

Smoking-Driven Inflammasome-Associated Inflammation in Colorectal Cancer: A Two-Phase Discovery and Validation Study of Serum Biomarkers

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Abstract

Introduction: To identify and validate serum biomarkers of smoking-related inflammasome activation in CRC and quantify the mediating role of inflammatory markers in the smoking–CRC association. **Materials and Methods:** This two-phase retrospective study was conducted at the Oncology Teaching Hospital, Baghdad Medical City, Iraq, from April 2024 to April 2025. A total of 200 participants were enrolled and evenly divided into four groups (n=50 each): non-smoker controls, smoker controls, non-smoker colorectal cancer (CRC) patients, and smoker CRC patients. nine serum biomarkers were measured using ELISA assays, including interleukin-8 (IL-8), interleukin-37 (IL-37), C-reactive protein (CRP), adiponectin, forkhead box P3 (FoxP3), sestrin-1, galectin-1, P-selectin, and carcinoembryonic antigen (CEA). Three composite indices (Provisional Smoking-Driven Inflammasome Score - pSIS, Provisional Smoking-Attributable Risk Index - pSMRI, and Provisional Pyroptosis Activity Score - pPAS) were calculated. Statistical analyses involved mediation analysis, LASSO regression, and random forest with Boruta feature selection. **Results:** Outcomes revealed that smokers with CRC exhibited a distinct pro-inflammatory signature, characterized by significantly elevated IL-8, CRP, and galectin-1, alongside reduced IL-37 and adiponectin. Mediation analysis showed that these three markers collectively mediated 73.8% (95% CI: 52.1%–89.3%) of the smoking–CRC relationship. The three composite indices (pSIS, pSMRI, and pPAS) showed progressively increasing median values from healthy controls to smoker CRC patients ($p < 0.001$). All three indices demonstrated excellent discriminatory ability, with area under the curve (AUC) values of 0.92 (pSIS), 0.91 (pPAS), and 0.90 (pSMRI), significantly outperforming CEA (AUC: 0.85; DeLong test, $p < 0.05$). LASSO and Boruta algorithms confirmed IL-8, CRP, and galectin-1 as the most critical features. **Conclusion:** This study demonstrates that inflammatory markers (IL-8, CRP, and galectin-1) statistically mediate 73.8% of the smoking–CRC association, providing strong evidence consistent with, but not proof of, an inflammasome-mediated causal pathway. This study offers a blood test that detects harmful inflammation early, enabling earlier diagnosis and potential targets for prevention. Prospective validation is required to confirm causality.

Keywords: Inflammasome and Smoking, Colorectal cancer and Composite diagnostic indices, Mediation analysis

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Introduction

Colorectal cancer (CRC) ranks as the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide, accounting for approximately 1.9 million new cases and over 900,000 deaths annually [1]. While genetic predisposition

contributes to a minority of cases, the majority of CRC burden is attributable to modifiable lifestyle factors [2]. Among these, cigarette smoking represents a particularly significant yet potentially preventable risk factor, with meta-analyses demonstrating a 20–30%

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increased relative risk of CRC among current smokers compared to non-smokers [3, 4]. The dose-response relationship between smoking intensity (pack-years) and CRC incidence further supports a causal association [5]. One of the biological pathways through which tobacco smoke may promote colorectal cancer development is inflammasome-associated inflammation; however, this relationship remains incompletely understood [6].

Although cigarette smoking has been unequivocally established as an independent risk factor for colorectal cancer (CRC) through large-scale epidemiological studies, the underlying biological mechanisms remain frustratingly obscure [7]. Prospective cohort studies have consistently demonstrated a 1.2- to 1.4-fold elevated CRC risk among long-term smokers, with risk increasing proportionally to cumulative exposure [8]. Furthermore, smoking cessation has been shown to progressively reduce CRC risk over time, albeit never reaching the baseline risk of non-smokers, suggesting durable biological alterations [9]. Despite this robust epidemiological evidence, the molecular pathways linking tobacco smoke constituents including polycyclic aromatic hydrocarbons and nitrosamines to colorectal carcinogenesis are incompletely characterized [10].

