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Review

Liquid Biopsy in Crohn's Disease: From Circulating Biomarkers to Multi-Omics Integration for Precision Medicine

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Abstract

Inflammatory bowel diseases (IBD) are chronic inflammatory conditions of the digestive tract, mainly represented by Crohn's disease (CD) and ulcerative colitis (UC). CD is driven by complex aetiopathophysiological mechanisms which include immune dysfunction, alteration in gut microbiota, genetic predispositions and environmental factors. Diagnosis and severity scoring, through imaging and endoscopy, are often limited by the subjective determination of disease activity indices, with scores dictating management. Liquid biopsy (LB) represents an emerging non-invasive approach for objective and continuous disease assessment. This methodology facilitates the isolation of circulating cell-free DNA (ccfDNA) from peripheral blood, enabling the simultaneous assessment of circulating exosomes, their cargo (microRNA) and inflammation-related molecules. Recent progress incorporates fragmentomics, the structural analysis of ccfDNA and the interpretation and integration of multi-omics models. These powerful combined approaches, driven by machine learning, could become essential for modern precision medicine, supporting the development of highly specific, individualised and predictive biomarkers for therapeutic response. This review discusses the role of LB in the management of patients with IBD and explores the future opportunities in precision medicine.

Keywords: Crohn's disease; inflammatory bowel diseases; cell-free nucleic acids; microRNAs; exosomes; biomarkers; liquid biopsy

1. Pathophysiological Background of Crohn's Disease and Rationale for Liquid Biopsy-Based Approaches

1.1 Crohn's Disease

Crohn's disease (CD) and ulcerative colitis (UC) are both forms of inflammatory bowel diseases (IBD) affecting the gastrointestinal tract [1]. CD can affect the whole gastrointestinal tract and it is now accepted that several factors govern its aetiopathophysiology including genetics, immunity, microbiota and environment factors [2] (Fig. 1). Various studies have also demonstrated the crucial role of dysregulated components of the innate and adaptive immunity in the pathogenesis of CD, in particular the T-cells [3,4,5].

The clinical management of CD necessitates frequent and objective monitoring of inflammatory activity and response to treatment. An ileocolonoscopy, combined with biopsy, has long been regarded as the gold standard for diagnosing and determining the severity and activity of mucosal diseases [6]. However, this is an invasive procedure and is not feasible for routine and repetitive monitoring [7]. Existing non-invasive markers such as faecal calprotectin (FC) and C-reactive protein (CRP) have several drawbacks relating to the lack of specificity for intestinal inflammation, with the diagnostic capabilities linked to the disease location [7]. They cannot predict disease relapse or therapeutic response when used individually, highlighting the need for more precise and disease-specific biomarkers. The limitations of current standards draw attention to a critical objective gap in CD monitoring, particularly, the absence of a minimally invasive and objective instrument [7]. The 2023 American Gastroenterological Association (AGA) recommendations are driving the adoption of a treat-to-target (T2T) approach, emphasising the use of these objective markers and highlighting the critical need for highly specific non-invasive tools that go beyond traditional CRP and FC [8,9,10,11].

1.2 Liquid Biopsy

Liquid biopsy (LB) represents a non-invasive emerging approach for the identification of disease-specific biomarkers. Described over a decade ago [12], it initially served to collect circulating tumor cells (CTCs) and has

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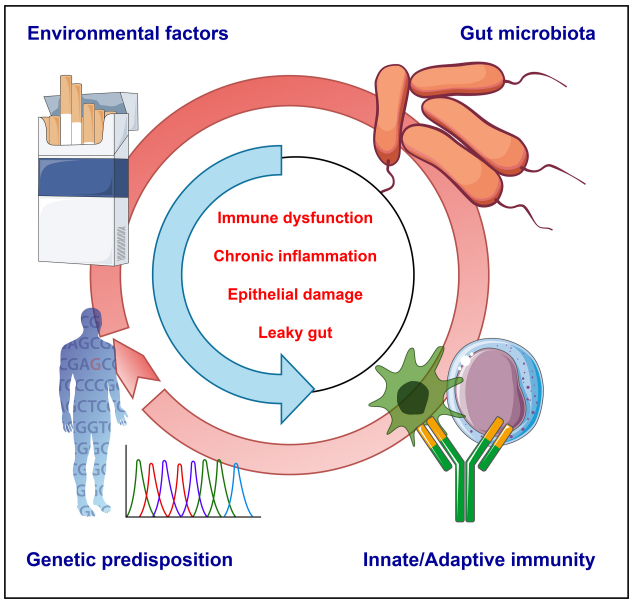


Fig. 1. Pathogenesis of Crohn's disease. Schematic diagram illustrating the complex interplay between genetic predisposition, gut microbiota dysbiosis, innate and adaptive immune responses and environmental triggers. These factors converge to drive chronic intestinal inflammation and epithelial barrier dysfunction (leaky gut). The icons used in the image were obtained from Bioicons and SciDraw.

now evolved to also assess additional tumor-derived components, such as circulating cell-free DNA (ccfDNA) [13], circulating cell-free RNA (ccfRNA) and even extracellular vesicles (EV) [14] (Fig. 2).

Circulating tumor DNA (ctDNA) extracted from the plasma of patients with cancer is enriched with DNA fragments [15] and molecular analysis of these fragments showed that they carry genetic and epigenetic alterations strictly related to the disease, allowing immune-profiling and targeted therapy [16] (Fig. 3A). LB allows to collect also exosomes, EVs characterised by having a diameter of about 30–150 nm. EVs, produced by the inward cell membrane budding and then merging with the endosomal compartment [17], carry distinct biological macromolecules deriving from their own mother cells, such as DNA, RNA, proteins and lipids [18]. Depending on the condition of the organ engaged in their formation, circulating exosomes might be specifically charged with physiologically active molecules, such as microRNAs (miRNAs) [19] (Fig. 3B), small single-stranded non-coding RNAs (ncRNAs) with a significant impact on human physiology and pathology [20].

While some scientific publications have explored the application of LB in IBD, this review adopts a distinct integrative perspective. Rather than focusing on single biomarker categories, it combines circulating nucleic acids, fragmentomics, exosomal miRNA profiling and emerging epigenetic signatures within a unified framework. In addition, it places particular emphasis on the integration of multi-omics datasets and computational approaches, highlighting how molecular and microbial features can be jointly analysed to enable patient stratification and support precision medicine strategies. Finally, this review also links circulating biomarkers to underlying disease mechanisms, providing a systems-level view that connects genetic susceptibility, regulatory pathways and non-invasive dynamic disease monitoring.

2. Molecular and Genetic Background of Crohn's Disease

2.1 Genetic Susceptibility, Autophagy Alteration and Environmental Triggers

The pathophysiology of CD involves a complicated interplay in which a dysregulated immune response to environmental stimuli and the intestinal microbiota is orchestrated by genetic susceptibility. Compared to the general population, first-degree relatives of an affected person have a markedly higher chance of acquiring CD due to an enrichment of genetic risk alleles [21]. Studies in monozygotic twins, similarly corroborate this link [22,23]. More than 200 genetic loci have been linked to the risk of IBD through genetic research, including genome-wide association studies (GWAS) [24]. These loci encode genes affecting innate immunity, autophagy and mucosal barrier function, which in turn contribute to the genetic propensity to CD. The most significant inherited loci consist of nucleotide-binding oligomerization domain 2 (*NOD2*), autophagy-related 16-like 1 (*ATG16L1)* and immunity-related GTPase M (*IRGM*) [25,26]. Other significant innate immune-related genes include caspase recruitment domain family member 9 (*CARD9*) and IL18 receptor accessory protein (*IL18RAP*) [27].

Since its discovery in 2001, the *NOD2* gene has been repeatedly found to be the most significant susceptibility gene in the aetiology of CD [28,29]. In terms of physiology, wild type *NOD2* is essential for immunological homeostasis, intestinal barrier integrity and autophagy mechanisms [30,31]. Muramyl dipeptide (MDP) and other components of bacterial walls are detected by the intracellular pattern recognition receptor that *NOD2* codes for [30,31]. The prevalent inherited *NOD2* risk variants R702W, G908R and the L1007fs frameshift mutation, are characterised as loss-of-function mutations. These variants impair the protein ability to recognise harmful or commensal microorganisms due to reduced autophagy and deficient nuclear factor-kappa B (NF-κB) activation [28,29,32]. This failure in bacterial clearance leads to the persistent intestinal inflammation and dysregulated immune responses characteristic of CD [29,32]. Clinically, these mutations predispose patients to a specific phenotype marked by ileal involvement (ileitis), stricturing disease behavior (B2) and a significantly increased risk of requiring surgery [28,29].

