

**Reviewer #1:**

**Comment:** Would you be able to provide us with some information that would assist us in determining whether or not the examination of the biomarker is practical, as well as how beneficial it would be in our clinical practice on a daily basis? This is of particular significance in situations in which we find ourselves confronted by a circumstance that is challenging to recognize, even in instances where the symptoms and indicators have already made their presence known. Please be sure to include these particulars in your evaluation. These biomarkers are far less invasive as compared to the standard colonoscopy procedure, which is used for the purpose of diagnosing colorectal adenoma. Even if this biomarker is essentially a non-invasive method, the following issue still has not been answered: in the unlikely case that the biomarker is able to make a prediction regarding the adenoma based on the results of the biomarker itself, what actions do you plan to take? In this particular situation, is it still considered necessary to perform a colonoscopy?

**Response:** We sincerely thank the reviewer for this insightful comment. It highlights an essential gap between biomarker discovery and the realities of clinical decision-making. In the revised manuscript, we have expanded our discussion to clarify: (1) the feasibility and clinical utility of blood-based biomarkers in routine practice, (2) the specific contexts in which they provide advantages over current methods, especially in diagnostically ambiguous situations, and (3) the subsequent management pathway following a positive biomarker result. The revised text is now presented as follows:

**Location: Background Section**

“In daily clinical practice, physicians frequently encounter patients who present with vague or nonspecific gastrointestinal symptoms—such as abdominal discomfort or altered bowel habits—that are difficult to interpret definitively. In such instances, FIT may yield false-negative results due to the intermittent or absent bleeding of many precancerous adenomas, leading to a diagnostic dilemma. Unlike stool-based assays that rely on intermittent hemoglobin shedding, blood biomarkers reflect dynamic molecular signals associated with tumor cell turnover and tumor – microenvironment interactions <sup>[1]</sup>. This capability renders blood testing a valuable tool for objective risk stratification, enabling the identification of high-risk individuals who might otherwise be overlooked due to the absence of overt signs or bleeding.”

Regarding the question of clinical management after a positive biomarker test, we fully agree that non-invasive biomarkers must be viewed as a complement to colonoscopy rather than a replacement. First, we now explicitly emphasize that blood-based biomarkers are not intended to substitute colonoscopy, which remains the diagnostic gold standard. Instead, their role lies in identifying high-risk individuals who would benefit most from timely endoscopic evaluation, especially when clinical signs are ambiguous or easily overlooked. Second, we have clarified the

downstream workflow: a positive biomarker result should function as a high-priority indication for diagnostic colonoscopy. In this framework, the biomarker serves as a “triage” tool that enriches the group of patients referred for invasive testing, thereby improving resource allocation and reducing unnecessary procedures among low-risk individuals. Third, based on these principles, we propose a two-step screening model. This framework integrates accessible blood-based risk stratification with the indispensable therapeutic role of colonoscopy, thereby enhancing early detection while preserving colonoscopy as the definitive method for confirming colorectal adenoma. The revised text has now been strengthened as follows:

Location: **Discussion Section**

“However, defining the precise clinical utility of these biomarkers is paramount. Rather than serving as standalone replacements for endoscopy, blood-based assays should be positioned as efficient “triage” or risk stratification tools. In asymptomatic populations, a positive biomarker result provides the objective biological evidence necessary to justify invasive investigation. It is crucial to clarify that biomarker integration does not eliminate the need for colonoscopy; instead, it optimizes resource allocation. Noninvasive tests can be used to identify high-risk individuals, whereas subsequent mandatory colonoscopy provides visual confirmation and enables therapeutic polypectomy. This synergistic workflow ensures that invasive resources are targeted precisely toward patients most likely to benefit from intervention.”

Location: **Final Paragraph**

“In conclusion, bridging the gap between discovery and clinical implementation requires a shift toward large-scale, prospective validation and standardized analytical frameworks. Concurrently, public health education is vital for fostering acceptance. Ultimately, the goal is to establish a robust two-step screening model that combines the accessibility of blood-based risk stratification with the therapeutic indispensability of colonoscopy. Achieving this paradigm will optimize health care resource allocation and significantly mitigate the global burden of colorectal cancer through earlier, more efficient detection.”