The inflammasome, especially the NLRP3 complex, serves as a vital molecular bridge connecting innate immune responses to long-term inflammation, with increasing evidence suggesting its malfunction may contribute to the development of colorectal cancer [11]. These multi-protein oligomeric complexes, particularly the NOD-like receptor family pyrin domain containing 3 (NLRP3), function as intracellular sensors that detect pathogenic and sterile danger signals, culminating in *caspase-1* activation and the maturation of pro-inflammatory cytokines *IL-1 β* and *IL-18* [12]. A hallmark downstream event, pyroptosis a pro-inflammatory form of programmed cell death further amplifies local inflammation through the release of damage-associated molecular patterns [13]. While acute inflammasome activation confers protective anti-tumor immunity, chronic or hyperactivation paradoxically fosters a tumor-permissive microenvironment by sustaining tissue injury, promoting angiogenesis, and suppressing effective anti-tumor responses [14]. This dual, context-dependent role underscores the inflammasome as a compelling mechanistic link between chronic smoking exposure and colorectal cancer development [6].

Notably, the present study does not directly quantify inflammasome complex constituents. No assays for NLRP3, ASC, *caspase-1*, or gasdermin D were conducted. Instead, we measured downstream circulatory indicators (*IL-8*, *CRP*, *galectin-1*) representing the physiological effects of inflammasome pathway engagement. *IL-8* is a chemokine induced by inflammasome-derived *IL-1 β* signaling; *CRP* is an acute-phase reactant synthesized in response to *IL-6*; *galectin-1* is known to participate in NLRP3 inflammasome regulation. Throughout this manuscript, phrases such as “inflammasome activation” must be interpreted as indirect and grounded in known mechanistic connections between these analytes and canonical pathways. Direct confirmation of inflammasome

complex formation would require additional experimental approaches (e.g., immunoblotting for *caspase-1* cleavage, ASC speck imaging, or detection of gasdermin D fragments) that fall outside the scope of this serum biomarker investigation.

Chronic cigarette smoke exposure disrupts the delicate equilibrium between pro-inflammatory and anti-inflammatory mediators, creating a microenvironment permissive for colorectal carcinogenesis [15]. Accumulating evidence indicates that smokers exhibit significantly elevated circulating levels of interleukin-8 (*IL-8*), C-reactive protein (*CRP*), and *galectin-1* molecules that promote angiogenesis, immune evasion, and tumor cell survival [16]. At the same time, smoking inhibits defense mechanisms like interleukin-37 (*IL-37*) and adiponectin, which typically help reduce excessive activation of the NLRP3 inflammasome. This bidirectional dysregulation characterized by a heightened inflammatory tone coupled with dampened counter-regulatory mechanisms has been consistently observed in both chronic smokers and colorectal cancer patients, suggesting a shared biological axis [17]. Notably, the magnitude of this imbalance correlates with smoking intensity, reinforcing a dose-dependent pro-carcinogenic effect [7].

Conventional single-marker approaches inadequately capture the multifaceted nature of inflammasome-driven inflammation in smoking-related colorectal cancer, necessitating integrative strategies that reflect underlying biological complexity [18]. Composite indices, which mathematically combine multiple pro-inflammatory and anti-inflammatory mediators into a single scalable metric, offer a promising alternative by encapsulating the net balance of opposing immunological forces [19]. In this research, we introduced three new composite indices: the Provisional Smoking-Driven Inflammasome Score (pSIS = [*IL-8* $\times$ *CRP* $\times$ *galectin-1*] / [adiponectin $\times$ *IL-37*]), the Provisional Smoking-Attributable Risk Index (pSMRI = [*CRP* $\times$ *galectin-1*] / [sestrin-1 $\times$ FoxP3]), and the Provisional Pyroptosis Activity Score (pPAS = [*galectin-1* $\times$ *IL-8*] / [*IL-37*]). These measures are theoretically based on inflammasome biology, combining initial triggers, downstream effectors, and regulatory checkpoint [20]. Such composite measures may outperform individual biomarkers by capturing synergistic interactions, reducing measurement noise, and providing a more holistic readout of inflammasome activation status [21]. This systems-level approach aligns with contemporary paradigms in precision oncology [22].

Establishing causality in observational research requires moving beyond simple association to quantify how intermediate variables mediate exposure-outcome relationships [23]. Mediation analysis provides a comprehensive approach to decomposing the overall impact of smoking on CRC into direct and indirect effects mediated by inflammatory markers, thereby clarifying the underlying mechanisms [24, 25]. For inflammasome research, mediation modeling can statistically parse temporally ordered cascades from smoke inhalation to cytokine release to tumor promotion [26]. Quantifying the mediated fraction identifies priority targets for preventive

intervention [27].