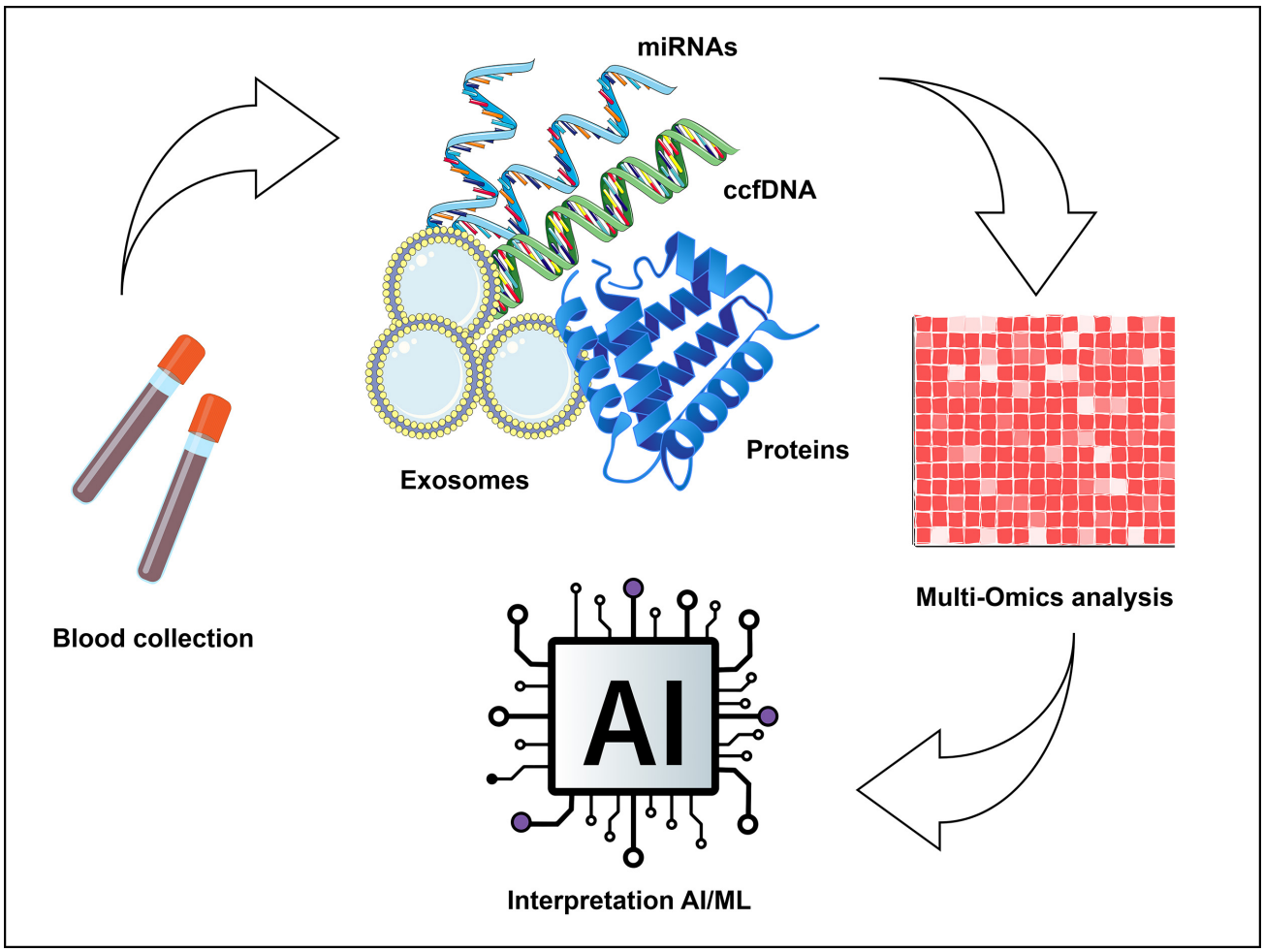


Fig. 2. Liquid biopsy workflow. Schematic representation of the LB pipeline: from peripheral blood collection and the isolation of ccfDNA, exosomes, miRNAs and proteins, to multi-omics profiling and AI-driven data interpretation for precision diagnostics. The icons used in the image were obtained from Bioicons and SciDraw. LB, liquid biopsy; AI, artificial intelligence; ML, machine learning; miRNA, microRNA; ccfDNA, circulating cell-free DNA.

The function of ATG16L1 is closely related to autophagy, an essential cellular mechanism for breaking down and recycling cytoplasmic components as well for getting clear of intracellular bacteria [33,34]. ATG16L1 [24] causes structural and functional abnormalities in Paneth cells that hinder their ability to generate antimicrobial peptides and eliminate intracellular bacteria. The T300A substitution (rs2241880), which causes aminoacidic substitution, is the most often researched genetic variant of *ATG16L1* linked to CD risk [35,36]. This variation impairs the ability of epithelial cells to encapsulate and eliminate endocytosed bacteria within autophagosomes [34]. Therefore, it is responsible of morphological abnormalities (abnormal secretory granules) and changes in the synthesis of antimicrobial peptides, which lead to dysbiosis and disruption of mucosal homeostasis [33,37]. ATG16L1 plays a joint role with NOD2 in innate immune defense and autophagy since MDP stimulation of NOD2 can draw ATG16L1 to the microbial infection site to start the autophagy process and destroy the germs. This mechanism is particularly altered when CD risk variants are present in both genes [34]. In addition, IRGM plays a role in autophagy too, and variations in this gene contribute to immunological dysregulation again by hindering the effective removal of intracellular pathogens [35,38,39].

Together, these genetic insights underscore autophagy as a critical convergent pathway in CD pathogenesis [24]. Rather than operating in isolation, NOD2, ATG16L1 and IRGM form a coordinated functional axis in which genetic defects inherently compromise intracellular pathogen clearance and mucosal defense [24]. At the molecular level, this impaired cellular clearance directly intersects with endoplasmic reticulum (ER) stress. While physiological autophagy dynamically resolves ER pathways to restore cellular equilibrium, CD risk variants disrupt this homeostatic balance, leaving ER stress unresolved and leading to persistent epithelial cell stress [24]. Importantly, these inherited innate immune defects are insufficient to trigger pathogen-

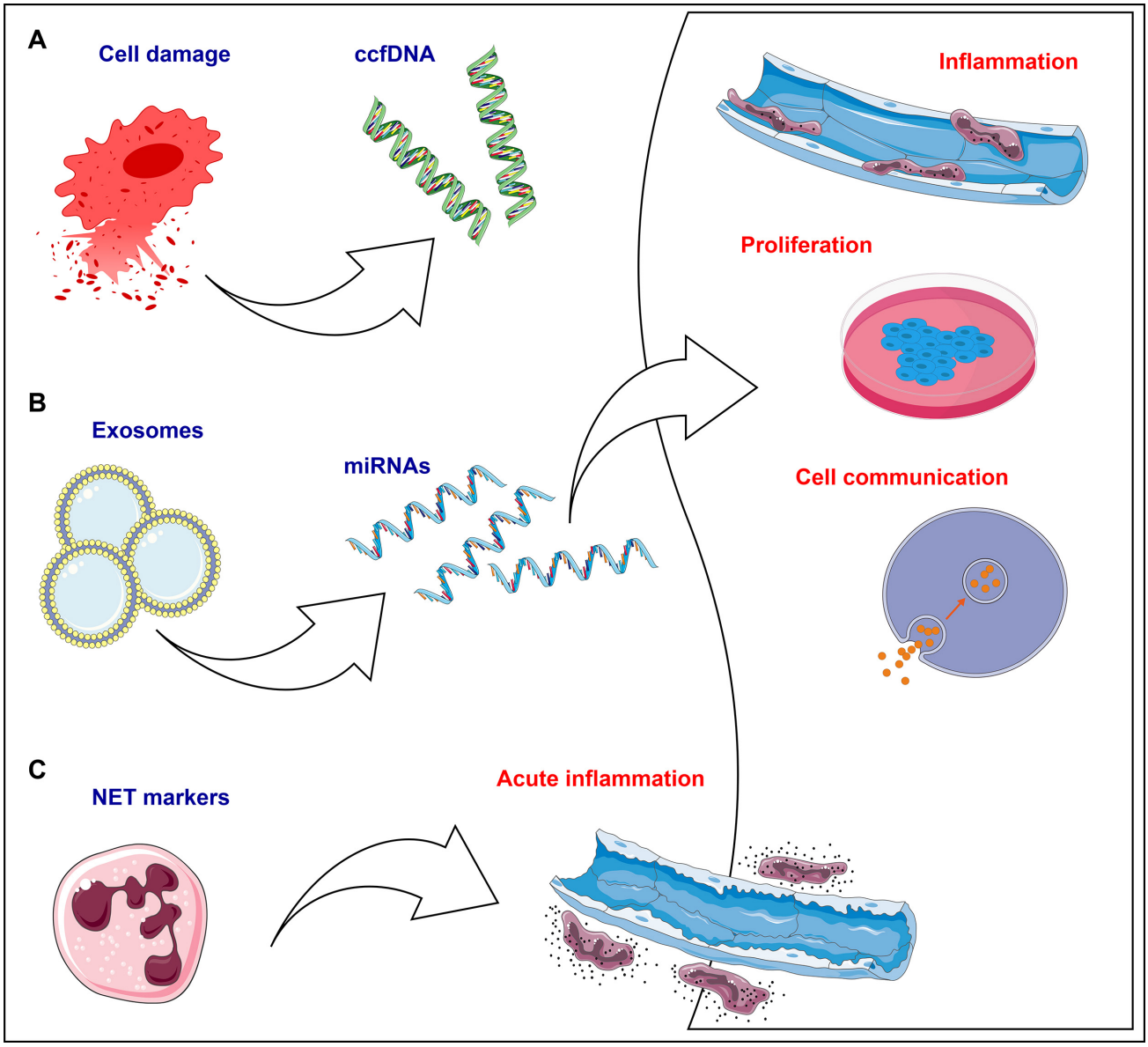


Fig. 3. Key biomarkers and their pathophysiological roles. Diagram illustrating the primary LB biomarkers and their clinical significance. (A) ccfDNA as an indicator of cell damage. (B) miRNAs as mediators of inflammation and exosomes in intercellular communication. (C) NET markers as signatures of acute inflammation. The icons used in the image were obtained from Bioicons and SciDraw. LB, liquid biopsy; ccfDNA, circulating cell-free DNA; miRNA, microRNA; NET, neutrophil extracellular trap.

esis on their own. Instead, they basically reshape the host-microbiota interface, lowering the threshold for environmental factors to trigger and perpetuate the non-resolving inflammatory state characteristic of CD [35,38].

Beyond the canonical susceptibility genes commonly described in CD, recent high-resolution genomic studies have expanded the spectrum of loci implicated in disease pathogenesis, highlighting additional pathways involved in immune regulation and epithelial homeostasis [40]. These loci encode additional genes involved in key biological processes, including innate immune responses, autophagy and maintenance of epithelial barrier integrity. The functional consequences of these genetic alterations are increasingly understood to drive dysregulated molecular pathways that extend beyond local intestinal inflammation and are reflected in circulating molecular signals. To precisely isolate these genetic drivers, recent large-scale efforts have transitioned from broad loci identification to high-density statistical fine-mapping [40]. By leveraging dense genotyping data from custom platforms like the Immunochip, researchers have rigorously evaluated the causal probabilities of variants within complex loci, including those encompassing smad family member 3 (*SMAD3*), interferon induced with helicase C domain 1 (*IFIH1*), leucine rich repeat kinase 2 (*LRRK2*), janus kinase (*JAK*)*2*, pr/set domain 1 (*PRDM1*), ikaros family zinc finger 1 (*IKZF1*) and nk2

homeobox 3 (*NKX2-3*) [40]. Rather than relying on speculative biological extrapolation, this fine-mapping framework constructs 95% credible sets to mathematically narrow down the specific genetic variants responsible for the association signals. For instance, within these prioritised regions, the resolution of linkage disequilibrium has allowed for the differentiation between missense variants, which alter protein-coding sequences and non-coding variants that likely exert cell-type-specific regulatory effects [40]. Furthermore, many of these resolved loci highlight a complex genetic architecture characterised by extensive pleiotropy, revealing shared causal mechanisms between CD, UC and other systemic immune-mediated inflammatory diseases [40]. Consequently, identifying these specific variations clarifies the precise genetic landscape necessary for targeted functional validation, moving past generalised locus descriptions into high-resolution causal variant discovery.

Collectively, the identification of these additional genes supports a model in which CD is not driven solely by defects in microbial recognition or barrier integrity, but rather by a broader network of dysregulated signalling pathways integrating innate immunity, adaptive immune differentiation and transcriptional regulation. This expanded genetic framework provides a more comprehensive basis for interpreting downstream molecular phenotypes and for developing targeted therapeutic strategies [41], and these emerging loci also provide potential links between genetic susceptibility and circulating molecular signatures, supporting their integration into biomarker discovery frameworks.