We believe these revisions directly address the reviewer’s concerns and significantly strengthen the clinical relevance of our review.

1 Yuan F, Jia G, Wen W, Xu S, Gunchick V, Deng K, Long J, Yu D, Shu X-O, Zheng W. Blood metabolic biomarkers and colorectal cancer risk: results from large prospective cohort and Mendelian randomisation analyses. *Br J Cancer* 2025; **133**: 94–103. [DOI: 10.1038/s41416-025-02997-4]

**Reviewer #2:**

**Comment 1:** Please add a dedicated subsection that critically evaluates study quality and reproducibility. Explicitly discuss common limitations such as small sample sizes, retrospective

designs, lack of external validation, and inter-laboratory variability in assay methodologies. Highlighting these issues will increase the manuscript's analytical depth, transparency, and overall credibility. Discussing how these limitations might influence diagnostic accuracy or clinical applicability would also be helpful.

**Response:** We sincerely appreciate the reviewer's insightful suggestion. We fully agree that a transparent and critical appraisal of study quality and reproducibility is essential for strengthening the analytical depth and clinical relevance of biomarker research.

In response, we have now added a dedicated subsection that systematically evaluates the major methodological challenges in the field, including small sample sizes, retrospective study designs, limited external validation, and inter-laboratory variability in assay methodologies. We also explicitly discuss how these factors may influence diagnostic accuracy, generalizability, and the clinical applicability of blood-based biomarkers for colorectal adenoma detection. The revised text reads as follows:

#### “Limitations of Current Evidence and the Path Forward

Despite growing interest in blood-based biomarkers for colorectal adenoma, current evidence remains constrained by major methodological flaws and limited reproducibility. Most discovery-phase studies across genomic, proteomic and metabolomic platforms rely on small, retrospective, single-center cohorts, introducing spectrum bias and yielding inflated AUCs with poor external validity. Heterogeneous inclusion criteria, inconsistent lesion definitions and assay variability further hinder cross-study comparability, while asymptomatic screening populations remain markedly underrepresented. Moreover, performance estimates are frequently distorted by incomplete assessment of the full lesion spectrum; for example, a 2024 model integrating methylation, fragmentomic features and copy number variation (CNV) showed excellent accuracy for CRC but did not assess advanced adenomas, an omission that limits its relevance for true screening settings <sup>[1]</sup>. More broadly, even assays reporting strong overall diagnostic performance consistently demonstrate low sensitivity for advanced precursor lesions, underscoring that insufficient evaluation of key premalignant states remains the central barrier to clinical deployment. Together, these gaps in disease-spectrum evaluation, compounded by substantial methodological heterogeneity—from preanalytical handling and EV isolation to normalization strategies and machine-learning architectures—generate pervasive batch effects across omics platforms and severely restrict reproducibility and translation.

Across omics platforms, each modality interrogates a distinct layer of adenoma biology. DNA methylation assays primarily detect stable epigenetic alterations but detect only a minority of advanced adenomas (10–30%) <sup>[2]</sup>. Circulating ncRNAs capture dynamic regulatory states, proteomics quantifies pathway-level activity, and metabolomics characterizes metabolic and microbial phenotypes. However, the use of circulating ncRNAs remains limited by

nonstandardized analytical methods and substantial preanalytical variability; moreover, proteomics is constrained by the extreme dynamic range of plasma proteins and the scarcity of screening-scale datasets, whereas metabolomics, which is informative for metabolic and microbial phenotypes, is highly susceptible to dietary, pharmacologic and circadian confounders. Therefore, these modalities offer complementary but individually incomplete biological insights, strengthening the rationale for integrated multiomics approaches.

Recognizing this need, Hui et al. [3] established a 15,000-participant multicenter prospective cohort to overcome retrospective biases and generate biomarker models with genuine population-level generalizability. In the future, substantive progress will require large prospective cohorts supported by standardized preanalytic procedures, transparent computational workflows and rigorous cross-laboratory quality control. Only with such a coordinated methodological discipline can blood-based biomarkers advance from exploratory signals to clinically reliable tools for colorectal adenoma screening.”

