making these techniques essential for assessing disease extent and extra-luminal complications (Maino et al., 2024). MRI offers the advantage of superior soft-tissue contrast and the absence of ionizing radiation, while CT remains faster and more widely available in clinical practice. Intestinal ultrasound (IUS) is emerging as a valuable, non-invasive tool for monitoring IBD activity by measuring bowel wall thickness and vascularity (Rimola et al., 2022). Capsule endoscopy can directly visualize the small bowel mucosa and detect ulcers or inflammation in areas not accessible to endoscopy or imaging, but it carries a risk of retention if strictures are present; therefore, cross-sectional imaging such as MRE or CTE is often performed first to check for obstructions (Hudesman et al., 2014).

1.5.5.3 Laboratory and Stool Tests

While not diagnostic on their own, lab tests support IBD diagnosis and help exclude other causes. Stool examinations are mandatory to rule out infections that can mimic IBD. Once infection is excluded, certain stool biomarkers are very useful in practice: fecal calprotectin (a neutrophil protein) and fecal lactoferrin are elevated in intestinal inflammation. Fecal calprotectin is particularly sensitive for active IBD; a normal calprotectin makes active IBD unlikely (negative predictive value >99% in some studies) (Lynch & Hsu, 2025). It's a non-invasive marker to distinguish IBD from irritable bowel syndrome (IBS), as IBS does not cause calprotectin elevation (Walsham & Sherwood, 2016). Blood tests often show inflammatory markers—elevated C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR)—during IBD flares. Anemia is common (from chronic blood loss or anemia of chronic disease), and patients may have low albumin if there is malabsorption or severe inflammation. Serologic tests for the aforementioned antibodies (ASCA, pANCA) can provide supportive evidence: for example, a positive ASCA and negative pANCA favor CD, whereas the opposite suggests UC

(Lynch & Hsu, 2025). However, these tests are not definitive or disease specific, so they are used to support diagnoses in combination with other studies rather than as primary diagnostics.

1.5.6 Current Immune Targets and Treatments for IBD

Advances in the treatment of IBD have been driven by improved understanding of the immune pathways that sustain intestinal inflammation. Current therapeutic strategies largely focus on targeting key components of the inflammatory response, including tumor necrosis factor- α (TNF- α), the IL-12/IL-23 axis, and mechanisms regulating leukocyte trafficking to the gut, as well as broader immune processes involved in mucosal inflammation and lymphocyte activation. In parallel, conventional immunosuppressive agents such as corticosteroids and thiopurines remain widely used, particularly for induction of remission. Collectively, these approaches reflect the central role of immune dysregulation in IBD and highlight the clinical relevance of characterizing immune activity beyond overt inflammation.

In routine clinical practice, the immune pathways implicated in IBD are targeted through a limited set of established therapeutic classes. 5-aminosalicylic acid (5-ASA) derivatives, such as mesalamine, are primarily used in UC and exert local anti-inflammatory effects through inhibition of cyclooxygenase and lipoxygenase pathways, suppression of NF- κ B-mediated transcription, and activation of PPAR γ , contributing to reduced mucosal inflammation and improved epithelial integrity.

Immunomodulators such as thiopurines (e.g. azathioprine) and methotrexate are used in the management of IBD as steroid-sparing maintenance therapies, particularly for maintenance of remission. These agents suppress adaptive immune activation by targeting proliferating immune cells, through interference with nucleic acid synthesis in the case of thiopurines and through inhibition of cell proliferation, induction of apoptosis, and modulation of pro-inflammatory cytokine production in the case of methotrexate.

Systemic corticosteroids, including prednisone, are employed for induction of remission in moderate-to-severe flares of IBD, though they are not suited for long term disease maintenance. These drugs exert broad immunosuppressive effects by downregulating pro-inflammatory cytokine transcription, limiting leukocyte recruitment, and inducing lymphocyte apoptosis.

In a subset of patients with more severe or corticosteroid-refractory disease, biologic and small-molecule therapies are indicated. Among biologics, agents targeting TNF- α , such as infliximab or adalimumab, are used to neutralize this central pro-inflammatory cytokine and attenuate downstream inflammatory cascades (Neurath, 2017). In parallel, small-molecule therapies include Janus kinase (JAK) inhibitors, which modulate intracellular signaling pathways by blocking cytokine-induced JAK–STAT activation, thereby suppressing transcription of pro-inflammatory genes across multiple cytokine axes. Unlike biologics, which act extracellularly, small molecules exert their effects within immune cells and offer oral administration and low immunogenicity (Jefremow & Neurath, 2023).

Together, these therapies illustrate how both targeted and broad immunosuppressive strategies are applied to modulate immune pathways central to IBD pathogenesis, while also underscoring the heterogeneity of immune responses observed in clinical practice.

1.5.7 Global Incidence and Prevalence of IBD

IBD currently affects millions globally. As of 2017, about 6.8 million people were described as living with IBD worldwide (Aqilion, n.d.). The global incidence is roughly 10 new cases per 100,000 people per year, and prevalence about 230 per 100,000 (0.23% of the population) (Heydari et al., 2025). Rates vary greatly by region—highest in Western industrialized nations and rising rapidly in newly industrialized regions (*Therapeutic Focus Areas*, n.d.) (summarized in Table 2).

1.5.7.1 High-Incidence Regions: North America, Europe, and Oceania

These regions have the world's highest reported IBD rates. Incidence surged in the late 20th century and now appears to plateau or even slight decline in recent years. Prevalence is high (often >0.3% of the population), reflecting the accumulation of cases over time. Key figures for each region include:

- **North America:** Among the highest IBD burdens globally. Incidence is around 20 new cases per 100,000 people per year, and prevalence roughly 250–300 per 100,000 ($\approx$ 0.25–0.3% of the population). (For example, UC incidence alone is $\sim$ 9–10/100k in North America.)

- **Europe:** IBD rates comparable to North America. Annual incidence is typically in the low teens per 100,000 (e.g. ~10–15/100k in many countries). Prevalence is around 350 per 100,000, with many Western European nations now exceeding 0.3% of the population with IBD.

- **Oceania (Australia/New Zealand):** Incidence and prevalence are on par with Western Europe and North America. Incidence is about 20–21 per 100,000 (among the highest globally). Prevalence is roughly 300–330 per 100,000—for instance, Australia and New Zealand report IBD prevalence in this range. On the other hand, Oceania has the highest CD prevalence of any continent, ~174 per 100,000 (Heydari et al., 2025).

1.5.7.2 Emerging Regions: East Asia, Middle East, Africa, Latin America

Historically, East Asia, the Middle East, Africa, and Latin America had very low IBD rates, but incidence has been accelerating in recent decades as countries undergo industrialization and adopt Westernized environments. Prevalence in these areas is still lower than in the West (owing to the more recent emergence of IBD), but it is steadily climbing. These trends suggest that environmental factors associated with modernization play a central role in IBD pathogenesis.

- **East Asia:** IBD was rare in East Asia in the 20th century, but cases have risen sharply in the 21st. Incidence remains lower than Western levels but is now on the order of a few per 100,000. For example, 1–3 new cases per 100,000 yearly were typical in China a decade ago, and some areas (e.g. South Korea) now report ~5–6 per 100,000. Prevalence is still in the tens per 100,000, with a pooled estimate ~51 per 100,000 across Asia (Heydari et al., 2025), but this is a dramatic increase from near-zero a few decades prior.

- **Middle East:** Countries in the Middle East and North Africa (MENA) region are experiencing a clear rise in IBD. The incidence in 2019 was about 3–4 per 100,000 on average, up from ~2/100k in 1990. Some Middle Eastern countries now approach mid–single-digit incidence rates (e.g. Jordan ~6.9/100k, one of the highest in the region). Prevalence remains relatively low—on the order of 40–60 per 100,000 in most MENA populations—but is steadily growing as more cases accumulate (Alsakarneh et al., 2024).

- **Africa:** IBD remains rare in most of Africa, but the continent is in an early stage of the epidemiologic transition. Reported incidence rates are generally <1 per 100,000 in

sub-Saharan Africa, and only a few per 100,000 in North African nations. For example, Morocco's IBD incidence was ~1.6/100k in 2019. Correspondingly, prevalence is very low—recent global data show only about 10–11 per 100,000 in sub-Saharan African regions. Despite these low absolute numbers, there is evidence of increasing incidence in Africa's more urbanized areas (Oshi, 2025). Health systems in African countries are now beginning to recognize and track this growth in IBD cases (Wood, 2025).

- **Latin America:** IBD rates in Latin American countries have historically been very low. Incidence is generally still under 1–2 per 100,000 in many areas. The prevalence is correspondingly modest (for example, around 50 per 100,000 in South America as a whole). However, studies confirm that incidence is rising in this region. Some urban centers that have undergone rapid socio-economic change are now reporting significantly higher numbers, signaling an emerging public health challenge in parts of Latin America (Heydari et al., 2025).

Table 2. Global incidence and prevalence of IBD by region

Values represent approximate incidence (new cases per 100,000 population per year) and prevalence (total cases per 100,000 population), based on recent epidemiological reviews and regional studies. Incidence is highest in North America, Europe, and Oceania, and increasing across East Asia, the Middle East, Latin America, and Africa. Prevalence remains lower in emerging regions but is steadily rising.

Region	IBD Incidence (/100k/year)	IBD Prevalence (/100k)	Trend Summary
North America	≈20	250–300	High and stable or plateaued
Europe	≈10–15	≈350	High and plateauing
Oceania (Australia/NZ)	20–21	300–330 (CD: ~174)	Very high; highest CD prevalence globally
East Asia	1–6	~51	Rising sharply from historically low levels
Middle East	3–7	40–60	Steadily increasing, especially in urban centers
Africa	<1 (SSA), ~1.6 (North Africa)	10–11	Emerging; still very low but rising in urban areas
Latin America	<1–2	≈50	Low but increasing, especially in urbanizing regions

1.5.8 Regional Distribution of UC and CD

Although CD and UC share similar global epidemiological trends, regional differences in their relative distribution have been observed. In Latin America and parts of Asia, both conditions were historically uncommon, reflecting the traditionally low overall incidence of IBD in these regions (Tian et al., 2025; Selvaratnam et al., 2019). Available data suggest that, during the initial emergence of IBD, UC was often detected earlier and remains more frequently reported than CD,

rather than being highly prevalent in absolute terms. In Uruguay, a retrospective, registry-based study using data collected between 2007 and 2011—the most recent publicly available national data—reported an adjusted IBD incidence of 5.22/105 person-years with a UC/CD ratio of 7.7. However, the country is still considered a region of relatively low overall IBD incidence (Iade et al., 2018). Importantly, recent evidence indicates that incidence of both UC and CD is increasing, consistent with broader regional and global trends associated with ongoing socioeconomic and lifestyle changes (Ananthakrishnan et al., 2020).

1.5.8.1 IBD Incidence in South America and Uruguay

IBD has historically been less common in South America than in Europe or North America, although both incidence and prevalence have increased steadily over recent decades. A 2019 systematic review reported UC prevalence across South America of approximately 15–24 per 100,000 population, compared with 2–14 per 100,000 for CD. Because prevalence data are limited for Uruguay, national estimates rely primarily on incidence data. A registry-based study from 2007–2008 reported very low annual incidence rates, with approximately 2.3–4.3 cases per 100,000 for UC and 0.3–0.7 per 100,000 for CD, yielding a marked UC:CD ratio of nearly 6:1. This predominance of UC, which is characteristic of many regions undergoing early epidemiologic transition, likely reflects both true disease patterns and historical underdiagnosis of CD due to its more heterogeneous clinical presentation. Overall, these data confirm that Uruguay remains a low-incidence setting for IBD, with rates well below those reported in high-incidence regions (>10–20 per 100,000/year). However, trends across Latin America—including rising diagnoses in urban populations—suggest that IBD incidence is increasing, underscoring the importance of generating local data on disease distribution and phenotype (Selvaratnam et al., 2019).

1.5.9 Environmental and Genetic Contributors to Rising IBD Incidence

Proposed contributors to rising diagnoses include adoption of Western dietary patterns characterized by high fat and sugar intake and low fiber consumption, widespread antibiotic use with consequent disruption of the gut microbiota, reduced exposure to enteric infections during early childhood consistent with the hygiene hypothesis, increasing urbanization, environmental pollution, and alterations in sunlight exposure and vitamin D status. The importance of

environmental influence is further supported by migration studies, which demonstrate that individuals relocating from low-incidence to high-incidence regions—particularly first-generation offspring—often develop IBD at rates comparable to the host population within a single generation (M’Koma, 2013).

While environmental exposures are thought to be the major drivers of recent increases in IBD incidence, genetic susceptibility remains an important contributor to geographic and individual risk patterns. IBD has a complex genetic architecture with over 200 susceptibility loci identified through genome-wide association studies, many of which influence pathways related to immune regulation and host–microbe interactions, indicating a biologically relevant genetic component to disease risk. Certain IBD-associated risk alleles vary by population, and many of these are more prevalent in European populations, partially explaining historically higher disease burdens in Western regions. Despite these genetic influences, the rapid rise of IBD in genetically diverse populations — including those without high frequencies of classic risk alleles — underscores the predominant influence of environmental and lifestyle factors interacting with underlying genetic susceptibility in driving disease emergence globally (Hadad et al., 2024).

1.5.10 Population Differences in IBD Research

1.5.10.1 Geographical and Ethnic Differences in IBD

IBD is not one uniform disease worldwide; its prevalence, typical presentation, and environmental risk factors vary across populations. IBD historically was concentrated in wealthy Western populations. Reviews report that North America, Northern Europe and Australasia have the highest IBD prevalence, whereas in the mid-20th century incidence in Asia, Africa and Latin America was very low (Bhayani et al., 2024; Kotze et al., 2020). For example, one systematic analysis found UC prevalence exceeded 0.3% in many European and North American countries (Kotze et al., 2020). In contrast, Latin American and Asian cohorts had negligible rates until recent decades. However, as regions industrialize and lifestyles westernize, IBD rates have surged. For example, Japan and urban China have rising rates of UC approaching those in the West. A recent review notes that IBD—once mainly a Western disease—now shows a “sizeable increase” in incidence across Asia (Bhayani et al., 2024). In Latin America, IBD was once considered rare, but countries like Brazil, Argentina, and Uruguay have documented growing

case numbers in recent decades as diagnostic capabilities improve and westernized living becomes common (Kotze et al., 2020). Ethnicity is also important: populations of Ashkenazi Jewish descent have long exhibited significantly higher IBD risk than neighboring groups, reflecting the strong role of genetic predisposition (Roth et al., 1989).

1.5.10.2 Disease Phenotype Differences Across Populations

The way IBD presents can vary by population. In East Asian CD patients, studies have reported more colonic involvement and frequent perianal fistulas than classically described in Western cohorts. Extraintestinal features also differ: for example, primary sclerosing cholangitis (PSC) and other immune-related complications appear less common in Asian IBD patients. Familial IBD has been reported less often in East Asian series, likely reflecting the historically lower incidence there. Experts emphasize the need to study these variations in disease location, complications, and incidence—one review concluded that more research should compare IBD phenotypes in Asian versus Western patients (Park et al., 2022)—to determine which differences are meaningful.

1.5.10.3 Diet, Westernization, and the Microbiome

The global rise of IBD tracks closely with adoption of a “Western” diet—high in animal fat, protein, simple sugars and ultra-processed foods, but low in fiber. Such diets drive gut dysbiosis (reduced microbial diversity, lower short-chain fatty acids, impaired mucus/barriers) that promotes intestinal inflammation. For example, Western-style diets (high in meat and low in fiber) are characteristic of affluent countries with high IBD, whereas plant-based, fiber-rich diets are associated with a richer microbiota and fewer IBD cases. Worldwide, increased consumption of ultra-processed foods (now ~58% of calories in rich nations) parallels surging IBD in Asia, Africa and other regions. In short, traditional high-fiber diets appear protective, while Westernized diets that disrupt the gut ecosystem are ubiquitous drivers of IBD risk across populations (Chiba et al., 2019; Spiller et al., 2025).

1.5.10.4 Environmental and Lifestyle Risk Modifiers

- **Smoking:** Smoking rates vary dramatically by region (e.g. ~29% of Europeans smoke versus ~10% of Africans). This exposure has opposite effects on IBD subtypes: current

smokers have roughly double the risk of CD but far lower risk of UC compared to never-smokers. In fact, one meta-analysis found current smokers had $\sim 1.76\times$ greater CD risk and an $OR \approx 0.58$ (protective) for UC onset. Clinically, smokers tend to have more aggressive CD (stricturing, penetrating disease, higher surgery rates) and milder or delayed UC. Because smoking prevalence and quitting trends differ globally, this helps explain regional IBD patterns. For example, Western countries have seen rising IBD in nonsmokers (as smoking declines), whereas some Asian cohorts have more smoking among IBD patients—reflecting how changing tobacco habits alter the Crohn’s-to-colitis ratio (Chen et al., 2022).

- **Urbanization, Rural Exposure, and Migration:** Living in cities (with higher hygiene, pollution and Western lifestyle) is linked to higher IBD rates, whereas rural/farm life is protective. Studies in North America and Europe consistently find lower IBD incidence among people raised in rural households or on farms. For example, a Canadian birth-cohort analysis showed that individuals from rural homes had significantly *lower* IBD risk (especially in children) than urban dwellers (Kuenzig et al., 2019). International migration reinforces the importance of the role of location in the development of IBD: immigrants from low-prevalence regions acquire the IBD risk of their new country. Notably, South Asian Canadians have IBD incidence (~ 14.6 cases/100k) several-fold higher than in India (~ 2.3 /100k) (D’Aloisio et al., 2025). In other words, moving into an urbanized, Western society (with its diet, lifestyle, and environmental exposures) quickly elevates IBD risk. These patterns show that urbanization and acculturation of lifestyle and microbiome can explain much of the regional IBD variation (Kuenzig et al., 2019; D’Aloisio et al., 2025).

- **Antibiotic Use and Pollution:** Modern environmental exposures such as antibiotics and pollutants can further perturb the gut and raise IBD risk. Heavy or early-life antibiotic use disrupts the developing microbiome, and epidemiological studies link prior antibiotic courses to higher IBD incidence. For instance, childhood antibiotic exposures have been associated with increased IBD risk in developed countries (and similarly in immigrant populations) (Leong et al., 2016). Likewise, air and water pollution can induce gut inflammation: particulate air pollutants alter the microbiota and are epidemiologically linked to IBD onset and flares. Recent reviews conclude that air pollution exposure can modulate gut immunity (via microbiome changes and systemic inflammation), suggesting that rising industrial pollution in

cities may contribute to the growing IBD burden (Olstrup et al., 2024). Therefore, excessive antibiotic use and environmental contaminants (common in urban/industrialized regions) further amplify the population-level IBD risk by promoting dysbiosis and mucosal injury.

- **Early-Life Microbial Exposure (Hygiene Hypothesis):** The “hygiene hypothesis” proposes that reduced microbial exposure in early life (from high sanitation, small families, fewer parasites, etc.) impairs immune tolerance and raises IBD risk. Global trends support this: as low-income countries modernize, improved hygiene and sanitation often precede steep IBD increases. In highly affluent Western populations, hygiene factors are saturated (everyone is exposed to similar high sanitation), so hygiene differences no longer explain much variation. In contrast, migrants from less-affluent regions tend to develop IBD (especially second-generation) when growing up in ultra-clean environments. Notably, the picture is not uniform: some infection-related exposures in parts of Asia (e.g. India) have been paradoxically associated with *higher* UC risk, not protection. Thus, early microbial and infection exposures clearly shape IBD risk, but their effects depend on the broader epidemiological context. In summary, populations undergoing rapid lifestyle change and hygiene improvement tend to see the greatest IBD surge, consistent with a modified hygiene hypothesis (Leong et al., 2016).

1.6 Other Gastrointestinal Conditions Included for Comparison

1.6.1 Irritable Bowel Syndrome

IBS is a functional gastrointestinal disorder defined by chronic abdominal pain with altered bowel habits, in the absence of obvious structural pathology. Historically, IBS was not considered an inflammatory condition; however, emerging evidence indicates a role for low-grade immune involvement. Many IBS patients exhibit subtle mucosal immune abnormalities—for example, an increased number of immune cells (e.g. lymphocytes, mast cells) in the colonic lining and elevated levels of inflammatory mediators—even though overt tissue damage is lacking. There is growing evidence of low-level chronic inflammation in IBS, and this immune activity is thought to interact with the enteric nervous system via the brain–gut axis. Perturbations of the gut microbiota (dysbiosis) have also been documented in IBS, which may further contribute to symptoms by influencing immune responses and gut neuronal signaling (Yuan et al., 2023).

Diagnosis of IBS is based on symptom-based criteria rather than identification of structural or biochemical abnormalities. Current diagnostic standards rely on the Rome IV criteria, which define IBS by recurrent abdominal pain occurring at least once per week over the preceding three months, associated with changes in stool frequency, stool form, or relation to defecation, with symptom onset at least six months before diagnosis. A positive diagnostic approach is recommended, supported by careful clinical assessment and exclusion of alarm features such as unintentional weight loss, gastrointestinal bleeding, anemia, nocturnal symptoms, or family history of IBD or colorectal cancer. IBS is further classified into subtypes based on predominant bowel habits, including diarrhea-predominant, constipation-predominant, mixed, and unclassified forms, which can inform therapeutic decision-making.

Management of IBS emphasizes a multidisciplinary, individualized approach targeting symptom burden and quality of life rather than disease modification. First-line strategies include patient education and reassurance, stress reduction, dietary modification, and physical activity. Dietary interventions such as a low fermentable oligosaccharides, disaccharides, monosaccharides, and polyol (FODMAP) diet have demonstrated efficacy in reducing abdominal pain, bloating, and bowel habit irregularities when implemented under nutritional supervision. Pharmacologic therapies are selected based on predominant symptoms and may include antispasmodics, antidiarrheals, laxatives, antidepressants with neuromodulatory effects, probiotics, and newer agents such as rifaximin or linaclotide in selected subtypes. Psychological interventions, including cognitive behavioral therapy, have also demonstrated efficacy in reducing symptom severity, reflecting the central role of brain–gut axis dysregulation in IBS pathophysiology (Hung et al., 2023).

In summary, IBS represents a functional bowel disorder in which immune and microbial factors may play a modulatory role, without the sustained mucosal inflammation characteristic of IBD.

1.6.2 Celiac Disease

Celiac disease is a chronic autoimmune disorder of the small intestine precipitated by dietary gluten (gliadin) in genetically predisposed individuals (Kowalski et al., 2025). In celiac disease, gluten ingestion triggers an antigen-specific immune response that damages the intestinal mucosa, leading to villous atrophy and malabsorption. A hallmark of celiac disease is the

presence of IgA-class autoantibodies against tissue transglutaminase (tTG) and endomysial antigens, which reflect loss of immune tolerance to a self antigen generated during the gluten-driven immune reaction (Kowalski et al., 2025; Pascual et al., 2014). These disease-specific IgA antibodies arise in the context of strong genetic susceptibility, most notably carriage of HLA-DQ2 and/or HLA-DQ8 alleles, and illustrate how a defined environmental trigger and host genetics can orchestrate a targeted autoimmune response in the gut (Kowalski et al., 2025).

From a clinical perspective, diagnosis of celiac disease is based on a combination of serologic testing and histologic confirmation in individuals consuming a gluten-containing diet. Serologic screening relies primarily on detection of IgA antibodies against tissue transglutaminase, with IgG-based assays used in cases of IgA deficiency or equivocal findings. Positive serology typically prompts upper gastrointestinal endoscopy with duodenal biopsy to confirm characteristic histopathologic features, including villous atrophy, crypt hyperplasia, and increased intraepithelial lymphocytes. Accurate diagnostic evaluation requires ongoing gluten exposure, as prior dietary restriction may result in false-negative findings.

The cornerstone of treatment for celiac disease is strict, lifelong adherence to a gluten-free diet, which eliminates the inciting antigen and typically leads to clinical improvement, normalization of serologic markers, and progressive mucosal healing. This direct relationship between antigen removal and immune remission highlights the antigen-specific nature of celiac disease and distinguishes it from other chronic inflammatory intestinal disorders. Ongoing follow-up is required to monitor dietary adherence, symptom resolution, and nutritional status, as inadvertent gluten exposure remains common even among treated patients (Blom et al., 2025).

From an immunological perspective, celiac disease therefore provides a useful point of comparison for distinguishing antigen-specific autoimmunity from immune responses associated with dysregulated host–microbiota interactions, such as those implicated in IBD.

1.7 IgA and IgG in Immunologically Mediated Intestinal Diseases

1.7.1 Overview of Humoral Immune Responses in IBD

It is important to note that humoral immune responses in IBD have been characterized across multiple analytical resolutions, ranging from measurements of total circulating Ig levels to highly specific analyses of antibody subclasses, antigen specificity, and microbial targeting patterns. Findings derived from these higher-resolution approaches have substantially advanced understanding of IBD heterogeneity and disease mechanisms. However, not all levels of immunological organization are equally accessible within every study design. The present work is positioned within this broader landscape and focuses on bulk IgA and IgG measurements and global antibody–microbiota interactions, with the aim of evaluating whether immunological distinctions described at finer resolution are reflected at a more integrated, population-level scale.

Both mucosal and systemic Ig responses—particularly those mediated by IgA and IgG—play integral roles in the pathophysiology of IBD. These antibody responses are now recognized as active participants in disease processes, rather than mere downstream effects (Lin et al., 2018). For example, Ig-coated gut bacteria are not bystanders: IgA-coated intestinal microbes from IBD patients have been shown to induce colitis when transferred to germ-free mice, underscoring that IgA flags microbes with pathogenic potential (Palm et al., 2014). Investigating the specificities of these antibodies, their bacterial coating patterns, and circulating antibody profiles—together with the effector mechanisms through which they mediate immune activation, inflammation, and tissue damage—provides valuable insight into IBD heterogeneity and pathogenesis.

1.7.1.1 Mucosal IgA and IgG Coating of the Gut Microbiota

One area of focus is how these antibodies interact with gut microbes at the mucosal surface. As noted above, patients with IBD exhibit an exaggerated antibody response against the commensal microbiota. Studies using flow cytometry and Ig-based bacterial sorting have shown that the proportion of fecal bacteria coated with IgA and IgG is markedly higher in individuals with CD and UC compared to healthy subjects (Lin et al., 2018). Since IgG-coated bacteria are virtually absent in healthy controls, the presence of IgG-bound microbes in the intestine of IBD patients highlights a breakdown of normal mucosal tolerance (Masu et al., 2021).

One advanced technique, IgA-SEQ, sorts and sequences bacteria according to the degree of IgA binding and has been used to identify the microbial taxa most strongly targeted by the mucosal

immune system. Using this approach, Palm et al. discovered that many bacterial species were uniquely coated in patients with IBD, as well as others unique to either CD or UC. Furthermore, they discovered that bacteria heavily coated with IgA in IBD (the so-called “high-IgA” taxa) are often pathobionts—normally harmless commensals that become inflammatory under disease conditions. When these IgA-coated bacteria were isolated from human IBD patients and transferred into germ-free mice, they induced colitis, demonstrating that IgA can selectively tag microbes with colitogenic potential (Palm et al., 2014). These findings indicate that IgA not only marks potentially harmful microbes but may actively contribute to disease by identifying pro-inflammatory members of the microbiota.

Complementary evidence from human studies supports these concepts. Using flow cytometric analysis (FACS), Masu et al. reported that fecal bacteria from IBD patients show markedly increased coating with both IgA and IgG compared to healthy controls, and that the degree of coating correlates with disease activity. Notably, UC exhibited a predominance of IgG-coated bacteria, whereas CD showed a mixed IgA/IgG pattern—consistent with the idea that epithelial barrier disruption in UC permits an IgG response that is rarely observed in the healthy gut (Masu et al., 2021). Importantly, the overall extent of IgA and IgG coating of gut bacteria correlates with disease activity and inflammation severity. Higher percentages of IgA- and IgG-coated fecal bacteria are associated with worse endoscopic inflammation and elevated systemic markers like CRP and ESR (Lin et al., 2018).

Multiple studies have confirmed that Ig coating of gut bacteria is increased in IBD compared with healthy controls. While some reports (such as the above FACS-based study) describe a predominant IgG-mediated bacterial coating in UC and more limited IgG in CD (Masu et al., 2021), other studies have observed elevated IgA- and IgG-coated bacteria in IBD overall—including in CD patients—especially during active disease (Lin et al., 2018). These differences may reflect variations in disease activity, cohort composition, and analytical methodologies (e.g. which IgG subclasses are assessed and the gating criteria used to define Ig-coated bacteria). Biologically, such heterogeneity suggests that immune responses to the intestinal microbiota in IBD are not uniform but instead vary across disease subtypes and inflammatory states. Collectively, these findings highlight the need for detailed, isotype-resolved analyses to better capture immunologic heterogeneity in IBD. Profiling the isotype and subclass

of Igs coating the gut microbiota—and how these patterns may vary across inflammatory states or clinical contexts—could provide a functional stratification of IBD beyond clinical classification alone. While the clinical utility of this approach remains to be validated in larger longitudinal cohorts, it holds promise for more personalized treatment strategies aligned with the movement toward precision medicine in IBD.

1.7.1.2 Systemic IgA and IgG responses in IBD

Beyond the mucosal compartment, patients with IBD mount distinct systemic IgA and IgG antibody responses that can be detected in the blood (e.g. via serum measurements or serologic assays). Total Ig levels and IgG subclass distributions in serum have provided additional insights into IBD immunopathology.

Overall, many IBD patients have serum IgA and IgG concentrations within normal ranges. In fact, one study found no significant difference in the total serum IgA or IgG levels of IBD patients versus healthy controls (Lin et al., 2018). Nevertheless, a substantial subset of IBD cases—often those with active or extensive disease—have exhibited elevated circulating Ig levels relative to healthy individuals. For example, in a multicenter cohort of over 600 patients with CD, 24.5% had hypergammaglobulinemia (elevated total IgG) and 17.4% had elevated serum IgA levels. The same study reported that polyclonal hypergammaglobulinemia was more frequently observed in patients with active disease, consistent with systemic immune activation (Song et al., 2021).