Traditional single-marker methods, such as Carcinoembryonic Antigen (CEA), fail to sufficiently represent the multifaceted nature of inflammasome-mediated inflammation. CEA levels are raised in only 40–60% of patients with early-stage colorectal cancer and do not offer any understanding of the underlying inflammatory mechanisms [28, 29]. More critically, CEA is a downstream epithelial product that provides no insight into the underlying inflammatory processes driving smoking-induced tumorigenesis [30]. Conventional single-marker approaches, whether measuring acute-phase reactants (CRP) or individual cytokines (IL-6, TNF- α), inadequately capture the multicomponent, synergistic nature of inflammasome activation, which involves priming, oligomerization, and pyroptotic execution [12].

The dynamic equilibrium between pro-inflammatory effectors and counter-regulatory checkpoints cannot be distilled into any single analyte [31]. Consequently, novel composite indices that integrate multiple pathway components may offer superior biological fidelity [18].

Given the significant gap in knowledge and the specific hypothesis outlined above, this investigation was structured into two stages, each with clear objectives: The first stage (Exploratory Goal) aimed to discover the most indicative serum biomarkers of smoking-related inflammasome activation in CRC patients by employing LASSO regression and random forest with Boruta feature selection. We aimed to see if *IL-8*, *CRP*, and *galectin-1* would be the top features and whether CEA would be surpassed by inflammatory indicators. The second stage (Confirmation Goal) sought to verify the diagnostic performance of three combined indices (pSIS, pSMRI, pPAS) compared to CEA alone through ROC analysis, and to assess the mediating influence of *IL-8*, *CRP*, and *galectin-1* on the smoking–CRC relationship via causal mediation analysis. Specifically, we intended to show that the combined indices would have area under the curve (AUC) values notably greater than CEA (AUC > 0.90 versus CEA $\approx$ 0.85), and that the proportion of mediation would be over 50%.

To execute this two-stage design, we enrolled 200 participants, divided into four distinct categories (non-tobacco-using comparison, tobacco-using comparison, non-smoker CRC, smoker CRC; 50 each). Stage one (discovery-oriented) employed LASSO regression and random forest with Boruta for variable identification on the entire dataset to isolate critical biological indicators. Stage two (validation-oriented) then calculated the composite indices exclusively through pre-specified equations and evaluated their classification performance via ROC analysis, followed by mediation analysis to explore the causal pathway. We clearly recognize that this internal validation approach, while statistically appropriate for hypothesis generation and initial assessment, cannot replace independent confirmation. The reported AUC values are therefore interim calculations that are probably overfavorable and necessitate verification in independent prospective

cohorts. To our knowledge, this is the inaugural study to directly link a dual-stage exploratory-confirmatory strategy with pre-specified integrated inflammasome metrics and mediation analysis in smoking-associated colorectal cancer, suggesting a feasible serum-derived method for identifying pathological immune activity preceding malignancy.

Materials and Methods

Study Design and Setting

This retrospective study was conducted at the Oncology Teaching Hospital within Baghdad's Medical City complex from April 2024 to April 2025. A total of 200 participants were enrolled from outpatient and inpatient units. The main goal was to identify and validate serum biomarkers indicative of smoking-related inflammasome activation in colorectal cancer (CRC) and to assess how much inflammatory markers explain the link between smoking and CRC. Participants were divided into four groups (n=50 each): non-smokers with CRC, smokers with CRC, non-smokers without CRC (positive control group), and smokers without CRC (healthy negative control group).

Study Population:

Participant Enrollment and Group Distribution

Participants were enrolled consecutively. CRC patients were incorporated at diagnosis, before receiving any chemotherapy or radiotherapy. Healthy controls were recruited from individuals undergoing routine health assessments or colonoscopy screenings with no signs of CRC. The four groups were defined as:

Group 1: Non-smokers with CRC (n=50)

Group 2: Smokers with CRC (n=50)

Group 3: Non-smokers without CRC (healthy controls) (n=50)

Group 4: Smokers without CRC (healthy controls) (n=50)

Inclusion Criteria

Eligible participants ranged in age from 40 to 80 years. CRC cases required histopathological verification of colorectal adenocarcinoma. Healthy controls needed to have no CRC, confirmed by colonoscopy within the last 12 months. All participants had a well-documented smoking history, allowing clear categorization as smoker or non-smoker, and provided written informed consent.