The complex interaction between environmental variables and host genes, immune system and variations in the gut microbiota also plays a key role in the development and progression of CD [42,43]. Cigarette smoking and *NOD2* genetic mutations are major independent risk factors for CD, particularly affecting the small intestine [44]. While a "second hit" from the environment (like smoking) is often needed to trigger the illness in genetically predisposed people [42], recent studies show a more complex relationship. Surprisingly, the statistical interaction between smoking and the main *NOD2* mutation is negative, likely because younger patients carry more genetic risks, while older patients have a longer history of smoking [44]. Still, the absolute risk remains higher for a smoker who also carries the genetic mutation.

2.2 Dysregulation of Immune Responses, Epithelial Barrier Integrity and Epigenetic Control

Chronic inflammation is sustained by a cascade of dysregulated adaptive immunity resulting from innate immunological dysfunction [45]. T helper (Th) cells are polarised toward the Th1 and Th17 phenotypes by pro-inflammatory cytokines generated by hyperactive macrophages, such as interleukin (IL)-12 and IL-23, the latter of which is a recognised therapeutic target [45]. Tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma are the main products of Th1 cells. The transmural inflammation and neutrophil recruitment, characteristic of CD, are mediated by IL-17, which is produced by Th17 cells. TNF-alpha is a crucial molecular mediator that causes immune cells to migrate into the intestinal mucosa and activates inflammatory signalling pathways by attaching to its receptors [45].

Interestingly, recent transcriptomic and mechanistic studies further support the integration of molecular profiling approaches in IBD research. Notably, Li and colleagues [46] in 2025 demonstrated that the early growth response factor 1 (EGR1)-a disintegrin and metalloproteinase 17 (ADAM17)-TNF-alpha signalling axis plays a central role in macrophage-mediated intestinal inflammation, as shown through RNA-sequencing and functional validation experiments. In this study, modulation of EGR1 activity was associated with downstream effects on *ADAM17* transcription and TNF-alpha production, ultimately impacting inflammatory responses and intestinal barrier integrity. These findings provide a clear example of how transcriptomic data can be integrated with functional analyses to elucidate regulatory pathways relevant to disease pathogenesis and highlight the potential of combining molecular and computational approaches for a deeper understanding of IBD biology [46]. Interestingly, it has also been demonstrated that variations in miRNA expression control the release of various pro-inflammatory chemokines, which in turn controls inflammatory responses. Mediators of the inflammation (TNF-alpha and -beta and NF-κB), microbial constituents (lipopolysaccharide and flagellin) and a wide range of toll-like receptor (TLR) ligands can regulate particular miRNAs, as for example *miR-146*, *miR-155* and *miR-132*, causing granulocytes and monocytes to proliferate and spread during inflammation [47].

This persistent immune activation disrupts epithelial integrity, leading to the characteristic "leaky gut". This dysfunction represents both a consequence of chronic inflammation and a factor that perpetuates the inflammatory vicious cycle, with increased intestinal permeability exposes the immune system to a higher load of luminal antigens, including microbial components, triggering and amplifying the inflammatory response. A key element of this dysfunction lies in the alterations of the tight junctions (TJs), complex protein structures that regulate paracellular permeability [48]. A reduced expression of essential TJ proteins, such as occludin and claudins (particularly claudin-2), favors the passage of molecules and pathogens across the epithelium [49]. This defect is also linked to genetic changes, as in the case of polymorphisms in genes coding for epithelial junction and mucus layer proteins, such as mucin-2 (MUC2) that contribute to individual susceptibility [48,49].

As the epithelial and immune alterations reinforce one another, they create a permissive environment for deeper

molecular dysregulation. Epigenetic modifications, such as DNA methylation, histone modifications and ncRNA-mediated regulation have recently been linked to the pathogenesis of CD. A significant increase in histone deacetylases (HDAC) 1, 2 and 7 expressions have been reported in intestinal fibroblasts associated with strictures, leading to histone H3 lysine 27 (H3K27) hypoacetylation and fibrotic activation [50]. Braga-Neto and colleagues [51] characterised the deregulation of several lincRNAs in CD4+ lamina propria lymphocytes and found that XLOC_000261 was highly expressed in patients, negatively modulating RAR-related orphan receptor gamma t (*RORγt*) and influencing Th17 cell differentiation. The interplay between DNA methylation, histone modifications and ncRNAs by modulating gene expression, appears to be a crucial mechanism not only in chronic inflammation, but also in the progression toward intestinal fibrosis in CD, with relevant implications for the development of epigenetic biomarkers and targeted therapeutic strategy. Together, these interconnected layers (immune dysregulation, epithelial barrier breakdown and epigenetic alterations) form a self-reinforcing pathogenic circuit that underlies CD chronicity.

3. Liquid Biopsy in Crohn's Disease

While the invasive gold standard remains central to IBD diagnosis and monitoring [52], LB is rapidly gaining traction as a promising non-invasive alternative. By analysing circulating biomarkers in body fluids, such as host and microbial ccfDNA, exosomes and ncRNAs [53], LB enables non-invasive diagnosis, sub-class differentiation and early surveillance of IBD-associated neoplasia [54]. This capacity to capture downstream molecular outputs of genomic and epigenomic dysregulation shifts the perspective of LB from a static diagnostic tool to a dynamic readout of disease biology, driving precision medicine in IBD.

Within this framework, EVs and specifically exosomes (about 30–150 nm) represent a crucial component of the LB landscape due to their role as essential mediators of intercellular communication [55]. Released by immune and epithelial cells, these vesicles carry a specific molecular cargo including proteins, lipids and RNAs, that precisely reflects the pathophysiological state of their cell of origin. In the context of IBD, decoding the data within circulating exosomes offers a unique window into mucosal immunity dysregulation, a driving factor in CD onset and evolution [56]. For instance, exosomes isolated from IBD patients exhibit elevated levels of pro-inflammatory cytokines (such as TNF-alpha, IL-8 and IL-6) that directly correlate with disease severity scores and can stimulate colonic epithelial cells to release further IL-8 [56]. Furthermore, the serum exosomal profile is significantly altered in intestinal illnesses [57]. *In vivo* models of dextran sulphate sodium (DSS)-induced colitis have shown that while visceral adipose tissue-derived exosomes can exacerbate inflammation *via* miR-155 pro-inflammatory action, targeting this pathway, such as through exosome-mediated miR-155 inhibitor treatment, can arrest colitis progression and accelerate wound healing [58,59]. Beyond diagnosis and dynamic monitoring, this dual nature highlights a promising shift for exosomes within the LB paradigm, suggesting that they could serve not only as highly sensitive biomarkers, but also as potential bio-engineered vehicles for controlled drug delivery and targeted disease management [60,61].

3.1 Circulating Cell-Free DNA and Fragmentomics

The ccfDNA is a crucial component of both disease pathogenesis and emerging non-invasive biomarkers. Elevated levels of ccfDNA, primarily fragments of nuclear DNA (ncDNA) and mitochondrial DNA (mtDNA), are consistently observed in the plasma of IBD patients [62], reflecting the widespread inflammation and tissue damage. High rates of cellular stress, apoptosis and necrosis in the inflammatory intestinal tissue are caused by genetic predisposition and resulting altered molecular pathways (such as defective autophagy and chronic ER stress). This persistent cellular turnover is the primary cause of nucleic acid release into the peripheral circulation. The release of host ccfDNA is thus a direct and quantifiable result of the genetically induced tissue damage, which is frequently marked by distinct fragmentation patterns and elevated quantities of mtDNA. Release mechanisms are thought to include high rates of apoptosis and necrosis in the severely inflamed gut mucosa, alongside the potential active extrusion of chromatin *via* neutrophil extracellular traps (NETs) from activated immune cells [53,63]. This establishes a clear molecular relationship where inherited genetic risk factors result in persistent disease, which is subsequently reflected and measurable through LB biomarkers (Fig. 3C). Therefore, this process is not only a consequence of tissue damage, but also reflects underlying genetic susceptibility and altered regulatory pathways, such as defective autophagy and chronic inflammatory signalling. As a result, ccfDNA represents a molecular footprint of disease-related genomic activity. Beyond serving as mere damage markers, ccfDNA (particularly mtDNA) and apoptosis-derived extrachromosomal circular DNA (eccDNA) act as potent damage-associated molecular patterns (DAMPs). By engaging immune receptors such as TLR9 and the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon response cGAMP interactor 1 (STING) pathway, these extracellular DNA fragments cease to be passive indicators and become active drivers of the chronic inflammatory cascade characteristic of IBD, ultimately triggering the production of pro-inflammatory cytokines and type I IFNs [53,62].

Hence, in addition to being a biomarker indicating tissue damage, ccfDNA is known to be a potential cause of inflammation in the context of IBD. The intricate processes of ccfDNA synthesis and fragmentation, which are linked to DNA repair and cell death mechanisms, affect the size and

functionality of the fragments [64]. Fragmentomics adds a new level of extremely specific diagnostics by using non-mutational markers, like methylation patterns and fragment size ratios, to improve our understanding of gene regulation and tissue-specific functions in illnesses [64]. Beyond quantitative assessment, fragmentomic features provide insights into nucleosome positioning, chromatin accessibility and tissue-specific epigenetic regulation. These characteristics enable a more refined interpretation of the genomic origin of circulating DNA fragments and their relationship with disease-specific cellular processes. Interestingly, in parallel to quantitative alterations, a critical and often underexplored aspect of circulating DNA in CD is its structural and organisational complexity [65]. Rather than representing a uniform pool of degraded nucleic acids, ccfDNA consists of highly structured fragments whose size distribution, nucleosomal positioning and molecular topology reflect the chromatin landscape and the mechanisms of release from specific cell populations. This fragmentomic dimension, therefore, provides strong biological insights since fragment length periodicity and nucleosome footprints retain tissue-specific signatures that reveal cellular origins and inflammatory contexts beyond mere bulk concentrations [65].

An emerging layer of complexity concerns the physical form of circulating DNA. In addition to linear fragments, eccDNA has been identified as a distinct component of the circulating nucleic acid pool. Unlike linear ccfDNA, eccDNA appears to exhibit enhanced immunostimulatory properties that are dependent not only on sequence composition, but also on its circular topology, which may facilitate more efficient recognition by DNA-sensing pathways. This suggests that structural features of ccfDNA, rather than abundance alone, may critically shape its biological activity in chronic inflammatory settings [65]. Consequently, ccfDNA and its various forms including eccDNA, alongside with circulating microbial cell-free DNA (cfmDNA), are actively being developed as components of LB for improved diagnosis, real-time assessment of activity and stratification of patients for targeted therapies [53,63].