Beyond total circulating Ig levels, differences in systemic Ig subclass distributions have been reported in IBD. While systemic IgA alterations have also been reported, the most detailed and phenotype-informative findings to date regarding subclasses concern those of IgG. IgG has four subclasses (IgG1–4) with distinct effector functions (e.g. IgG1 is the most abundant and pro-inflammatory) (Castro-Dopico & Clatworthy, 2019). Several studies have measured serum IgG1–4 in IBD to see if UC and CD differ. Philipsen et al. found UC patients tend to have higher circulating IgG1 (and often IgG4) while CD patients have relatively higher IgG2 (1995). Gouni-Berthold et al. similarly reported higher IgG1 in UC and higher IgG2 in CD (1999). IgG3 levels have shown no consistent UC/CD difference. In short, several studies report a tendency

toward IgG1 predominance in UC, whereas CD more often exhibits a relatively greater contribution of IgG2, though these patterns are not uniform across cohorts.

Several studies link IgG subclasses to IBD severity and phenotype. Gouni-Berthold et al. found that certain IgG subclasses correlate with activity: in CD patients, higher IgG1 and IgG2 levels tracked with more active disease ($p<0.001$), whereas in UC patients higher IgG3 correlated inversely with activity ($p<0.01$). In other words, IgG1/IgG2 rise with active CD, and IgG3 falls with active UC. Total IgG or IgA showed some correlations too, but subclass shifts were notable (Gouni-Berthold et al., 1999).

Unlike IgG1 and IgG2, which are more directly linked to inflammatory activity, IgG4 appears to reflect immune regulation and disease phenotype rather than a simple inflammation gradient. Recent work highlights IgG4 as a marker of severity. Koutroumpakis et al. reported that about 20% of IBD patients have low serum IgG4 (an IgG4 “deficiency”) and these patients had markedly worse courses—more hospitalizations, surgeries, long-term steroid use and biologic use—than patients with normal/high IgG4 (2021). Chase et al. similarly found that patients with low IgG4 were significantly more likely to require advanced therapies (vedolizumab, azathioprine, steroids) (2023). In contrast, serum IgG4 above normal ranges, though uncommon, was linked to PSC in IBD (Koutroumpakis et al., 2021). In summary, low IgG4 identifies a “severe IBD” subgroup (regardless of UC/CD), whereas elevated IgG4 tends to associate with IBD phenotypes like PSC.

These subclass findings suggest IgG profiling can sharpen IBD immunophenotyping. Gouni-Berthold et al. concluded that IgG subclass concentrations “correlate...with disease activity and therefore could be used as additional markers” (1999). In other words, measuring IgG1–4 adds nuance beyond total IgG. For example, a predominance of IgG1—and in some contexts altered IgG4—has been reported in UC-like systemic immune responses, whereas an IgG2-skew might hint at CD pathology (Philipsen et al., 1995; Song et al., 2021). Likewise, identifying an IgG4-deficient profile immediately flags a high-risk, severe-IBD subgroup (Koutroumpakis et al., 2021). Overall, IgG subclass analysis has been proposed as a way to help resolve IBD heterogeneity: it provides biomarkers correlated with phenotype and activity that broader tests (such as ESR/CRP or total Ig) miss.

Traditional serologic assays for IgA/IgG (including both total levels and disease-specific antibodies) have already been employed to aid in distinguishing IBD from non-IBD mimics, but they also shed light on each patient's underlying immunological phenotype. These antibody profiles—from broad changes in Ig levels or IgG subclass distributions to specific anti-microbial IgG and IgA titers—underscore the breakdown of tolerance and chronic immune stimulation in IBD. Increasingly, they are being recognized for their potential to predict disease course and guide management (Song et al., 2021).

Taken together, these findings illustrate that systemic humoral immune responses in IBD are expressed across multiple layers of biological organization. While total circulating IgA and IgG levels provide a global measure of systemic antibody abundance, finer distinctions in immune activity are often revealed through shifts in antibody specificity, subclass distribution, or functional phenotype. IgG subclass profiling, in particular, has been shown to enhance immunophenotypic resolution beyond that afforded by bulk serologic measures.

Within this framework, subclass-specific observations have informed broader efforts to integrate clinical parameters with serological and immunological profiling, with the long-term goal of improving prognosis and supporting more personalized approaches to IBD management. Such advances depend on immune measurements with sufficient biological resolution to distinguish meaningful differences between patients, and on validation of these measurements across disease activity and inflammatory states, treatment contexts, and populations.

Importantly, changes in IgG subclass composition may occur independently of substantial variation in total IgG concentration. As a consequence, analyses based on bulk IgA or IgG abundance capture integrated immune output but may not reflect redistribution among subclasses or shifts in antibody function. Preserved or overlapping total Ig levels therefore represent a distinct level of immunological information rather than an absence of underlying immune heterogeneity.

Despite extensive evidence of Ig dysregulation in IBD, as has been demonstrated above, findings based on total IgA and IgG concentrations have been inconsistent across studies. Some cohorts demonstrate normal total serum Ig levels, whereas others report elevation primarily in subsets of patients with active or extensive disease. Similarly, increased bacterial Ig coating has been

reported in active disease, but the magnitude and isotype distribution vary across studies and analytical approaches. These discrepancies highlight the importance of analytical resolution and cohort context when interpreting humoral immune measurements.

More broadly, many disease-associated antibody patterns described in IBD have been identified using approaches that emphasize antigen specificity, microbial taxonomic resolution, or Ig subclass differentiation, frequently in cohorts enriched for active or severe disease. Whether these distinctions are consistently preserved when antibody responses are assessed at a more integrated level—through measurements of total IgA and IgG abundance or global antibody coating of the gut microbiota—has been less systematically examined, particularly in clinically heterogeneous and treated outpatient populations in a South American context. Addressing this gap is essential for understanding which aspects of humoral immune dysregulation represent conserved features of IBD and which reflect context-dependent phenomena observable only at higher analytical resolution.

1.7.2 Humoral Immune Responses in IBS

Ig alterations in IBS are generally modest and compartment-specific, consistent with the functional nature of the disorder and the absence of overt mucosal inflammation.

Population-based studies assessing circulating antibodies have not demonstrated evidence of systemic IgG- or IgG4-mediated food or yeast hypersensitivity in IBS, with serum Ig levels largely comparable between IBS patients and healthy controls after adjustment for dietary intake and clinical variables. Associations between selected food-specific IgG levels and symptom severity appear to reflect dietary modification rather than disease-driven immune activation, supporting the interpretation that circulating IgG profiles in IBS primarily mirror antigen exposure rather than pathogenic immune responses (Ligaarden et al., 2012).

In contrast to the relative stability of systemic Ig levels, emerging evidence indicates localized mucosal immune engagement in diarrhea-predominant IBS (IBS-D). Patients with IBS-D exhibit increased fecal IgA concentrations, selective upregulation of IgA1 production in the terminal ileum, and an increased proportion of IgA-coated fecal bacteria, without corresponding elevations in circulating IgA (Liu et al., 2020). These findings suggest that IBS, while not characterized by generalized humoral immune dysregulation, may involve targeted IgA-mediated

interactions with the gut microbiota at the mucosal surface. This distinction between limited, localized immune activation in IBS and the broader immunological alterations observed in IBD provides an important conceptual framework for interpreting Ig profiles across gastrointestinal disease states.

1.7.3 Humoral Immune Responses in Celiac Disease

Celiac disease is characterized by a humoral immune profile in which IgA-mediated autoantibody responses predominate in most patients during active disease, while IgG responses become clinically informative in specific contexts (Dahlbom et al., 2005). Clinical cohort studies have demonstrated that selective or partial IgA deficiency occurs more frequently in celiac disease than in the general population, affecting approximately 2% of patients, and is associated with distinct serological patterns (Chow et al., 2012).

In individuals with IgA deficiency, IgG-based autoantibodies—particularly IgG antibodies directed against tissue transglutaminase and endomysial antigens—serve as reliable alternative markers for disease detection, showing high concordance and diagnostic performance despite absent IgA responses (Dahlbom et al., 2005). Importantly, IgG celiac-associated antibodies may remain persistently elevated in IgA-deficient patients even after histologic recovery on a gluten-free diet, indicating that IgG serological responses do not necessarily parallel mucosal healing (Chow et al., 2012). Together, these findings support a dual Ig framework in celiac disease, characterized by dominant IgA responses in most patients and diagnostically compensatory IgG responses in the setting of IgA deficiency.

1.8 Rationale and Study Objectives

Because of the aforementioned differences in IBD epidemiology across countries and regions, findings from one population may not directly translate to another. Many landmark IBD studies and clinical trials have predominantly involved North American or European cohorts; their results might not account for genetic backgrounds or environmental contexts present elsewhere.

Therefore, analyzing variables such as IgA/IgG patterns in different populations is invaluable, as it can help determine whether a potential biomarker or mechanism is universally present or varies by ethnicity or region. Population-specific data can inform tailored healthcare approaches.

By comparing cohorts (e.g., Europe vs. Latin America vs. Asia), scientists can disentangle which aspects of IBD are fundamental versus which are modulated by local factors. This project, for instance, focusing on Uruguayan patients, will fill a gap in South American data and either reinforce or challenge findings from other regions. Moreover, studying populations at different stages of epidemiologic transition (from low to high IBD incidence) may shed light on environmental triggers, including protective or predisposing factors that could inform preventive strategies.

Additionally, despite substantial advances in the clinical management of IBD, these conditions remain associated with significant long-term morbidity. CD frequently leads to complications such as strictures and fistulas due to transmural inflammation, while extensive or long-standing UC is associated with severe flares, increased colorectal cancer risk, and extraintestinal manifestations affecting the joints, skin, eyes, and hepatobiliary system. Many patients require lifelong immunosuppressive or biologic therapy, yet disease courses remain highly variable, with periods of remission punctuated by unpredictable relapses (Tian et al., 2025; Lynch & Hsu, 2025).

These clinical realities highlight a persistent limitation in current diagnostic and monitoring strategies. Conventional tools, such as clinical indices and systemic inflammatory markers including CRP, primarily reflect overt inflammation and provide limited insight into underlying immune heterogeneity. In particular, they may fail to capture ongoing mucosal immune activity during clinically inactive disease or to distinguish between inflammatory and non-inflammatory gastrointestinal disorders with overlapping symptoms.

At the population level, important gaps remain in the immunologic characterization of IBD outside high-incidence regions. Most studies examining immune mechanisms in IBD, including analyses of Ig responses to the intestinal microbiota, have been conducted in North America, Europe, or East Asia. In contrast, data from South America—and Uruguay specifically—are scarce. This represents a critical gap, as regional differences in genetics, environment, diet, microbial exposures, and healthcare access may shape immune responses and disease expression. Whether Ig patterns described in other populations are conserved in Uruguayan patients remains largely unknown.

In parallel, relatively few studies have compared systemic and intestinal humoral immune responses across IBD and other immune-mediated digestive conditions within the same population. Disorders such as IBS and celiac disease can present with chronic gastrointestinal symptoms similar to IBD but differ fundamentally in their underlying immune mechanisms and management. Comparative immune profiling across these conditions may help clarify which antibody patterns are specific to intestinal inflammation versus reflective of broader immune activation.

From a mechanistic perspective, IgA and IgG represent complementary facets of intestinal immunity. IgA plays a central role in mucosal immune homeostasis and microbial containment, whereas IgG responses are often associated with loss of tolerance and inflammatory immune activation. Measuring both soluble antibody levels and the fraction of gut bacteria coated by IgA or IgG provides a functional readout of host–microbiota interactions that may capture immune processes not reflected by standard clinical markers. Assessing these responses in both plasma and fecal compartments further allows differentiation between systemic and mucosal immune activity.

1.8 Hypothesis

This study is based on the hypothesis that patients with IBD in Uruguay exhibit distinct patterns of systemic and intestinal IgA and IgG responses compared with individuals with IBS, celiac disease, and healthy controls. It further hypothesizes that variation in these Ig measures within the IBD cohort relates to clinical context, including self-reported symptom presence and CRP-defined systemic inflammatory status at the time of sampling.

1.9.1 General Objective

The general objective is to characterize systemic and intestinal humoral immune responses in a Uruguayan cohort through a preliminary and exploratory study, by evaluating IgA and IgG levels and bacterial-coating patterns across patients with IBD, IBS, celiac disease, and healthy controls. In addition, this study aims to explore whether these antibody profiles can differentiate between these conditions, with the goal of contributing to the development of more specific, non-invasive measures of disease presence and activity.

1.9.2 Specific Objectives

1. To quantify plasma IgA and IgG concentrations in patients with IBD, IBS, celiac disease, and healthy controls using direct ELISA.
2. To quantify fecal IgA and IgG concentrations in the same diagnostic groups using direct ELISA.
3. To determine the proportion of fecal bacteria coated with IgA and IgG in each group using flow cytometry.
4. To compare Ig levels and bacterial-coating patterns between patients with IBD and non-IBD comparison groups (IBS, celiac disease, and healthy controls).
5. To evaluate differences in intestinal IgA and IgG levels and bacterial-coating patterns within the IBD cohort according to self-reported symptom presence at the time of sampling.
6. To examine whether intestinal IgA and IgG levels and bacterial-coating patterns differ according to CRP-defined inflammatory status in patients with IBD.

2. Materials and Methods

2.1 Ethical Considerations

All procedures involving human participants were conducted in accordance with the ethical standards of the institutional research committee and with the principles of the Declaration of Helsinki and its later amendments. Written informed consent was obtained from all participants prior to inclusion in the study (Supplementary Material S2).

The study protocol, titled “Caracterización de la respuesta inflamatoria en patologías digestivas y su asociación con la disbiosis de la microbiota intestinal”, was reviewed and approved by the Comité de Ética de la Investigación of the Hospital de Clínicas “Dr. Manuel Quintela” (Universidad de la República). Approval was granted on 2 May 2022 and remained valid for the duration of the project (Supplementary Material S3).

Biological samples collected under this approved protocol contributed to multiple related research activities within the scope of the project and institutional oversight. The analyses presented in this thesis were conducted in accordance with the approved protocol and the informed consent provided by participants.

2.2 Study Design and Participants

This was a cross-sectional observational study including 82 adult participants (≥ 18 years) divided into four groups. The cohort consisted of 17 patients with IBD, 15 patients diagnosed with IBS, 12 individuals with celiac disease, and 38 healthy controls. CD and UC analyzed together due to sample size, with only 2 patients diagnosed with CD versus 15 patients with UC. Participants were recruited from the Gastroenterology Department at Hospital de Clínicas, Montevideo, Uruguay, between June 2022 and March 2025.

Due to differences in sample availability and timing of enrollment, not all participants contributed data to every experimental assay. Plasma antibody quantification, fecal antibody analyses, complete blood count (CBC), and CRP measurements were therefore performed on partially overlapping subsets of the cohort. Accordingly, sample sizes vary slightly across

analyses; the specific n for each assay and diagnostic group is indicated in the corresponding Results sections, as well as in Table 3 below.

Table 3. Sample availability by assay and diagnostic group

Distribution of enrolled participants and assay-specific sample availability across diagnostic groups. Total enrolled reflects all participants recruited into the study. Plasma Ig refers to participants included in plasma IgA and IgG ELISA analyses. Fecal Ig refers to participants with available stool samples used for both soluble Ig quantification by ELISA and bacterial fraction Ig assessment by flow cytometry (IgA- and IgG-associated bacterial signal). CBC reflects hematological measurements obtained from the same peripheral blood draw used for plasma antibody quantification when applicable, as performed through routine clinical laboratory testing. CRP values were available for a subset of participants.

Diagnostic Group	Total Enrolled	Plasma Ig	Fecal Ig	CBC	CRP
IBD	17	15	12	16	16
IBS	15	15	2	14	13
Celiac Disease	12	12	4	12	9
Healthy Controls	38	37	18	32	20

2.3 Clinical Data Collection and Disease Classification

Diagnosis of IBD (CD and UC) was established based on an integrated assessment of clinical, endoscopic, histopathological, radiological, and evolutive criteria, in accordance with standard clinical practice at Hospital de Clínicas.

IBS was diagnosed according to the Rome IV criteria. Celiac disease was diagnosed based on the presence of disease-specific serological markers (anti-transglutaminase or anti-endomysial antibodies) and compatible duodenal histology.

Participants with IBS were required to meet Rome IV diagnostic criteria and have no known diagnosis of celiac disease, confirmed by negative anti-transglutaminase antibodies with normal total IgA levels within the previous year.

Participants with celiac disease had a confirmed diagnosis based on at least one positive disease-specific serological marker and compatible duodenal biopsy findings.

Healthy controls were defined as adults (>18 years) with no personal history of IBD, IBS, or celiac disease, who did not meet Rome IV criteria for IBS, had no known celiac disease, and were not receiving antibiotic treatment at the time of enrollment or within the preceding two months.

CRP measurements were obtained from routine clinical laboratory records at Hospital de Clínicas as part of standard patient care at the time of study enrollment. Three participants initially recruited as healthy controls were excluded following predefined study criteria prior to statistical analysis due to elevated CRP levels (>10 mg/L), suggestive of acute inflammatory or infectious processes incompatible with the definition of a healthy control.

Demographic and clinical data were collected from medical records and participant questionnaires, including age, sex, disease duration, and current treatment status (Supplementary Material S1). Due to the heterogeneity and overlap of therapies, treatment status was analyzed as a binary variable (receiving treatment at the time of sampling: yes/no), without stratification by treatment type. For celiac disease, treatment refers to adherence to a gluten-free diet at the time of sampling.

General exclusion criteria across all study groups included pregnancy, unwillingness to participate, and antibiotic use within the two months prior to sample collection.

2.4 Sample Collection and Processing

2.4.1 Blood Sample Collection and Processing

Peripheral blood (~5 mL) was collected by venipuncture into anticoagulant tubes. Within 36 hours of collection, samples were transferred under sterile conditions to 15-mL Falcon tubes and centrifuged at 1700 rpm for 20 minutes at 4 °C to separate plasma. The plasma supernatant was aliquoted into sterile microtubes and stored at -80 °C until analysis.

A portion of each collected blood sample, as well as an aliquot of plasma obtained after centrifugation, was used for hematological assessment through a CBC. The workflow for blood plasma sample collection and processing is shown in Figure 2.

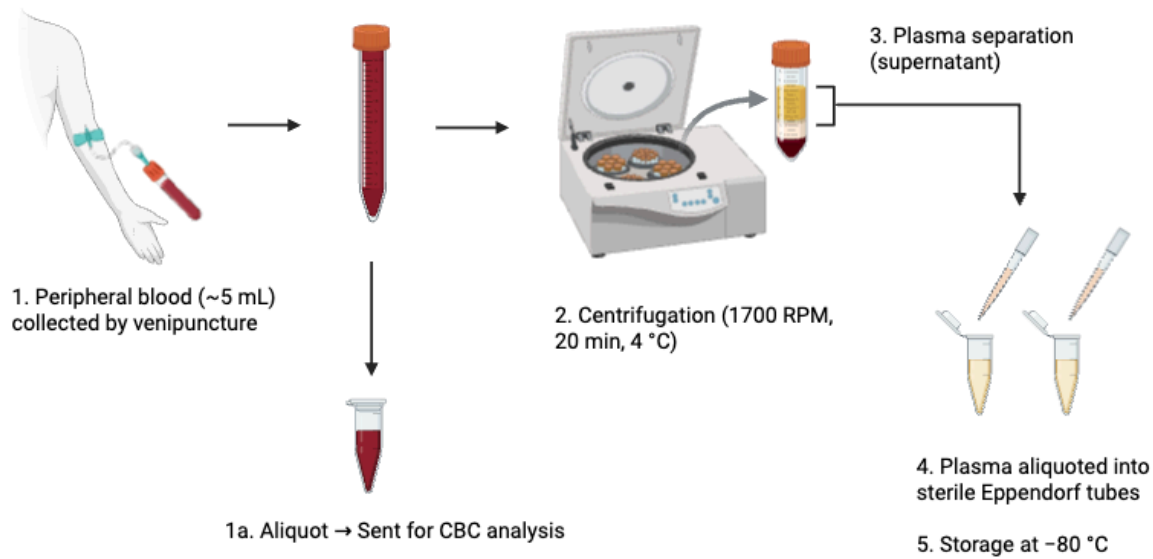


Figure 2. Overview of plasma sample processing workflow

(1) Peripheral blood (~5 mL) was collected by venipuncture into anticoagulant tubes. (1a) A portion of the sample was aliquoted and sent for complete blood count (CBC) analysis. (2) The remaining blood was centrifuged at 1700 rpm for 20 min at 4 °C. (3) Plasma (supernatant) was separated from cellular components. (4) Plasma was aliquoted into sterile Eppendorf tubes. (5) Samples were stored at -80 °C until analysis.

2.4.2 Fecal Sample Collection and Processing

Participants provided approximately 1 g of fresh stool, collected in sterile containers and stored at -80 °C until analysis.

To account for procedural variability and intra-sample heterogeneity, fecal samples were processed with technical replicates. Given that fecal material cannot be fully homogenized prior to subsampling, independent aliquots from the same sample were processed in parallel through downstream steps. Technical replicates were used as a quality-control measure and were not considered independent observations.

For each replicate, approximately 100 mg of each fecal sample was weighed and homogenized in 1 mL of phosphate-buffered saline (PBS, pH 7.4) supplemented with 10 mM EDTA, 0.4 mg/mL soybean trypsin inhibitor (aprotinin; Sigma-Aldrich, SRE0078), and a broad-spectrum protease inhibitor cocktail targeting serine, cysteine, and metalloproteases (Sigma-Aldrich, P2714; 10x

stock; used at 1x final concentration) to prevent proteolytic degradation prior to Ig quantification. To prepare this buffer, 900 μL of PBS–EDTA–trypsin inhibitor solution was mixed with 100 μL of the protease inhibitor cocktail to obtain a total volume of 1 mL per sample.

Samples were vortexed until visibly homogenized and then kept under continuous agitation at 4 °C for 30–60 min to ensure complete solubilization. The homogenates were centrifuged at 1500 x g for 20 min at 4 °C, and the resulting supernatant was carefully transferred to a clean microtube. The remaining pellet was discarded.

A second centrifugation step was performed at 10000 x g for 10 min at 4 °C to separate bacterial cells from soluble components. The resulting supernatant, containing soluble Igs, was collected and stored at –20 °C until ELISA analysis.

The remaining bacterial pellet was reserved for subsequent flow cytometric analysis. The workflow for stool sample collection and processing is shown in Figure 3.

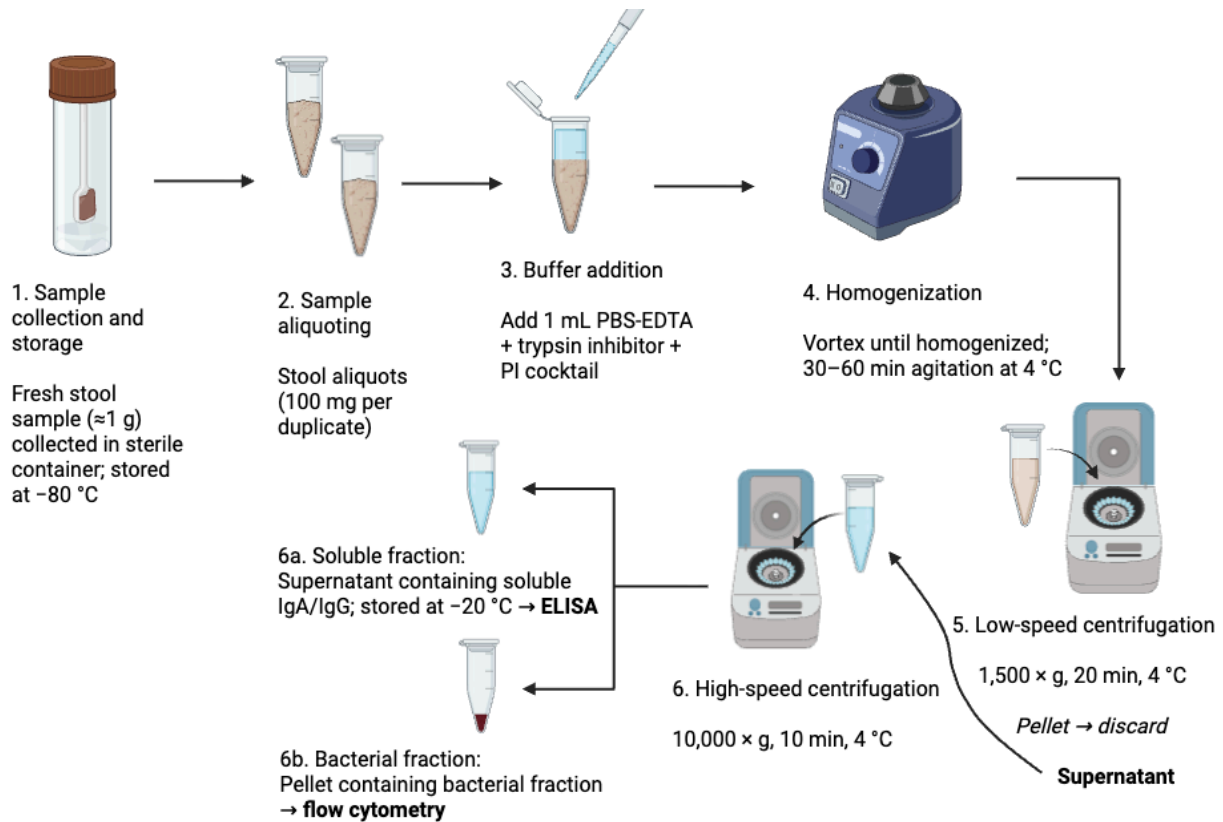


Figure 3. Overview of stool sample processing workflow

(1) Fresh stool samples (~1 g) were collected in sterile containers and stored at -80 °C until processing. (2) To account for intra-sample heterogeneity, each sample was processed in technical duplicate, with independent aliquots of approximately 100 mg prepared in parallel. (3) Aliquots were resuspended in PBS-EDTA buffer supplemented with trypsin inhibitor and a protease inhibitor cocktail. (4) Samples were homogenized by vortexing and agitation for 30–60 min at 4 °C until visibly uniform. (5) Homogenates were subjected to low-speed centrifugation (1,500 × g, 20 min, 4 °C) to remove coarse particulate material. (6) The resulting supernatant was centrifuged at high speed (10,000 × g, 10 min, 4 °C) to separate soluble and bacterial fractions. (6a) The final supernatant, containing soluble IgA and IgG, was stored at -20 °C for ELISA analysis. (6b) The bacterial pellet was reserved for flow cytometric analysis.

2.5 Immunoglobulin Quantification by ELISA

Direct ELISA experiments were used to quantify IgA and IgG concentrations in plasma and fecal samples, in which samples were adsorbed directly onto the plate and total Igs were detected using enzyme-conjugated anti-human antibodies. Separate assays were optimized for each biological matrix to account for differences in sample composition and expected antibody concentration ranges.

2.5.1 Protocol Optimization

Prior to sample analysis, ELISA conditions were systematically optimized to ensure reliable detection and quantification of Igs in both plasma and fecal samples. Optimization focused on key parameters influencing assay performance, including antibody concentrations, sample dilution ranges, and substrate development conditions. These adjustments were necessary to achieve an appropriate balance between signal intensity, background noise, and assay linearity.

2.5.1.1 Optimization of Plasma ELISA

Preliminary plasma ELISA experiments were conducted to optimize assay conditions prior to sample analysis. Initial attempts yielded inconsistent results, characterized by low or near-background signal detection in IgA assays, variable signal intensity in IgG assays, and elevated background levels that reduced assay sensitivity.

Systematic adjustments were therefore implemented, including optimization of detection antibody dilutions, standard curve ranges, and development conditions. In particular, detection antibody concentration was identified as a critical factor influencing signal-to-noise ratio, with a dilution of approximately 1:2000 providing the most consistent balance between signal intensity and background noise, whereas higher concentrations (e.g., 1:1000) increased background signal and lower concentrations ($\geq 1:4000$) reduced assay sensitivity. Standard curve ranges were adjusted to ensure linear signal detection across the dynamic range of the assay.

Variation in substrate development kinetics, likely related to differences in hydrogen peroxide reagent performance, resulted in substantial differences in signal intensity and development time across experiments, underscoring the importance of consistent reagent preparation and the use of freshly prepared or properly stored aliquots.

Through iterative optimization, assay conditions were refined to achieve reproducible standard curves with appropriate dynamic range and clear separation from background signal, which were subsequently applied to all experimental samples.

2.5.1.2 Optimization of Fecal ELISA

Preliminary experiments were conducted to optimize detection conditions prior to patient sample antibody analysis. Early assays using more concentrated stool preparations frequently resulted in plateaued absorbance values and limited dilution-dependent signal changes in several samples, suggesting matrix-associated interference.

Systematic testing of stool dilutions demonstrated that more diluted preparations improved assay interpretability by reducing matrix-associated interference. A stool dilution of 1:500 was identified as providing the most consistent balance between signal detectability and avoidance of saturation effects, and was therefore selected for subsequent analyses.

Despite optimization, sample behavior remained heterogeneous. Some samples consistently produced strong signals, while others remained near or below background levels, and several exhibited limited responsiveness across dilution series, with minimal changes in absorbance despite increasing dilutions. Standard curves for both IgA and IgG showed appropriate performance, indicating that limitations in signal detection were primarily driven by sample characteristics rather than calibration parameters.

Detection sensitivity remained limited, as signals approached background levels at higher dilution factors. This likely reflects a combination of true biological variability and technical challenges associated with measuring antibodies in fecal samples. In particular, the use of minimally processed stool extracts may introduce matrix-associated interference, reducing assay sensitivity compared to more extensively purified or fractionated sample preparations.

2.5.2 Plasma IgA and IgG Measurement

Direct ELISAs were performed to quantify circulating IgA and IgG levels in plasma and to compare antibody concentrations across diagnostic groups. The assays included 15 patients with IBD, 15 with IBS, 12 with celiac disease, and 37 healthy controls. Separate 96-well plates were used for each Ig isotype. Key reagents, concentrations, buffer compositions, and incubation conditions used for the direct ELISA for plasma samples are summarized in Table 4, while the workflow for plasma antibody quantification is shown in Figure 4.

2.5.2.1 Plate Coating and Sample Preparation

On Day 1, microplates were coated with serial dilutions of purified human Ig standards diluted in carbonate–bicarbonate buffer (pH 9.0).

As referenced in Section 2.5.1.1, the concentrations and dilution ranges described below were established through prior optimization experiments to ensure optimal signal detection within the linear range of the assay and to yield reliable and reproducible standard curves.