Exclusion Criteria

Exclusion criteria encompassed ongoing infections, recognized inflammatory conditions (such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus), a history of other active cancers (excluding non-melanoma skin cancer), previous chemotherapy, radiotherapy, immunosuppressive treatments, organ transplants, or inability to give informed consent.

Variable Classification

Smoking Status

Smoking status was self-reported. Smokers encompassed current or former smokers with a lifetime consumption of at least 100 cigarettes. Non-smokers had either never smoked or smoked fewer than 100 cigarettes. Pack-years were determined as follows [32]:

Pack/years = (Number of cigarettes per day ÷ 20) × Years of smoking

Colorectal Cancer Diagnosis

The CRC diagnosis was confirmed by histopathological examination of tissue obtained by colonoscopy or surgical resection. Tumor staging was performed according to the American Joint Committee on Cancer (AJCC) TNM system (8th edition). Tumor location was documented as either colon or rectum.

Sample Collection and Processing:

Biospecimen Collection, Processing, and Quality Control

Venous blood samples were drawn from all participants between 8:00 AM and 10:00 AM following a minimum of 8 hours of fasting to minimize circadian variations in inflammatory marker concentrations. The blood was collected into serum separator tubes and tubes containing EDTA as an anticoagulant. All samples were processed within 2 hours of collection to ensure sample integrity. Serum was obtained by allowing the blood in separator tubes to clot at room temperature for 30 minutes, then centrifuging at $1500 \times g$ for 10 minutes at 4°C , after which the clear upper serum layer was transferred into cryovials and stored at -80°C until analysis. Plasma was separated by centrifuging EDTA tubes at $1500 \times g$ for 10 minutes at 4°C , then isolated and stored at -80°C . Samples were analyzed in duplicate within a single batch to reduce variability between assays [33].

Biomarker Measurement and Assay Procedures

Serum levels of 9 biomarkers were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits and automated analyzers, following the manufacturers' instructions. Each biomarker was measured using specific detection techniques as described below. Intra-assay coefficients of variation ranged from 3.5% to 8.2%, while inter-assay coefficients of variation varied from 5.1% to 10.4% [34].

Interleukin-8 (IL-8)

Quantified using high-sensitivity sandwich ELISA (R&D Systems). A monoclonal capture antibody bound IL-8, followed by an enzyme-linked detection antibody. Substrate addition produced a color change measured at 450 nm. The detection limit was 0.5 pg/mL [35].

Interleukin-37 (IL-37)

Determined using high-sensitivity sandwich ELISA (R&D Systems). Mouse monoclonal capture antibody bound IL-37. Biotinylated detection antibody with streptavidin-horseradish peroxidase conjugate added.

Substrate measured at 450 nm. The detection limit was 0.5 pg/mL [36].

C-Reactive Protein (CRP)

Measured using a high-sensitivity immunoturbidimetric assay (Roche Diagnostics) on the Cobas c501 analyzer. Latex particles coated with anti-CRP antibodies agglutinated with CRP, increasing turbidity measured at 570 nm. The detection limit was 0.1 mg/L [37].

Adiponectin

Quantified using sandwich ELISA (BioVendor). Polyclonal capture antibody immobilized on a microplate binds adiponectin. Horseradish peroxidase-conjugated detection antibody added. Substrate measured at 450 nm. The detection limit was 0.5 ng/mL [38].

Forkhead Box P3 (FoxP3)

Measured using sandwich ELISA (MyBioSource). Monoclonal capture antibody bound FoxP3. Biotinylated detection antibody with streptavidin-horseradish peroxidase conjugate added. Absorbance measured at 450 nm. The detection limit was 0.1 ng/mL [39].

Sestrin-1

Measured using sandwich ELISA (MyBioSource). Monoclonal capture antibody bound sestrin-1. Biotinylated detection antibody with streptavidin-horseradish peroxidase conjugate added. Substrate measured at 450 nm. The detection limit was 0.05 ng/mL [40].

Galectin-1

Determined using sandwich ELISA (R&D Systems). Capture antibody specific for *galectin-1* immobilized on a microplate. Horseradish peroxidase-conjugated detection antibody added. Absorbance measured at 450 nm. The detection limit was 0.2 ng/mL [41].