The compartmentalisation of ccfDNA within EV introduces an additional regulatory dimension since EV-associated DNA is protected from enzymatic degradation and can be selectively delivered to recipient cells, thereby acting as a vehicle for intercellular communication. Notably, double-stranded DNA carried by EV has been shown to activate intracellular sensing pathways such as STING in target immune cells, directly contributing to macrophage activation and amplification of inflammatory responses. This EV-mediated transfer supports the view that circulating DNA is not merely a passive marker but an active participant in the propagation of mucosal inflammation [65]. The persistence of circulating DNA is tightly regulated by degradation mechanisms, and alterations in this balance may have functional consequences. Evidence of reduced DNase activity in IBD suggests that impaired clearance contributes to the accumulation of structurally intact and potentially immunogenic DNA species, prolonging their interaction with immune receptors. This imbalance between release and degradation provides a mechanistic explanation for sustained exposure of the immune system to endogenous nucleic acids, reinforcing chronic inflammatory circuits even in the absence of overt tissue damage [62].

Altogether, these observations indicate that the biological relevance of ccfDNA in CD resides not only in its concentration, but in its structural diversity, mode of transport and persistence in circulation. Integrating these parameters into ccfDNA analysis may therefore reveal previously unrecognised mechanistic links between tissue damage, immune activation and systemic signalling.

Epigenomics

Beyond inherited genetic variation, epigenetic modifications are increasingly recognised as important contributors to the pathophysiology and clinical outcomes of CD [66]. DNA methylation and histone alterations control gene expression levels and are essential elements of inflammatory signalling [67]. Analysis in the clinical context has demonstrated that peripheral blood DNA methylation patterns are highly correlated with acute inflammation and return to normal as patients respond to treatment [68]. The Epigenetic Predictors of Biologic Therapy Response in Crohn's Disease (EPIC-CD) project, which sought to create and confirm peripheral blood DNA methylation patterns capable of predicting response to biological therapy in CD, thoroughly investigated the clinical potential of epigenetic profiling [69]. To predict combined endoscopic and clinical/biochemical response to vedolizumab and ustekinumab in patients with CD, the EPIC-CD study developed panels of baseline peripheral blood DNA methylation signatures, which subsequently demonstrated high temporal stability [69]. Specifically, a panel of 68 CpG sites was identified for ustekinumab response and 25 CpG sites for vedolizumab response, both yielding a validated area under the curve (AUC) of 0.75 in an independent external validation cohort (95% CI 0.65–0.87 for ustekinumab and 0.65–0.85 for vedolizumab) [69]. Since the CpG panels are linked to genes implicated in the mechanisms of action of the corresponding medicines, these epigenetic markers have a high biological relevance. In line with vedolizumab role as an integrin inhibitor, the vedolizumab markers are connected to pathways linked to integrin-dependent T-cell homing [69]. Similar to how ustekinumab affects IL-12/IL-23 signalling, the ustekinumab markers are associated with pathways in innate immunity and Th17 Treg differentiation [69]. Regardless of inflammatory status, these methylation levels showed high hyper-stability (intraclass correlation coefficient values $\geq$0.90) across treatment phases, highlighting their robustness as reliable molecular predictors for therapeutic non-response, especially in biologically naive patients [69].

Chromatin alterations, particularly histone modifications, are part of the molecular regulation controlled by epigenetics [67]. Histone and DNA methylation-related factors including SET domain containing 2 (*SETD2*), methyl-CpG binding domain protein 2 (*MBD2*) and ubiquitin like with PHD and ring finger domains 1 (*UHRF1*), are essential for regulating inflammatory processes in the gut [67]. Finally, ncRNAs are also known to be important epigenetic modulators and it has been observed that they work as tumor suppressors or oncogenes in intricate regulatory networks to regulate gene expression in inflammatory diseases [70]. They are promising non-invasive targets for LB in monitoring these systemic regulatory changes because of their stability in circulation, frequently within microvesicles [70].

3.2 Microbial Factors in Liquid Biopsy: From Circulating Signatures to Functional Microbiome Readouts

CD is strongly associated with gut microbiota dysbiosis that extends beyond compositional changes, involving deep functional and metabolic alterations. Multi-omics analyses have shown a shift from obligate anaerobic and short-chain fatty acid-producing bacteria toward facultative anaerobes, accompanied by coordinated disruptions in microbial transcriptional activity and metabolite pools. Dysbiosis, therefore, represents a dynamic, system-level reprogramming closely linked to host inflammation rather than a static taxonomic imbalance [71]. Within the LB framework, these alterations are partially retained by cfmDNA. The cfmDNA points to a compromised intestinal barrier and subsequent microbial translocation into the systemic circulation, a key factor in IBD aetiology [72]. Analysing the specific composition of this cfmDNA could provide a unique molecular understanding of the underlying gut dysbiosis. Moreover, it has been demonstrated that certain bacterial cfmDNA fragments, especially those rich in CpG structures, directly activate TLR9, thereby worsening colitis [65]. As a non-invasive diagnostic, evaluation of cfmDNA provides a highly specific molecular readout of disease status and microbial insult by directly connecting the gut microbiome (a key component of CD) to the systemic inflammatory response [72].

However, cfmDNA alone underestimates the complexity of microbiome-derived signals since functional outputs, particularly circulating and faecal metabolites, provide a more direct readout of microbial activity and its systemic consequences. Integrative microbiome-metabolome studies are supporting this concept as observed by Li and colleagues [73] that reported corresponding alterations in microbial composition and circulating metabolites associated with intestinal fibrosis in CD, including amino acids and bile acids. Importantly, specific metabolites such as L-aspartic acid were shown to exert causal pro-fibrotic effects, establishing a mechanistic link between microbial metabolism and disease progression. Consistently, data from the Integrative Human Microbiome Project demonstrate that disease activity is associated with global reshaping of metabolite pools, including depletion of beneficial metabolites, like short-chain fatty acids, and accumulation of pro-inflammatory compounds [71]. Microbiome alterations in CD are also temporally dynamic, occurring in episodic bursts characterised by coordinated shifts in microbial composition, gene expression and metabolite production, which are mirrored by fluctuations in circulating molecular profiles. These findings highlight the potential of LB to capture real-time disease activity rather than static snapshots [71].

Beyond individual biomarkers, the integration of microbiome composition with host metabolomics provides deeper insight into disease heterogeneity. Zdanowicz and colleagues [74], studying treatment-naïve paediatric IBD patients, showed that microbiome profiles alone poorly discriminate CD from UC, whereas combined microbiome-metabolome analysis identified distinct biological subgroups characterised by differential metabolite signatures, including lipid species and microbial-derived compounds such as p-cresol. Similarly, it was also reported that baseline microbiome structure and metabolic output are strongly associated with response to biologic therapy, with higher microbial enrichment and increased levels of secondary bile acids correlating with treatment achievement [75]. Large-scale and cross-cohort analyses further confirm that CD is characterised by reproducible depletion of butyrate-producing commensals (for example *Faecalibacterium prausnitzii*) and enrichment of pro-inflammatory taxa (such as *Escherichia coli*), together with alterations in metabolic pathways related to amino acids, energy metabolism and host-microbe signalling [76]. Importantly, integrated models combining microbial and metabolomic features achieve high diagnostic accuracy, highlighting the added value of multi-omics approaches.

From a mechanistic perspective, microbial metabolites represent the key interface between microbiota and host physiology. Metabolites such as short-chain fatty acids, bile acids and tryptophan derivatives regulate immune homeostasis, epithelial integrity and inflammatory signalling [77]. Their dysregulation contributes directly to CD pathogenesis, affecting T-cell responses, barrier function and mucosal immunity. Moreover, microbiome-dependent metabolic pathways may influence therapeutic response through drug biotransformation, further supporting their relevance in precision medicine [78]. Overall, microbial factors in LB should be viewed as a multi-layered system in which cfmDNA represents only one component. The integration of microbial DNA, metabolite profiles and functional microbiome readouts provides a more comprehensive and mechanistically informative framework. Therefore, this would support a transition from a host-centred to a hybrid host-microbiome LB model, enabling improved disease stratification, monitoring and personalised therapeutic approaches.

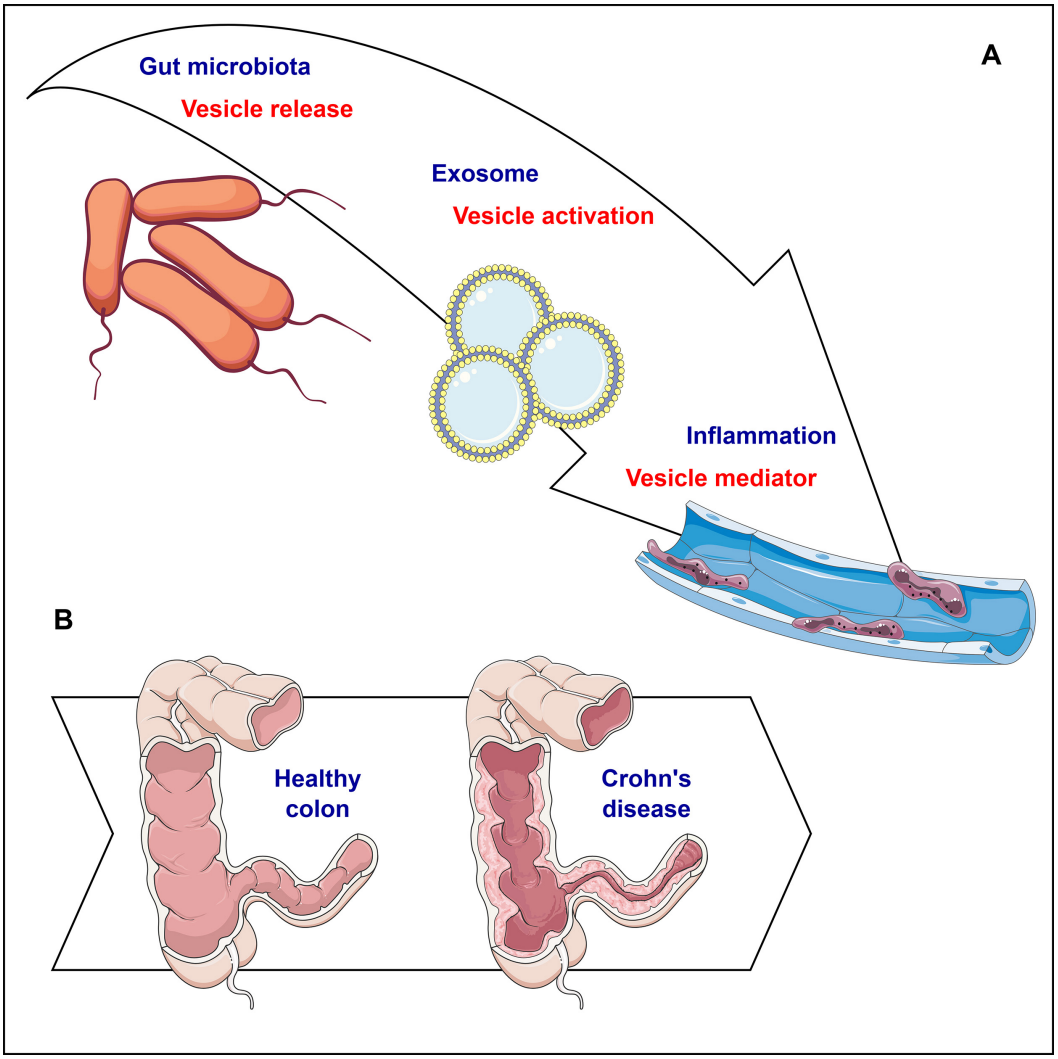


Fig. 4. The exosome–microbiota–immune axis. (A) Flowchart depicting the release of microbiota-derived vesicles and their role in modulating immune signalling. (B) This process ultimately drives the chronic inflammatory cascade characteristic of CD. The icons used in the image were obtained from Bioicons and SciDraw. CD, Crohn's disease.