The IgA standard was prepared from human IgA (Sigma-Aldrich, I4036-1MG) at an initial concentration of 10 µg/mL. Four consecutive 1:2 serial dilutions in carbonate–bicarbonate buffer (pH 9.0) were performed to obtain standards of 5, 2.5, 1.25, and 0.625 µg/mL, followed by two additional 1:4 dilutions yielding 0.15625 and 0.0390625 µg/mL.

The IgG standard was prepared from human IgG (Sigma-Aldrich, I4506-10MG) at an initial concentration of 40 µg/mL. Six consecutive 1:4 serial dilutions were performed, yielding standards of 10, 2.5, 0.625, 0.15625, 0.0390625, and 0.009765625 µg/mL.

Each well received 50 µL of coating solution. Well H1 was left with buffer only to serve as a background and blocking control.

Plasma samples were diluted 1:2000 in carbonate–bicarbonate buffer and applied to both plates. Plates were incubated overnight at 4 °C to allow antigen binding.

2.5.2.2 Blocking and detection

On Day 2, plates were washed three times with PBS-Tween (0.1%, wash buffer) and then blocked with 1% gelatin in PBS for 1–2 h at 37 °C. After blocking, plates were washed three times with wash buffer and incubated with horseradish peroxidase (HRP)-conjugated detection anti-human IgG or IgA antibodies diluted in PBS-Tween–Gelatin (PTG, 0.05% Tween-20, 0.5% gelatin).

Plates were incubated for 1 h at 37 °C, then washed three times with PBS-Tween (0.05%).

2.5.2.3 Substrate development and reading

The chromogenic substrate was prepared fresh by dissolving 0.02 g *o*-phenylenediamine dihydrochloride (OPD) in 40 mL citrate–phosphate buffer and adding 80 µL 3% hydrogen peroxide (H₂O₂). The substrate solution (50 µL/well) was added to each plate and incubated until color development was visually apparent (approximately 90–120 s). The reaction was stopped by adding hydrochloric acid (HCl; final concentration 3 M) and optical density (OD) was measured at 492 nm using a Thermo Scientific Multiskan GO Microplate Spectrophotometer.

Standard curves were generated using the IgA and IgG standards and with background-subtracted OD values. Curves were constructed by applying a 4-parameter logistic (4PL) fit to the non-zero standards only, using background-subtracted OD readings and log-transformed concentrations; the blank was therefore excluded from the regression.

Antibody concentrations in plasma samples were calculated by nonlinear regression using a four-parameter logistic (4PL) model in GraphPad Prism 8. Resulting concentrations were subsequently analyzed using non-parametric statistical methods as described below.

While absolute plasma Ig concentrations varied between independent assay runs, the relative differences between samples were preserved across experiments. To account for inter-assay variability, data are presented as interpolated concentrations for primary analyses, and normalized fold-change values relative to the median healthy control value from the same assay run were calculated as a secondary analysis to assess the robustness of observed group-level differences. These data are presented as fold change relative to controls.

Reported plasma Ig concentrations represent values interpolated from in-house ELISA standard curves under the specified dilution and assay conditions. These measurements are intended for relative comparisons within this study and are not directly equivalent to clinical serum reference ranges obtained using standardized diagnostic platforms.

The same overall ELISA workflow was adapted for use with stool supernatants, as described in Section 2.5.3.

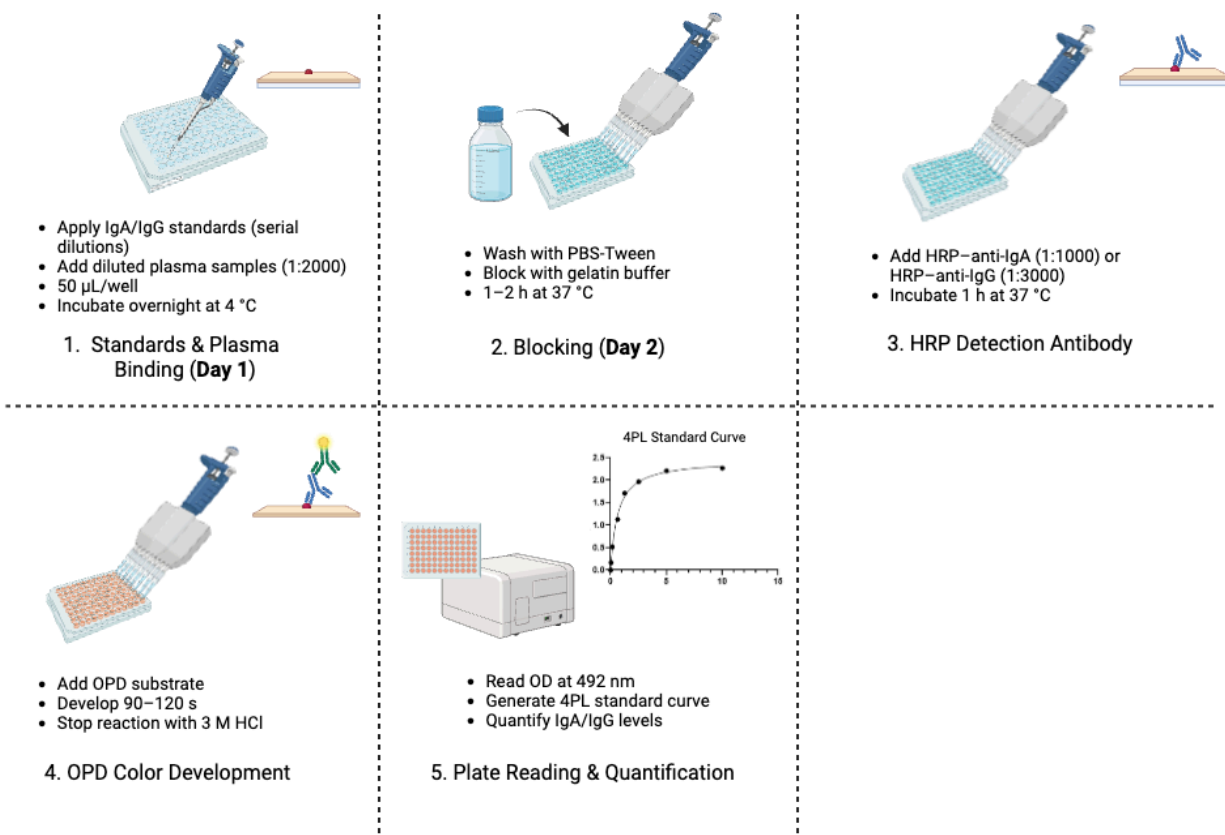


Figure 4. Workflow of direct ELISA for plasma antibody quantification

(1) Plates were coated with serial dilutions of purified human IgA or IgG standards and diluted plasma samples (1:2000) and incubated overnight at 4 °C. (2) Following washing with PBS–Tween, plates were blocked with gelatin buffer for 1–2 h at 37 °C. (3) Bound Igs were detected using HRP-conjugated anti-human IgA (1:1000) or anti-human IgG (1:3000) antibodies and incubated for 1 h at 37 °C. (4) Color development was achieved using OPD substrate and the reaction was stopped with 3 M HCl. (5) Optical density was measured at 492 nm, and IgA and IgG concentrations were quantified by interpolation from four-parameter logistic (4PL) standard curves.

Table 4. Reagents and conditions used for direct ELISA of IgA and IgG

The table summarizes the key reagents, concentrations or dilutions, buffer compositions, incubation conditions, and notes for each step of the direct ELISA protocol used to quantify plasma and fecal IgA and IgG.

Step / Reagent	Description	Concentration or Dilution	Buffer / Composition	Incubation Conditions	Notes
Coating antigen	Human IgA and IgG standards (Sigma-Aldrich, I4036-1MG and I4506-10MG)	IgA: 10 µg/mL starting concentration ($4 \times 1:2$ and $2 \times 1:4$ serial dilutions); IgG: 40 µg/mL starting concentration ($6 \times 1:4$ serial dilutions)	Carbonate–bi carbonate buffer (pH 9.0)	Overnight at 4 °C	50 µL per well; H1 well = buffer only (blank)
Sample dilution	Human plasma; Human stool	1:2000; 1:500	Carbonate–bi carbonate buffer (pH 9.0)	Overnight at 4 °C	Co-incubated with coating
Wash buffer	PBS-Tween	N/A	PBS + Tween-20 (0.01%)	Between incubation steps	3 × washes after coating/blocking; 5 × before substrate
Blocking buffer	PTG buffer	N/A	PBS + 0.05% Tween-20 + 0.5% gelatin	1–2 h at 37 °C	Prevents non-specific binding
Detection antibodies	HRP-Goat anti-human IgA (BioLegend 411002); HRP-Donkey anti-human IgG (BioLegend 410902)	IgA 1:400; IgG 1:2000	PTG buffer	1 h at 37 °C	Diluted fresh each use
Substrate solution	OPD + H ₂ O ₂	2 plates: 0.02 g OPD / 40 mL buffer + 80 µL H ₂ O ₂	Citrate–phosphate buffer (pH 5)	RT for 90–120 s	Protect from light

Stop solution	HCl	3 M final	N/A	Immediate after color development	Added when color sufficiently developed
Reading	Optical density measurement	N/A	N/A	N/A	492 nm (Thermo Scientific Multiskan GO)

2.5.3 Soluble Fecal IgA and IgG Measurement

Direct ELISAs were performed to quantify soluble IgA and IgG present in the fecal supernatant and to compare antibody concentrations across diagnostic groups, providing a measure of mucosal antibody responses to microbial antigens. Separate 96-well plates were used for each Ig isotype. This assay followed a protocol adapted from the plasma assay described above. Key reagents, concentrations, buffer compositions, and incubation conditions used for the direct ELISA are summarized in Table 4, while the workflow for fecal antibody quantification is shown in Figure 5.

The analysis included a total of 36 fecal samples (18 healthy controls, 12 IBD, 2 IBS, and 4 celiac disease), each tested in 2–4 technical replicates. For stool ELISA analyses, the median of technical replicate values was calculated to obtain a single per-sample concentration used for statistical analysis. This approach was selected to provide a robust summary measure and to maintain consistency with the non-parametric statistical framework applied throughout the study.

2.5.3.1 Plate coating and sample preparation

Stool supernatants used in this assay were obtained as described in Section 2.4.2 following differential centrifugation.

Microplates were coated separately for IgG and IgA quantification.

For the IgA assay, the standard curve was prepared from human IgA (Sigma-Aldrich, I4036-1MG) and diluted to an initial concentration of 0.5 µg/mL (carbonate–bicarbonate buffer, pH 9.0). Six consecutive 1:2 serial dilutions were generated, producing standards of 0.25, 0.125,

0.0625, 0.03125, 0.015625, and 0.0078125 $\mu\text{g/mL}$. The dilution series yielded sufficient volume for the two IgA microplates required for sample processing.

For the IgG assay, the standard curve was prepared from human IgG (Sigma-Aldrich, I4506-10MG) and began at a concentration of 0.25 $\mu\text{g/mL}$. Six consecutive 1:2 serial dilutions were performed, yielding standards of 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, and 0.00390625 $\mu\text{g/mL}$. The prepared standards provided sufficient volume for the two IgG microplates processed.

Fecal supernatants were diluted 1:500 in carbonate–bicarbonate buffer, and 200 μL of each dilution was added per well. Plates were incubated overnight at 4 $^{\circ}\text{C}$ to allow antigen adsorption.

2.5.3.2 Blocking and detection

Blocking, washing, and detection steps were performed exactly as described for the plasma ELISAs (Section 2.5.2.2). The only differences were the detection antibodies used: HRP-donkey anti-human IgG (BioLegend 410902; 1:500) for IgG plates and HRP-goat anti-human IgA (BioLegend 411002; 1:1000) for IgA plates.

2.5.3.3 Substrate development and reading

Substrate development, reaction stopping, plate homogenization, and OD reading followed the same procedure as described for plasma ELISAs (Section 2.5.2.3). For each isotype, standard curves were derived from the corresponding dilution series and fitted with a 4PL model in GraphPad Prism 8, as previously described, to determine sample concentrations.

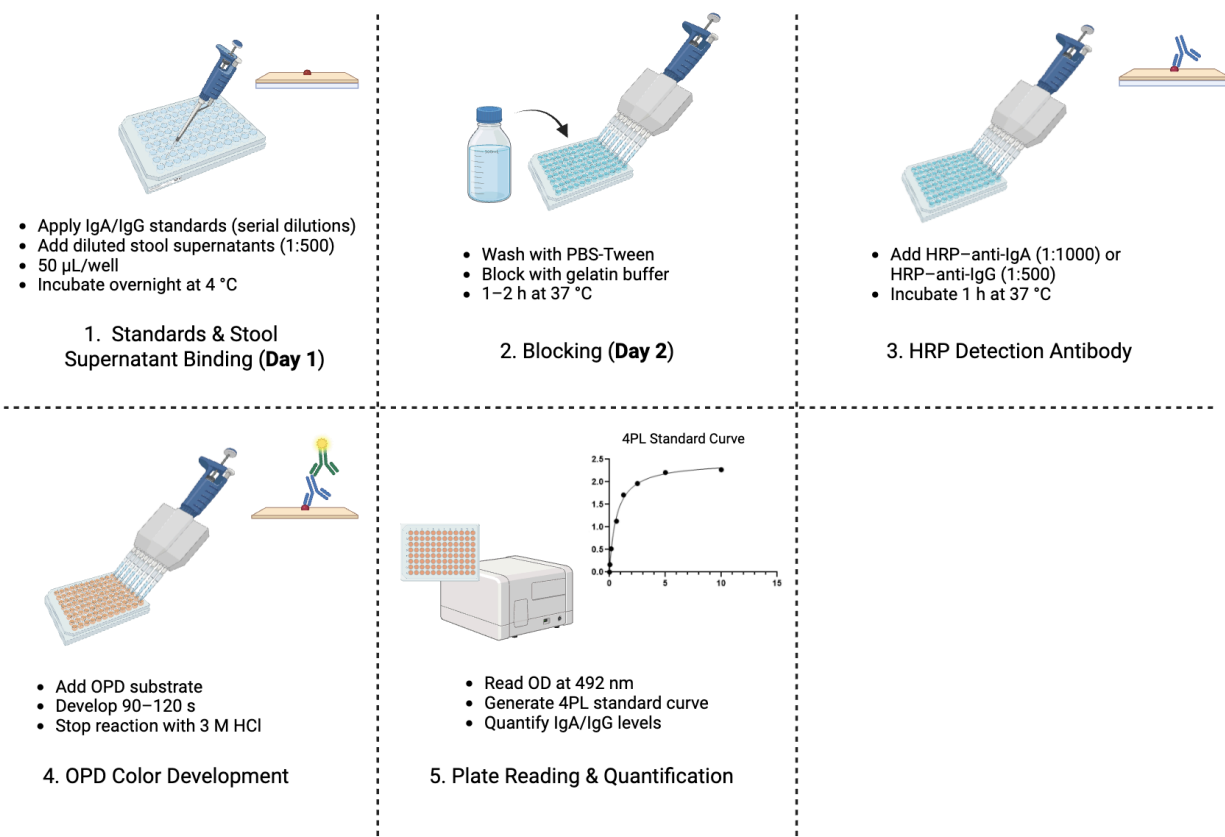


Figure 5. Workflow of direct ELISA for fecal antibody quantification.

(1) Plates were coated with serial dilutions of purified human IgA or IgG standards together with diluted fecal supernatant samples (1:500) and incubated overnight at 4 $^{\circ}$ C. (2) Following washing with PBS–Tween, plates were blocked with gelatin buffer for 1–2 h at 37 $^{\circ}$ C. (3) Bound Igs were detected using HRP-conjugated anti-human IgA (1:1000) or anti-human IgG (1:500) antibodies and incubated for 1 h at 37 $^{\circ}$ C. (4) Color development was achieved using OPD substrate and the reaction was stopped with 3 M HCl. (5) Optical density was measured at 492 nm, and soluble IgA and IgG concentrations were quantified by interpolation from four-parameter logistic (4PL) standard curves.

2.6 Flow Cytometric Analysis of Antibody-Coated Bacteria

Flow cytometry was used to assess the relative abundance of fecal bacteria bound by IgA or IgG, reflecting host antibody interactions with the gut microbiota. The analysis included 36 participants, comprising 12 patients with IBD, 2 patients with IBS, 4 patients with celiac disease, and 18 healthy controls. Key reagents, concentrations, buffer compositions, and incubation conditions used for flow cytometry analysis of Ig-coated bacteria are summarized in Table 5, while the workflow is shown in Figure 6.

IgA- and IgG-associated fluorescence signals were quantified and expressed as ratios normalized to the background signal derived from the unlabeled (unstained) fraction of each processed sample. All normalized ratio values, including those <1 , were retained for statistical analyses.

2.6.1 Flow Cytometric Analysis Sample Preparation

For flow cytometric analyses, a subset of fecal samples was processed and analyzed in additional technical replicates across independent assay runs to assess inter-assay consistency. All valid measurements obtained for these samples were included in the per-sample median calculation. These additional replicates were used for quality control and were not treated as independent observations.

The bacterial pellet used for flow cytometric staining corresponds to the microbial fraction isolated during stool processing (Section 2.4.2).

Following the second centrifugation step described above for stool samples, the bacterial pellet was used for flow cytometric analysis of antibody-coated bacteria. The pellet was resuspended in 100 μ L of phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T) and protease inhibitor cocktail (10x stock; final concentration 1x, corresponding to 10% of total volume). The suspension was gently vortexed to ensure complete resuspension.

Samples were either stored at -20°C for later analysis or processed immediately. When analyzed the same day, bacterial suspensions were kept on ice in PBS-T with protease inhibitors prior to staining.

2.6.2 Antibody Incubation and Dilution Optimization

For detection of surface-bound Igs, bacterial pellets were incubated with fluorescently labeled secondary antibodies specific for human IgA and IgG. Two staining conditions were applied for each sample: antibody dilution 1:50 and an unstained control (bacteria without antibody).

Fluorescent antibodies were diluted in phosphate-buffered saline containing 1% Tween-20 (PBS-T 1%). Samples were incubated for 1 h at 4°C in the dark, followed by washing steps as described below.

The antibodies used were as follows:

- **FITC anti-human IgG Fc** (clone M1310G05, Rat IgG2a, BioLegend, cat. no. 410720) — green fluorescence
- **APC anti-human Igα** (clone HM47, Mouse IgG1, BioLegend, cat. no. 333506) — red fluorescence

As described in Table 5, antibodies were used at final working dilutions of 1:50 and stored at 2–8 °C according to manufacturer recommendations.

Table 5. Flow cytometry staining conditions and antibody specifications

The table summarizes the fluorescent secondary antibodies used for flow cytometry, including their target specificity, fluorophore, clone, isotype, working dilutions, preparation conditions, and storage recommendations. Antibodies were used to detect surface-bound human IgA or IgG on fecal bacteria.

Name of Reagent	Function in Experiment	Manufacturer and Catalog No.	Clone	Isotype	Stock Concentration	Final Working Dilution	Preparation Method	Storage Conditions
FITC anti-human IgG Fc	Detection of IgG-coated bacteria (green fluorescence)	BioLegend, 410720	M1310 G05	Rat IgG2a	100 µg/mL	1:50	Diluted in PBS-T 1%	2–8 °C
APC anti-human Igα	Detection of IgA-coated bacteria (red fluorescence)	BioLegend, 333506	HM47	Mouse IgG1	50 µg/mL	1:50	Diluted in PBS-T 1%	2–8 °C

2.6.3 Comparison of Washed vs. Unwashed Samples

As part of protocol optimization, an initial comparison was performed to assess whether additional washing steps affected fluorescence signal, background levels, or bacterial population profiles during flow cytometric analysis. For this purpose, aliquots of antibody-labeled fecal bacterial suspensions were analyzed either directly or following washing.

After incubation, the suspensions were gently mixed to resuspend any settled bacteria. Each reaction ($\approx 60 \mu\text{L}$ total volume) was divided into two equal portions:

- **Unwashed fraction:** Diluted with $100 \mu\text{L}$ PBS-T 1% and analyzed directly on the flow cytometer.
- **Washed fraction:** The remaining volume was washed twice by adding $200 \mu\text{L}$ PBS-T 1%, centrifuging at 4,000 rpm (maximum for plate adapter) for 10 min at 4°C , discarding the supernatant, and resuspending the pellet in $100 \mu\text{L}$ PBS-T.

Comparison of washed and unwashed fractions revealed no appreciable differences in the distribution of bacterial populations or relative fluorescence patterns, although washing resulted in a reduction in total event counts. Based on these observations, subsequent flow cytometric analyses were performed using unwashed samples. Data from washed samples were not included in the final analyses.

2.6.4 Data Acquisition, Gating Strategy, and Interpretation

Forward scatter (FSC) and side scatter (SSC) parameters were used to identify bacterial-sized events and exclude debris. An initial broad gate (referred to as the major gate) was applied in FSC/SSC space to capture the principal bacterial population, which consistently comprised $\geq 90\%$ of total events and served as the parent population for downstream analyses (Figure 7a).

Doublets and aggregated events were excluded using a singlet discrimination gate based on scatter pulse geometry (SSC-A versus SSC-H) (Figure 7b).

To minimize nonspecific fluorescence, singlet events were subsequently examined in non-target fluorescence channels (PE and PerCP). A distinct population exhibiting low and correlated signal in these channels was identified and retained, while events displaying elevated or diffuse fluorescence profiles were excluded from downstream analyses as nonspecific (Figure 7c).

Fluorescence signals were collected in the APC and FITC channels to detect IgA- and IgG-associated fluorescence, respectively (Figure 7d-e). Data were acquired on a BD Accuri C6 Plus flow cytometer, with up to 10,000 events collected within the final gated bacterial population. IgA- and IgG-associated fluorescence was quantified as signal normalized to

background derived from the unlabeled fraction of each processed sample. Background fluorescence was defined using the corresponding unlabeled (unstained) fraction for each sample, and antibody-associated signal was assessed by comparison of fluorescence intensity distributions following gating.

Data acquisition and analysis were performed using BD Accuri C6 Plus software (BD Biosciences) and GraphPad Prism version 8.

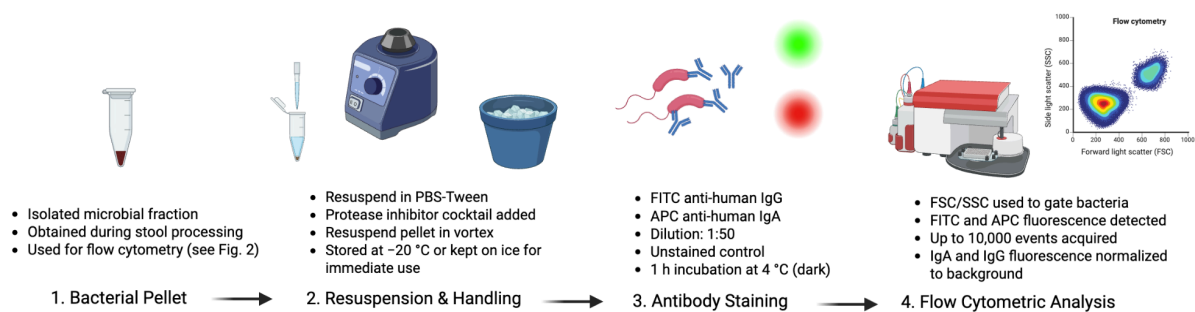


Figure 6. Workflow for flow cytometric analysis of antibody-coated fecal bacteria

(1) Fecal bacterial pellets obtained during stool processing were used for flow cytometric analysis. (2) Pellets were resuspended in PBS–Tween supplemented with a protease inhibitor cocktail and either kept on ice for immediate use or stored at -20°C prior to staining. (3) Bacterial suspensions were incubated with fluorescently labeled antibodies specific for human IgG (FITC) and IgA (APC) at a dilution of 1:50, with unstained fractions included for background normalization, and incubated for 1 h at 4°C in the dark. (4) Samples were analyzed by flow cytometry, with forward and side scatter (FSC/SSC) parameters used to gate the bacterial population and FITC and APC fluorescence used to detect IgG- and IgA-associated signals. Antibody binding was quantified as fluorescence intensity normalized to background within the gated bacterial population.

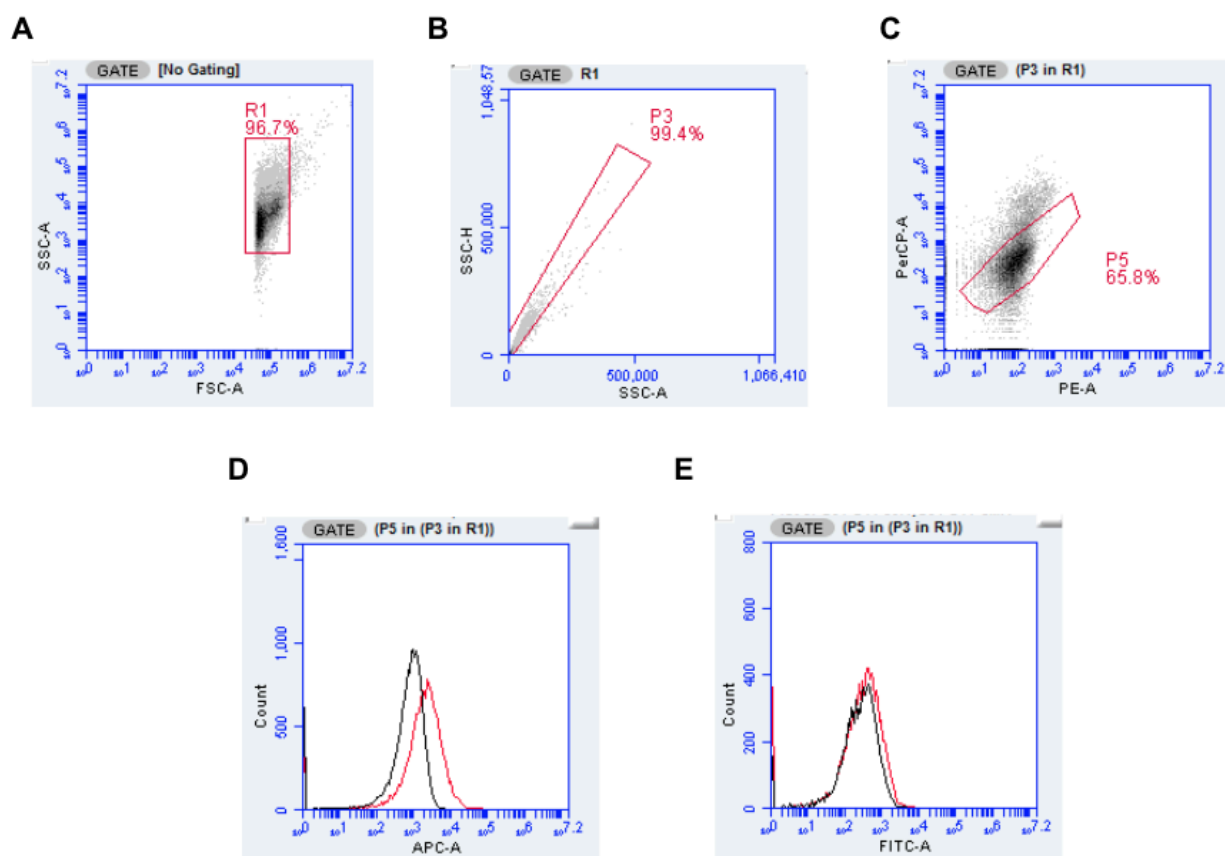


Figure 7. Flow cytometry gating strategy and fluorescence quantification illustrated using healthy control samples

(A) Forward scatter (FSC) versus side scatter (SSC) plot showing bacterial-sized events. A broad “major gate” was applied to define the primary bacterial population and exclude debris; this population served as the parent gate for all downstream analyses.

(B) SSC-A versus SSC-H plot of events within the major gate, showing the singlet discrimination gate used to exclude doublets and aggregated events.

(C) PE-A versus PerCP-A plot of singlet events showing the fluorescence-based quality control gate used to exclude events with nonspecific fluorescence in non-target channels prior to antibody-specific analysis.

(D) Representative APC-A fluorescence histogram comparing the labeled sample (anti-IgA staining; red) and the corresponding unlabeled (unstained) fraction (black) within the final gated bacterial population, shown to illustrate background normalization and assessment of IgA-associated fluorescence.

(E) Representative FITC-A fluorescence histogram comparing the labeled sample (anti-IgG staining; red) and the corresponding unlabeled (unstained) fraction (black) within the final gated bacterial population, shown to illustrate background normalization and assessment of IgG-associated fluorescence.

Control samples are shown to illustrate the gating and normalization strategy and are not intended to represent the central tendency of IgA or IgG fluorescence across the control group.

Internal gate labels shown in plots reflect instrument software nomenclature.

2.7 Hematological and Inflammatory Marker Assessment

Hematological and systemic inflammatory parameters were obtained from routine clinical laboratory testing performed at the Hospital de Clínicas at the time of study enrollment. A CBC was available for most participants using peripheral blood samples collected by venipuncture, and CRP concentrations were incorporated as systemic indicators of immune and inflammatory status, as described in Section 2.2.

Hematological values were treated as single time-point measurements per participant. CRP concentrations were reported in mg/L. Because CRP measurements were performed using non-uniform clinical assays with potentially differing reference ranges and analytical sensitivities, absolute CRP concentrations were not analyzed as continuous variables. Instead, CRP values were categorized according to the standard clinical cutoff used at Hospital de Clínicas, with values > 5 mg/L classified as elevated and values ≤ 5 mg/L classified as non-elevated. Values reported as below the assay detection limit were retained within the non-elevated category. As described above, control participants with markedly elevated CRP levels (>10 mg/L) were excluded from the study.

2.8 Statistical Analysis

Statistical analyses were designed to compare antibody concentrations and bacterial Ig coating patterns across diagnostic groups and to assess relationships between selected systemic and mucosal immune responses.

Statistical analyses were performed using GraphPad Prism version 8 unless otherwise specified. The multivariable Spearman correlation matrix was generated using jamovi version 2.6.44. Given the non-normal distribution of immunological variables and heterogeneous group sizes, non-parametric statistical methods were applied throughout.