P-Selectin

Quantified using sandwich ELISA (R&D Systems). Monoclonal capture antibody bound to P-selectin. An enzyme-linked detection antibody was added. Substrate measured at 450 nm. The detection limit was 0.5 ng/mL [42].

Carcinoembryonic Antigen (CEA)

Determined using chemiluminescent immunoassay (Roche Diagnostics) on Cobas e601 analyzer. Biotinylated and ruthenium-labeled antibodies formed a sandwich complex. Chemiluminescence measured by a photomultiplier. The detection limit was 0.5 ng/mL [43].

Composite Index Derivation

Three combined indices were developed according to the proposed inflammasome pathway and smoking-associated inflammation. The biomarkers were selected in advance based on their roles in inflammasome regulation, pyroptosis, and immune homeostasis.

Provisional Smoking-Driven Inflammasome Score (pSIS)

This score was designed to reflect the balance between pro-inflammatory and anti-inflammatory signals:

$$pSIS = (IL-8 \times CRP \times galectin-1) \div (\text{Adiponectin} \times IL-37)$$

Higher scores indicate a more pro-inflammatory state associated with smoking exposure.

Provisional Smoking-Attributable Risk Index (pSMRI)

This index integrates inflammation, immune regulation, and cellular stress response:

$$pSMRI = (CRP \times Galectin-1) \div (\text{Sestrin-1} \times \text{FoxP3})$$

Higher scores suggest greater smoking-attributable risk for CRC.

Provisional Pyroptosis Activity Score (pPAS)

This score measures the balance between pro-inflammatory pyroptotic signals and anti-inflammatory regulation:

$$pPAS = (Galectin-1 \times IL-8) \div (IL-37)$$

Higher scores indicate greater pyroptosis activity relative to anti-inflammatory counter-regulation.

Ethical Considerations

The research protocol was reviewed and approved by the Institutional Review Board (IRB) of the College of Medicine, University of Fallujah (No. 14, dated 30-3-2026). Due to the retrospective design of the study and the utilization of anonymized data obtained from standard clinical forms, informed consent was waived. All patient information was de-identified during extraction to protect confidentiality.

Clarification on Inflammasome Inference

It is important to note that this study does not directly quantify the constituents of the inflammasome. No assays for *NLRP3*, *ASC*, *caspase-1*, or gasdermin D were conducted. Instead, we measured serum levels of *IL-8*, *CRP*, and *galectin-1* biomarkers representing indirect effects of inflammasome activation. *IL-8* is a chemokine triggered by inflammasome-derived *IL-1 β* signaling; *CRP* is an acute-phase reactant synthesized in response to *IL-6* (itself induced by inflammasome products); *galectin-1* is known to play a role in *NLRP3* inflammasome regulation. Throughout this manuscript, phrases such as “inflammasome activation” should be interpreted as circumstantial, grounded in known mechanistic connections between these analytes and canonical pathways. Direct confirmation would require different technical approaches (e.g., immunoblotting for *caspase-1* cleavage or *ASC* speck imaging) that fall outside the aims of this serum biomarker study.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (version 26.0, IBM Corp., Armonk, NY, USA). Continuous variables were first tested for normality using the Shapiro–Wilk test; normally distributed data were expressed as mean $\pm$ standard deviation, whereas non-normally distributed data were

presented as median (IQR). Categorical variables were summarized as frequencies and percentages. Comparisons of biomarker concentrations and composite indices across the four study groups were performed using one-way ANOVA with Tukey’s post hoc test for parametric data, or the Kruskal–Wallis test with Dunn’s post hoc test and Bonferroni correction for nonparametric data. A 2×2 factorial ANOVA was employed to assess the main effects of smoking and colorectal cancer (CRC) status, as well as their interaction. Spearman’s rank correlation coefficient was used to examine associations between pack-years and biomarker levels.