Exosome-Mediated Host-Microbe Interactions

Within this multi-layered framework, exosomes and EVs emerge as pivotal biological vehicles that bridge the gap between microbial activity and host cellular responses. While metabolites diffuse as chemical signals, exosomes function as specialised cargo-delivery systems, trafficking complex molecular messages directly into the systemic circulation. Consequently, circulating exosomes represent an ideal substrate for LB, capturing real-time crosstalk at the intestinal barrier.

The gut microbiota is a key factor in the development of IBD [79] and exosomes facilitate crucial molecular interactions that contribute to the establishment of chronic inflammation. Intestinal mucosa-derived exosomes bearing high quantities of the chemokine C-C motif chemokine ligand 20 (CCL20) and prostaglandin E2 (PGE2) are released when *Bacteroides fragilis* (intestinal pathogenic bacteria) shed vesicles from the outer membrane [57]. In the intestinal mucosa of patients affected by CD in comparison to control people, invasive *Escherichia coli* strains, known as adherent-invasive *Escherichia coli* (AIEC), are more prevalent [80,81]. Human intestinal epithelial cells and macrophages have been demonstrated to release exosomes when AIEC is infected [82]. Intestinal epithelial and macrophage-naive cells can respond to exosomes by secreting more pro-inflammatory cytokines and activating NF-κB and the mitogen-activated protein kinase (MAPK) pathways, according to research on the exosomes produced by AIEC-infected cells [82]. Exosomes generated by AIEC-infected cells also increased intracellular bacterial proliferation in human intestinal epithelial cells and macrophages compared to exosomes secreted from uninfected cells [82]. This shows that exosomes have a role in the host innate immune responses being activated during AIEC infection and most likely during CD inflammation [17,82] (Fig. 4A). Therefore, exosome-mediated signalling links exosome ac-

tivity to the persistence of chronic diseases by promoting both intracellular bacterial proliferation and inflammation (Fig. 4B).

The preservation of intestinal mucosal homeostasis relies on a balanced local immune system, which must simultaneously foster tolerance toward dietary proteins and commensal microbiota while remaining ready to trigger effective defenses against enteric pathogens [83]. Exosomes immunosuppressive action is one of the numerous ways that support immunotolerance by controlling activated T-cells. In contrast, pro-inflammatory IL-8 levels rise when exosomes from patients affected by IBD are transferred to the human colon carcinoma DLD-1 cells [56]. Other mechanisms of maintaining intestinal homeostasis are regulated by circulating exosomes that are involved in communication between cells, by modulating macrophage activity [84] and annexin A1 (ANXA1). This latter is stimulated through epithelial damage and is strongly induced during the inflammatory response including in patients with CD [85]. ANXA1 is released following its incorporation in EVs deriving from epithelial cells of the intestine, and patients affected by active IBD have elevated levels of these secreted vesicles in their serum [86]. Consequently, ANXA1-containing EVs potentially serve as a biomarker for intestinal mucosal inflammation. Interestingly, exosomes produced from intestinal CD14+ macrophages in CD patients contribute to persistent immune activity by protecting CD4+ T-cells from activation-induced cell death *via* exosomal membrane TNF. Recent research has demonstrated that immune system responses in the intestine of CD patients are exacerbated by aberrant immune cell alterations and the cytokines they produce [87,88]. For example, CD14+ macrophages contribute to CD by making T CD4+ cells resistant to cell death induced by the activation itself [87]. Finally, it has been noted that epithelial cells from CD patients in the active phase overexpress IL-6, IL-8 and IL-1 [88] proving that immune cells cause persistent inflammation in CD patients. A growing number of microarray investigations of linked diseases have generated a significant amount of data because of advancements in genomic sequencing technology.

3.3 MicroRNAs as Circulating Biomarkers in Crohn's Disease

The pathophysiology of IBD is closely linked to the dysregulation of miRNAs, a class of short ncRNAs which causes inflammation, fibrosis and abnormal autophagy signalling. Since miRNAs are nucleic acids, they can be detected with great specificity and are not degraded in the extracellular matrix, which makes them useful biomarkers [89]. The option to use miRNAs as possible biomarkers is influenced by these two properties, particularly in bodily fluids like serum. Their stability in circulation is also given by their extremely short length in terms of nucleotides (20–22 bases) and, recently, it has also been found that they are delivered into circulation by exosomes [89], this contributing to their remarkable durability in systemic circulation. It has been reported that miRNAs dysregulated in IBD can exacerbate fibrosis, activate NF-κB and alter autophagy, all of which can lead to intestinal inflammation [90]. A list of miRNAs associated with IBD may provide useful insights on pathogenicity and treatment response, with correlation between IBD and miRNA expression levels being proven [91,92]. The analysis carried out by Lewis and colleagues [93] on 372 miRNAs in serum from 6 CD patients with severe illness (as determined by the Montreal criteria) and 11 patients with less severe disease, as well as 5 healthy control patients, revealed 94 miRNAs that were differentially expressed, including miR-19-3p, miR-29a-3p and miR-29c-3p.

In 2008, Wu and colleagues [94] studied the expression of miRNAs in the colonic mucosa deriving from patients affected by UC (active and inactive), chronic active CD, irritable bowel syndrome and microscopic colitis, in comparison to healthy control participants. They showed that patients with UC had different levels of miRNA expression in their mucosa when compared to the other patients [94]. They found that miR-192 was highly expressed in the epithelial cells of the colon and had the ability to inhibit the expression of macrophage inflammatory protein-2 (*MIP-2*). Wu and colleagues [94] also hypothesised that miRNAs have important roles in several IBD pathogenetic mechanisms.

The discovery of potential biomarkers indicative of therapeutic impact is a critical factor to consider in this scenario. Let-7d and let-7e have been found by Fujioka and colleagues [92] as potential therapeutic biomarkers in CD patients using anti-TNF drugs such infliximab. According to the therapeutic effects of infliximab, it was discovered that these miRNAs exhibit a similar pattern of expression. In the group of patients who used infliximab to achieve clinical remission, the levels were dramatically raised [92]. Chen and colleagues [95] described the ability of miR-200b to specifically modulate the fibrotic process stimulated by transforming growth factor (TGF)-beta 1 and how its expression was increased in the serum of CD patients with a stenosing phenotype. Additionally, inflammatory-responsive miRNAs, specifically miR-146, miR-155 and miR-132, serve as key molecular markers of active inflammation. Furthermore, the differential expression of miR-19b, miR-106a and miR-629 between UC and active CD highlights their high potential as diagnostic biomarkers for distinguishing these disease subtypes [47].

Rather than relying on single marker, miRNA profiling in CD has revealed reproducible multi-miRNA signatures associated with specific clinical phenotypes. For instance, serum-based analyses have shown that panels including miR-125b, miR-25 and miR-486 can discriminate CD patients from controls with high diagnostic accuracy, with reported AUC values approaching 0.886 for miR-125b

and 0.945 for miR-486, highlighting the advantage of multiplex profiling over individual markers [96]. This principle is further strengthened in the context of EVs. High-throughput sequencing of serum-derived exosomes in CD has identified up to 176 distinct miRNAs, with 55 significantly downregulated in stricturing patients compared to healthy controls, demonstrating that disease-associated changes involve coordinated shifts in miRNA networks rather than isolated alterations [97]. Among these, EV-miRNA profiling has revealed clinically actionable signatures. Notably, miR-205-5p levels in serum exosomes are reduced, with respect to the healthy controls, in stricturing CD and receiver operating characteristic (ROC) analysis indicates a moderate diagnostic performance (AUC ~0.8) for identifying fibrotic complications. Consequently, these findings demonstrate that EV profiling can effectively track disease-specific molecular pathways associated with tissue remodelling [97].

Importantly, this profiling study also highlighted that EV-miRNA signatures reflect coordinated targeting of biological pathways. In stricturing disease, multiple downregulated miRNAs are predicted to converge on fibrogenic regulators such as zinc finger E-box-binding homeobox 1 (*ZEB1*) and collagen-related genes (for example collagen type I alpha 1 chain, *COL1A1*), supporting the concept that network-level dysregulation, rather than single-miRNA effects, underlies fibrotic progression [97]. Another key insight emerging from EV-miRNA profiling is its ability to provide indirect cellular information. Reduced circulating exosomal miR-205-5p levels correlate with decreased expression of the same miRNA in peripheral blood mononuclear cells, indicating that EV cargo mirrors intracellular regulatory states and can be used to infer immune cell contributions without direct tissue sampling [97]. At a broader level, comparative profiling across studies suggests that distinct EV-miRNA signatures can stratify disease phenotypes, including inflammatory *vs* fibrotic behaviour, and may complement other LB components such as ccfDNA. However, despite these promising results, variability in vesicle isolation techniques and sequencing pipelines remains a major limitation, underscoring the need for standardised workflows to enable reproducible, clinically applicable miRNA profiling [96,97].

In CD, exosomal cargo can also monitor vascular pathology, in fact, it has been reported that the function of human umbilical vein endothelial cells (HUVECs) is inhibited by circulating exosomal miR-144-3p levels (targeting fibronectin 1, FN1), which are markedly higher in active CD patients [98]. The genetic characterisation of these exosomal components enables LB to concurrently track various aspects of CD pathology, including vascular damage, fibrosis and inflammation, then illustrating the complexity of such disorder. Within this framework, exosomal miRNAs act as key mediators of intercellular communication and represent a circulating extension of gene regulatory networks. Their expression profiles reflect transcriptional reprogramming associated with inflammation, immune activation and fibrosis. This highlights how circulating molecular signatures can directly link underlying genetic and regulatory mechanisms to clinically relevant disease phenotypes.