2.8.1 Group Comparisons

Group differences across diagnostic categories were assessed using a non-parametric one-way analysis framework, with diagnostic group as the independent variable. Because data were not normally distributed, direct ELISA measurements (plasma and stool), flow cytometry

parameters, and hematological values were analyzed using the Kruskal–Wallis test, followed by Dunn’s multiple comparison test for post-hoc pairwise comparisons between diagnostic groups when applicable.

2.8.2 Handling of Technical Replicates

For direct ELISA experiments (plasma and stool), each sample was measured in a minimum of two and up to four technical replicates. For each subject, the median of replicate measurements was calculated and used as the representative value for statistical analysis. This approach was selected to reduce the influence of technical variability and outlier measurements.

For flow cytometry experiments, each sample was analyzed in a minimum of two independent technical runs. For each subject, the median of all valid technical replicates was used as the final flow cytometry value included in downstream statistical analyses, minimizing the impact of run-to-run variability.

Blood count parameters were obtained as single experimental measurements per subject.

2.8.3 Correlation Analyses

An exploratory multivariable Spearman correlation matrix was constructed to evaluate pairwise associations among systemic Ig measures, soluble fecal Ig concentrations, and bacteria-associated antibody fluorescence signals across all available paired observations.

Based on statistically significant associations identified in the multivariable analysis, targeted pairwise Spearman correlation analyses were subsequently performed to further examine selected relationships of interest. These included correlations between plasma IgA and IgG concentrations, fecal IgA and IgG concentrations, IgA- and IgG-coated bacterial fluorescence signals, and associations between soluble fecal Ig concentrations and IgG-coated bacterial fluorescence. All correlation analyses were conducted using paired measurements, with the number of paired observations varying by assay combination.

For correlation analyses involving soluble fecal Ig concentrations measured by ELISA, all available values were included, with non-detectable measurements retained as zero to reflect absence of measurable antibody signal.

For fold-change visualizations relative to healthy controls, analyses were restricted to samples with detectable fecal Ig levels. Because fold-change calculations require division by the median value of the control group, inclusion of zero-valued samples would result in undefined or non-informative ratios. Therefore, fold-change plots were generated using detectable samples only.

Exploratory post hoc correlation analyses stratified by diagnostic group were performed where sample size permitted.

CRP was not included in correlation analyses, as CRP values were not available as a continuous variable across all participants and were therefore analyzed categorically in subgroup comparisons.

2.8.4 Subgroup Analyses

Subgroup analyses within the IBD cohort were performed using the Mann–Whitney U test when comparing two groups.

Participants with IBD were classified as symptomatic or asymptomatic at the time of sampling based on self-reported gastrointestinal symptoms to explore whether differences in symptom burden may be associated with variation in fecal Ig patterns. This classification was used for exploratory subgroup analyses of flow cytometry parameters and soluble fecal Ig measurements and was not intended to represent objective disease activity or remission status. Interpretation of these comparisons was limited by small subgroup sizes.

Within the IBD cohort, participants were additionally stratified according to CRP status (elevated versus non-elevated) based on a predefined clinical cutoff. Flow cytometry parameters and soluble fecal Ig concentrations were compared between CRP-defined subgroups. Interpretation of these comparisons was limited by small subgroup sizes.

Plasma Ig concentrations were not emphasized in subgroup analyses, as they reflect longer-term systemic immune status and are less likely to vary in relation to short-term clinical symptoms or fluctuations in CRP.

2.8.5 Demographic and Clinical Variables

Demographic and clinical variables, including age, body mass index (BMI), and CRP, were summarized using descriptive statistics and compared between groups using a one-way analysis framework (Kruskal–Wallis test), with diagnostic group as the independent variable. Sex distribution across diagnostic groups was compared using the Pearson chi-square test.

CRP measurements were used for descriptive purposes and categorical subgrouping but were not analyzed as a continuous variable. The proportion of individuals with elevated CRP was compared across diagnostic groups using Fisher’s exact test. Participants with CRP values exceeding predefined thresholds were excluded from the healthy control group or classified into elevated versus non-elevated CRP subgroups within the IBD cohort, as described previously.

2.8.6 Statistical Thresholds and Data Presentation

A significance threshold of $p < 0.05$ was applied for all analyses, and all tests were two-tailed. Quantitative continuous variables are presented as individual data points with median and interquartile range (IQR), consistent with the use of non-parametric statistical tests. CBC parameters, which were approximately normally distributed, are presented as mean $\pm$ SD for clinical interpretability.

For descriptive visualization purposes, $\log_2$ -transformed ratios of group mean values relative to the mean of the healthy control group were calculated to generate heatmaps illustrating relative biomarker patterns across diagnostic categories. Because these heatmaps were intended for qualitative comparison rather than hypothesis testing, mean values were used solely for visualization and were not employed in inferential statistical analyses.

3. Results

3.1 Participant Characteristics

As described previously, the objective of this study was to evaluate systemic and fecal Ig profiles and antibody-coated gut bacteria across distinct diagnostic groups, including patients with IBD, IBS, celiac disease, and healthy controls. To address this objective, participants meeting predefined inclusion criteria were enrolled, and their baseline demographic and clinical characteristics were documented to provide context for subsequent immunological analyses.

Baseline characteristics of the study population are summarized in Table 6. Age and BMI are reported as median and interquartile range, and sex is reported as number and percentage of participants per category. Normality of continuous variables was assessed using the Shapiro–Wilk test; age did not follow a normal distribution, whereas BMI did not significantly deviate from normality. For consistency across variables and diagnostic groups, both age and BMI are presented as median and interquartile range.

Median age was 58 years (IQR 36–61.5) in patients with IBD, 59 years (IQR 31–71) in patients with IBS, 59 years (IQR 53.25–67.75) in patients with celiac disease, and 63 years (IQR 58–69.25) in healthy controls.

Median BMI was 30.46 kg/m² (IQR 25.20–35.70) in patients with IBD, 27.55 kg/m² (IQR 20.89–31.64) in patients with IBS, 29.14 kg/m² (IQR 20.82–30.87) in patients with celiac disease, and 28.81 kg/m² (IQR 25.35–33.50) in healthy controls.

Sex distribution was female-predominant across all diagnostic groups, with females comprising approximately 71% of the IBD group, 93% of the IBS group, 83% of the celiac disease group, and 76% of healthy controls. This distribution is broadly consistent with the known epidemiology of these conditions in Uruguay and in the broader literature.

In Uruguay, population-based incidence data indicate a significantly higher incidence of IBD among women compared with men, with adjusted incidence rates of 6.59 and 3.80 per 10⁵ person-years for females and males, respectively ($p = 0.028$), supporting the female predominance observed in the present IBD cohort (Iade et al., 2018).

Similarly, IBS has been consistently reported as markedly more prevalent in women in regional studies, with female representation approaching 90% in Uruguayan samples, in line with the strong female predominance observed in this study (Guido et al., 2024).

For celiac disease, sex-specific prevalence data in Uruguay remain limited; however, international and Latin American studies report a female-to-male ratio of approximately 2.8:1. The female predominance observed in the celiac disease group in this cohort is therefore consistent with established global patterns, despite the absence of robust national prevalence estimates (Parra-Medina et al., 2015).

Based on self-reported symptoms at the time of sampling, the proportion of participants reporting symptomatic disease varied across diagnostic groups. Among patients with IBD, 9 of 17 individuals (53%) reported ongoing symptoms. In the IBS group, 7 of 15 participants (47%) reported ongoing symptoms, while 4 of 12 patients with celiac disease (33%) reported symptomatic disease at the time of sample collection. Symptom status was not applicable for healthy controls.

For exploratory analyses, participants with IBD were classified as symptomatic or asymptomatic based on self-reported symptoms at the time of sampling. Symptom status was recorded for descriptive purposes only and was not intended to represent objective disease activity or remission.

At the time of sample collection, the majority of patients across diagnostic groups were receiving treatment for their condition, although the nature and definition of treatment varied by diagnosis. Downstream analyses were applied only within the IBD cohort.

Most patients with IBD were receiving standard-of-care pharmacological therapies, most commonly 5-aminosalicylic acid (oral and/or topical mesalazine), either as monotherapy or in combination with azathioprine. A smaller subset of patients with IBD was receiving biologic therapy targeting tumor necrosis factor (anti-TNF), and some individuals were receiving systemic corticosteroids at the time of sampling. For descriptive analyses, IBD treatments were grouped into broad therapeutic categories due to overlapping mechanisms of action and frequent use of combination regimens.

Among patients with celiac disease, treatment status was defined by self-reported adherence to a gluten-free diet, which represents the standard and primary therapeutic intervention for this condition. Patients reporting adherence were therefore considered treated, whereas those not adhering to a gluten-free diet were considered untreated.

In the IBS group, treatment was defined as the use of symptom-directed pharmacological agents at the time of sampling, including pinaverium bromide, mebeverine, and/or simethicone, either alone or in combination. Under these criteria, 14 of 17 patients diagnosed with IBD (82%) were undergoing treatment. In the IBS group, 9 of 15 participants (60%) reported receiving treatment, while 9 of 12 patients with celiac disease (75%) were receiving treatment at the time of sampling. Treatment status was not applicable for healthy controls.

Baseline demographic and clinical characteristics did not differ significantly across diagnostic groups. Age and BMI were comparable between groups (Kruskal-Wallis test, $p = 0.1404$ and $p = 0.4290$, respectively). Sex distribution also did not differ significantly among groups ($\chi^2 = 2.907$, $df = 3$, $p = 0.4061$).

Table 6. Study groups, participant demographics, and clinical characteristics

Age and BMI are presented as median and IQR. Sex distribution is reported as number (percentage). No statistically significant differences were observed between diagnostic groups for age, BMI, or sex. Treatment status and disease symptoms are reported descriptively for patient groups only. Disease symptoms reflect self-reported symptoms at the time of sample collection.

Diagnostic Group	n	Age (years), median (IQR)	Male, n (%)	Female, n (%)	BMI (kg/m ²), median (IQR)	Receiving treatment, n (%)	Disease Symptoms, n (%)
Healthy Controls	38	63 (58–69.25)	9 (24%)	29 (76%)	28.81 (25.35–33.50)	N/A	N/A
IBD	17	58 (36–61.5)	5 (29%)	12 (71%)	30.46 (25.20–35.70)	14/17 (82%)	9/17 (53%)
IBS	15	59 (31–71)	1 (7%)	14 (93%)	27.55 (20.89–31.64)	9/15 (60%)	7/15 (47%)
Celiac Disease	12	59 (53.25–67.75)	2 (17%)	10 (83%)	29.14 (20.82–30.87)	9/12 (75%)	4/12 (33%)

3.2 Hematological and Inflammatory Parameters (CBC and CRP)

Complete blood count (CBC) parameters (WBC, hemoglobin, hematocrit, platelet count, and red cell indices) and CRP concentrations were evaluated across diagnostic groups as summarized in Table 7. CBC data were available for 74 participants: 32 healthy controls, 16 patients with IBD, 14 patients with IBS, and 12 patients with celiac disease.

CRP data were available for a subset of participants ($n = 58$), reflecting routine clinical testing practices, as CRP measurements were not systematically obtained across all diagnostic groups, particularly among healthy controls. CRP values were available for 20 healthy controls, 16 patients with IBD, 13 patients with IBS, and 9 patients with celiac disease.

Because CRP measurements were obtained as part of routine clinical care using assays for which the specific testing kits were not consistent across patients, absolute CRP concentrations were not considered directly comparable across individuals. As reference ranges and analytical sensitivity may vary between assays, CRP values were therefore not analyzed as continuous variables.

Instead, CRP results were categorized using the clinical cutoff routinely applied at Hospital de Clínicas, with values > 5 mg/L classified as elevated and values ≤ 5 mg/L classified as non-elevated. This dichotomous classification reflects standard clinical interpretation in this setting and allows for consistent comparison across participants despite assay heterogeneity.

Using this approach, elevated CRP was observed in 9 of 20 healthy controls, 6 of 16 patients with IBD, 3 of 13 patients with IBS, and 3 of 9 patients with celiac disease, while the remaining participants in each group subset exhibited CRP values at or below the clinical cutoff (Figure 8). As explained previously, healthy controls were defined as individuals without a diagnosed gastrointestinal disorder among those studied here at the time of enrollment. Mild CRP elevations in this group likely reflect the use of routine clinical laboratory data rather than strict exclusion of all inflammatory states, and may capture transient or subclinical inflammatory activity unrelated to the chronic diseases evaluated in this cohort. Elevated CRP values were observed across all diagnostic groups, and no statistically significant differences in the proportion of individuals with elevated CRP were detected between groups (Fisher's exact test, $p = 0.5706$). Within each group, elevated CRP reflected a subset of individuals rather than a consistent shift across the cohort as a whole.

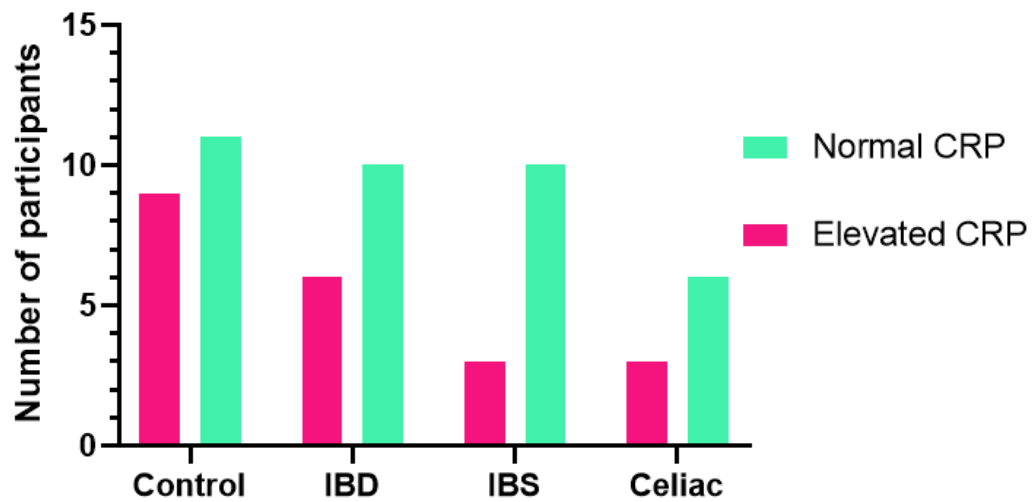


Figure 8. CRP status across diagnostic groups

Bars represent the number of individuals classified as having non-elevated/normal (≤ 5 mg/L; light green) or elevated (> 5 mg/L; fuchsia) CRP within each diagnostic group. Statistical comparisons between groups were performed using Fisher's exact test ($p = 0.5706$).

No statistically significant differences were observed between diagnostic groups for most CBC parameters ($p > 0.05$) (Supplemental Material S4).

For mean corpuscular hemoglobin concentration (MCHC) and monocyte percentage (Mon%), the Kruskal–Wallis test indicated a significant overall difference between groups ($p = 0.0374$; $p = 0.0289$). Post hoc pairwise comparisons using Dunn's test revealed higher MCHC values in patients with IBD compared with healthy controls ($p = 0.0334$) and higher Mon% values in patients with IBD compared with patients with celiac disease ($p = 0.0285$) (Table 7). A representative three-panel composite displaying white blood cell count, hemoglobin concentration, and platelet count is shown in Figure 9.

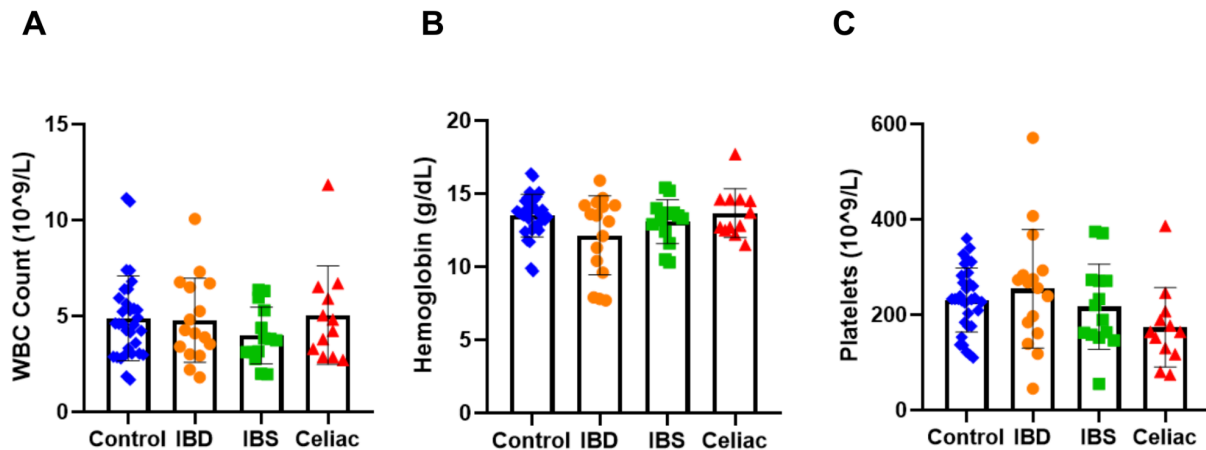


Figure 9: CBC parameters across diagnostic groups

(A) White blood cell count (WBC), (B) hemoglobin concentration, and (C) platelet count for individuals in the IBD, IBS, celiac disease, and healthy control groups. Individual points represent participant values; error bars indicate the mean $\pm$ standard deviation. No statistically significant differences were observed across groups for the parameters shown (Kruskal–Wallis test, $p > 0.05$). These results indicate broadly comparable hematological status across diagnostic categories.

Table 7. Hematological parameters and C-reactive protein status across diagnostic groups

White blood cell count (WBC), hemoglobin, platelet count, neutrophil count, lymphocyte count, mean corpuscular hemoglobin concentration (MCHC), and monocyte percentage (Mon%) are presented as mean $\pm$ standard deviation (SD). C-reactive protein (CRP) was analyzed as a categorical variable and is reported as the number and percentage of participants with elevated CRP (>5 mg/L) within each diagnostic group. CRP data were available for a subset of participants (healthy controls, $n = 20$; IBD, $n = 16$; IBS, $n = 13$; celiac disease, $n = 9$).

Comparisons of hematological parameters across diagnostic groups were performed using the Kruskal–Wallis test. Differences in the proportion of individuals with elevated CRP were assessed using Fisher’s exact test.

No statistically significant differences were observed for most hematological parameters. However, MCHC values were significantly higher in patients with IBD compared with healthy controls, and Mon% values were significantly higher in IBD patients compared with celiac patients in post hoc pairwise analyses ($p = 0.0374$; $p = 0.0289$). Reference ranges for adult values were as follows: WBC $4.5\text{--}11.0 \times 10^9/\text{L}$; hemoglobin $12.0\text{--}16.0$ g/dL in females and $13.5\text{--}17.5$ g/dL in males; platelets $140\text{--}450 \times 10^9/\text{L}$; MCHC $310\text{--}360$ g/L; and monocytes $4.0\text{--}12\%$.

No statistically significant differences were observed in the proportion of individuals with elevated CRP across diagnostic groups (Fisher’s exact test, $p = 0.5706$).

Parameter	Healthy Controls (n = 32)	IBD (n = 16)	IBS (n = 14)	Celiac (n = 12)	p-value
WBC ($\times 10^9/\text{L}$)	4.88 ± 2.21	4.79 ± 2.20	3.99 ± 1.49	5.05 ± 2.56	0.6533
Hemoglobin (g/dL)	13.51 ± 1.46	12.16 ± 2.71	13.09 ± 1.50	13.68 ± 1.66	0.5018
Platelets ($\times 10^9/\text{L}$)	230.90 ± 67.34	254.80 ± 124.5	217.00 ± 89.15	173.80 ± 83.21	0.0958
Neutrophils ($\times 10^9/\text{L}$)	1.36 ± 1.5	1.26 ± 0.75	0.94 ± 0.49	1.45 ± 1.43	0.7722
Lymphocytes ($\times 10^9/\text{L}$)	2.36 ± 0.80	2.39 ± 1.43	2.19 ± 0.73	2.55 ± 0.84	0.5116
MCHC (g/L)	308.10 ± 29.06	330.40 ± 25.36	317.30 ± 25.79	303.10 ± 39.46	0.0374
Monocytes (%)	10.53 ± 6.82	11.53 ± 4.18	8.41 ± 4.42	7.07 ± 4.10	0.0289
CRP >5 mg/L, n (%)	9 (45.0)	6 (37.5)	3 (23.1)	3 (33.3)	0.5706

3.3 Plasma Antibody Quantification

Plasma Ig analyses were performed on a total of 79 samples (15 IBD, 15 IBS, 12 celiac disease, and 37 healthy controls). Two IBD participants were enrolled after completion of the plasma antibody quantification assays and therefore could not be included due to logistical constraints.

Additionally, one healthy control participant provided only a stool sample and was excluded from all plasma-based and complete blood count analyses. These inclusion criteria were applied consistently across plasma IgA and IgG analyses.

Plasma concentrations of soluble IgA and IgG were quantified using direct ELISA to compare circulating Ig levels across diagnostic groups. Each Ig isotype was measured in a single experimental run using a 96-well plate, and standard curves were generated for each plate to calculate concentrations.

Although absolute plasma Ig concentrations varied between independent pilot runs during assay optimization, relative differences between samples were consistent, and group-level patterns were preserved when data were normalized to healthy control values. Accordingly, analyses focused on within-experiment comparisons and normalized data where appropriate.

During assay development, multiple ELISA formats were evaluated for plasma Ig quantification. The final assay design was selected based on its ability to produce consistent dilution-dependent behavior and reproducible relative differences between samples, rather than identical absolute concentrations across independent runs.

3.3.1 Assay Format Selection for Plasma Immunoglobulin Quantification

During the assay optimization phase, both sandwich and direct ELISA formats were evaluated for the quantification of total IgA and IgG in plasma samples. However, the sandwich ELISA format yielded inconsistent and non-reproducible results for both Ig isotypes across multiple optimization attempts.

For both IgA and IgG, plasma dilution series frequently failed to exhibit the expected concentration-dependent relationship with the standard curve, including non-monotonic and inverted signal patterns across dilutions. In several experiments, signal development kinetics were highly variable, ranging from unusually prolonged substrate development times to rapid overdevelopment, while in other cases absorbance values remained near background levels, precluding reliable quantification. Additionally, sporadic signal development was observed in blank wells, indicating non-specific signal generation.

Due to the lack of reproducibility, irregular dilution behavior, and evidence of non-specific signal, the sandwich ELISA format was deemed unsuitable for reliable quantification of plasma IgA and IgG under the conditions tested. Consequently, a direct ELISA format was selected and optimized for all subsequent plasma Ig measurements. This decision was made prior to the initiation of fecal sample processing and was based exclusively on assay performance during plasma optimization.

3.3.2 Plasma IgA Levels

The IgA ELISA displayed appropriate model fitting for its standard curve, with an R^2 value of 0.9961. The curve was used to interpolate IgA concentrations for all samples.

Plasma IgA concentrations showed variability across diagnostic groups. Median IgA concentrations were 593.9 $\mu\text{g/mL}$ (IQR 376.5–1293) in patients with IBD, 650.9 $\mu\text{g/mL}$ (IQR 448.0–719.6) in patients with IBS, 789.6 $\mu\text{g/mL}$ (IQR 455.5–1211) in patients with celiac disease, and 715.7 $\mu\text{g/mL}$ (IQR 557.1–923.1) in healthy controls (Table 8, Figure 10).

No statistically significant differences were observed between diagnostic groups when plasma IgA concentrations were analyzed as absolute values (Kruskal–Wallis test, $p = 0.6063$) or after normalization to the control median and expressed as fold change (Kruskal–Wallis test, $p = 0.5661$).

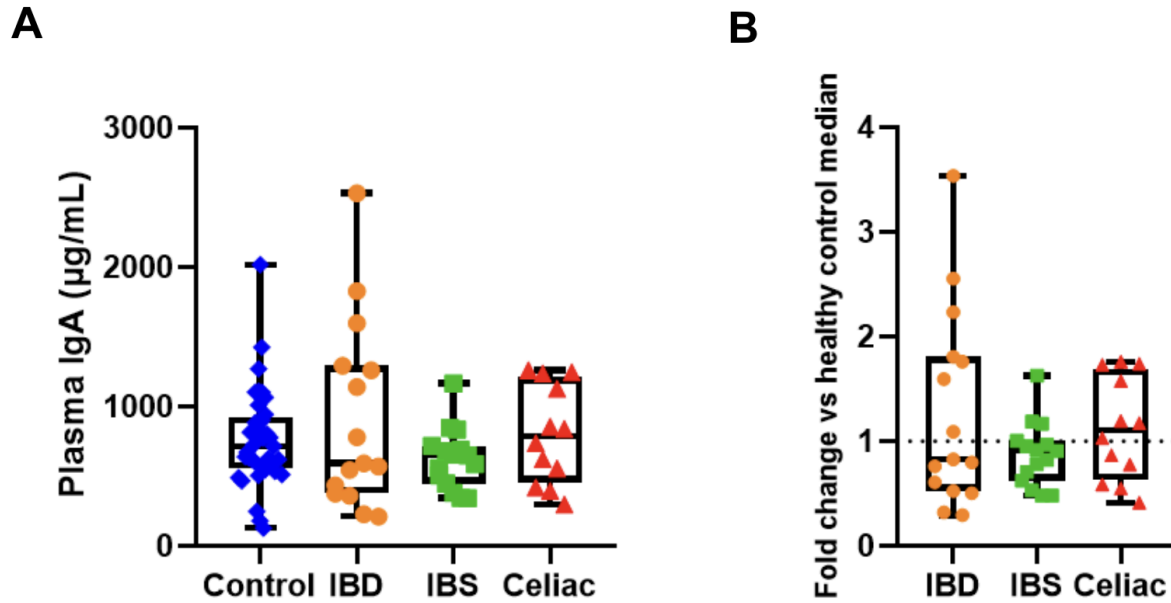


Figure 10. Plasma IgA concentrations across diagnostic groups

(A) Plasma IgA concentrations shown as absolute values (µg/mL) and (B) plasma IgA concentrations normalized to the median value of healthy control samples and expressed as fold change relative to controls for individuals in the IBD, IBS, celiac disease, and healthy control groups. Individual data points represent samples. Box-and-whisker plots display the median (center line), interquartile range (box, 25–75%), and range (whiskers). No statistically significant differences were observed between diagnostic groups when data were analyzed as absolute concentrations or as normalized fold-change values (Kruskal–Wallis test; $p = 0.6063$ and $p = 0.5661$, respectively).

3.3.3 Plasma IgG Levels

The IgG ELISA standard curve exhibited an R^2 value of 0.997. Plasma IgG concentrations were calculated accordingly.

Plasma IgG concentrations varied across diagnostic groups. Median IgG concentrations were 1718.9 µg/mL (IQR 1535.0–2044.8) in patients with IBD, 1570.0 µg/mL (IQR 1450.1–1810.9) in patients with IBS, 1615.9 µg/mL (IQR 1491.4–2169.3) in patients with celiac disease, and 1837.4 µg/mL (IQR 1347.6–2423.1) in healthy controls (Table 8, Figure 11).

Statistical analysis showed no significant difference across groups for analysis of absolute plasma IgG concentrations (Kruskal–Wallis test, $p = 0.2847$) or after normalization to the control median and expressed as fold change (Kruskal–Wallis test, $p = 0.6371$).

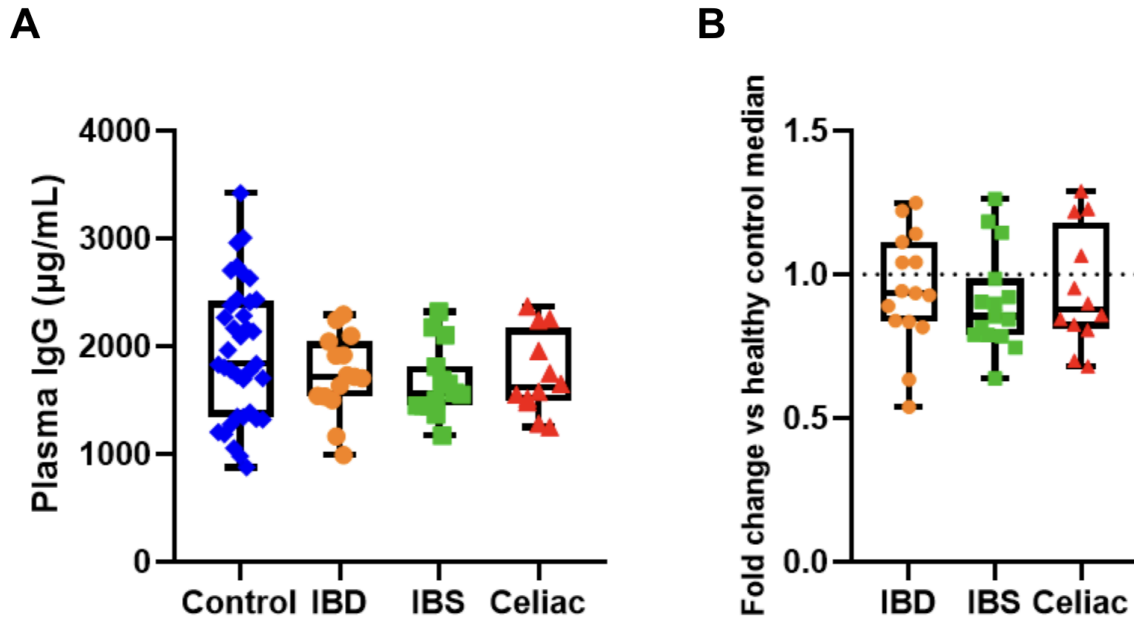


Figure 11. Plasma IgG concentrations across diagnostic groups.

(A) Plasma IgG concentrations expressed as absolute values (µg/mL). (B) Plasma IgG concentrations normalized to the median value of healthy control samples and expressed as fold change relative to controls. Data are shown for individuals in the IBD, IBS, celiac disease, and healthy control groups. Individual data points represent samples. Box-and-whisker plots display the median (center line), interquartile range (box, 25–75%), and range (whiskers). No statistically significant differences were observed between diagnostic groups when data were analyzed as absolute concentrations or as normalized fold-change values (Kruskal–Wallis test; $p = 0.2847$ and $p = 0.6371$, respectively).