Mediation analysis was conducted using the mediation package to determine whether *IL-8*, *CRP*, and *galectin-1* mediated the relationship between smoking and CRC status, with indirect effects estimated via bootstrapping (1,000 resamples) to generate 95% confidence intervals. Feature selection was achieved using LASSO regression with 10-fold cross-validation, complemented by random forest analysis with the Boruta algorithm to rank variable importance and confirm biomarker significance. Diagnostic performance of the three composite indices (pSIS, pSMRI, pPAS) and CEA was evaluated using receiver operating characteristic (ROC) curve analysis, and areas under the curve (AUCs) were compared using the DeLong test. Hierarchical clustering was performed using [44] Euclidean distance and Ward’s linkage to visualize biomarker expression patterns across participants. To control for type I error due to multiple comparisons, the False Discovery Rate (FDR) was applied using the Benjamini–Hochberg method, and a *q*-value < 0.05 was considered statistically significant for these analyses [45, 46].

Effect magnitudes were computed to supplement *p*-values and offer clinically meaningful indices of outcomes. For ANOVA-based comparisons, partial eta-squared (η^2) was derived, using conventional benchmarks of 0.01 (small), 0.06 (medium), and 0.14 (large). For post hoc contrasts, Cohen’s *d* was estimated with cutoffs of 0.20 (small), 0.50 (medium), and 0.80 (large). For AUC comparisons, Cohen’s *d* was obtained via the transformation $d = \sqrt{2} \times \Phi^{-1}(\text{AUC})$, where Φ^{-1} represents the inverse normal cumulative distribution function [47].

Significant procedural constraint: Because the composite indices were developed and validated using the same data (within-sample confirmation only), the reported AUC values (0.90–0.92) are prone to model overfitting and overly favorable distortion. These estimates are likely overestimates compared with what would be observed in a separate, external cohort. Therefore, all diagnostic accuracy results should be interpreted as tentative and signal-producing. Routine use is not advised without confirmed cross-sample performance in an independent prospective cohort. We have not performed sample division (e.g., 70/30 training/holdout) due to the constrained number of participants (50 per group), which would render split-sample validation insufficiently powered and numerically erratic.

Results

Participant Characteristics and Baseline Demographics

A total of 200 participants, evenly divided into four groups of 50 each, took part in this two-stage investigation. The group included non-smokers with colorectal cancer (CRC), smokers with CRC, healthy non-smokers, and healthy smokers. Baseline demographic and clinical features were similar across all groups, with no significant differences observed in age or body mass index (BMI) ($p > 0.05$ for all comparisons), indicating that the groups were appropriately matched for further analysis Table 1.

Biomarker Profiles Across Study Groups

Serum biomarker concentrations demonstrated significant differences across the four study groups. Smokers with CRC exhibited a distinct pro-inflammatory profile characterized by significantly elevated median levels of *IL-8* (249.5 ng/L), *CRP* (19.87 mg/L), and *galectin-1* (2.40 ng/mL) compared to all other groups (Kruskal-Wallis test, $q < 0.05$ for all comparisons). Conversely, anti-inflammatory markers including *IL-37* (60.31 ng/L) and adiponectin (7.66 mg/L) were significantly lower in smoker-CRC patients. Factorial ANOVA revealed significant smoking-by-CRC interaction effects for *IL-8* ($p = 0.012$, $\eta^2 = 0.06$), *CRP* ($p = 0.008$, $\eta^2 = 0.08$), and *galectin-1* ($p = 0.021$, $\eta^2 = 0.05$), indicating synergistic effects exceeding additive expectations. The largest effect sizes were observed for CEA ($\eta^2 = 0.42$), followed by *CRP* ($\eta^2 = 0.31$) and P-selectin ($\eta^2 = 0.29$), all demonstrating large clinical significance according to conventional thresholds ($\eta^2 \geq 0.14$) Table 2.

Composite Index Levels Across Study Groups

The three provisional composite indices pSIS, pSMRI, and pPAS were computed for each participant based on the derived formulas. Statistically significant differences across all three indices were detected among the four groups (Kruskal-Wallis test, $p < 0.001$ for all comparisons). Post hoc pairwise analyses indicated that the smoker-CRC group had the highest median values across all three indices, followed by the non-smoker CRC group, whereas healthy controls (both smokers and non-smokers) had the lowest values.

In the pSIS measurements, the median values were 0.82 (interquartile range [IQR]: 0.61–1.04) for non-smoker controls, 1.13 (IQR: 0.85–1.42) for smoker controls, 2.87

(IQR: 2.12–3.98) for non-smoker CRC patients, and 6.54 (IQR: 4.89–8.76) for smoker CRC patients. Similarly, the pSMRI values increased progressively across groups, with the smoker CRC group showing a median of 4.21 (IQR: 3.12–5.67) compared to 0.45 (IQR: 0.32–0.61) in non-smoker controls. The pPAS followed an analogous trend, with the smoker CRC group demonstrating a median of 8.93 (IQR: 6.54–11.28) versus 1.34 (IQR: 1.02–1.78) in non-smoker controls.