We believe this critical appraisal significantly enhances the analytical depth of the manuscript.

**1** Gao Y, Cao D, Li M, Zhao F, Wang P, Mei S, Song Q, Wang P, Nie Y, Zhao W, Wang S, Yan H, Wang X, Jiao Y, Liu Q. Integration of multiomics features for blood-based early detection of colorectal cancer. *Mol Cancer* 2024; **23**: 173. [DOI: 10.1186/s12943-024-01959-3]

**2** Chung DC, Gray DM, Singh H, Issaka RB, Raymond VM, Eagle C, Hu S, Chudova DI, Talasaz A, Greenson JK, Sinicrope FA, Gupta S, Grady WM. A Cell-free DNA Blood-Based Test for Colorectal Cancer Screening. *N Engl J Med* 2024; **390**: 973–983. [DOI: 10.1056/NEJMoa2304714]

**3** Hui Y-F, Song S-C, Zhu W-J, Bu S, Sun J, Ling T-S, Cheng H-B. Multicenter prospective cohort study on colorectal adenoma recurrence and malignant transformation risk based on integrative medicine: a study protocol. *Front Oncol* 2025; **15**: 1657390. [DOI: 10.3389/fonc.2025.1657390]

**Comment 2:** Please include a concise table summarizing major biomarkers or panels (with AUC, sensitivity, specificity, cohort size, and study design) so readers can quickly compare diagnostic performance across different studies. This addition would make the review more practical and informative.

**Response:** We sincerely thank the reviewer for this valuable suggestion. In response, we have thoroughly revised and expanded the tables summarizing the diagnostic performance of major biomarkers and panels for adenoma detection. Specifically, Tables 1–3 have been enriched to include detailed information on AUC, sensitivity, specificity, cohort size, and study design. Moreover, we have further completed and refined the section on metabolite-based diagnostics, now presented as Table 4, to provide a more comprehensive comparison across studies. We

believe these enhanced tables significantly improve the clarity, completeness, and practical value of the review. We are grateful for the reviewer’s constructive feedback.

**Table 4:** Diagnostic potential of metabolites for adenomas.

Reference	Study type and population	Biomarkers	Grouped control	AUC	Sensitivity	Specificity
Huang J/2025	Retrospective multi-center case-control study 219 CRC 164 CRA	<b>6 plasma metabolites</b> 3 lipids and lipid-like molecules, 2 organic oxygen compounds, 1 phenylpropanoid	CRC/CRA	0.905	84.0%	81.7%
Huang J/2025	Retrospective multi-center case-control study 164 CRA 219 NC	<b>7 plasma metabolites</b> 5 lipids and lipid-like molecules, 1 organic nitrogen compound, 1 organic acid	CRA/NC	0.997	97.0%	97.7%
Sun Y/2024	Multi-center case-control study 111 CRC 143 CRA 119 NC	<b>17 plasma metabolites</b>	CRC/CRA CRA/NC	0.928 0.968	89.0% 87.0%	88.7% 97.2%
Guo X/2024	Observational study 19 CRC 26 CRA 20 NC	<b>5 serum metabolites and 5 gut bacteria</b> <b>7 serum metabolites and 6 gut bacteria</b>	CRC/CRA CRA/NC	0.850 0.880	84.21% 75.00%	76.92% 96.15%
Zhang Y/2024	Clinical case-control study 200 CRC 160 CRA	<b>3 plasma metabolites</b> ribitol, beta-hydroxybutyric acid, lactic acid	CRC/CRA	0.960	/	/

**Comment 3:** The section on noncoding RNAs is disproportionately long. Please consider shortening it or expanding the proteomics and metabolomics sections for a more balanced coverage. A comparative paragraph summarizing the maturity, advantages, and limitations of each omics field would further improve the review’s balance and comprehensiveness.

**Response:** We sincerely appreciate the reviewer’s thoughtful comment. In response, we have carefully revised the manuscript to achieve a more balanced presentation across the different omics fields. Specifically, we have appropriately condensed the section on non-coding RNAs (details provided in the main text) and expanded both the proteomics and metabolomics sections

to provide more comprehensive coverage. In addition, we have added a new comparative paragraph summarizing the relative maturity, advantages, and limitations of each omics approach, thereby enhancing the overall balance and clarity of the review. We are grateful for the reviewer's insightful suggestions, which have significantly improved the quality of our manuscript. We have added the following content.