Table 8. Plasma IgA and IgG concentrations across diagnostic groups

Plasma IgA and IgG concentrations were quantified by direct ELISA in healthy controls and patients with IBD, IBS, and celiac disease. Data are presented as median concentrations ($\mu\text{g/mL}$) with IQR (25–75%). Fold-change values represent median Ig concentrations normalized to the median value of the healthy control group. No statistically significant differences were observed between diagnostic groups for plasma IgA or IgG concentrations when analyzed as absolute values or as normalized fold-change values (Kruskal–Wallis test; IgA: $p = 0.6063$ and $p = 0.5661$; IgG: $p = 0.2847$ and $p = 0.6371$).

Diagnostic Group	n	Plasma IgA ($\mu\text{g/mL}$), median (IQR)	IgA Fold Change vs Control	Plasma IgG ($\mu\text{g/mL}$), median (IQR)	IgG Fold Change vs Control
Healthy Controls	37	715.7 (557.1–923.1)	N/A	1837.4 (1347.6–2423.1)	N/A
IBD	15	593.9 (376.5–1293)	0.83	1718.9 (1535.0–2044.8)	0.94
IBS	15	650.9 (448.0–719.6)	0.91	1570.0 (1450.1–1810.9)	0.85
Celiac Disease	12	789.6 (455.5–1211)	1.1	1615.9 (1491.4–2169.3)	0.88

3.4 Soluble Fecal Antibody Quantification

Soluble fecal IgA and IgG in stool samples were quantified using direct ELISA. Fecal Ig analysis included 36 samples (12 IBD, 2 IBS, 4 celiac disease, and 18 healthy controls). The reduced sample size reflects limited availability of stool specimens, as not all participants were able to provide a stool sample during the collection period.

Fecal Ig measurements were available for only two IBS participants ($n = 2$) and are therefore presented descriptively. Marked inter-individual variability was observed within this subset, including one sample with elevated fecal IgA and IgG concentrations. Similarly, fecal Ig data for celiac disease participants were limited ($n = 4$) and are interpreted descriptively. Given these sample sizes, no formal group-level conclusions were drawn for IBS or celiac disease based on fecal antibody measurements. Selected high-responding individuals are further contextualized in a subsequent case-level analysis.

Due to the number of samples and technical replicates analyzed, two 96-well plates were required for each Ig isotype, and a separate standard curve was generated and applied for each plate to calculate antibody concentrations.

Although soluble fecal antibody concentrations showed acceptable inter-assay consistency, results are presented as absolute concentrations with additional normalization to healthy control values to be consistent with plasma antibody concentration presentation.

3.4.1 Fecal IgA Concentrations and Relative Fold Change

Fecal IgA concentrations were first analyzed across all available samples to characterize overall detectability and absolute concentration values measured by ELISA. IgA was detectable in 38.9% of samples across diagnostic groups. Absolute fecal IgA concentrations for all samples are shown in Figure 12a, with undetectable samples represented as zero values.

Considering all samples together, median concentrations were 1.3 $\mu\text{g/mL}$ for patients with IBD, 46.4 $\mu\text{g/mL}$ for patients with IBS, 2.0 $\mu\text{g/mL}$ for patients with celiac disease, and 0.0 $\mu\text{g/mL}$ for healthy controls.

After isolating samples with detectable IgA, median fecal IgA concentrations were 11.6 $\mu\text{g/mL}$ (IQR 2.9–36.5) in patients with IBD, 46.4 $\mu\text{g/mL}$ (IQR 14.8–77.9) in patients with IBS, 5.9 $\mu\text{g/mL}$ (IQR 3.9–7.8) in patients with celiac disease, and 17.6 $\mu\text{g/mL}$ (IQR 9.5–42.5) in healthy controls (Table 9).

To assess relative differences in fecal IgA levels between diagnostic groups while minimizing the influence of undetectable values, a secondary analysis was performed using only samples with detectable IgA. For this analysis, IgA concentrations were normalized to the median value of the healthy control group and expressed as fold change. Fold-change values for samples with detectable IgA are shown in Figure 12b.

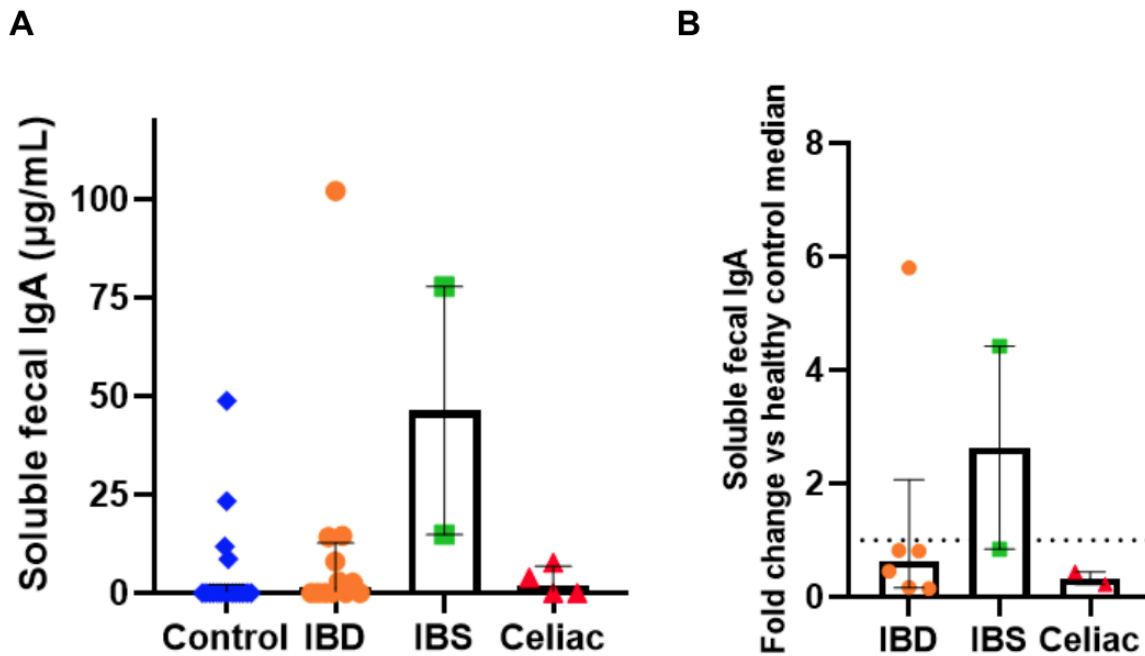


Figure 12. Soluble fecal IgA concentrations across diagnostic groups, shown as absolute values and relative fold change

(A) Soluble fecal IgA concentrations measured by ELISA are shown as absolute values (µg/mL) for individuals in the healthy control, IBD, IBS, and celiac disease groups. Individual data points represent samples. No statistically significant differences were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.0700$).

(B) Soluble fecal IgA concentrations from detectable samples were normalized to the median value of the healthy control group and expressed as fold change. Only samples with detectable IgA were included in this analysis. Individual data points represent samples. Fecal IgA was detectable in 14 out of 36 samples across all diagnostic groups (38.9%). Bars indicate median fold-change values, and error bars represent the IQR (25–75%). The dotted line denotes a fold change of 1, corresponding to the healthy control median. No statistically significant differences in fold-change values were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.2857$).

No statistically significant differences were observed between diagnostic groups for either absolute fecal IgA concentrations including all samples ($p = 0.0700$) or fold-change values restricted to detectable samples ($p = 0.2857$). Group-specific detection frequencies and absolute fecal IgA concentrations are reported in Table 9.

3.4.1.1 Subgroup Analysis of Fecal IgA Concentrations by Self-Reported Symptom Status

Subgroup analyses were performed to explore differences in fecal IgA concentrations according to the presence or absence of self-reported gastrointestinal symptoms at the time of sampling in patients with IBD. This symptom-based classification was used for exploratory purposes only and was not intended to represent objective disease activity.

Among the total IBD cohort, 9 patients reported ongoing symptoms and 7 reported no symptoms. Fecal IgA measurements by ELISA were available for 7 symptomatic patients and 4 asymptomatic patients.

To allow comparison of fecal IgA concentrations across symptom-defined subgroups, analyses were performed including all available samples, with non-detectable values retained as zero to reflect absence of measurable antibody signal. Differences between symptomatic ($n = 7$) and asymptomatic participants ($n = 4$) were assessed using the Mann–Whitney U test due to non-normal distribution and small sample size. No statistically significant difference in fecal IgA concentration was observed between symptomatic and asymptomatic participants (Mann–Whitney $U = 13$, $p = 0.9242$). Individual fecal IgA concentrations stratified by symptom status are shown in Figure 13.

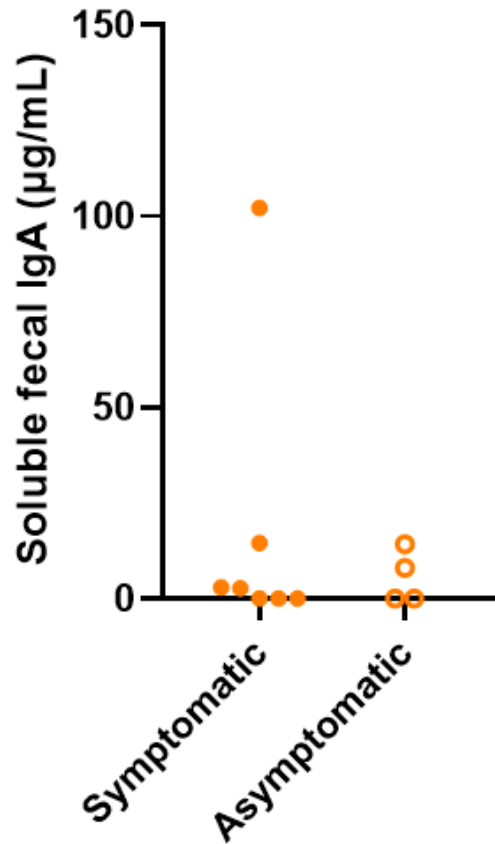


Figure 13. Fecal IgA concentrations by self-reported symptom status in patients with IBD

Fecal IgA concentrations measured by direct ELISA are shown according to self-reported symptom status at the time of sample collection (symptomatic vs asymptomatic) in patients with IBD. All available samples were included in the analysis, with non-detectable values retained as zero to reflect absence of measurable antibody signal (symptomatic, $n = 7$; asymptomatic, $n = 4$). Each point represents an individual participant. Differences between subgroups were assessed using the Mann–Whitney U test due to non-normal distribution, and no statistically significant difference was observed (Mann–Whitney $U = 13$, $p = 0.9242$).

3.4.2 Fecal IgG Concentrations and Relative Fold Change

Fecal IgG concentrations were first analyzed across all available samples to characterize overall detectability and absolute concentration values measured by ELISA. IgG was detectable in 52.7% of samples across diagnostic groups. Absolute fecal IgG concentrations for all samples are shown in Figure 14a, with undetectable samples represented as zero values.

When analyzing all samples, median concentrations were 1.4 µg/mL for patients with IBD, 21.3 µg/mL for patients with IBS, 0.6 µg/mL for patients with celiac disease, and 0.0 µg/mL for healthy controls (Table 9).

After isolating only samples with detectable IgG, median fecal IgG concentrations were 7.4 µg/mL (IQR 2.3–16.1) in patients with IBD, 21.3 µg/mL (IQR 1.7–40.9) in patients with IBS, 1.5 µg/mL (IQR 1.1–1.9) in patients with celiac disease, and 3.5 µg/mL (IQR 0.9–12.4) in healthy controls (Table 9).

To assess relative differences in fecal IgG levels between diagnostic groups while minimizing the influence of undetectable values, a secondary analysis was performed using only samples with detectable IgG. For this analysis, IgG concentrations were normalized to the median value of the healthy control group and expressed as fold change. Fold-change values for samples with detectable IgG are shown in Figure 14b.

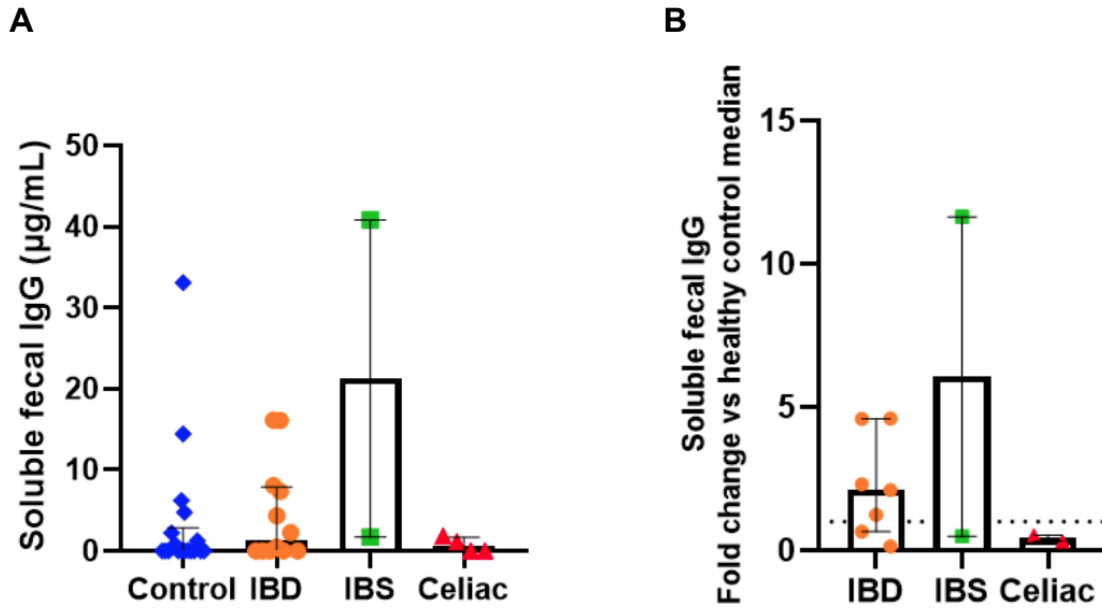


Figure 14. Soluble fecal IgG concentrations across diagnostic groups, shown as absolute values and relative fold change

(A) Soluble fecal IgG concentrations measured by ELISA are shown as absolute values (µg/mL) for individuals in the healthy control, IBD, IBS, and celiac disease groups. Individual data points represent samples. Bars indicate median values, and error bars represent the IQR (25–75%). No statistically significant differences were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.2803$).

(B) Soluble fecal IgG concentrations from detectable samples were normalized to the median value of the healthy control group and expressed as fold change. Only samples with detectable IgG were included in this analysis. Fecal IgG was detectable in 19 out of 36 samples across all diagnostic groups (52.7%). Bars indicate median fold-change values, and error bars represent the IQR (25–75%). The dotted line denotes a fold change of 1, corresponding to the healthy control median. No statistically significant differences in fold-change values were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.4182$).

No statistically significant differences were observed between diagnostic groups for either absolute fecal IgG concentrations including all samples ($p = 0.3394$) or fold-change values restricted to detectable samples ($p = 0.4182$). Group-specific detection frequencies and absolute fecal IgG concentrations are reported in Table 9.

Table 9. Soluble IgA and IgG detectable concentrations in stool samples across diagnostic groups

Soluble fecal IgA and IgG concentrations were quantified by ELISA in healthy controls and patients with IBD, IBS, and celiac disease. The total number of samples analyzed per group, along with the number and percentage of samples with detectable IgA or IgG, are shown.

Concentration values are presented both (1) including all samples, with non-detectable values retained as zero, and (2) restricted to samples with detectable Ig levels only. Values are reported as median (IQR, 25–75%).

Group comparisons were performed using the Kruskal–Wallis test. No statistically significant differences were observed between diagnostic groups for fecal IgA ($p = 0.0700$) or fecal IgG ($p = 0.2803$) when all samples were included.

Diagnostic Group	n (total)	n detectable (% IgA % IgG)	Fecal IgA (μg/mL), median (IQR)	Detectable fecal IgA (μg/mL), median (IQR)*	Fecal IgG (μg/mL), median (IQR)*	Detectable fecal IgG (μg/mL), median (IQR)
Healthy Controls	18	4 (22.2%) 8 (44.4%)	0.0 (0.0–2.2)	17.6 (9.5–42.5)	0.0 (0.0–2.9)	3.5 (0.9–12.4)
IBD	12	6 (50.0%) 7 (58.3%)	1.3 (0.0–12.7)	11.2 (2.9–36.5)	1.4 (0.0–7.9)	7.4 (2.3–16.1)
IBS	2	2 (100%) 2 (100%)	46.4 (14.8–77.9)	46.4 (14.8–77.9)	21.3 (1.7–40.9)	21.3 (1.7–40.9)
Celiac Disease	4	2 (50.0%) 2 (50.0%)	2.0 (0.0–6.8)	5.9 (3.9–7.8)	0.6 (0.0–1.7)	1.5 (1.1–1.9)

3.4.2.1 Subgroup Analysis of Fecal IgG Concentrations by Self-Reported Symptom Status

Fecal IgG concentrations were similarly examined by presence or absence of self-reported symptoms in patients with IBD. Of the 9 patients who reported ongoing symptoms and 7 patients without symptoms, fecal IgG samples were available for 7 symptomatic patients and 4 asymptomatic patients.

As with fecal IgA, subgroup analyses of fecal IgG concentrations were performed including all available samples, with non-detectable values retained as zero to reflect absence of measurable antibody signal. Fecal IgG concentrations were compared between symptomatic ($n = 7$) and asymptomatic ($n = 4$) participants using the Mann–Whitney U test due to non-normal distribution and small sample size. No statistically significant difference was observed between subgroups (Mann–Whitney $U = 9$, $p = 0.3939$). Individual fecal IgG concentrations stratified by self-reported symptom status are shown in Figure 15.

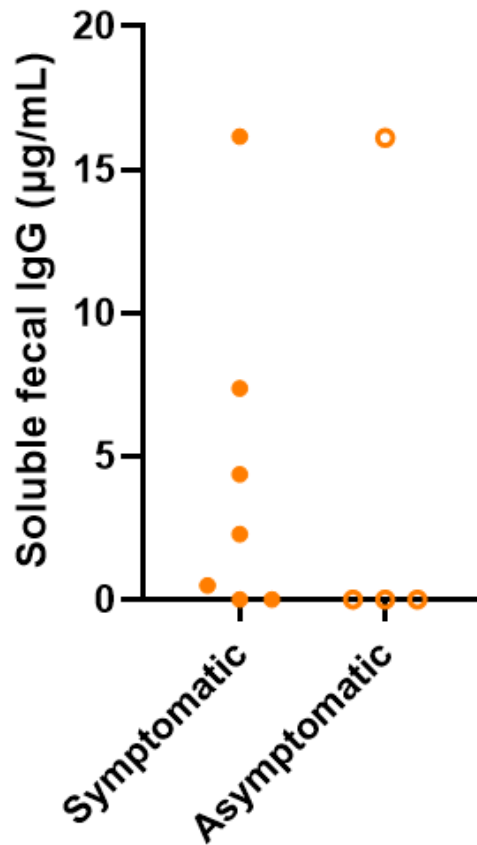


Figure 15. Fecal IgG concentrations by self-reported symptom status in patients with IBD

Fecal IgG concentrations measured by direct ELISA are shown according to self-reported symptom status at the time of sample collection (symptomatic vs asymptomatic) in patients with IBD. All available samples were included in the analysis, with non-detectable values retained as zero to reflect absence of measurable antibody signal (symptomatic, $n = 7$; asymptomatic, $n = 4$). Each point represents an individual participant. Differences between subgroups were assessed using the Mann–Whitney U test due to non-normal distribution and small sample size, and no statistically significant difference was observed (Mann–Whitney $U = 9$, $p = 0.3939$).

3.4.3 Plate-to-Plate Consistency

Because the total number of samples exceeded the capacity of a single microplate, fecal IgA and IgG measurements were performed across two plates per isotype. To assess plate-to-plate consistency, several identical control samples were included on both plates. Absorbance values and corresponding concentrations for these repeated controls were highly similar across plates, supporting the consistency of relative concentration estimates between assays. These findings indicate that plate-to-plate variability did not materially affect group-level comparisons.

3.5 Flow Cytometry Identification of Antibody-Coated Bacteria

Flow cytometry was used to characterize IgA- and IgG-coated fecal bacteria in human stool samples and to compare the relative abundance of antibody-bound bacterial populations across diagnostic groups. This analysis included values below 1, reflecting signal at or below sample background. A total of 36 fecal samples were analyzed, including 12 patients with IBD, 2 patients with IBS, 4 patients with celiac disease, and 18 healthy controls. IgA- and IgG-associated fluorescence signals were quantified and normalized to background to assess antibody binding at the bacterial level.

As stated previously, flow cytometric analysis of antibody-coated fecal bacteria was available for only two IBS participants ($n = 2$) and four celiac disease participants ($n = 4$). Given these group sizes, flow cytometry-derived IgA and IgG coating ratios for these groups are presented descriptively only, and no formal group-level conclusions were drawn.

This analysis was performed as a comparative assessment to determine whether the relative abundance of antibody-coated bacteria differed between diagnostic groups and to complement ELISA-based measurements of soluble IgA and IgG. In particular, flow cytometry results were evaluated in relation to stool-based ELISA measurements to assess whether differences in soluble fecal Ig levels were reflected in antibody binding to the gut microbiota.

3.5.1 Gating Strategy and Controls

IgA- and IgG-associated fluorescence was analyzed within the final gated bacterial population using the gating strategy described in Materials and Methods (Section 2.6.4; Figure 7).

A sequential gating strategy was applied to quantify IgA⁺ and IgG⁺ bacterial populations. Initial gating was performed on forward scatter (FSC) and side scatter (SSC) parameters to identify bacterial events and exclude debris. A major gate was then defined to capture the principal bacterial population, which consistently comprised approximately 90% of total events and served as the parent population for downstream analyses. Doublets and aggregated events were excluded in a subsequent gate based on scatter characteristics.

To minimize the contribution of nonspecific signal, fluorescence detected in channels other than FITC and APC was used to identify and exclude autofluorescent events. In samples where a secondary population with elevated signal across multiple channels was observed, these events

were interpreted as autofluorescent and were excluded from analysis. IgA⁺ and IgG⁺ bacteria were subsequently quantified based on APC and FITC fluorescence, respectively, within the final gated bacterial population.

3.5.2 IgA-Coated Bacterial Fraction

The relative abundance of IgA-coated bacteria differed modestly across diagnostic groups, as assessed by the ratio of IgA flow cytometry–derived fluorescence from technical replicates normalized to background (Figure 16).

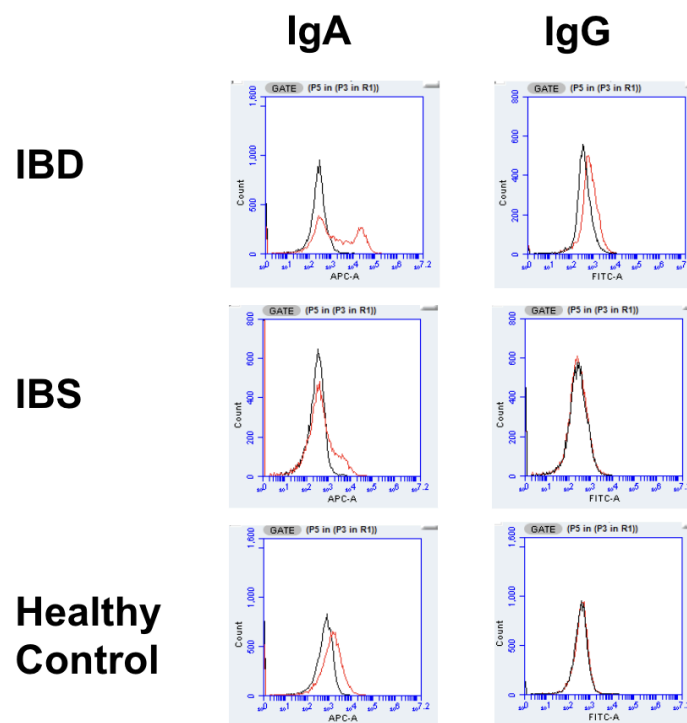


Figure 16. Representative flow cytometry histograms illustrating IgA- and IgG-associated fecal bacteria across diagnostic groups

Columns correspond to antibody isotype (IgA, left; IgG, right), and rows correspond to diagnostic categories (IBD, IBS, healthy controls). For each panel, black curves represent unstained controls, while red curves represent antibody-stained samples (anti-IgA or anti-IgG, as indicated). Within each diagnostic group, histograms are shown for individual samples obtained from a single representative participant, illustrating within-sample staining patterns and differences in fluorescence shift between IgA and IgG labeling. Examples were selected to demonstrate both pronounced and minimal rightward shifts relative to unstained controls. These histograms are provided for qualitative visualization purposes only and are not intended to represent inter-individual variability or group-level comparisons. Quantitative analyses of Ig-coated bacterial fractions across participants are presented elsewhere.

For each sample, background fluorescence was defined as the median fluorescence intensity (MFI) of the same bacterial suspension processed in parallel in the absence of anti-human IgA antibody, reflecting bacterial autofluorescence and nonspecific signal. Median IgA signal/background ratios were 1.15 in patients with IBD, 1.00 in patients with IBS, 1.13 in patients with celiac disease, and 1.18 in healthy controls (Table 10, Figure 17).

No statistically significant differences were observed across groups (Kruskal–Wallis test, $p = 0.3759$).

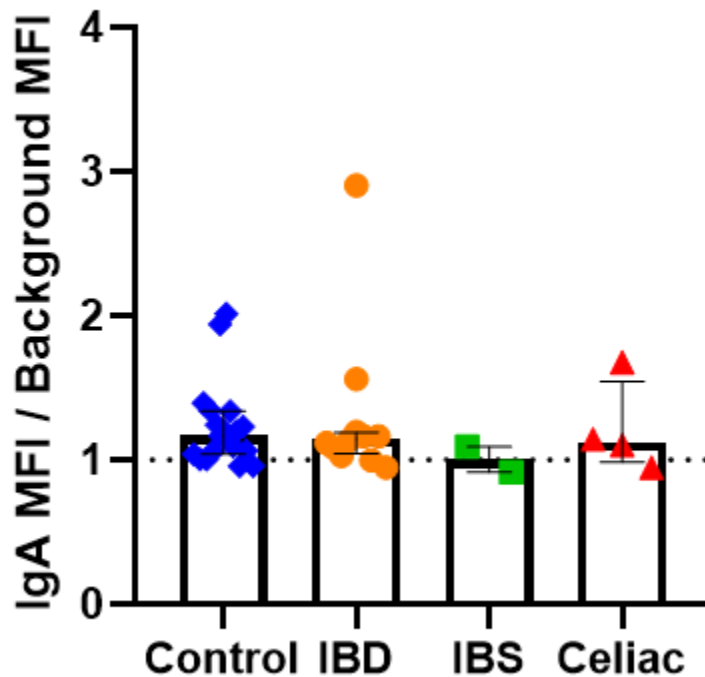


Figure 17. Relative abundance of IgA-coated fecal bacteria assessed by flow cytometry

IgA binding to fecal bacteria was quantified by flow cytometry and expressed as the ratio of IgA median fluorescence intensity (MFI) from technical replicates normalized to background signal. Individual data points represent samples from healthy controls ($n = 18$), patients with IBD ($n = 12$), IBS ($n = 2$), and celiac disease ($n = 4$). Individual data points represent samples. Bars indicate median values, and error bars represent the IQR. No statistically significant differences were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.3759$).

3.5.2.1 Subgroup Analysis of IgA-Coated Bacteria by Self-Reported Symptom Status

Flow cytometric analyses were conducted to assess whether the proportion of IgA-coated fecal bacteria differed according to self-reported symptom status at the time of sample collection in patients with IBD. Within the IBD cohort, 9 patients reported ongoing symptoms, while 7 reported no symptoms at the time of sampling. Stool samples suitable for IgA flow cytometric analysis were available for 7 symptomatic patients and 4 asymptomatic patients.

No statistically significant difference was observed in the proportion of IgA-coated bacteria between symptomatic and asymptomatic IBD patients (Mann–Whitney test, $p = 0.2303$).

Individual values for IgA-coated bacterial fractions stratified by self-reported symptom status are shown in Figure 18.

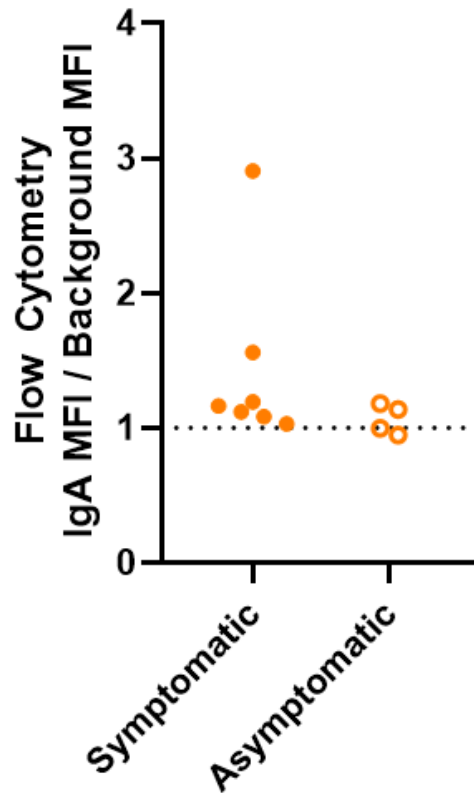


Figure 18. Proportion of IgA-coated fecal bacteria by self-reported symptom status in patients with IBD

The proportion of IgA-coated fecal bacteria measured by flow cytometry is shown according to self-reported symptom status at the time of sample collection (symptomatic vs asymptomatic) in patients with IBD. Stool samples suitable for IgA flow cytometric analysis were available for 7 symptomatic patients and 4 asymptomatic patients. Each point represents an individual participant. No statistically significant difference was observed between groups (Mann–Whitney test, $p = 0.2303$). The dashed horizontal line at $y = 1$ indicates the reference value corresponding to background-level fluorescence.

3.5.3 IgG-Coated Bacterial Fraction

The relative abundance of IgG-coated bacteria differed modestly across diagnostic groups, as assessed by the ratio of IgG flow cytometry–derived fluorescence from technical replicates normalized to background (Figure 16). In the same manner as described above, background fluorescence for each sample was defined as the MFI of the same bacterial suspension processed in parallel in the absence of anti-human IgG antibody, reflecting bacterial autofluorescence and nonspecific signal. Median IgG signal/background ratios were 1.13 in patients with IBD, 1.09 in patients with IBS, 1.02 in patients with celiac disease, and 1.05 in healthy controls (Table 10, Figure 19).