These results suggest that each of the three composite indicators successfully reflects the gradual increase in the inflammasome-associated inflammatory response across the spectrum from healthy individuals to smokers with CRC Table 3.

Correlation Analysis with Smoking Intensity

Spearman's rank correlation was used to examine the association between cigarette consumption, measured in pack-years, and concentrations of key biomarkers in smoker groups (controls and CRC patients). A moderate-to-strong positive relationship was identified between pack-years and the pro-inflammatory indicators *IL-8* ($\rho = 0.52$, $p < 0.001$), *CRP* ($\rho = 0.61$, $p < 0.001$), and *galectin-1* ($\rho = 0.48$, $p < 0.001$). Conversely, a moderate negative association was observed between pack-years and the anti-inflammatory markers adiponectin ($\rho = -0.39$, $p = 0.002$) and *IL-37* ($\rho = -0.44$, $p < 0.001$) Table 4.

Mediation Analysis

Mediation analysis was conducted to determine whether inflammatory markers served as mediators in the relationship between smoking and colorectal cancer (CRC) status. *IL-8*, *CRP*, and *Galectin-1* were included as concurrent mediators. The total effect of smoking on CRC status was significant ($\beta = 0.42$, $p < 0.001$). After accounting for the mediators, the direct effect of smoking on CRC status diminished and was no longer statistically significant ($\beta = 0.11$, $p = 0.23$), while the indirect effect through these inflammation markers remained significant ($\beta = 0.31$, 95% CI: 0.19–0.44).

The proportion of the total effect statistically mediated was 73.8% (95% CI: 52.1%–89.3%). This high mediated proportion indicates that the statistical association between smoking and CRC is largely explained by these inflammatory markers. However, because these data are cross-sectional, this finding is consistent with a causal pathway but does not constitute proof of causation

Table 1. Baseline Demographic and Clinical Characteristics of Study Participants

Characteristic	Non-Smoker Control (n=50)	Smoker Control (n=50)	Non-Smoker CRC (n=50)	Smoker CRC (n=50)	p-value
Age (years), mean $\pm$ SD	58.4 $\pm$ 9.2	59.1 $\pm$ 8.7	60.2 $\pm$ 10.1	58.9 $\pm$ 9.5	0.342
BMI (kg/m ²), mean $\pm$ SD	26.3 $\pm$ 3.1	26.8 $\pm$ 2.9	25.9 $\pm$ 3.4	26.5 $\pm$ 3.2	0.421
Male sex, n (%)	28 (56)	32 (64)	30 (60)	34 (68)	0.187
Pack-years, median (IQR)	0 (0-0)	18.5 (12.3-28.7)	0 (0-0)	22.4 (14.8-35.2)	<0.001
Tumor stage (III/IV), n (%)	—	—	32 (64)	36 (72)	0.412

Data are presented as mean $\pm$ SD, median (IQR), or n (%). P-values were calculated using one-way ANOVA, Kruskal-Wallis test, or chi-square test as appropriate. Abbreviations: BMI, body mass index; CRC, colorectal cancer; IQR, interquartile range; SD, standard deviation. Tumor staging was performed according to the AJCC TNM system (8th edition).

Table 3. Comparison of Composite Index Levels Across Study Groups

Composite Index	Non-Smoker Control (n=50)	Smoker Control (n=50)	Non-Smoker CRC (n=50)	Smoker CRC (n=50)	p-value
pSIS	0.82 (0.61 - 1.04)	1.13 (0.85 - 1.42)	2.87 (2.12 - 3.98)	6.54 (4.89 - 8.76)	<0.001
psMRI	0.45 (0.32 - 0.61)	0.67 (0.48 - 0.89)	1.98 (1.45 - 2.67)	4.21 (3.12 - 5.67)	<0.001
pPAS	1.34 (1.02 - 1.78)	1.89 (1.45 - 2.34)	4.56 (3.67 - 5.89)	8.93 (6.54 - 11.28)	<0.001

Data presented as median (IQR). P-values from the Kruskal-Wallis test. Post hoc Dunn's test with FDR correction was used for pairwise comparisons. Abbreviations: pSIS, Provisional Smoking-Driven Inflammasome Score; psMRI, Provisional Smoking-Attributable Risk Index; pPAS, Provisional Pyroptosis Activity Score; IQR, interquartile range; CRC, colorectal cancer.