4. The Identification and Evaluation of Novel Biomarkers

Circulating Immune and Inflammatory Biomarkers in Crohn's Disease

Three serological indicators deriving from 13 different studies were evaluated by a meta-analysis in 2021: anti-*Saccharomyces cerevisiae* antibodies (ASCA), anti-flagellin (CBir1) antibodies and CRP [99]. A prognostic relationship for ASCA and anti-CBir1 was observed. Patients positive for ASCA had considerably greater odds of having severe illness (OR 2.29, 95% CI 1.31–3.99) and hazard ratio analysis revealed a significant correlation between anti-CBir1 and severe CD. Proteasome 20S subunit alpha 7 (PSMA7) and pregnancy zone protein (PZP) are further novel indicators currently under consideration. In a 2017 study, Zheng and colleagues [100] reported that UC and CD patients, in comparison to healthy individuals, showed significantly different salivary exosomal levels of PSMA7, while Shao and colleagues [101] reported higher exosomal PZP levels in serum of IBD patients. Table 1 (Ref. [7,53,66,92,94,99,100,101,102,103,104,105,106,107]) summarises the biomarkers that might be relevant for IBD patients.

Together with these serological indicators, additional layers of immune-related biomarkers have emerged, particularly those reflecting neutrophil-driven inflammatory mechanisms. Activated neutrophils contribute to inflammation through NETosis, the active extrusion of chromatin and proteins to form NETs, web-like structures consisting of nuclear DNA and proteins (Fig. 3C). Through the employment of LB circulating NET markers have been identified as promising diagnostic and predictive biomarkers for IBD [103]. Specifically, individuals with active IBD showed significantly higher amounts of circulating markers reflecting this process, such as citrullinated histone H3 (CitH3), ccfDNA and myeloperoxidase (MPO)-DNA complexes, than patients with inactive IBD and healthy controls ($p < 0.001$) [103]. These NETs indicators propose a precise assessment of acute systemic inflammation, showing a positive correlation with traditional inflammatory cytokines like CRP and IL-6 [103].

Beyond neutrophil-derived signals, other circulating molecules involved in metabolic and immunological regulation further expand the biomarker landscape of IBD. Studies are currently confirming the validity of soluble protein indicators, in addition to conventional cytokines [104,105]. IBD patients have considerably higher levels of chemerin, an adipokine associated with inflammation

Table 1. Summary of biomarkers in inflammatory bowel disease patients.

Biomarker	Type	Clinical utility	Recent findings/implications	Reference
ASCA, anti-CBir1	Antibody	Prognostic marker for severe CD	Associated with increased odds of severe illness (ASCA) or significant correlation with severe CD (anti-CBir1)	[99]
CRP	Protein	General inflammation index	Not completely specific, CRP shows conflicting data in predicting post-operative recurrence or relapse	[7,102]
PSMA7, PZP	Protein	Diagnostic and prognostic validity	Elevated in CD/UC (PZP exosomal levels significantly greater in IBD patients)	[100,101]
miRNAs (miR-192, let-7d/e)	ncRNA	Diagnosis, differentiation (CD *vs* UC) and therapeutic response (anti-TNF)	Dysregulated in IBD, let-7d/e raised in patients achieving clinical remission with infliximab	[92,94]
ccfDNA fragmentomics	Nucleic acid	Early diagnosis, disease monitoring and precision therapy guidance	Qualitative analysis of non-mutational signatures (size, methylation) to improve specificity, ccfDNA is a potential driver of inflammation	[53]
NET markers (MPO-DNA, CitH3)	Protein, DNA complex	Diagnostic and prognostic in active IBD	Significantly elevated in active IBD group, positively correlated with conventional inflammatory indices (CRP, IL-6)	[103]
Chemerin	Adipokine	Diagnosis and monitoring of IBD activity	Significantly higher in active IBD patients *vs* non-active, supporting potential as a non-invasive biomarker	[104]
LRG (leucine-rich alpha-2 glycoprotein)	Protein	Monitoring of IBD activity	Systematic review supports its usefulness in monitoring IBD	[105]
Lymphocyte subpopulations (CD4+, CD8+, Tm cells)	Cellular, immune	Prognostic for disease progression and predictive for response to biologics	Prognostic capacity demonstrated, treatment affects frequency/function of memory T-cell subsets	[106,107]
Pharmacoepigenetics (ncRNAs, DNA methylation)	Epigenetic	Predictive biomarker for treatment response	Emerging field exploring epigenetic profiles to guide personalised use of biologics and small molecules	[66]

LB, liquid biopsy; ASCA, anti-Saccharomyces cerevisiae antibody; CBir1, flagellin; CRP, C-reactive protein; PSMA7, proteasome 20S subunit alpha 7; PZP, pregnancy zone protein; miRNA, microRNA; ccfDNA, circulating cell-free DNA; NET, neutrophil extracellular trap; MPO, myeloperoxidase; CitH3, citrullinated histone H3; LRG, leucine-rich alpha-2 glycoprotein; Tm, memory T; ncRNA, non-coding RNA; CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; TNF, tumor necrosis factor.

and immunological modulation, than healthy controls (standardised mean difference, SMD: 0.61, 95% CI: 0.46–0.76, $p < 0.001$) [104] and, more importantly, individuals with active IBD had considerably greater levels of chemerin than those in a non-active state (SMD: 0.36, 95% CI: 0.15–0.57, $p < 0.001$), indicating that chemerin has a great deal of promise as a non-invasive biomarker for CD activity monitoring and diagnosis [104]. Additionally, in a 2025, the value of leucine-rich alpha-2 glycoprotein (LRG) as a new serum biomarker for tracking IBD activity was also confirmed [105].

In parallel with soluble protein mediators, alterations in lymphocyte populations provide yet another dimension of insight into disease activity and therapeutic responses. It has been recently reported that several T-cell subpopulations, such as CD4+ and CD8+ T-cells could also predict the course of IBD [107] with the frequency and function of memory T (Tm) cell subsets having greatly impacted by anti-inflammatory or immunosuppressive drug treatment. In this perspective, one novel therapeutic approach for the treatment of CD is the pursuit of a better knowledge of how these drugs alter circulating Tm cell responses [106,107].

5. Clinical Applications in Diagnosis, Prognosis and Monitoring

5.1 Diagnosis and Differential Diagnosis Using Liquid Biopsy (CD vs UC)

One of the principal utilities of LB within the context of IBD is the achievement of an early, accurate and non-invasive diagnosis. Since the distinction is essential for individualised therapy, the first step in managing IBD involves not only verifying the existence of chronic inflammation, but also correctly subtyping the illness as CD, UC or IBD-unclassified (IBDU) [108]. The pathophysiology of CD is closely related to the release of ccfDNA fragments following intestinal epithelial cell death, damage and high cellular turnover linked to gut inflammation. This

Table 2. The comparative use of well-established and novel non-invasive Crohn's disease markers.

Marker type	Example marker	Clinical utility	Advantage-limitation (CD specific)	Reference
Faecal (established)	Faecal calprotectin (FC)	Activity monitoring, rule-out IBD	High specificity for gastrointestinal inflammation and reduced sensitivity in small bowel (L1) CD	[7,111,112]
Systemic (traditional)	C-reactive protein (CRP)	Acute inflammation, monitoring	Widely available, low cost, low specificity and poor relapse prediction alone	[7,102]
Circulating (LB)	ccfDNA (microbial/host)	Diagnosis, disease activity index correlation	High sensitivity in diagnosing active IBD and specificity challenges in systemic inflammation	[53,65]

FC, faecal calprotectin; CRP, C-reactive protein; LB, liquid biopsy; CD, Crohn's disease; IBD, inflammatory bowel disease; ccfDNA, circulating cell-free DNA.

leads to a greater translocation of host and microbial ccfDNAs into circulation directly reflecting the severity and activity of the underlying disease [53]. Therefore, ccfDNA testings have shown remarkable diagnostic efficacy, particularly those evaluating microbial ccfDNA presence in plasma. The ccfDNA had high sensitivity and specificity for differentiating between asymptomatic people and active IBD patients and, because of its high sensitivity, it may be used as a first-line non-invasive triage method to identify patients who need invasive endoscopic examination in the future. More importantly, work is underway to develop assays for measuring cfmDNA in plasma. These tests look for biomarkers of microbial species and use classification algorithms to accurately discriminate CD from UC [109] since each subtype is linked to distinct patterns of inflammation and microbial dysbiosis.

As mentioned before, the stability of circulating exosomal miRNAs (Fig. 3B) in biological fluids makes them especially promising [110] and, interestingly, it has been reported that patients with active CD had considerably higher levels of circulating exosomal miR-144-3p than controls [98]. It may be possible to identify disease subtypes based on distinct biological pathways by analysing panels of circulating miRNAs, such as those that are regularly increased in active disease (miR-21, miR-126 and miR-223 in UC) [110]. Several miRNAs (such as miR-19b, miR-106a and miR-629) may be used to differentiate CD from UC. Since miRNAs can express themselves differently during active and inactive disease, some authors have specifically proposed that miRNAs could be novel biomarkers in CD, useful for both diagnosis and monitoring [47].

5.2 Monitoring Disease Activity and Therapeutic Response

The most popular non-invasive gut sampling indicator for routine monitoring is FC, a protein secreted by activated neutrophils [7]. When it is applied in the identification of active *vs* remission periods of CD, FC shows a good correlation with disease activity [111]. However, FC levels frequently remain higher even in individuals whose Crohn's disease activity index (CDAI) indicates remission, then becoming a powerful indicator of persistent endoscopically detectable histological inflammation [112] (Table 2, Ref. [7,53,65,102,111,112]).

Beyond faecal markers, additional circulating indicators provide complementary information on systemic inflammatory processes that FC alone cannot capture. FC has a higher predictive power in UC than in CD [113] and, similarly, in patients with endoscopically and clinically inactive disease, CRP by itself is not very good at predicting relapse [102]. Given CD variety and intricate pathophysiology, it is likely that a panel of biomarkers, rather than just one, is needed to reliably differentiate between disease states and estimate evolution [113]. When choosing the best biological or small molecule drug for the treatment of IBD, predictive biomarkers are essential [114]. Circulating LB biomarkers such as ccfDNA and selected protein panels have a complementary role to FC, with ccfDNA offers better insight into systemic inflammation and the severity of L1 disease where luminal markers fall short [7]. It has been demonstrated that in both IBD patients and murine models of colitis, the amount of total ccfDNA and, more especially, EV-derived ccfDNA correlates well with disease activity index (DAI) [65], in contrast to simple inflammatory markers (CRP or FC) (Table 2). Monitoring ccfDNA release offers a direct and non-invasive readout of the cellular stress within the inflammatory process since it is linked to increased cell turnover, genomic instability and apoptosis, indicating disease activity and development [65].

Markers from LB are being assessed to predict how well specific treatments would work. Several circulating indicators, including transmembrane TNF-alpha-bearing lymphocytes, CD4+ and CD8+ T-cells and plasma cells, have been found to have the ability to predict response to anti-TNF-alpha drugs [107]. The response to integrin inhibitors such as vedolizumab may also be predicted by Tm cells, CD4+ T-cells and naïve B cells [106,107].