No statistically significant differences were observed across groups (Kruskal–Wallis test, $p = 0.4921$).

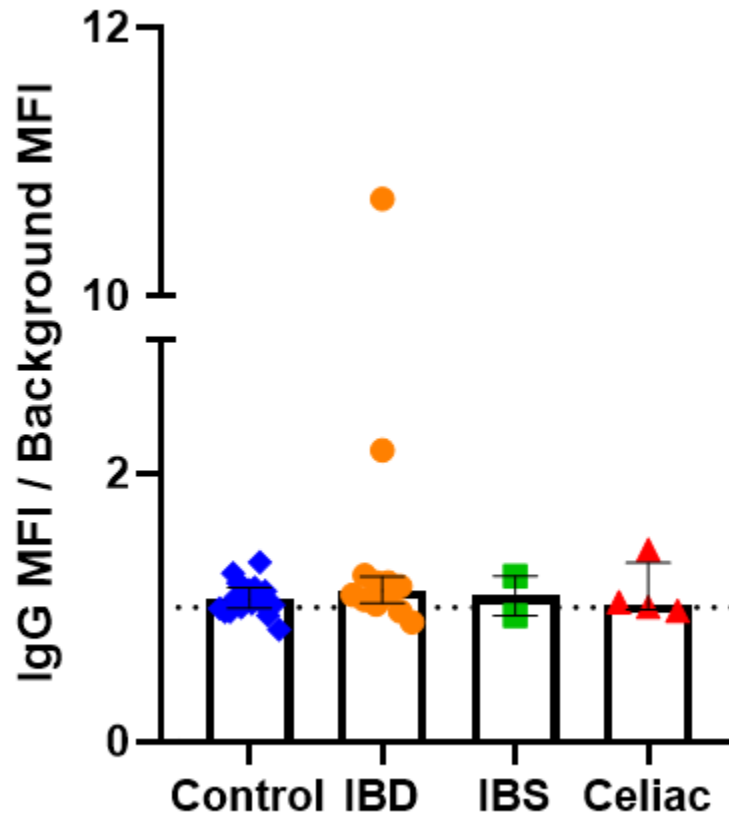


Figure 19. Relative abundance of IgG-coated fecal bacteria assessed by flow cytometry

IgG binding to fecal bacteria was quantified by flow cytometry and expressed as the ratio of IgG median fluorescence intensity (MFI) from technical replicates normalized to background signal. Individual data points represent samples from healthy controls ($n = 18$), patients with IBD ($n = 12$), IBS ($n = 2$), and celiac disease ($n = 4$). Individual data points represent samples. Bars indicate median values, and error bars represent the IQR. No statistically significant differences were observed between diagnostic groups (Kruskal–Wallis test, $p = 0.4921$).

Table 10. Relative abundance of IgA- and IgG-associated fluorescence by flow cytometry

The relative abundance of IgA- and IgG-coated fecal bacteria was quantified by flow cytometry and expressed as MFI ratios normalized to the background (unlabeled) fraction within each processed sample. Data are shown for healthy controls and patients with IBD, IBS, and celiac disease. Values are presented as median with IQR (25–75%). Group comparisons were performed using the Kruskal–Wallis test and revealed no statistically significant differences between diagnostic groups for either IgA- or IgG-associated fluorescence ($p = 0.3759$, $p = 0.4921$).

Diagnostic Group	n	IgA background-normalized MFI, median (IQR)	IgG background-normalized MFI, median (IQR)
Healthy Controls	18	1.18 (1.06–1.34)	1.05 (0.99–1.15)
IBD	12	1.15 (1.04–1.19)	1.13 (1.03–1.23)
IBS	2	1.00 (0.92–1.09)	1.09 (0.94–1.23)
Celiac Disease	4	1.13 (0.99–1.55)	1.02 (0.98–1.33)

3.5.3.1 Subgroup Analysis of IgG-Coated Bacteria by Self-Reported Symptom Status

Differences in IgG-coated fecal bacteria according to self-reported symptom status at the time of sample collection were evaluated using flow cytometry in patients with IBD. As with the IgA analyses, stool samples suitable for IgG flow cytometric analysis were available for 7 symptomatic patients and 4 asymptomatic patients.

The proportion of IgG-coated bacteria did not differ significantly between symptomatic and asymptomatic IBD patients (Mann–Whitney test, $p = 0.5273$). Individual IgG-coated bacterial fractions stratified by self-reported symptom status are shown in Figure 20.

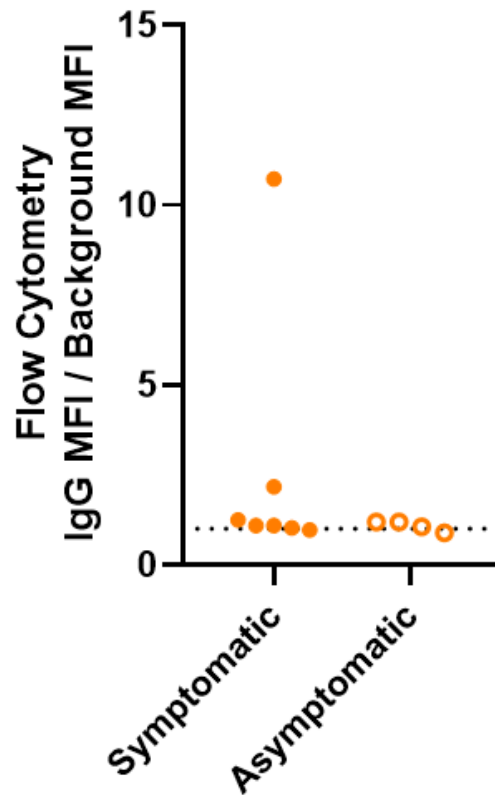


Figure 20. Proportion of IgG-coated fecal bacteria by self-reported symptom status in patients with IBD

The proportion of IgG-coated fecal bacteria measured by flow cytometry is shown according to self-reported symptom status at the time of sample collection (symptomatic vs asymptomatic) in patients with IBD. Stool samples suitable for IgG flow cytometric analysis were available for 7 symptomatic patients and 4 asymptomatic patients. Each point represents an individual participant. No statistically significant difference was observed between groups (Mann–Whitney test, $p = 0.5273$). The dashed horizontal line at $y = 1$ indicates the reference value corresponding to background-level fluorescence.

3.6 Relative Biomarker Patterns Across Diagnostic Groups

Given the general absence of statistically significant results in the group-level comparisons described above, a heatmap was generated to provide an integrated overview of relative biomarker patterns across diagnostic categories (Figure 21).

Log₂-transformed ratios were calculated from the mean values of each patient group relative to the mean values of healthy controls for each biomarker. Positive values (red) indicate higher mean levels compared to controls, whereas negative values (blue) indicate lower mean levels.

In the IBD group, mean plasma and fecal IgA levels were modestly elevated relative to controls, whereas plasma and fecal IgG levels were comparable to control values. Both IgA-coated and

IgG-coated bacterial measures were modestly increased compared with controls. CRP levels were elevated in the IBD group relative to controls.

In the IBS group, plasma IgA and IgG levels were similar to control values. In contrast, fecal IgA and fecal IgG levels were markedly elevated relative to controls. IgA- and IgG-coated bacterial levels were near control values. CRP levels in the IBS group were comparable to controls.

In the celiac disease group, plasma IgA levels were slightly elevated relative to controls, while plasma IgG levels were similar to control values. Mean fecal IgA and fecal IgG levels were reduced relative to controls, with IgG fecal levels showing the greatest relative decrease. IgA-coated and IgG-coated bacterial measures were approximately comparable to control values. CRP levels were elevated relative to controls.

Overall, these group-level patterns suggest differing distributions of systemic and mucosal humoral markers across the three disease categories, indicating that distinct antibody-associated profiles are present at the population level.

Because these values are derived from group mean ratios and presented for visualization purposes, this heatmap reflects relative group-level tendencies and does not represent statistical testing.

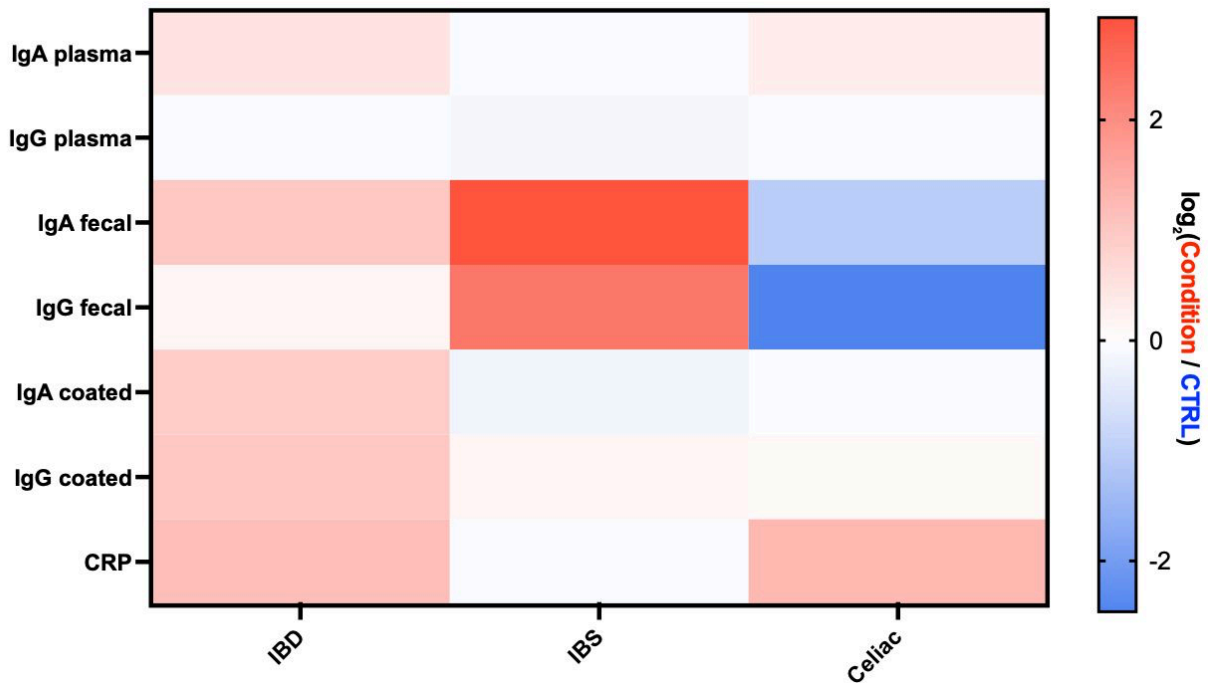


Figure 21. Heatmap of relative biomarker levels across diagnostic groups

Heatmap depicting $\log_2$ -transformed ratios of mean biomarker levels in patient groups (IBD, IBS, and celiac disease) relative to healthy controls. Values were calculated as $\log_2(\text{mean_condition} / \text{mean_control})$ for each biomarker, including plasma IgA and IgG (ELISA), fecal IgA and IgG (ELISA), IgA- and IgG-coated bacteria measured by flow cytometry, and CRP. Positive values (red) indicate higher mean levels compared to controls, whereas negative values (blue) indicate lower mean levels. This visualization is presented for descriptive comparison of group-level tendencies and does not represent statistical testing.

While this visualization summarizes relative biomarker patterns across diagnostic groups, subsequent correlation analyses evaluated inter-variable associations across individual paired observations.

3.7 Correlation Analyses Between Systemic, Fecal, and Bacteria-Associated Antibody Measures

To evaluate relationships between systemic antibody levels, soluble fecal Ig concentrations, and bacteria-associated antibody binding, Spearman rank correlation analyses were performed.

An initial exploratory Spearman multivariable correlation matrix was constructed including all primary Ig variables: plasma IgA and IgG (ELISA), fecal IgA and IgG (ELISA), and IgA- and IgG-associated bacterial fluorescence (flow cytometry) (Table 11). This matrix was generated using jamovi (version 2.6.44) and calculated using all available paired observations.

Significant positive correlations were observed between plasma IgA and plasma IgG concentrations ($\rho = 0.454, p < .001$), indicating coordinated systemic Ig responses.

Within fecal measurements, fecal IgA and fecal IgG concentrations were also positively correlated ($\rho = 0.476, p < .01$).

IgG-associated bacterial fluorescence demonstrated significant positive correlations with fecal IgA ($\rho = 0.336, p < .05$) and fecal IgG concentrations ($\rho = 0.428, p < .01$). In addition, IgA- and IgG-associated bacterial fluorescence signals were positively correlated with one another ($\rho = 0.335, p < .05$).

No significant correlations were observed between plasma Ig concentrations and bacteria-associated fecal antibody measures.

Table 11. Spearman correlation matrix among systemic, fecal, and bacteria-associated Ig measures

Values represent Spearman's rank correlation coefficients (ρ) calculated using paired observations across the full dataset. Plasma IgA and IgG concentrations were measured by ELISA. Fecal IgA and IgG concentrations were measured by ELISA. IgA- and IgG-coated bacteria were quantified by flow cytometry (FC) as antibody-associated bacterial fluorescence signal. The number of paired observations varied by assay combination due to sample availability.

* $p < .05$

** $p < .01$

*** $p < .001$

	IgA Plasma (ELISA)	IgG Plasma (ELISA)	IgA Fecal (ELISA)	IgG Fecal (ELISA)	IgA-coated bacteria (FC)
IgG Plasma (ELISA)	0.454***	—			
IgA Fecal (ELISA)	0.158	0.077	—		
IgG Fecal (ELISA)	-0.105	-0.138	0.476**	—	
IgA-coated bacteria (FC)	-0.253	-0.312	-0.063	0.165	—
IgG-coated bacteria (FC)	-0.16	-0.239	0.336*	0.428**	0.335*

To visually illustrate the direction and relative magnitude of observed associations, pairwise scatterplots with fitted regression lines are shown in Figure 22.

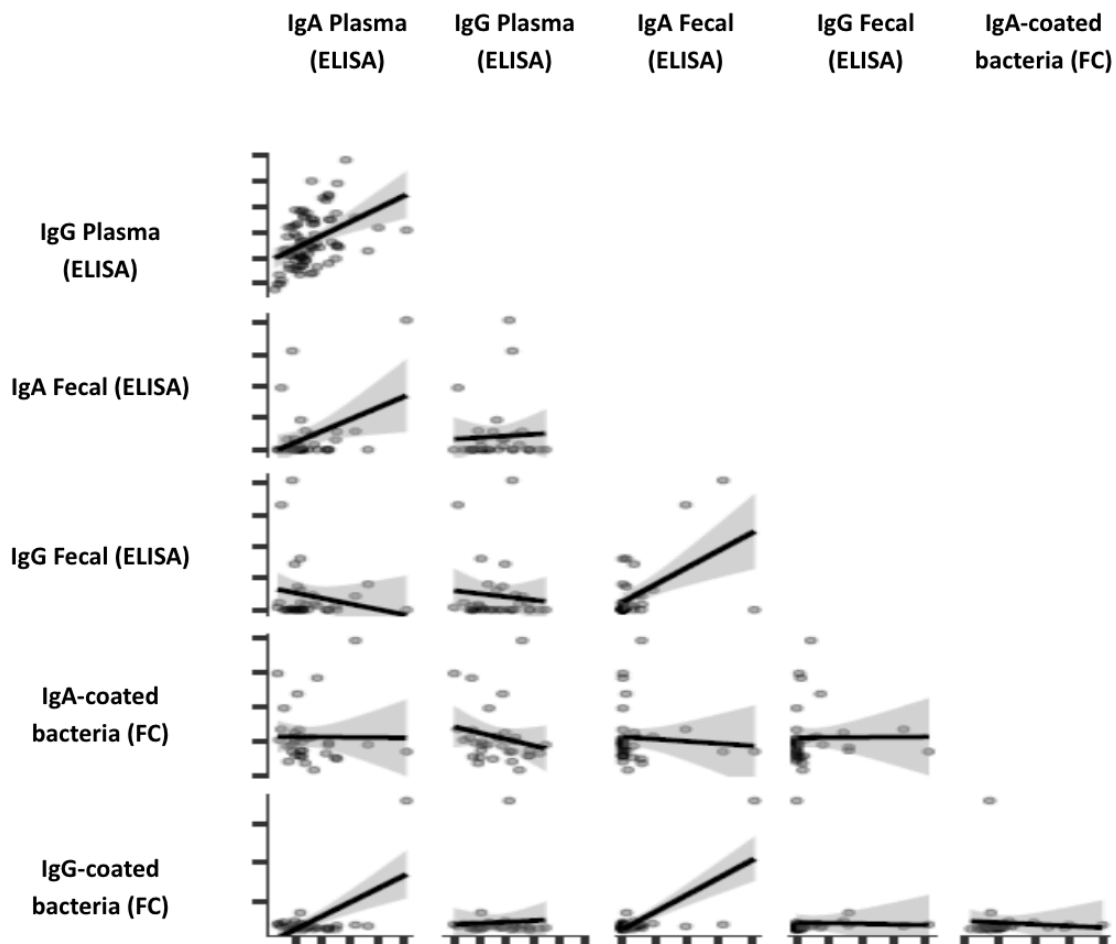


Figure 22. Pairwise relationships among systemic, fecal, and bacteria-associated Ig measures
 Scatterplots depict pairwise associations between plasma IgA and IgG (ELISA), fecal IgA and IgG (ELISA), and IgA- and IgG-coated bacterial fluorescence measured by flow cytometry (FC). Each point represents an individual participant. Solid lines indicate linear fit with shaded 95% confidence intervals shown for visualization only. Statistical significance and correlation coefficients are reported in Table 11.

While the exploratory matrix provides a global overview of relationships among Ig measures, additional targeted Spearman correlation analyses were conducted to further examine specific variable pairs and cohort-level patterns, with corresponding scatterplots shown in Figures 23–26.

These analyses were performed using paired measurements, with the number of observations varying by assay combination.

3.7.1 Relationship Between Plasma IgA and IgG

Consistent with the multivariable analysis, plasma IgA and plasma IgG concentrations demonstrated a statistically significant positive correlation ($n = 79$; Spearman $\rho = 0.454$, $p < .0001$; Figure 23), indicating coordinated systemic Ig responses.

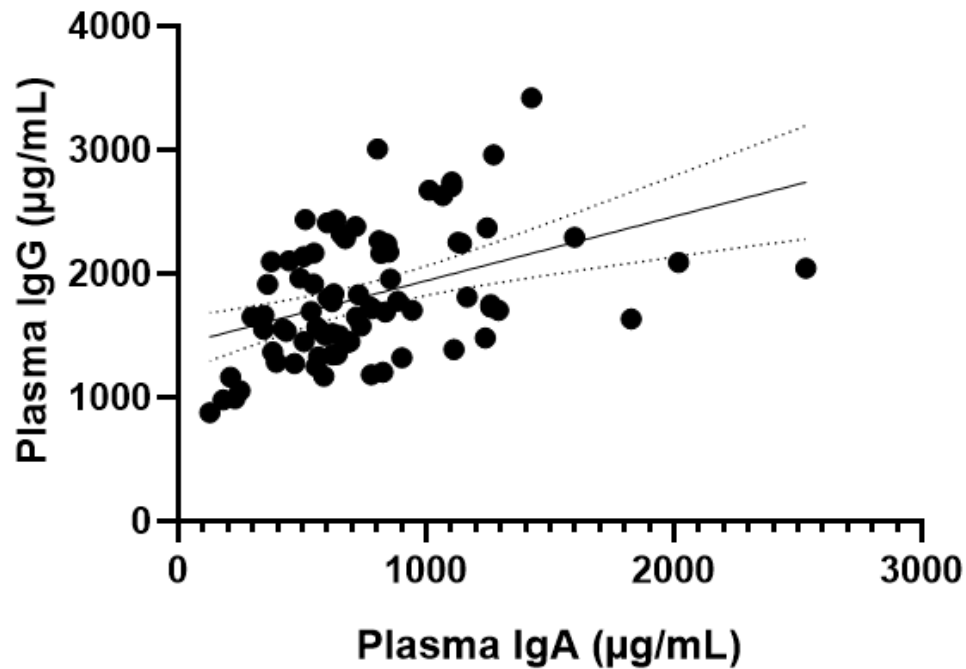


Figure 23. Correlation between plasma IgA and plasma IgG concentrations

Plasma IgA concentrations (µg/mL) plotted against plasma IgG concentrations (µg/mL) measured by ELISA ($n = 79$). Spearman rank correlation demonstrated a statistically significant positive association ($\rho = 0.454$, $p < .0001$). Each point represents an individual sample. Solid line indicates fitted linear trend line; dashed lines represent 95% confidence intervals.

3.7.2 Relationship Between Fecal IgA and IgG

Fecal IgA and fecal IgG concentrations were also positively correlated ($n = 36$; Spearman $\rho = 0.476$, $p = 0.0034$; Figure 24), reflecting coordinated mucosal Ig abundance across paired samples.

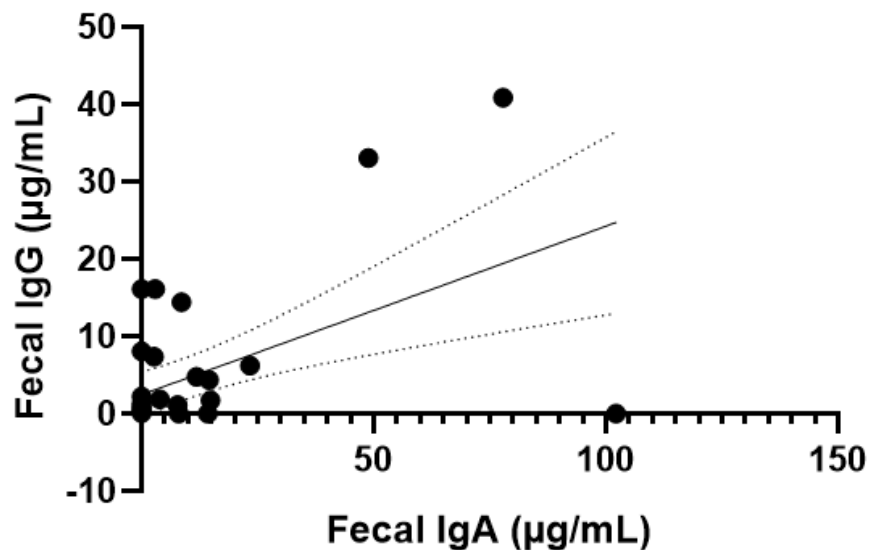


Figure 24. Correlation between fecal IgA and fecal IgG concentrations

Fecal IgA concentrations ($\mu\text{g/mL}$) plotted against fecal IgG concentrations ($\mu\text{g/mL}$) measured by ELISA ($n = 36$). Spearman rank correlation demonstrated a statistically significant positive association ($\rho = 0.476$, $p = 0.0034$). Each point represents an individual paired sample. Solid line indicates fitted linear trend line; dashed lines represent 95% confidence intervals.

3.7.3 Relationship Between IgA- and IgG-Coated Bacteria

To examine the relationship between antibody isotypes bound to the gut microbiota, Spearman correlation analysis was performed comparing IgA-coated and IgG-coated bacterial fluorescence.

A modest but statistically significant positive correlation was observed between IgA- and IgG-associated bacterial signals ($n = 36$; Spearman $\rho = 0.3354$, $p = 0.0455$; Figure 25), indicating partial concordance between IgA and IgG coating patterns across paired samples.

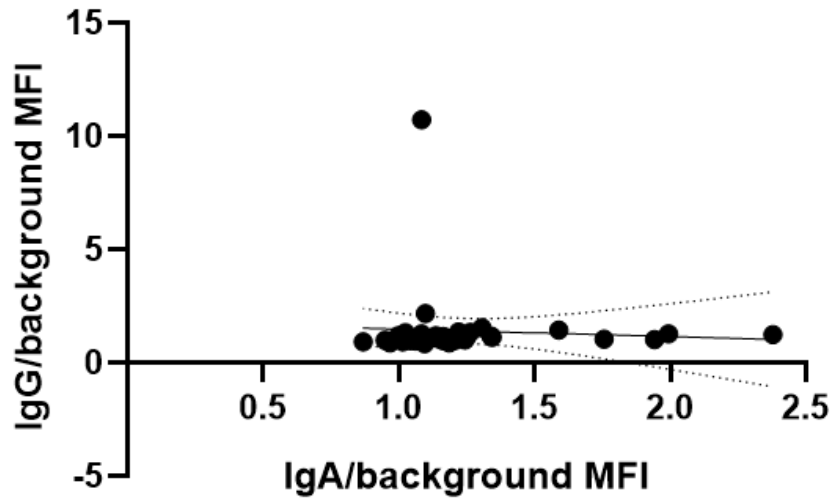


Figure 25. Association Between IgA- and IgG-Coated Bacterial Signal

IgA-associated bacterial mean fluorescence intensity (MFI) plotted against IgG-associated bacterial MFI measured by flow cytometry ($n = 36$). Spearman rank correlation demonstrated a modest but statistically significant positive association ($\rho = 0.3354$, $p = 0.0455$). Each point represents an individual paired sample. Solid line indicates fitted linear trend line; dashed lines represent 95% confidence intervals.

3.7.4 Relationship Between Fecal Ig measures and IgG-Coated Bacteria

To further examine associations between soluble fecal Ig concentrations and antibody binding to the gut microbiota, Spearman correlation analyses were performed comparing fecal IgA and IgG concentrations with IgG-coated bacterial fluorescence.

Fecal IgA concentrations demonstrated a modest but statistically significant positive correlation with IgG-coated bacterial fluorescence ($n = 36$; Spearman $\rho = 0.336$, $p = 0.0454$; Figure 26a). Similarly, fecal IgG concentrations were positively correlated with IgG-coated bacterial fluorescence ($n = 36$; Spearman $\rho = 0.428$, $p = 0.0092$; Figure 26b). These findings indicate partial concordance between soluble fecal Ig levels and IgG-associated bacterial signal across paired samples.

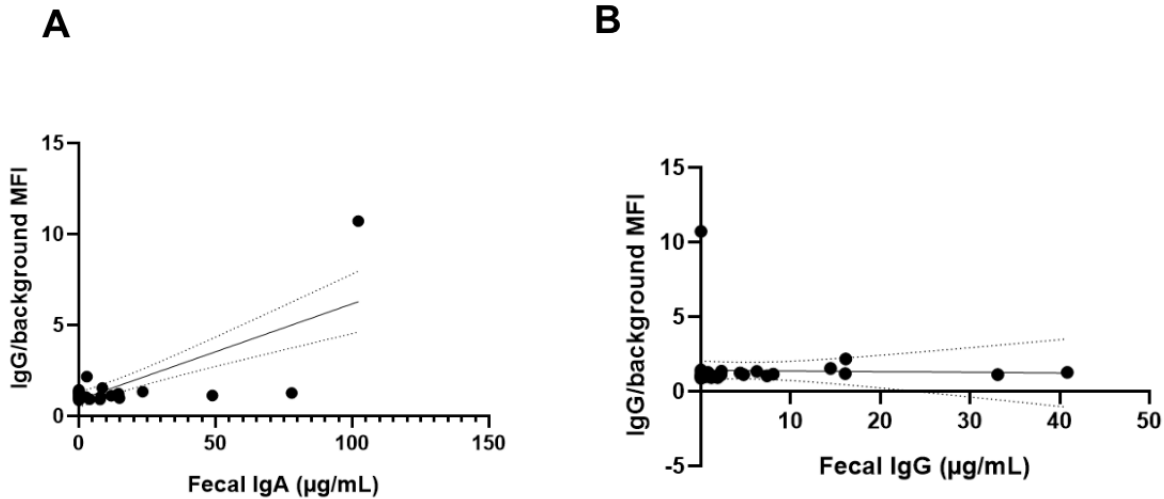


Figure 26. Correlations between soluble fecal Ig concentrations and IgG-coated bacterial signal

(A) Fecal IgA concentrations (µg/mL) measured by ELISA plotted against background-normalized IgG-associated mean fluorescence intensity (MFI) measured by flow cytometry (n = 36). Spearman rank correlation demonstrated a modest but statistically significant positive association ($\rho = 0.336$, $p = 0.0454$).

(B) Fecal IgG concentrations (µg/mL) measured by ELISA plotted against background-normalized IgG-associated MFI (n = 36). Spearman rank correlation demonstrated a statistically significant positive association ($\rho = 0.428$, $p = 0.0092$).

Each point represents an individual paired sample. Solid lines indicate fitted linear trend lines, with dashed lines representing 95% confidence intervals. Correlation coefficients and statistical significance were determined using Spearman rank correlation and are reported in the main text.

Beyond continuous correlation analyses, additional exploratory analyses were conducted to assess whether systemic inflammatory status, as defined by CRP category, was associated with mucosal antibody measures within the IBD cohort.

3.8 CRP-Stratified Analyses of Mucosal Antibody Measures in IBD

To explore the relationship between systemic inflammatory status and mucosal Ig measures in patients with IBD, Ig measures derived from fecal ELISA and flow cytometry were compared between individuals with elevated (>5 mg/L) and non-elevated CRP (≤ 5 mg/L) at the time of sampling. CRP was analyzed as a categorical variable due to heterogeneity in clinical assay methods and the absence of standardized kit information.

Comparisons were performed using the Mann–Whitney test due to small sample sizes and non-normal data distributions.

Among IBD participants, no significant difference in fecal IgA concentration was observed between individuals with elevated CRP ($n = 3$) and those with non-elevated CRP ($n = 8$; Mann–Whitney $U = 9$, $p = 0.6061$; Figure 27a).

Similarly, in the same cohort subgroups, no significant difference in fecal IgG concentration was observed between IBD participants with elevated CRP and those with non-elevated CRP (Mann–Whitney $U = 11$, $p = 0.9394$; Figure 27b).

Interpretation of these findings is limited by the small number of individuals with elevated CRP.

differences. However, a single statistically significant difference was observed in the proportion of IgA-coated bacteria measured by flow cytometry, with higher values in individuals with non-elevated CRP compared with those with elevated CRP. No corresponding difference was observed for IgG-coated bacteria.

Given the modest sample sizes, particularly within CRP-defined subgroups and for fecal ELISA analyses, these findings should be considered exploratory. The isolated significant result should be interpreted cautiously and viewed as hypothesis-generating rather than confirmatory evidence of a systematic relationship between systemic inflammation and mucosal antibody responses.