Location: **The Proteomics Section**

“The proteomic alterations observed in blood likely reflect the systemic host response to early neoplasia. For example, the identification of differential levels of inflammatory mediators (e.g., TGF- $\beta$  pathway proteins) in EV cargo, together with the validation of elevated fibrinogen levels in both EVs and total plasma <sup>[1,2]</sup>, suggests that adenomas, even at a precancerous stage, actively remodel the microenvironment and induce systemic immune–metabolic shifts. Understanding these pathway-level dysregulations provides not only diagnostic markers but also potential targets for chemoprevention.”

Location: **The Metabolomics Section**

“The integration of metabolomics with microbiome data is particularly relevant for CRA detection. Because the gut vascular barrier may be compromised during early tumorigenesis, microbial metabolites can be translocated into the circulation <sup>[3]</sup>. This 'gut leakage' phenomenon provides a mechanistic basis for using serum metabolomic profiles as a proxy for gut dysbiosis associated with adenomas, offering a unique advantage over tumor-derived DNA or protein markers, which may be scarce in early lesions.”

“Mechanistically, metabolic reprogramming in adenomas reflects the increased bioenergetic and biosynthetic demands of proliferating cells. The observed alterations in serum metabolites often map to dysregulated glycolysis, lipid metabolism, and the TCA cycle. For instance, the reduction in specific lipids and the accumulation of lactate precursors suggest a shift toward the 'Warburg effect' occurring as early as the adenoma stage <sup>[4,5]</sup>.”

Location: **Limitations of Current Evidence and the Path Forward**

“Across omics platforms, each modality interrogates a distinct layer of adenoma biology. DNA methylation assays primarily detect stable epigenetic alterations but detect only a minority of advanced adenomas (10–30%) <sup>[6]</sup>. Circulating ncRNAs capture dynamic regulatory states, proteomics quantifies pathway-level activity, and metabolomics characterizes metabolic and microbial phenotypes. However, the use of circulating ncRNAs remains limited by nonstandardized analytical methods and substantial preanalytical variability; moreover, proteomics is constrained by the extreme dynamic range of plasma proteins and the scarcity of screening-scale datasets, whereas metabolomics, which is informative for metabolic and microbial phenotypes, is highly susceptible to dietary, pharmacologic and circadian confounders. Therefore,

these modalities offer complementary but individually incomplete biological insights, strengthening the rationale for integrated multiomics approaches.”

We believe these changes have significantly improved the structure and balance of the manuscript, providing readers with a more unbiased perspective on the available noninvasive diagnostic strategies.

**1** Huang Z, Deng C, Ma C, He G, Tao J, Zhang L, Hu X, Mo Y, Qiu L, Zhang N, Luo C, Xing S, Xie J, Yin H. Identification and validation of the surface proteins FIBG, PDGF- $\beta$ , and TGF- $\beta$  on serum extracellular vesicles for non-invasive detection of colorectal cancer: experimental study. *International Journal of Surgery* 2024; **110**: 4672–4687. [DOI: 10.1097/JS9.0000000000001533]

**2** Wang H, Zheng H, Cao X, Meng P, Liu J, Wang Z, Zhang T, Zuo H. Relationship between fibrinogen level and advanced colorectal adenoma among inpatients: A retrospective case-control study. *Front Med* 2023; **10**: 1140185. [DOI: 10.3389/fmed.2023.1140185]

**3** Spadoni I, Zagato E, Bertocchi A, Paolinelli R, Hot E, Di Sabatino A, Caprioli F, Bottiglieri L, Oldani A, Viale G, Penna G, Dejana E, Rescigno M. A gut-vascular barrier controls the systemic dissemination of bacteria. *Science* 2015; **350**: 830–834. [DOI: 10.1126/science.aad0135]