In addition to improving diagnostic and disease-monitoring capabilities, circulating biomarkers are increasingly being investigated for their ability to predict therapeutic response. Among the potential clinical applications of advanced LB in CD, the most established is its capacity to predict disease progression and stratify patients according to their risk of relapse, thereby supporting more personalised treatment strategies. Expanding on this growing interest in personalised therapy, emerging pharmacoepigenetic approaches further integrate molecular signatures to

refine treatment stratification. The novel field of pharmacoepigenetics is focusing on using these epigenetic signatures in circulating blood as predictive biomarkers for treatment response in IBD, since environmental factors and epigenetic modifications (such as DNA methylation and ncRNAs) are critical in CD. By analysing molecular information that guides decisions about drug dosage or class change, this strategy seeks to assist in overcoming non-response effects [66].

5.3 Future Directions: Multi-Omics Integration and Precision Medicine

Moving LB from promise to utility requires advanced technology, including new miniaturising and microfluidic technologies for high-throughput sample analysis [115]. Strong computational methods are essential to manage the complexity of molecular signatures, such as ccfDNA fragmentomics or multi-protein panels [53]. Machine learning (ML) algorithms are vital for pattern identification, converting data into clinical recommendations and improving diagnostic accuracy, as observed in IBD subtyping [116]. Building on these technological and computational advances, their practical relevance becomes even clearer when considering how LB workflows enable the analysis of circulating nucleic acids for clinical decision-making. The analysis of circulating nucleic acids *via* the LB workflow is vital for developing customised and precision medical therapies [53]. Essentially, the LB workflow generates a continuous loop going between molecular diagnosis and the monitoring of targeted therapies [53]. Despite rapid advancement, large prospective clinical trials are necessary for full validation before routine adoption.

Beyond these workflow considerations, the integration of multi-omics methodologies represents a critical step toward fully exploiting the potential of liquid biopsy in CD. While individual circulating biomarkers such as ccfDNA and EV-miRNAs provide valuable yet partial insights, their combined interpretation within multi-layered analytical frameworks enables a systems-level characterisation of disease biology. Multi-omics approaches integrating genomic (ccfDNA), transcriptomic (miRNA), proteomic and microbiome-derived signals allow the identification of biologically distinct patient subgroups and uncover pathway-level dysregulation that cannot be captured by single-marker strategies alone [117]. In this integrated context, converging evidence demonstrates that key inflammatory and fibrogenic pathways such as TNF-alpha, IL-17 and IL-23 signalling, alongside janus kinase-signal transducer and activator of transcription (JAK/STAT) activation, operate across multiple biological layers, while parallel alterations in microbiome composition and host metabolism further shape disease progression and recurrence risk [118]. These findings reinforce the concept of CD as a multi-dimensional network disorder, requiring coordinated biomarker interrogation. Data published in 2025 from the Study of a Prospective Adult Research Cohort with IBD (SPARC IBD) reported that multi-omics signals were highly effective in differentiating between inflammatory profiles, demonstrating the potential for stratifying patients based on their molecular phenotype [119]. The future direction involves moving beyond single pathogenic components to comprehensive integrated multi-omics profiles that systematically combine data flows like genomics (ccfDNA), transcriptomics (miRNAs), proteomics and microbiome analysis [120], with the final aim to create a full IBD interactome (Fig. 5A). By integrating multiple layers of biological information, multi-omics approaches enable the identification of complex interactions between genetic susceptibility, gene expression and environmental factors. This systems-level perspective is essential for understanding disease heterogeneity and for defining molecular endotypes in CD.

Expanding from these multi-layered molecular insights, advanced computational approaches become indispensable for translating complex datasets into clinically meaningful predictions. This multi-omics strategy, coupled with longitudinal data, represents the technical base necessary for the reliable stratifying of patients into molecular subgroups, which is essential for achieving effective precision medicine in CD [120]. To analyse these complex datasets, select the optimal marker combinations and convert multi-omics profiles into reliable and customised predictive models for improved CD monitoring, artificial intelligence (AI) and ML are considered crucial [121]. However, managing these complex datasets requires multidisciplinary teamwork and sophisticated in silico methods (Fig. 5B) [120] and, integrating AI and ML into the analysis can reveal complex biomarker patterns that traditional statistics miss, leading to better therapeutic guidance [120] (Fig. 5C). AI and ML are therefore essential to translate this molecular complexity into clinically actionable models. By integrating heterogeneous datasets, including LB components, imaging, histology and clinical variables, AI-driven approaches can identify non-linear relationships and predictive patterns that are not accessible through conventional statistical methods. Notably, integrative models in IBD have already achieved predictive performances with AUC values in the range of 0.77–0.85 for treatment response, supporting their potential role in guiding therapeutic stratification [117]. Within this framework, LB assumes a central role as a minimally invasive and dynamic data source, linking systemic molecular signals to tissue-level processes. The convergence of circulating biomarkers, multi-omics profiling and AI-driven integration thus represents a paradigm shift from descriptive biomarker identification toward predictive, mechanism-based precision medicine. However, despite these promising advances, standardisation of pre-analytical workflows, harmonisation of multi-omics pipelines and prospective validation in large-scale cohorts remain essential prerequisites for clinical translation.

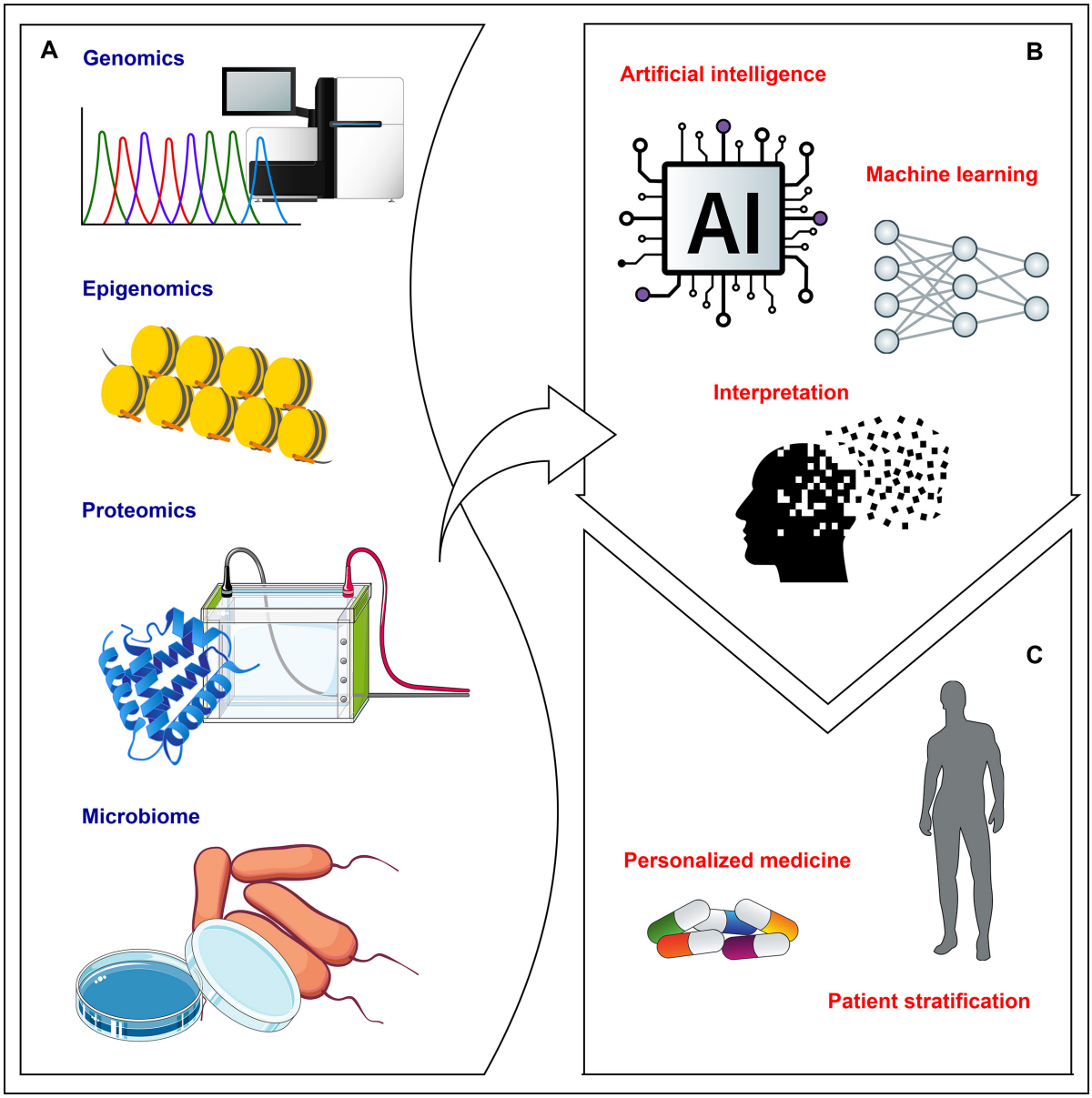


Fig. 5. Multi-omics integration and AI/ML framework. (A) A comprehensive framework integrating genomic, epigenomic, proteomic and microbiomic data. (B) These layers are processed through artificial intelligence and machine learning algorithms. (C) This flow enables robust patient stratification and personalised precision medicine strategies. The icons used in the image were obtained from Bioicons and SciDraw. AI, artificial intelligence.

Finally, to sustain the integration of AI-driven analytics, further technological innovation is required, particularly in automating and scaling sample-processing steps critical for LB applications. The current bottleneck in sample preparation must be solved by technological roadmaps prioritising high-throughput automation. In this perspective, the rapid development in microfluidic technologies is key to efficiently isolating analytes like ccfDNA and exosomes [122,123]. Future platforms must also address single-exosome analysis to manage biological heterogeneity [124], thereby enabling the rigorous and repetitive sample collection needed for prospective multi-omics cohort studies.

6. Challenges and Future Perspectives in the Clinical Translation of Liquid Biopsy for Crohn's Disease

By advancing diagnostics beyond the use of invasive endoscopy and static biomarkers, the emerging area of LB has revolutionary promise for the non-invasive treatment of CD. However, achieving this promise necessitates overcoming several challenging translational barriers, including clinical validation, technical standardisation and economic integration.

First, the core impediment to translate LB into routine CD management is the general lack of standardisation

Table 3. Technical and analytical hurdles in liquid biopsy for Crohn's disease.