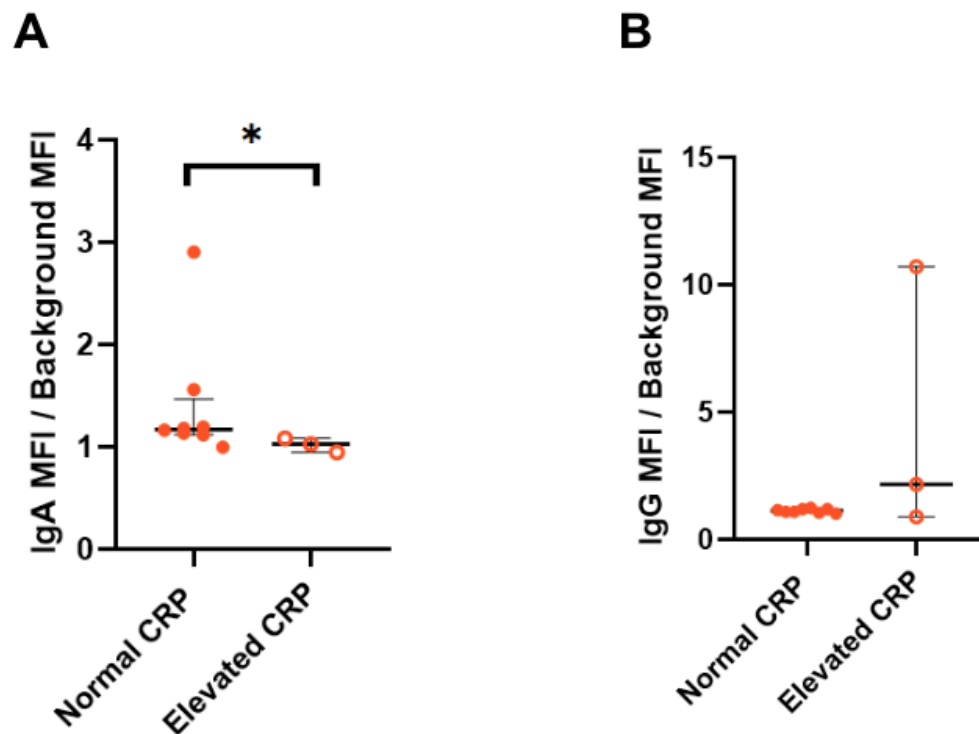


Figure 28. Flow cytometric measures of antibody-coated bacteria stratified by CRP status in patients with IBD

(A) Proportion of IgA-coated bacteria and (B) proportion of IgG-coated bacteria measured by flow cytometry in patients with IBD stratified by CRP status at the time of sampling. Individual data points are shown with median and interquartile range. Comparisons between CRP-defined subgroups were performed using the Mann–Whitney U test. A statistically significant difference was observed for IgA-coated bacteria ($p = 0.0485$), whereas no significant difference was observed for IgG-coated bacteria ($p = 0.4970$).

3.9 Characterization of high-responding individuals

To contextualize the variability observed in group-level analyses, selected high-responding individuals were compared with diagnostic group median values across systemic, fecal, and flow cytometric measures (Table 12). Individual antibody measurements were compared against diagnostic group medians to explore whether elevated systemic or fecal Ig levels aligned with clinical context.

Among IBD participants, two distinct high-response profiles were observed that differed in their systemic inflammatory status. IBD-HR1, a symptomatic individual receiving treatment with mesalazine at the time of sampling, exhibited elevated CRP, indicating the presence of systemic inflammation. This profile was accompanied by very high plasma IgA concentrations and substantially elevated fecal IgA levels. In addition, this individual demonstrated a pronounced increase in IgG-coated bacteria by flow cytometry, exceeding the IBD group median and values observed across other diagnostic groups. Taken together, this profile reflects the co-occurrence of systemic inflammation, heightened antibody abundance, and increased antibody binding to the fecal microbiota.

In contrast, IBD-HR2, who was also receiving mesalazine treatment but exhibited non-elevated CRP, showed elevated plasma IgA and fecal IgA concentrations as well as elevated IgA-coated bacterial fractions in the absence of systemic inflammatory elevation. However, IgG measurements and bacteria-coated fractions remained near group median values. This profile indicates that enhanced IgA responses and increased IgA coating of intestinal bacteria can occur independently of elevated CRP and without a corresponding increase in IgG systemic or mucosal responses, or IgG-associated bacterial binding.

Fecal Ig data for IBS were limited ($n = 2$) and are therefore presented descriptively. Within this subset, IBS-HR1 exhibited high fecal IgA and IgG concentrations relative to both the IBS ranges and other diagnostic groups, while plasma IgA and IgG levels remained within the range observed across groups. Clinical data for this individual were incomplete: CRP data were not available for this individual, and treatment status and symptom presence could not be determined. Flow cytometric IgA and IgG ratios were comparable to group medians, suggesting that elevated fecal Ig concentrations did not necessarily translate into increased bacterial coating.

Overall, these case-level comparisons highlight heterogeneity in Ig profiles within and across diagnostic categories and illustrate that elevated fecal or systemic antibody levels do not uniformly correspond to increased bacterial coating by IgA or IgG, nor do they consistently predict systemic inflammatory states as measured by CRP data.

Table 12. Characterization of high-responding individuals across diagnostic groups

Selected high-responding individuals were examined in relation to diagnostic group reference values for plasma Ig concentrations (IgA and IgG), soluble fecal Ig concentrations (IgA and IgG), and flow cytometric measures of IgA- and IgG-coated bacteria. Individual Ig measurements were compared against diagnostic group reference distributions to contextualize the magnitude of observed responses.

Group reference values are presented as median (IQR) unless otherwise indicated, while individual high-responders are shown as single values. For IBS, group reference values from fecal samples are descriptive only due to limited sample size (n = 2) and are reported as observed ranges.

CRP is reported as a categorical indicator of systemic inflammatory status (elevated vs non-elevated) and is included to provide clinical context rather than as a quantitative comparator. Treatment status and symptom presence is indicated for case studies where available.

Variable	IBD (Group Reference)	IBD-HR1	IBD-HR2	IBS (Group Reference)	IBS-HR1	Celiac Disease (Group Reference)	Healthy Control (Group Reference)
CRP Status Group: n (%) elevated; Individual: Elevated / Non-elevated	6 (37.5%)	Elevated	Non-elevated	3 (23.1%)	Not available	3 (33.3%)	9 (45.0%)
Plasma IgA (µg/mL)	593.9 (376.5–1293.0)	2531.7	1599.1	650.9 (448.0–719.6)	448	789.6 (455.5–1211.0)	715.7 (557.1–923.1)
Plasma IgG (µg/mL)	1718.9 (1535.0–2044.8)	2044.8	2295.6	1922.5 (1443.8–2104.1)	2104.1	1615.9 (1491.4–2169.3)	1837.4 (1347.6–2423.1)
Fecal IgA (µg/mL)	1.3 (0.0–12.7)	102.2	14.6	14.8–77.9	77.9	2.0 (0.0–6.8)	0.0 (0.0–2.2)
Fecal IgG (µg/mL)	1.4 (0.0–7.9)	0	4.4	1.7–40.9	40.9	0.6 (0.0–1.7)	0.0 (0.0–2.9)
IgA Background-normalized MFI	1.2 (1.0–1.2)	1.1	2.9	0.9–1.1	1.1	1.1 (1.0–1.6)	1.0 (1.0–1.1)
IgG Background-normalized MFI	1.1 (1.0–1.2)	10.7	1.2	0.9–1.2	1.2	1.0 (1.0–1.3)	1.1 (1.0–1.2)
Treatment Status (Yes/No)	N/A	Yes	Yes	N/A	Not available	N/A	N/A
Symptom Presence (Yes/No)	N/A	Yes	Yes	N/A	Not available	N/A	N/A

4. Discussion

4.1 Overview of Study Aims and Principal Findings

The present study examined Ig profiles measured in plasma, stool, and antibody-coated bacteria across patients with IBD, IBS, celiac disease, and healthy controls. The aim was to assess whether soluble or bacteria-coated IgA and IgG levels in plasma or in feces differ across diagnostic groups or reflect disease-related features.

This work was guided by the hypothesis that patients with IBD would exhibit distinct systemic and intestinal IgA and IgG response patterns relative to non-IBD comparison groups, and that these measures would associate with markers of inflammation.

Contrary to this expectation, the analyses did not reveal robust or consistent group-level differences in total IgA or IgG concentrations or in global bacterial-coating patterns between IBD and non-IBD groups. Similarly, most Ig measures did not show consistent associations with self-reported symptom presence or CRP stratification. One exception was a higher proportion of IgA-coated bacteria observed among IBD participants with elevated CRP, as assessed by flow cytometry; however, this finding was based on a small subgroup and was not accompanied by corresponding differences in IgG-coated bacteria or soluble fecal Ig concentrations. Overall, Ig levels and antibody–bacteria interactions were largely comparable across diagnostic groups and clinical strata.

These findings do not indicate an absence of immune dysregulation in IBD. Rather, they suggest that large shifts in total IgA or IgG abundance, or in broad patterns of bacterial coating, are not universal features of established, clinically managed disease. Instead, the results help define the contexts in which Ig-based measurements are informative, as well as the limits of what bulk quantitative and global coating metrics can capture in heterogeneous patient populations.

4.2 Interpretation of Group-Level IgA and IgG Profiles Across Diagnostic Categories

Therefore, many prior studies reporting disease-associated IgA or IgG differences have focused on antigen-specific responses or taxon-resolved antibody targeting—such as IgA-SEQ or subclass-resolved Ig coating—rather than on total antibody abundance measured across clinically

heterogeneous cohorts (Palm et al., 2014; Masu et al., 2021). The observed overlap across groups highlights substantial inter-individual variability and suggests that total Ig measures may reflect conserved aspects of baseline gut–immune interaction rather than clearly defined disease-specific inflammatory signatures.

As outlined in the Introduction, IBS and celiac disease were included as functional comparisons along the spectrum of gut immune dysregulation. IBS served as a non-inflammatory point of comparison to evaluate whether Ig-based measures distinguish IBD from gut conditions characterized by symptoms in the absence of overt intestinal inflammation. Inclusion of celiac disease enabled assessment of whether IgA and IgG measures reflect generalized immune activation across immune-mediated intestinal conditions or distinguish disease-specific immune contexts. In the present study, no statistically significant differences were observed between celiac disease, IBD, IBS, or healthy controls across plasma or fecal Ig measures. Consequently, these markers did not demonstrate disease-specific separation and are best interpreted as reflecting heterogeneous, individual-level immune responses rather than diagnostic group–defined patterns. Fecal Ig measures for celiac disease and IBS were interpreted descriptively due to limited sample size, and conclusions for these groups therefore rely primarily on plasma-based analyses.

4.3 Epidemiological Context and UC-Predominant IBD Representation

The predominance of UC over CD within the IBD cohort is consistent with epidemiological data from Uruguay and the broader South American region, where UC is substantially more prevalent than CD, with reported UC:CD ratios approaching 6:1 (Selvaratnam et al., 2019). This regional pattern provided important context for the present study and partially explains the limited representation of CD within the cohort. As a result, UC and CD were analyzed together for IBD-related analyses, and findings should be interpreted as reflecting IBD in a setting where UC predominates rather than as disease-specific comparisons. This epidemiological context is particularly relevant when interpreting Ig response heterogeneity in the IBD group, as the observed patterns may be more representative of IBD in a UC-predominant population rather than CD disease–specific immune features.

This epidemiological context is particularly relevant when interpreting Ig heterogeneity, as Ig subclass distributions and antibody–microbiota interactions have been reported to differ between UC- and CD-dominant cohorts, potentially shaping aggregate immune profiles in population-specific studies (Song et al., 2021; Masu et al., 2021).

Additionally, many prior studies have focused on antigen-specific responses or taxon-resolved patterns of antibody targeting, rather than on bulk Ig abundance measured across heterogeneous clinical populations, and were conducted in larger cohorts. In this context, the largely overlapping Ig profiles observed here likely reflect the clinically managed, heterogeneous outpatient population examined in the present study.

4.4 Systemic Hematological and Inflammatory Context

According to World Health Organization criteria, BMI values ≥ 25 kg/m² are classified as overweight and ≥ 30 kg/m² as obese (World Health Organization [WHO], 2025). By these standards, although BMI did not differ significantly between diagnostic groups, median values across all cohorts were in the overweight to obese range (Table 6). Given the known associations between adiposity, systemic inflammation, and immune modulation (Burak et al., 2024), BMI represents a potential contextual modifier when interpreting immunological readouts, even in the absence of between-group differences.

Baseline hematological parameters and systemic inflammatory markers were largely comparable across diagnostic groups, consistent with the outpatient and predominantly treated population represented in this cohort. Although statistically significant group-level differences were detected for mean corpuscular hemoglobin concentration (MCHC) and monocyte percentage (Mon%), these differences were modest in magnitude and limited in biological context.

Post hoc pairwise analyses revealed higher MCHC values in patients with IBD compared with healthy controls, though values remained close to established adult reference ranges (310–360 g/L), and were not accompanied by corresponding differences in hemoglobin concentration, hematocrit, or other red blood cell indices. In the absence of concordant alterations across related erythrocyte parameters, this isolated MCHC difference is likely of limited clinical significance within the context of this study.

Similarly, Mon% values were modestly higher in patients with IBD compared with patients with celiac disease. While many values fell within the expected reference range (4–12%), a subset of IBD participants exhibited Mon% values above the upper limit of this range. These elevations were not accompanied by coordinated shifts in other leukocyte populations or by a broader hematological profile indicative of overt systemic inflammation. Rather than reflecting frank hematological dysregulation, this pattern is consistent with heterogeneous, low-grade systemic immune activation or altered immune cell distribution in a subset of patients with IBD, potentially influenced by disease heterogeneity and treatment status.

Taken together, these observations indicate that systemic hematological and inflammatory profiles were broadly comparable across diagnostic groups, with only minor deviations detected that are unlikely to meaningfully confound or drive the Ig-focused analyses presented here.

In contrast, previous studies—particularly those enriched for active or untreated disease—have reported more pronounced hematological alterations in IBD, including lower hemoglobin levels, mild reductions in MCHC, and reactive thrombocytosis relative to healthy controls (Dong et al., 2020; Carrillo-Palau et al., 2020). For example, Dong et al. (2020) described significantly lower hemoglobin and MCHC values and higher platelet counts in IBD patients, although absolute values often remained within or near reference ranges and total white blood cell counts were largely comparable between groups. Such abnormalities are well documented in moderate-to-severe or active IBD but are less consistently observed in patients with quiescent disease or under treatment.

In the existing literature, alterations in circulating monocytes in IBD have most often been described in cohorts enriched for active or severe disease, where absolute monocytosis has been associated with elevated inflammatory markers, anemia, and adverse clinical outcomes. For example, Anderson et al. reported that a substantial subset of patients with IBD developed absolute monocytosis over longitudinal follow-up, which correlated with markers of systemic inflammation and disease burden. Importantly, such findings were derived from tertiary-care populations and were based on absolute monocyte counts rather than relative monocyte percentages (Anderson et al., 2021). In contrast, the present study evaluated Mon% at a single outpatient time point in a predominantly treated cohort and observed a more modest elevation limited to comparisons between IBD and celiac disease. This pattern suggests that subtle shifts in

leukocyte distribution may persist in IBD even in the absence of overt systemic inflammation or pronounced monocytosis, and that these changes may be more readily detected when compared with other immune-mediated gastrointestinal conditions characterized by different inflammatory mechanisms and treatment states.

A noteworthy factor that may explain the absence of pronounced hematological differences across diagnostic groups in the present study is the limited clinical stratification available at the time of sampling. Although symptom presence was recorded, objective measures of disease activity or severity were not used to define analytical subgroups. Numerous studies reporting anemia, thrombocytosis, or altered red cell indices in IBD have focused on patients with active, moderate-to-severe, or untreated disease, in whom systemic inflammation and iron deficiency are more prominent (Dong et al., 2025; Udvardy et al., 2001). In contrast, the present cohort consisted primarily of outpatients, many of whom were receiving treatment at the time of sampling, and hematological analyses were conducted without stratification by symptom status or treatment intensity. As a result, potential alterations in parameters such as hemoglobin, platelet count, or leukocyte levels that vary with clinical status or treatment context may have been diluted when analyzing diagnostic groups as a whole.

Similarly, the absence of overt hematological abnormalities in the celiac disease cohort contrasts with reports of frequent anemia in newly diagnosed or untreated disease (Seidita et al., 2022; Balaban et al., 2019), likely reflecting dietary treatment and disease control in the outpatient setting. The lack of significant hematological alterations in IBS is also consistent with prior reports describing generally normal blood counts in this functional disorder (Güven et al., 2022).

Overall, the minimal group-level differences observed here likely reflect disease activity, treatment status, and outpatient recruitment rather than absence of underlying inflammatory processes. In this context, the isolated and modest MCHC difference in IBD is consistent with previously reported trends (Dong et al., 2020), while preserved hemoglobin, platelet, and white blood cell parameters underscore the relatively stable systemic hematological profile of this cohort.

CRP, evaluated as a categorical indicator of systemic inflammatory status, showed substantial intra-group variability, and the proportion of individuals with elevated CRP did not differ

significantly between groups. The presence of elevated CRP among some healthy controls as well as patients with IBD, IBS, and celiac disease likely reflects outpatient recruitment, treatment effects, and transient or non-disease-specific inflammatory influences, highlighting the limited specificity of single time-point CRP measurements in clinically heterogeneous populations.

Within the IBD cohort, stratification by CRP status did not correspond to consistent differences in soluble fecal IgA or IgG concentrations, nor with IgG-associated bacterial fluorescence measured by flow cytometry (Figures 27 and 28). However, a statistically significant increase in the proportion of IgA-coated bacteria was observed among participants with non-elevated CRP. This finding suggests that higher levels of bacterial IgA coating do not necessarily correspond to systemic inflammatory activity and may instead reflect mucosal immune engagement independent of CRP-defined inflammation in a subset of individuals.

At the same time, this result must be interpreted cautiously. The elevated CRP subgroup was small, and the absence of parallel differences in soluble fecal Ig levels or IgG-coated bacterial fractions indicates that this pattern does not reflect a broad amplification of humoral responses across compartments. Rather, it may represent a localized or isotype-specific shift in mucosal immune targeting during systemic inflammatory states. Given the limited subgroup size and cross-sectional design, this observation should be considered hypothesis-generating and warrants confirmation in larger cohorts with longitudinal inflammatory assessment.

Taken together, these results indicate that systemic inflammatory status, as approximated by CRP at a single time point, does not broadly track with soluble mucosal Ig abundance or global antibody-microbiota interactions in this cohort. Although elevated CRP was associated with increased IgA-coated bacterial fractions, this relationship was isotype-specific and not accompanied by parallel changes in soluble Ig levels or IgG-coated bacteria. These findings suggest that CRP captures certain dimensions of inflammatory activity but does not comprehensively reflect mucosal humoral organization in this predominantly treated outpatient IBD cohort.

4.5 Preservation of Systemic Immunoglobulin Levels Across Diagnostic Groups

Plasma IgA and IgG concentrations did not differ significantly between diagnostic groups, whether analyzed as absolute values or after normalization to healthy control medians (Figures 10 and 11). This indicates that overall circulating Ig levels are broadly similar across patients with IBD, IBS, celiac disease, and healthy controls in this cohort. Although humoral immunity plays a key role in these conditions, the absence of differences in total Ig concentrations is biologically plausible. This interpretation should also be considered in light of the moderate sample sizes within each diagnostic group (15 IBD, 15 IBS, 12 celiac disease, and 38 healthy controls) and the substantial inter-individual variability observed, which together limit statistical power to detect modest group-level differences in circulating Ig levels.

Importantly, stable systemic Ig levels do not rule out disease-relevant immune alterations. In immune-mediated gastrointestinal diseases, dysregulation often involves changes in antibody specificity, subclass distribution, or functional targeting rather than changes in total antibody quantity. Many prior studies have therefore focused on antigen-specific responses or cohorts with more pronounced inflammatory activity. In contrast, the present study measured total IgA and IgG in a clinically heterogeneous and largely treated population, in which preserved systemic Ig levels may reflect regulatory balance or treatment effects rather than a lack of immune involvement.

Consistent with Lin et al. (2018), we observed substantial inter-individual variability and overlap in systemic IgA and IgG concentrations across diagnostic groups. In contrast to that study, the present analysis did not directly assess Ig associations with disease activity, as objective disease activity indices were not evaluated. This discrepancy likely reflects differences in cohort composition and analytical focus, as Lin et al. emphasized correlations within subsets of patients exhibiting higher inflammatory activity, whereas the present analysis prioritized group-level distributions within a clinically heterogeneous population (Lin et al., 2018).

Although elevated serum Ig levels have been reported in subsets of patients with CD, particularly those with complicated phenotypes (Song et al., 2021), we did not observe comparable enrichment at the cohort level. This divergence likely reflects both the limited representation of

CD within the present study and our emphasis on population-level distributions rather than extreme phenotypes.

4.6 Soluble Fecal Immunoglobulins and Mucosal Immune Variability

Soluble fecal IgA and IgG concentrations exhibited substantial variability across individuals and were detectable in only a subset of stool samples. Fecal Ig analyses were performed on 36 stool samples (12 IBD, 2 IBS, 4 celiac disease, and 18 healthy controls), reflecting limited stool availability and resulting in reduced statistical power for subgroup comparisons, as not all participants were able to provide a fecal sample during the collection period.

Detection frequencies and concentration ranges were consistent with the known biological and technical variability of fecal antibody measurements, which are influenced by factors such as stool consistency, transit time, proteolytic degradation, and localized secretion dynamics (Frehn et al., 2014).

No statistically significant differences in fecal IgA or IgG concentrations were observed between diagnostic groups, either when analyzing all samples or when restricting analyses to detectable values and normalized fold-change measures. Although descriptive differences in median concentrations and detection frequencies were observed—most notably within the IBS cohort—these apparent elevations were disproportionately influenced by a single high-responding individual among the limited number of IBS stool samples analyzed. This reflects substantial inter-individual variability in fecal Ig responses rather than a consistent group-level pattern. The small number of available stool specimens for the IBS and celiac disease cohorts therefore precludes meaningful inferential interpretation for these groups (Figure 12, Figure 14). Accordingly, fecal Ig values for IBS and celiac disease were retained in figures for transparency and descriptive context but were not interpreted as evidence of disease-specific differences.

Subgroup analyses stratified by the presence or absence of self-reported symptoms within the IBD cohort likewise did not reveal statistically significant differences in fecal IgA or IgG concentrations when all available samples were included and non-detectable values were retained as zero (Figures 13 and 15). Although symptomatic individuals tended to exhibit higher

detection frequencies for fecal antibodies, comparisons of overall concentration distributions between symptomatic and asymptomatic participants were not statistically significant. These findings suggest that self-reported symptom status at a single time point did not correspond to measurable differences in soluble fecal Ig abundance in this cohort.

These findings suggest that bulk soluble fecal Ig concentration alone may lack the sensitivity required to detect differences associated with self-reported symptom status in heterogeneous outpatient populations. Given the multifactorial nature of symptom expression in IBD, concentration-based measures may not fully capture localized immune activity or functional differences in antibody targeting.

While prior studies have reported associations between fecal IgA or IgG levels and objective measures of disease activity in IBD (Lin et al., 2018), the present study evaluated symptom-defined subgroups rather than endoscopic or histologic inflammation. In this context, differences in fecal Ig abundance were not consistently detectable. These findings suggest that soluble fecal Ig concentration alone may be insufficient to reflect symptom-level variation in mucosal immune activity within treated outpatient cohorts.

4.7 Antibody Coating of the Gut Microbiota Assessed by Flow Cytometry

Flow cytometric analysis of IgA- and IgG-coated fecal bacteria revealed modest variation across diagnostic groups but no statistically significant differences in background-normalized fluorescence ratios. Median values for both isotypes clustered near background levels across all cohorts, indicating broadly comparable degrees of antibody binding to gut bacteria (Figures 17 and 19).

An important methodological consideration when interpreting these results is the absence of an external reference standard or enrichment strategy to amplify antibody-coated bacterial populations, such as the internal gating and sorting approaches used to enrich IgA^{high} fractions in IgA-SEQ studies or the subclass-resolved analytical frameworks employed in other flow cytometry-based analyses (Palm et al., 2014; Masu et al., 2021). The methodological differences in the present study likely contributed to lower overall fluorescence signals and limits direct comparison of coating magnitude across platforms.

These findings are inconsistent with models in which disease states are defined by large-scale increases or decreases in total antibody coating of the gut microbiota. Instead, they support a more nuanced view in which disease-associated immune alterations may involve selective targeting of specific microbial populations or antigens rather than global shifts in coating intensity. Within the constraints of bulk, non-enriched measurements, subgroup analyses stratified by symptom presence similarly showed no significant differences in IgA- or IgG-coated bacterial fractions, suggesting that population-level antibody–microbiota interactions remain broadly comparable across symptomatic and asymptomatic individuals in this cohort (Figures 18 and 20).

In this respect, our results align with IgA-SEQ and subclass-resolved coating studies that emphasize selective immune targeting of specific microbial taxa rather than uniform increases in antibody binding across the microbiota (Palm et al., 2014; Masu et al., 2021). However, unlike IgA-SEQ and subclass-resolved coating studies that identify selective antibody targeting of specific microbial populations in IBD, we did not observe disease-associated increases in overall antibody coating intensity. This indicates that functionally relevant immune targeting can occur without detectable changes in bulk coating magnitude measured at the population level.

4.8 Cross-Assay Synthesis and Interpretation of Correlation Analyses

An exploratory multivariable correlation analysis provided a global overview of relationships among systemic, soluble fecal, and bacteria-associated Ig measures (Table 11, Figure 22). This analysis revealed coordinated patterns within compartments—plasma IgA and IgG were positively correlated, as were fecal IgA and IgG concentrations—while cross-compartment associations between systemic and mucosal measures were largely absent. At the mucosal–microbial interface, fecal IgA and IgG concentrations were modestly associated with IgG-coated bacterial signal, and IgA- and IgG-coated bacterial measures were positively correlated with one another, indicating partial concordance in antibody engagement with the gut microbiota.

The absence of significant correlations between plasma Ig concentrations and mucosal antibody measures is consistent with the concept that systemic and intestinal humoral responses are regulated through partially independent mechanisms. Circulating Ig levels may reflect systemic

immune activation, whereas fecal antibody levels are influenced by local mucosal production, epithelial transport, and luminal dynamics.

The observed association between fecal IgG concentrations and IgG-associated bacterial fluorescence further suggests that soluble IgG levels in stool partially reflect antibody engagement with the gut microbiota. However, the moderate magnitude of this correlation and substantial inter-individual variability indicate that total fecal IgG concentration does not fully capture patterns of microbial targeting.

The positive correlations observed between IgA and IgG within both plasma and fecal compartments suggest shared regulatory influences or parallel activation pathways within systemic and mucosal humoral immunity. These coordinated patterns may reflect generalized immune activation rather than isotype-specific dysregulation.

When considered alongside the descriptive heatmap visualization (Figure 21), these correlation patterns further underscore the heterogeneity of humoral organization across diagnostic groups. Although bulk Ig abundance did not robustly separate disease categories, structured relationships within compartments and partial coupling between soluble fecal Ig and bacteria-associated signal were evident across individuals. Together, these findings suggest that the organization of humoral responses—rather than absolute Ig quantity alone—may provide a more informative lens for understanding mucosal immune dynamics in this cohort.

Notably, many of the observed correlations were moderate in magnitude, and substantial inter-individual variability was present, indicating that these associations represent partial rather than deterministic relationships between humoral compartments.

In this context, the findings refine the original hypothesis by indicating that while altered humoral immune responses are biologically plausible in IBD, such alterations may manifest as shifts in compartmental coordination and mucosal–microbial interactions rather than as large-scale differences in total Ig abundance.

4.9 Interpretive Considerations and Scope of Inference

Across inferential group-level analyses, data were summarized using medians and interquartile ranges rather than means and standard deviations to reflect non-normal distributions, heterogeneous variance, and the presence of outliers, particularly in fecal Ig measurements. This reporting strategy supports conservative interpretation of group-level differences and minimizes the influence of extreme values on central tendency estimates.

For descriptive visualization purposes, however, mean-based $\log_2$ ratios were used in the generation of the heatmap to illustrate relative group-level tendencies compared to controls. Because this representation is intended for qualitative comparison rather than statistical inference, it does not replace the median-based analyses used for hypothesis testing.

Although structured relationships between humoral markers were observed, effect sizes were moderate and substantial inter-individual variability was present. These features, combined with the modest sample size and the inclusion of a treated outpatient population, limit the ability to generalize findings to untreated or more severe disease states.

Rather than contradicting established models of immune dysregulation in gastrointestinal disease, these results suggest that disease-relevant humoral immune alterations may occur at a finer scale than is captured by bulk quantitative measures. Features such as antigen specificity, microbial targeting, antibody subclass distribution, or functional properties of Igs may provide more sensitive insight into mucosal immune dysregulation than absolute concentrations alone.

Accordingly, while the study was designed to assess whether bulk systemic and mucosal IgA and IgG measures distinguish IBD from non-IBD conditions and vary across clinical context, including self-reported symptom presence and CRP-defined inflammatory status, the results indicate that such distinctions are not consistently captured by total Ig abundance in a treated outpatient population. These findings emphasize both the value and the limitations of bulk Ig measurements and underscore the importance of high-resolution and context-aware approaches for future investigations of humoral immunity in IBD.

4.10 Heterogeneity and High-Responding Immunoglobulin Profiles

Case-level analysis of selected high-responding individuals was conducted to illustrate extreme Ig response profiles observed at the upper end of the cohort distribution, rather than typical or representative response patterns, and were selected for descriptive purposes only. This heterogeneity underscores that elevated antibody levels do not reflect a uniform biological phenotype. In IBD, two distinct high-response patterns were selected for closer examination. One individual demonstrated concordant categorical elevation CRP, as well as elevated plasma IgA, fecal IgA, and IgG-coated bacteria by flow cytometry, consistent with a state of heightened inflammatory immune activation. In contrast, a second IBD high-responder exhibited elevated plasma IgA, fecal IgA, and IgA-coated bacteria in the absence of categorically elevated CRP and without marked expansion of IgG responses, suggesting that increased IgA production can occur independently of overt systemic inflammation or increased IgG responses.