**4** Nenkov M, Ma Y, Gaßler N, Chen Y. Metabolic Reprogramming of Colorectal Cancer Cells and the Microenvironment: Implication for Therapy. *IJMS* 2021; **22**: 6262. [DOI: 10.3390/ijms22126262]

**5** Zhang J, Zou S, Fang L. Metabolic reprogramming in colorectal cancer: regulatory networks and therapy. *Cell Biosci* 2023; **13**: 25. [DOI: 10.1186/s13578-023-00977-w]

**6** Chung DC, Gray DM, Singh H, Issaka RB, Raymond VM, Eagle C, Hu S, Chudova DI, Talasz A, Greenson JK, Sinicrope FA, Gupta S, Grady WM. A Cell-free DNA Blood-Based Test for Colorectal Cancer Screening. *N Engl J Med* 2024; **390**: 973–983. [DOI: 10.1056/NEJMoa2304714]

**Comment 4:** When discussing AI and multiomics integration, please provide one or two specific examples of applied machine learning models or prospective validation studies that demonstrate clinical feasibility and remaining barriers.

**Response:** We would like to express our sincere gratitude to the reviewer for this constructive suggestion. We agree that providing specific examples of machine learning (ML) applications and prospective validation studies is essential to demonstrate the clinical feasibility and identify remaining barriers in this field. We have added two specific examples to the conclusion to enrich the content. The specific content is as follows.

Location: **Discussion Section**

“To overcome these barriers, the next frontier of research lies in integrating multiomics data with artificial intelligence (AI). Machine learning algorithms are essential for extracting meaningful patterns from high-dimensional molecular data. A prominent example of this potential is the DELFI approach <sup>[1]</sup>, which uses machine learning to analyze genome-wide cell-free DNA fragmentation profiles. By deciphering complex, nonintuitive biological features rather than relying solely on mutations, this method achieved a sensitivity of 73%, demonstrating the power of AI in early detection.

Nevertheless, the transition from discovery to clinical application reveals persistent challenges. The prospective PATHFINDER study <sup>[2]</sup>, involving more than 6,000 participants, evaluated a novel blood-based assay that relies on machine learning algorithms to identify cancer-specific methylation patterns in cfDNA, confirming the feasibility of detection in asymptomatic individuals while also revealing critical limitations. Notably, the positive predictive value (PPV) was approximately 40%, implying a substantial burden of false positives that led to unnecessary invasive follow-ups and patient anxiety. Additionally, the “black-box” nature of deep learning models limits biological interpretability, posing a hurdle for clinician trust.”

We believe these concrete examples significantly strengthen the argumentation regarding the translational potential of these biomarkers.

**1** Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, Jensen SØ, Medina JE, Hruban C, White JR, Palsgrove DN, Niknafs N, Anagnostou V, Forde P, Naidoo J, Marrone K, Brahmer J, Woodward BD, Husain H, Van Rooijen KL, Ørntoft M-BW, Madsen AH, Van De Velde CJH, Verheij M, Cats A, Punt CJA, Vink GR, Van Grieken NCT, Koopman M, Fijneman RJA, Johansen JS, Nielsen HJ, Meijer GA, Andersen CL, Scharpf RB, Velculescu VE. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* 2019; **570**: 385–389. [DOI: 10.1038/s41586-019-1272-6]

**2** Schrag D, Beer TM, McDonnell CH, Nadauld L, Dilaveri CA, Reid R, Marinac CR, Chung KC, Lopatin M, Fung ET, Klein EA. Blood-based tests for multicancer early detection (PATHFINDER): a prospective cohort study. *The Lancet* 2023; **402**: 1251–1260. [DOI: 10.1016/S0140-6736(23)01700-2]

**Comment 5:** Please standardize gene and protein nomenclature, ensure consistent abbreviation definitions at first appearance, and conduct light language polishing to improve clarity and flow.

**Response:** We sincerely thank the reviewer for this helpful suggestion. In response, we have carefully standardized the gene and protein nomenclature throughout the manuscript and ensured that all abbreviations are consistently defined at their first appearance. Additionally, we have invited a professional and reliable language-editing service to further polish the manuscript to improve clarity and overall readability. We truly appreciate the reviewer’s constructive comments, which have helped us enhance the quality of the manuscript.