Target analyte	Technical challenge	Translational impact	Required advancement	Reference
ccfDNA (circulating, mitochondrial)	Pre-analytical instability (collection, tube type, storage) and loss of short fragments	Poor reproducibility and comparability across labs, increased risk of false negatives in early/mild disease	Mandatory standardisation of pre-analytical operating procedures and implementation of automated and closed-loop extraction systems	[125]
Extracellular vesicles (exosomes)	High heterogeneity, non-standardised and low-throughput isolation (ultracentrifugation)	Difficulty in achieving high purity, limited scalability for large validation cohorts and compromised quantification	Adoption of high-throughput microfluidics or chromatography and mandatory adherence to MISEV guidelines	[122,124,126]
Multi-omics data	Significant analytical and computational batch effects	Skewed diagnostic performance and inability to distinguish technical noise from actual biological variance	Implementation of advanced computational correction algorithms and collaborative data sharing	[127]

ccfDNA, circulating cell-free DNA; MISEV, Minimal Information for Studies of Extracellular Vesicles.

and reproducibility across the entire pipeline [125]. In the pre-analytical steps non-standardised procedures for handling ccfDNA, including variations in collection tubes, timing and storage, directly impact sample quality and quantity [125]. This technical noise tends to mask the already low biological signal present in early or residual disease, undermining the sensitivity needed for clinical utility [54,125] (Table 3, Ref. [122,124,125,126,127]).

For the preparation of EV/exosomes, technical obstacles emphasise the issue, since isolation methods like ultracentrifugation are not suitable for high-throughput clinical use [122] and a universally accepted scalable protocol is lacking [126]. Furthermore, the inherent biological heterogeneity of EVs complicates the establishment of universal diagnostic criteria and biomarker validation [124] (Table 3). Finally, large-scale omics studies are prone to analytical and computational "batch effects" that necessitate accurate statistical correction (for example, ComBat) to prevent technical noise from being erroneously interpreted as real biological variance, thereby protecting the integrity of biomarker validation [127].

Once these methodological issues are acknowledged, the next challenge lies in determining whether such biomarkers can achieve clinically meaningful performance in real-world patient populations. Beyond technical reproducibility, the successful clinical translation of LB biomarkers relies on demonstrating robust performance metrics (sensitivity and specificity) within heterogeneous patient cohorts [128] (Table 4, Ref. [120,129,130,131]).

Since the greatest clinical value of LB is prediction (for example, forecasting recurrence or anti-TNF response) [128], biomarkers must be validated in large, multi-centre, prospectively and repetitively collected datasets to establish causal inference and high predictive value [120]. Furthermore, CD requires biomarkers to perform accurately diagnosis across diverse clinical endpoints, such as achieving mucosal healing or predicting complications. This necessitates a shift toward developing biomarkers that accurately reflect the unique pathophysiology of CD, requiring a fundamental and costly shift toward rigorous, longitudinal trial designs [53,120].

After evaluating both technical and clinical constraints, it becomes equally important to consider the economic and regulatory realities that ultimately determine whether these tools can enter routine practice. Even technically and clinically robust LB assays face major obstacles regarding health economics and regulatory acceptance [130,131,132] (Table 4). Given the significant economic burden of IBD, estimated in the US alone to exceed $6.8 billion annually [132], novel LB tools must demonstrate compelling cost savings or improved quality-adjusted life years (QALYs) gained [133]. While LB has proven cost-effective in oncology for treatment selection [130], in CD economic sustainability depends on preventing costly and unnecessary interventions. For colorectal cancer screening, health economic models show that although LB increases life-years gained, the incremental cost-effectiveness ratios (ICERs) can sometimes exceed the US willingness-to-pay (WTP) threshold [131]. Therefore, LB economic value lies not in primary diagnosis, but in enabling therapeutic precision, accurately predicting non-response to biologics or post-operative recurrence [128,130]. The major regulatory barrier is that no biomarker has yet been formally qualified by the Food and Drug Administration (FDA) biomarker qualification program (BQP) for IBD clinical trial use [129] (Table 4). However, the FDA has encouraged the study of non-invasive blood-based markers (for example the calprotectin fragment CPa9-HNE) as enrichment tools in clinical trials, suggesting that validated prognostic markers could accelerate drug development [129].

From an economic and utility standpoint, current IBD diagnostic tools present contrasting profiles. Conventional

Table 4. Clinical and economic barriers to liquid biopsy integration in inflammatory bowel diseases.

Hurdle category	Specific barrier	Consequence/Limitation	Required solution	Reference
Clinical validation	Reliance on cross-sectional or retrospective patient data	Inability to establish causal inference or generate robust predictive models for relapse/response	Mandatory shift to large-scale, multi-center and longitudinal cohort studies	[120]
Regulatory pathway	Absence of FDA BQP-qualified IBD biomarkers and lack of consensus on gold-standard clinical endpoints	Slow translation and uncertainty for drug development, validation studies yielding ambiguous results	Formal validation and consensus on primary endpoints (standardised mucosal healing assessment)	[129]
Cost-effectiveness	High testing costs resulting in ICERs often exceeding WTP thresholds for screening	Limited reimbursement potential, failure to demonstrate economic value compared to existing cheap diagnostics	Focus LB on high-value applications such as treatment selection, monitoring therapeutic drug management and preventing expensive complications	[130,131]

FDA, Food and Drug Administration; BQP, biomarker qualification program; IBD, inflammatory bowel disease; ICER, incremental cost-effectiveness ratios; WTP, willingness-to-pay; LB, liquid biopsy.

biomarkers like FC and CRP are highly accessible for routine monitoring, with typical costs under €50, but lack disease specificity. Conversely, endoscopy with biopsy, the gold standard, carries significant patient burden and substantial costs, often exceeding €1000, precluding frequent longitudinal use [134]. LB frameworks currently require higher upfront investments due to sequencing and bioinformatics demands, often ranging from several hundred to over €1000 per test [135]. However, their economic value is expected to emerge in high-stake scenarios, such as predicting therapeutic response, where precise patient stratification can ultimately offset these initial technological costs [134,135].

7. Conclusions

For patients with CD, LB has the potential to be a fundamental tool for reliable diagnosis and non-invasive effective monitoring. Its clinical value exceeds the simple identification of inflammation and moves toward complex molecular stratification, addressing the limitations of the existing gold standard ileocolonoscopy [6].

Traditional tools such as FC are limited in patients with CD restricted to the small bowel (L1). The advantage of novel developments in ccfDNA fragmentomics as well as the discovery of NET markers, such as MPO-DNA and CitH3, are that they offer a more specific indicator of inflammatory activity. Furthermore, markers can also aid with vascular pathogenesis (exosomal miRNAs) [98] and disease severity (ccfDNA) [65]. The ccfDNA demonstrates good sensitivity and specificity [109] for differentiating between the types of IBD as well as whether the patient is likely to be symptomatic.

LB achieves strong clinical utility in prognostic assessment and therapeutic decision-making. It surpasses traditional markers such as FC and CRP in predicting short-term and mid-/long-term relapse [136]. This capability enables specific risk stratification and guides therapy reduction. In the future, it could support risk stratification and, through multi-omics modelling, generate algorithmic management plans for patients with IBD.

Abbreviations

ADAM17, a disintegrin and metalloproteinase 17; AGA, American Gastroenterological Association; AI, artificial intelligence; AIEC, adherent-invasive Escherichia coli; ANXA1, annexin A1; ASCA, anti-Saccharomyces cerevisiae antibody; ATG16L1, autophagy-related 16-like 1; AUC, area under the curve; BQP, biomarker qualification program; CARD9, caspase recruitment domain family member 9; CBir1, flagellin; ccfDNA, circulating cell-free DNA; ccfRNA, circulating cell-free RNA; CCL20, C-C motif chemokine ligand 20; CD, Crohn's disease; CDAI, Crohn's disease activity index; cfmDNA, circulating microbial cell-free DNA; cGAS, cyclic GMP-AMP synthase; CitH3, citrullinated histone H3; CPa9-HNE, calprotectin fragment; CRP, C-reactive protein; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; DAI, disease activity index; DAMP, damage-associated molecular pattern; DSS, dextran sulphate sodium; eccDNA, extrachromosomal circular DNA; EGR1, early growth response factor 1; EPIC-CD, Epigenetic Predictors of Biologic Therapy Response in Crohn's Disease; ER, endoplasmic reticulum; EV, extracellular vesicle; FC, faecal calprotectin; FDA, Food and Drug Administration; FN1, fibronectin 1; GWAS, genome-wide association study; HDAC, histone deacetylase; HUVEC, human umbilical vein endothelial cell; H3K27, histone H3 lysine 27; IBD, inflammatory bowel disease; IBDU, IBD-unclassified; ICER, incremental cost-effectiveness ratio; IFIH1, interferon induced with helicase C domain 1; IFN, interferon; IKZF1, ikaros family zinc finger 1;

IL, interleukin; IL18RAP, IL18 receptor accessory protein; IRGM, immunity-related GTPase M; JAK, janus kinase; JAK-STAT, janus kinase-signal transducer and activator of transcription; LB, liquid biopsy; LRG, leucine-rich alpha-2 glycoprotein; LRRK2, leucine rich repeat kinase 2; MAPK, mitogen-activated protein kinase; MBD2, methyl-CpG binding domain protein 2; MDP, muramyl dipeptide; MIP-2, macrophage inflammatory protein 2; miRNA, microRNA; MISEV, Minimal Information for Studies of Extracellular Vesicles; ML, machine learning; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; MUC2, mucin 2; ncRNA, non-coding RNA; NET, neutrophil extracellular trap; NF-κB, nuclear factor-kappa B; NKX2-3, nk2 homeobox 3; NOD2, nucleotide-binding oligomerization domain 2; PGE2, prostaglandin E2; PRDM1, pr/set domain 1; COL1A1, collagen type I alpha 1 chain; PSMA7, proteasome 20S subunit alpha 7; PZP, pregnancy zone protein; QALY, quality-adjusted life year; ROC, receiver operating characteristic; RORγt, RAR-related orphan receptor gamma t; SETD2, SET domain containing 2; SMAD3, smad family member 3; SMD, standardised mean difference; SPARC IBD, Study of a Prospective Adult Research Cohort with IBD; STING, stimulator of interferon response cGAMP interactor 1; TGF, transforming growth factor; Th, T helper; TJ, tight junction; TLR, toll-like receptor; Tm, memory T; TNF, tumor necrosis factor; T2T, treat-to-target; UC, ulcerative colitis; UHRF1, ubiquitin like with PHD and ring finger domains 1; WTP, willingness-to-pay; ZEB1, zinc finger E-box-binding homeobox 1.

Author Contributions

GP, FS and PP conceived and designed the study. GF, CQ, FE, and KS conducted an extensive and systematic review of the relevant literature. FS and PP provided critical guidance and expert input on the key topics addressed in the manuscript. All authors contributed substantially to the drafting and development of the manuscript. Specifically: GF, GP, KS and FS authored sections pertaining to the clinical management and pathological features of Crohn's disease; CQ was responsible for the sections focused on extracellular vesicles; FE and PP prepared the content related to liquid biopsy and circulating nucleic acids. All authors participated in the editorial revision process, ensuring accuracy and clarity throughout the manuscript. All authors have read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the drafting of this manuscript, the authors utilised Gemini and Copilot 365 solely for language editing and proofreading purposes. All AI-assisted suggestions were critically evaluated and revised by the authors, who remain fully responsible for the scientific content and conclusions of this study.

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