These contrasting IBD profiles emphasize that Ig elevations may reflect different underlying processes, including systemic inflammatory activity or localized mucosal immune responses. IBD-HR1 demonstrated concurrent systemic inflammation, elevated IgA levels in feces and plasma, and markedly increased IgG-associated bacterial binding, suggesting coordinated immune activation across systemic and mucosal immune compartments. In contrast, IBD-HR2 exhibited elevated plasma and fecal IgA concentrations along with increased IgA-associated bacterial binding despite non-elevated CRP and without parallel IgG response expansion.

These cases illustrate that Ig responses within IBD are not uniform and may follow distinct immunological patterns. Enhanced mucosal IgA production and increased bacterial coating can occur independently of systemic inflammatory elevation, and isotype-specific differences in bacterial targeting further highlight variability in host–microbiota immune interactions.

Similar inter-individual heterogeneity in humoral immune profiles has been described in prior IBD studies, in which subsets of patients exhibit discordant systemic and mucosal antibody responses reflecting distinct underlying immune states rather than a uniform disease-associated pattern (Palm et al., 2014; Lin et al., 2018).

Although fecal Ig data for IBS were limited, the presence of a high-responding IBS individual with markedly elevated fecal IgA and IgG but plasma antibody levels within the range observed across groups suggests that strong mucosal antibody responses can occur outside of classical

inflammatory disease. The absence of CRP data for this participant precludes assessment of systemic inflammation; however, the lack of elevated antibody-coated bacteria by flow cytometry indicates that high soluble fecal Ig levels do not uniformly correspond to increased antibody-mediated microbial coating. This observation aligns with emerging evidence that IBS encompasses heterogeneous immune phenotypes, including subsets characterized by mucosal immune activation without systemic inflammatory markers.

Across diagnostic categories, celiac disease and healthy control groups showed more constrained distributions of plasma and fecal Igs and flow cytometry ratios, reinforcing that extreme antibody phenotypes were uncommon outside selected individuals. Taken together, these findings support a model in which both systemic and mucosal antibody responses are highly individualized and variably coupled to microbial binding. Importantly, the presence of high-responding individuals cautions against overinterpretation of group-level medians and highlights the value of integrating case-level analyses to better understand immune heterogeneity in gastrointestinal disorders. These observations further emphasize that group-level summaries based on aggregate cross-sectional analyses may incompletely capture biologically relevant extremes, particularly in modestly sized cohorts.

5. Conclusions

This study evaluated systemic and intestinal IgA and IgG responses in patients with IBD within a Uruguayan clinical context, in comparison with individuals with IBS, celiac disease, and healthy controls. The primary aim was to evaluate whether bulk antibody measures and global antibody–microbiota interactions distinguish IBD from non-IBD conditions and reflect clinically relevant inflammatory states.

With the results obtained, the original hypothesis could not be confirmed. At the population level, the results indicate that total circulating and fecal IgA and IgG concentrations, as well as global antibody coating of fecal bacteria, do not consistently distinguish IBD from non-IBD conditions in a predominantly treated, clinically heterogeneous outpatient cohort. Stratification by systemic inflammatory status, assessed using categorical CRP classification, similarly revealed no consistent differences in soluble fecal Ig concentrations or global bacterial-coating patterns across diagnostic groups. The absence of consistent group-level separation does not invalidate the biological plausibility of altered humoral responses in IBD but suggests that bulk concentration-based metrics capture only limited aspects of immune dysregulation in this cohort and clinical context, under real-world outpatient conditions.

Despite the absence of robust group-level separation, multivariable correlation analyses revealed structured patterns of humoral organization across compartments. IgA and IgG levels were coordinated within both systemic and mucosal compartments, and soluble fecal Ig measures demonstrated modest but significant associations with IgG-coated bacterial signal. However, plasma Ig concentrations were not correlated with mucosal or microbiota-associated measures, indicating partial compartmental independence of systemic and intestinal humoral responses. These findings suggest that the internal organization of antibody responses may be more informative than absolute abundance alone.

Importantly, the lack of consistent group-level separation does not imply immunological uniformity. Substantial inter-individual variability was observed across systemic, fecal, and microbiota-associated antibody measures, including the presence of high-responding individuals within both patient and control groups. Within the IBD cohort, CRP stratification identified a single significant increase in IgA-coated bacterial fractions among individuals with non-elevated

CRP; however, this signal was restricted to a small subgroup and was not accompanied by corresponding differences in IgG-coated bacteria or soluble Ig levels. This pattern reinforces the view that systemic inflammatory markers and mucosal antibody responses are only partially aligned and may reflect distinct, compartmentalized aspects of immune regulation.

Taken together, these findings refine the original hypothesis by suggesting that disease-relevant humoral immune alterations in IBD are unlikely to be captured by total IgA or IgG abundance alone. Instead, meaningful immunopathological differences are more likely to emerge at finer levels of resolution, including antibody specificity, subclass distribution, microbial targeting, or functional properties, which are not assessed by bulk quantitative or global coating measures.

Overall, this work underscores both the value and the limitations of commonly used immunological readouts in heterogeneous, treated outpatient populations. By situating systemic and intestinal antibody responses within a Uruguayan clinical setting, the study provides a grounded framework for interpreting Ig-based measurements and highlights the need for integrative, high-resolution approaches to the study of mucosal immunity in IBD, particularly those capable of resolving antigen specificity, microbial targeting patterns, and functional antibody properties beyond bulk quantitative measures.

6. Limitations

6.1 Sample Size and Subgroup Representation

Interpretation of stool Ig levels in IBS is constrained by the small sample size ($n = 2$), and observed variability should therefore be interpreted with caution. Similar constraints apply to the celiac disease cohort ($n = 4$). Patient recruitment and biological sample availability—particularly for stool-based analyses—limited the number of fecal specimens available for Ig quantification and symptom-based subgroup comparisons. Consequently, statistical power to detect small-to-moderate differences in fecal IgA and IgG concentrations and bacteria-associated antibody binding was reduced, restricting inference regarding subtle subgroup effects, especially within the IBS and celiac disease cohorts.

Nevertheless, the consistency of observed patterns across multiple analytical approaches and the concordance between ELISA- and flow cytometry-based measures support the robustness of the primary conclusion that large-scale differences in bulk Ig measures are not a dominant feature of this cohort.

6.2 Use of Total Rather Than Antigen-Specific Immunoglobulin Measurements

This study quantified total IgA and IgG concentrations in plasma and stool and assessed global antibody binding to fecal bacteria. While this approach captures overall antibody abundance and coating intensity, it does not assess antigen specificity or distinguish which microbial targets are being recognized. Consequently, disease-relevant alterations in antibody targeting may be present without manifesting as differences in total Ig levels or overall bacterial coating, and therefore would not be detected by the methods employed here.

Several prior studies in IBD have instead focused on antigen-specific antibody responses, demonstrating that immune dysregulation in IBD is often directed toward defined microbial antigens rather than reflected in bulk Ig levels. For example, serum IgA and IgG responses against microbial antigens such as *Saccharomyces cerevisiae* mannan (ASCA), *Escherichia coli* outer membrane protein C (OmpC), and bacterial flagellins (e.g., CBir1) have been repeatedly associated with CD and with specific disease phenotypes, severity, or complications, despite

substantial overlap in total Ig concentrations across patients (Targan et al., 2005; Rieder et al., 2010).

Antigen-specific antibody measurements have also been applied at the mucosal level. Studies assessing fecal IgA and IgG directed against defined bacterial species or microbial lysates have shown that elevated antibody responses can occur locally in the gut even in the absence of marked systemic inflammation, highlighting compartment-specific immune activation that is not captured by total Ig measurements alone (Frehn et al., 2014). Similarly, approaches such as IgA-SEQ have demonstrated that bacteria heavily targeted by IgA in IBD are selectively enriched for colitogenic potential, despite the absence of large differences in overall IgA abundance (Palm et al., 2014).

Taken together, these findings illustrate that antigen-specific antibody profiling provides biological resolution that is not accessible through measurements of total Ig abundance. The present study therefore captures global humoral activity but cannot resolve whether observed immune responses are directed toward disease-relevant microbial antigens. Future studies integrating total Ig quantification with antigen-specific assays—using defined microbial proteins, polysaccharides, or patient-derived bacterial targets—may better delineate the specificity and functional relevance of humoral immune responses in IBD.

6.3 Treatment Status and Clinical Heterogeneity

Most patients were receiving treatment at the time of sample collection, which likely attenuated systemic and mucosal inflammatory signals. Although this clinical heterogeneity limits conclusions regarding Ig patterns in untreated or newly diagnosed disease, it enhances the ecological validity of the findings by reflecting immune profiles in clinically managed populations under real-world conditions. Accordingly, the results are most appropriately interpreted as representative of immune states under treatment rather than untreated disease.

6.4 Control Group Variability and Definition of “Healthy” Controls

Inter-individual variability was observed within the healthy control group across systemic inflammatory markers, hematological parameters, and fecal Ig measures, reflecting the inclusion of community-based controls rather than highly selected “super-healthy” individuals. In

particular, a substantial proportion of healthy controls exhibited evidence of low-grade systemic inflammation at the time of sampling: 9 of 20 control participants (45%) had CRP values above the institutional cutoff of 5 mg/L, despite lacking diagnosed gastrointestinal inflammatory disease.

As described in the Materials and Methods, three individuals initially recruited as healthy controls were excluded prior to analysis due to markedly elevated CRP levels (>10 mg/L), reflecting a predefined criterion to remove control participants with overt systemic inflammation. Although all control participants met predefined inclusion criteria and lacked diagnosed gastrointestinal inflammatory disease, some individuals exhibited values outside established reference ranges for certain parameters. Such deviations do not necessarily indicate underlying pathology, as reference ranges are population-based and single time-point measurements may be influenced by transient physiological, environmental, or lifestyle-related factors. Notably, several associations observed in this study—particularly between soluble fecal IgG concentrations and IgG-coated bacterial fractions—were most evident within the healthy control group, suggesting that inter-individual variability among controls reflects meaningful biological heterogeneity rather than random noise.

Exclusion of control participants based on post hoc identification of out-of-range immunological values was not specified a priori and would be incompatible with the study design, as such exclusion could artificially inflate apparent disease–control differences. Future studies could improve control group characterization not by removing individuals with atypical immune profiles, but by incorporating more detailed phenotyping, repeated longitudinal sampling, or stratification based on subclinical inflammatory markers to better contextualize baseline immune variability.

7. Future Directions

7.1 Increased Sample Size and Improved Subgroup Representation

Future studies would benefit from increased sample sizes, particularly for stool-based analyses, through more consistent and structured fecal sample collection across participants. Expanding the number of available stool specimens would improve statistical power for detecting modest

differences in fecal Ig concentrations and bacteria-associated antibody binding and would enable more robust subgroup analyses by disease classification and symptom presence. Improved representation of IBS and celiac disease cohorts would be especially important for assessing whether subtle mucosal immune differences exist that were not detectable in the present study. In addition, larger cohorts would facilitate the application of more refined stratification approaches that move beyond bulk antibody measurements, including analyses focused on antibody specificity or microbial targeting patterns. In such settings, further subgrouping by treatment exposure, disease phenotype or location, or known inflammatory markers (e.g., CRP or fecal calprotectin) may help clarify whether selective immune targeting underlies disease-associated variation that is not captured by absolute Ig concentration-based measures alone.

7.1.1 Optimization of Fecal Sample Collection Workflow

A major constraint on stool-based analyses in the present study was inconsistent fecal sample collection, reflecting practical and patient-related barriers rather than lack of eligibility. Experience from the present study indicates that reliance on stool provision at the time of clinic visits is often impractical, as many participants were unable to arrive with samples or provide samples on demand.

Future studies could therefore improve fecal sample yield by implementing a more structured, patient-centered, home-based collection workflow. This may include advance patient education on the importance of stool-based immunological analyses, provision of standardized collection kits with clear written and visual instructions, flexible pre/post-visit collection windows aligned with patients' normal bowel habits, and coordinated drop-off, pickup, or mail-in return options.

Follow-up contact after enrollment to troubleshoot collection difficulties could further improve compliance and sample yield.

Together, these measures would increase the availability of fecal material, enhance statistical power for mucosal immune analyses, and reduce bias introduced by differential sample availability.

7.2 Integration of Functional Antibody Measures

While this study focused on quantitative measures of Ig abundance and bacterial coating, future work incorporating functional antibody assays may provide deeper insight into the biological relevance of antibody–microbiota interactions. Functional readouts such as complement activation, opsonophagocytic activity, or antibody affinity profiling could help distinguish between protective, neutral, and potentially pathogenic antibody responses within the gut. In addition, emerging evidence suggests that structural features of antibodies—such as Fc glycosylation patterns—can substantially influence effector function and immune activation (Castañeda-Casimiro et al., 2025). Assessing whether such features differ across patient groups may clarify functional consequences of antibody responses that are not apparent from concentration-based measurements alone.

7.3 Antigen-Specific and Taxon-Resolved Analyses of Antibody Targeting

Future studies should extend beyond bulk Ig measurements to assess antigen-specific and microbiota-resolved antibody responses. Coupling flow cytometric sorting of IgA⁺ and IgG⁺ bacterial populations with sequencing-based taxonomic identification would enable characterization of the microbial communities preferentially targeted by host antibodies. This approach would allow determination of whether disease-associated immune alterations are concentrated within specific microbial subsets rather than reflected in global changes in antibody abundance or overall bacterial coating, and would directly build on the flow cytometry framework established in this study.

7.4 Expansion to Untreated or Early-Disease Cohorts

Examining Ig profiles in untreated or newly diagnosed patients may help determine whether the preserved antibody patterns observed in this cohort reflect treatment-mediated normalization or intrinsic features of disease biology. Longitudinal sampling across disease onset, treatment initiation, and remission would further clarify how systemic and mucosal antibody responses evolve over time and in relation to clinical intervention.

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Supplementary Materials

Supplementary Material S1. Participant Questionnaire

Questionnaire administered to study participants at the time of enrollment, including demographic information, self-reported symptoms, and clinical background data used for descriptive analyses.

ASLAMIENTO Y CULTIVO DE BLASTOCYSTIS SPP

POLICLÍNICA _____

IDENTIFICACIÓN Y ASPECTOS SOCIO-DEMOGRÁFICOS

1. Fecha de entrevista dd ____ mm ____ aa ____ 3. Nombre completo _____ 5. Teléfono _____ 6. Fecha de nacimiento dd ____ mm ____ aa ____ 8. Género <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>Femenino</td></tr><tr><td>2</td><td>Masculino</td></tr><tr><td>3</td><td>Otro.</td></tr></table> Cuál? _____ 10. Profesión _____	1	Femenino	2	Masculino	3	Otro.	2. Código _____ 4. Cédula de identidad _____ 7. Edad (años) _____ 9. Nivel educativo máx. alcanzado <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>Primaria incompleta</td></tr><tr><td>2</td><td>Primaria completa</td></tr><tr><td>3</td><td>Secundaria incompleta</td></tr><tr><td>4</td><td>Secundaria completa</td></tr><tr><td>5</td><td>UTU incompleto</td></tr></table> 11. Dirección (aclarar barrio) _____	1	Primaria incompleta	2	Primaria completa	3	Secundaria incompleta	4	Secundaria completa	5	UTU incompleto
1	Femenino																
2	Masculino																
3	Otro.																
1	Primaria incompleta																
2	Primaria completa																
3	Secundaria incompleta																
4	Secundaria completa																
5	UTU incompleto																

12. Ubicación de la vivienda <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>Urbana</td></tr><tr><td>2</td><td>Rural</td></tr></table> 13. Cerca de la vivienda existen <table border="1" style="display: inline-table; vertical-align: middle;"><tr><th>Sí</th><th>No</th><th>No sabe</th></tr><tr><td>1</td><td>2</td><td>3</td></tr></table> Basureros <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>2</td><td>3</td></tr><tr><td>1</td><td>2</td><td>3</td></tr></table> Insectos <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>2</td><td>3</td></tr><tr><td>1</td><td>2</td><td>3</td></tr></table> Roedores	1	Urbana	2	Rural	Sí	No	No sabe	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	14. El piso que predomina en la vivienda es de: <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>1</td><td>Tierra</td></tr><tr><td>2</td><td>Madera</td></tr><tr><td>3</td><td>Cemento</td></tr></table> <table border="1" style="display: inline-table; vertical-align: middle;"><tr><td>4</td><td>Baldosa</td></tr><tr><td>5</td><td>Otro</td></tr></table> Cuál: _____	1	Tierra	2	Madera	3	Cemento	4	Baldosa	5	Otro
1	Urbana																																
2	Rural																																
Sí	No	No sabe																															
1	2	3																															
1	2	3																															
1	2	3																															
1	2	3																															
1	2	3																															
1	Tierra																																
2	Madera																																
3	Cemento																																
4	Baldosa																																
5	Otro																																

15. ¿Convive con animales?

1	Si
2	No
3	No sabe

16. Los animales con que convive son:

1	Perro
2	Gato
3	Gallinas y otras aves
4	Vacas y otros bovinos
5	Cerdos
6	Caballos
7	Otros

Cuál: _____

17. ¿Como realiza la eliminación final de excretas?

1	Pozo séptico
2	Saneamiento
3	Inodoro sin conexión
4	Letrina (hoyo seco)
5	Campo abierto
6	Rio o quebrada
7	Otra

Cuál: _____

18. ¿De donde proviene principalmente el agua que consume?

1	Agua corriente urbana (OSE)
2	Agua corriente urbana (OSE) filtrada
3	Agua lluvia
4	Fuente de agua natural
5	Pozo con bomba
6	Otra

Cuál: _____

19. La persona que cocina: ¿Lava las frutas o verduras antes de consumirlas?

1	Siempre
2	Casi siempre
3	Casi nunca
4	Nunca
5	No sabe

20. ¿Cómo lava las verduras?

1	Por arrastre
2	De remojo en un recipiente
3	Otra

Cuál: _____

21. Come carne poco cocida? ¿De que animal?

1	Siempre
2	Casi siempre
3	Casi nunca
4	Nunca
5	No sabe

22. Anda descalzo:

1	Siempre
2	Casi siempre
3	Casi nunca
4	Nunca
5	No sabe

23. ¿Tiene contacto por trabajo/recreación con suelo o tierra?

1	Siempre
2	Casi siempre
3	Casi nunca
4	Nunca
5	No sabe

24. Consume bebidas calientes?

1	Siempre
2	Casi siempre
3	Casi nunca
4	Nunca
5	No sabe

25. ¿Alguna vez le diagnosticaron parásitos?

- 1 Si ¿Hace cuánto? meses
2 No
3 No sabe

27. ¿A realizado viajes al exterior en el último año?

- 1 Si ¿Dónde?:
2 No
3 No recuerda

29. ¿Es alérgico a?:

- 1 Gluten
2 Lactosa
3 Látex
4 Polen
5 Polvo
6 Animales
7 Frutos secos
8 Huevo
9 Pescado y frutos de mar
10 Otra:
11 Ninguna

31. ¿Ha sido vacunado contra el COVID-19?:

- 1 No
2 Si (especificar tipo de vacuna y número de dosis):

26. ¿Le han hecho un coproparasitario?

- 1 Si ¿Hace cuánto? meses
2 No
3 No sabe

28. a) ¿Cómo defines tu dieta?

- 1 Completa (carnes, verduras, cereales frutas)
2 Vegetariano
3 Vegano
4 Sin lactosa
5 Sin gluten
6 Otra (especificar):

30. ¿Ha consumido alguna de las siguientes drogas en los últimos 6 meses?:

- 1 AINES
2 Antibiótico
3 Corticoides
4 Antimicótico
5 Antidepresivo
6 Ansiolítico
7 Antiácido
8 Probióticos
9 Otra:
10 Ninguna

32. Enfermedades diagnosticadas. Marque si tiene diagnosticada alguna de estas enfermedades:

- 1 Enfermedad de Crohn
- 2 Colitis ulcerosa
- 3 Enfermedad celíaca
- 4 Síndrome de intestino irritable
- 5 COVID 19
- 6 Enfermedades autoinmunes
- 7 Divertículos de colon
- 8 HIV/SIDA
- 9 Diabetes
- 10 Síndrome metabólico
- 11 Cáncer (especificar)
- 12 Otra: _____
- 13 Ninguna

33. Ud nació por

- 1 Parto
- 2 Cesárea
- 3 Desconoce

34. Peso
Talla
IMC

35.a Es celíaco?:

- 1 Si
- 2 No
- 3 No sabe

35.b Fecha del diagnóstico

35.c.1 Biopsia al diagnóstico

- 1 Si
- 2 No

35.d.1 Anticuerpos positivos al diagnóstico

- 1 Si
- 2 No
- 3 No sabe

35.c.2 Resultado biopsia

- 1 Marsh II
- 2 Marsh III
- 3 Desconoce

35.d.2 Resultados anticuerpos (si corresponde)

- 1 tTG
- 2 EmA
- 3 PDG

35.e Cumple DLG estricta

- 1 Si
- 2 No
- 3 No sabe

Fecha de inicio

35.f.1 Anticuerpos positivos durante última consulta

- 1 Si
- 2 No
- 3 No sabe

35.h.1 Síntomas al momento de la consulta

- 1 Si
- 2 No
- 3 No sabe

35.f.2 Resultados anticuerpos en última consulta (si corresponde)

- 1 tTG
- 2 EmA
- 3 PDG

35.h.2 Qué síntomas

- 1 Distensión abdominal
- 2 Diarrea
- 3 Constipación
- 4 Vómitos
- 5 Cefaleas

36. a Usted tiene EI? ☐ 1 Si ☐ 2 No Fecha del diagnóstico.....

36.b Presentación ☐ 1 CU ☐ 2 Enf. De Crohn

36.c.1 Ud. ha recibido tratamiento en los últimos 6 meses: ☐ 1 Si ☐ 2 No

36.d.1 Síntomas al momento de la consulta ☐ 1 Si ☐ 2 No

36.c.2 Tratamiento? ☐ 1 Mesalazina oral/tópica
☐ 2 Aza
☐ 3 Mercaptopurina
☐ 4 Prednisona
☐ 5 Budesonida
☐ 6 Hidrocortisona
☐ 7 Metrotexato
☐ 8 Anti TNF
☐ 9 Metronidazol
☐ 10 Ciprofloxacina
☐ 11 Rifaximina

36.d.2 Cuáles síntomas? ☐ 1 Diarrea
☐ 2 Sangrado
☐ 3 Dolor abdominal
☐ 4 Fiebre

36.e.1 Extensión de la CU ☐ 1 Si ☐ 2 No

36.f.1 Extensión de la EC ☐ 1 Si ☐ 2 No

36.g.1 Patrón de la EC ☐ 1 Si ☐ 2 No

36.e.2 Extensión? ☐ 1 E1
☐ 2 E2
☐ 3 E3

36.f.2 Extensión? ☐ 1 L1
☐ 2 L2
☐ 3 L3
☐ 4 L4

36.g.2 Patrón? ☐ 1 B1
☐ 2 B2
☐ 3 B3

36.h Enfermedad perianal ☐ 1 Si ☐ 2 No

37. a Ud tiene Síndrome de intestino irritable: ☐ 1 Si ☐ 2 No Fecha del diagnóstico.....

37.b ☐ 1 SII tipo D
☐ 2 SII tipo C
☐ 3 SII inclasificable

37.c.1 Síntomas actuales ☐ 1 Si ☐ 2 No

37.d.1 Recibió en los últimos 6 meses tratamiento? ☐ 1 Si ☐ 2 No

37.c.2 Cuáles síntomas? ☐ 1 Diarrea
☐ 2 Constipación
☐ 3 Dolor abdominal

37.d.2 Cuál? ☐ 1 Pinaverio
☐ 2 Mebevirina
☐ 3 Trimebutina
☐ 4 Rifaximina
☐ 5 Probióticos

38.a Grupo control

1	Si
2	No

38.b Patología por lo que se asiste en la policlínica

1	ERGE
2	Dispepsia/Ulcera
3	Enf. Hepáticas
4	Enf. rectoanal y colónica
5	Otra:
5	Ninguna

38.a.1 ¿Se toma el momento de la consulta?

1	Si
2	No

38.a.2 ¿Cuáles síntomas?

1	Dolor
2	Sangrado
3	Dolor abdominal
4	Hepático

38.a.3 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.4 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.5 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.6 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.7 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.8 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.9 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.10 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.11 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.12 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.13 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.14 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.15 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.16 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.17 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.18 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.19 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.20 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.21 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

38.a.22 ¿Cuál es el diagnóstico?

1	Enfermedad
2	Enfermedad
3	Enfermedad
4	Enfermedad

Supplementary Material S2. Informed Consent Form (Template)

Template of the written informed consent form provided to all participants prior to study enrollment. The version shown corresponds to the Ethics Committee–approved document and does not include participant-specific identifiers or signatures.



UNIVERSIDAD
DE LA REPÚBLICA
URUGUAY



CLÍNICA de
Gastroenterología



Universidad de la República – Facultad de Medicina

Hospital de Clínicas "Dr. Manuel Quintela" – Clínica de Gastroenterología

Facultad de Medicina Departamento de Inmunobiología.

Institut Pasteur Montevideo - Laboratorio de apicomplejos.

Consentimiento informado

Proyecto: Caracterización de la respuesta inflamatoria en patologías digestivas y su asociación con la disbiosis de la microbiota intestinal.

Como paciente fui informado de manera clara y completa sobre la finalidad de todos los procedimientos clínicos a realizarse y toma de muestras. Habiendo obtenido la información necesaria por parte del equipo investigador, decido participar en el presente estudio luego de haber leído y comprendido la información recibida.

En este contexto, expreso mi consentimiento para: la revisión de mi ficha clínica, procesamiento de mi información clínica recabada a través de un cuestionario telefónico y acceso a resultados de mis estudios. Autorizo también la publicación de los resultados de esta investigación, en congresos y revistas científicas, siempre que mis datos personales se mantengan confidenciales.

Esta autorización se mantiene vigente hasta ser cancelada de manera escrita por mí

Fecha: _____

Nombre de paciente: _____

Cédula: _____

Firma: _____

	Docentes responsables	
	Prof. Agda.Dra. Teresa Freire	Prof. Dra. Carolina Olano

Supplementary Material S3. Ethics Committee Approval

Official ethics approval issued by the Comité de Ética de la Investigación del Hospital de Clínicas “Dr. Manuel Quintela”, Universidad de la República (Montevideo, Uruguay), for the project “Caracterización de la respuesta inflamatoria en patologías digestivas y su asociación con la disbiosis de la microbiota intestinal.” Approval granted on 2 May 2022 and valid for the duration of the study.

UNIVERSIDAD DE LA REPÚBLICA
HOSPITAL DE CLÍNICAS
“DR. MANUEL QUINTELA”
SECRETARÍA GENERAL
DEPARTAMENTO DE COMISIONES
COMITÉ DE ÉTICA DE LA INVESTIGACIÓN

Montevideo, 2 de Mayo de 2022

Se transcribe resolución del Comité de Ética del Hospital de Clínicas de fecha 2 de Mayo de 2022

En relación al proyecto presentado por la Clínica de Gastroenterología

“Caracterización de la respuesta inflamatoria en patologías digestivas y su asociación con la disbiosis de la microbiota intestinal”

Investigadora responsable: Prof. Dra. Carolina Olano

El Comité de Ética de la Investigación del Hospital de Clínicas resuelve aprobar la realización de este proyecto en esta Institución.

La aprobación otorgada por este Comité de Ética es desde el 2 de Mayo de 2022 hasta la fecha de finalización del mismo.



Prof. Dr. Raúl Ruggia
Coordinador del Comité de Ética de la Investigación

Supplementary Material S4. Complete Blood Count (CBC) Data Visualizations

Supplementary figures showing CBC parameter distributions across diagnostic groups that are not included in the main text.

Parameters shown include:

(A) Mean corpuscular hemoglobin concentration (MCHC, g/L)

(B) Monocyte percentage (Mon%, %)

(C) Absolute neutrophil count (Neu#, $\times 10^9/L$)

(D) Absolute lymphocyte count (Lym#, $\times 10^9/L$)

(E) Absolute monocyte count (Mon#, $\times 10^9/L$)

(F) Absolute eosinophil count (Eos#, $\times 10^9/L$)

(G) Absolute basophil count (Bas#, $\times 10^9/L$)

(H) Neutrophil percentage (Neu%, %)

(I) Lymphocyte percentage (Lym%, %)

(J) Eosinophil percentage (Eos%, %)

(K) Basophil percentage (Bas%, %)

(L) Red blood cell count (RBC, $\times 10^{12}/L$)

(M) Mean corpuscular volume (MCV, fL)

(N) Mean corpuscular hemoglobin (MCH, pg)

(Q) Red cell distribution width – coefficient of variation (RDW-CV, %)

(R) Red cell distribution width – standard deviation (RDW-SD, fL)

(S) Mean platelet volume (MPV, fL)

(T) Platelet distribution width (PDW, fL)

(U) Plateletcrit (PCT, %)

Data are shown as individual values with group medians and interquartile ranges. Statistically significant pairwise differences are indicated by brackets and asterisks (* $p < 0.05$), as determined by post hoc Dunn's multiple comparison test following Kruskal–Wallis analysis.

Adult reference ranges: Reference intervals are provided for parameters shown in this figure: MCHC (31.0–36.0 g/L), Mon% (4.0–12.0%), absolute neutrophils ($1.80\text{--}7.70 \times 10^9/L$), absolute lymphocytes ($1.00\text{--}4.80 \times 10^9/L$), absolute monocytes ($0.20\text{--}1.00 \times 10^9/L$), absolute eosinophils ($0.10\text{--}0.60 \times 10^9/L$), absolute basophils ($<0.20 \times 10^9/L$), neutrophil percentage (50.0–70.0%), lymphocyte percentage (25.0–40.0%), eosinophil percentage (2.0–4.0%), basophil percentage ($<1.0\%$), red blood cell count ($4.10\text{--}5.10 \times 10^{12}/L$), mean corpuscular volume (78.0–98.0 fL), mean corpuscular hemoglobin (25.0–35.0 pg), red cell distribution width ($<12.0\%$), mean platelet volume (5.0–15.0 fL), platelet distribution width (reference range not specified by institution), and plateletcrit (reference range not specified by institution). Parameters not displayed in this figure (e.g., hemoglobin, total white blood cell count, platelet count) are listed in the main text in the description of Table 7.