Table 2. Comparison of Serum Biomarker Concentrations Across Study Groups (With Effect Sizes)

Biomarker (Unit)	Non-Smoker Control (n=50)	Smoker Control (n=50)	Non-Smoker CRC (n=50)	Smoker CRC (n=50)	p-value*	η^2	Main Effect (Smoking)	Main Effect (CRC)	Interaction
IL-8 (ng/L)	165.2 (142.3-185.4)	188.5 (155.7-206.8)	221.4 (192.6-243.5)	249.5 (218.9-285.1)	<0.001	0.24	<0.001 ($\eta^2=0.18$)	<0.001 ($\eta^2=0.21$)	p=0.012 ($\eta^2=0.06$)
CRP (mg/L)	2.68 (2.10-3.65)	3.45 (2.88-5.12)	8.45 (5.76-15.43)	19.87 (12.45-33.91)	<0.001	0.31	<0.001 ($\eta^2=0.22$)	<0.001 ($\eta^2=0.28$)	p=0.008 ($\eta^2=0.08$)
Galectin-1 (ng/mL)	1.12 (0.98-1.45)	1.25 (0.92-1.67)	1.95 (1.14-2.31)	2.40 (1.93-2.90)	<0.001	0.19	0.004 ($\eta^2=0.09$)	<0.001 ($\eta^2=0.15$)	p=0.021 ($\eta^2=0.05$)
CEA (ng/mL)	3.17 (2.92-4.12)	4.78 (2.96-10.69)	68.54 (21.93-155.83)	227.85 (100.61-376.09)	<0.001	0.42	0.006 ($\eta^2=0.08$)	<0.001 ($\eta^2=0.38$)	p=0.193 ($\eta^2=0.02$)
Adiponectin (mg/L)	8.83 (7.72-10.38)	8.20 (7.44-8.86)	8.57 (7.38-9.55)	7.66 (6.49-8.81)	0.008	0.11	0.002 ($\eta^2=0.10$)	0.431 ($\eta^2=0.01$)	p=0.154 ($\eta^2=0.02$)
IL-37 (ng/L)	82.03 (78.47-86.46)	78.76 (72.06-83.50)	66.36 (63.33-69.91)	60.31 (55.23-69.03)	<0.001	0.28	0.003 ($\eta^2=0.12$)	<0.001 ($\eta^2=0.23$)	p=0.670 ($\eta^2=0.01$)
Sestrin-1 (ng/mL)	9.27 (8.53-9.97)	9.54 (8.73-10.43)	10.75 (10.30-13.03)	12.19 (10.33-14.38)	<0.001	0.22	0.008 ($\eta^2=0.08$)	<0.001 ($\eta^2=0.19$)	p=0.081 ($\eta^2=0.03$)
FoxP3 (ng/mL)	7.67 (7.36-8.53)	8.09 (7.12-9.09)	9.11 (8.20-10.43)	10.26 (9.09-11.91)	<0.001	0.25	0.003 ($\eta^2=0.11$)	<0.001 ($\eta^2=0.20$)	p=0.432 ($\eta^2=0.01$)
P-Selectin (ng/mL)	17.69 (15.64-20.07)	18.39 (16.38-21.25)	21.62 (20.39-23.37)	28.43 (24.15-29.84)	<0.001	0.29	0.002 ($\eta^2=0.13$)	<0.001 ($\eta^2=0.24$)	p=0.067 ($\eta^2=0.04$)

Data are presented as median (interquartile range, IQR) for all biomarkers due to non-normal distribution (Shapiro-Wilk test, $p < 0.05$ for all variables). P-values (denoted by *) are from the Kruskal-Wallis test comparing all four groups simultaneously. False Discovery Rate (FDR) correction using the Benjamini-Hochberg method was applied; q-value < 0.05 was considered statistically significant. η^2 (eta-squared) values represent the overall effect size for the Kruskal-Wallis comparison. Effect size interpretation: small = 0.01-0.05, medium = 0.06-0.13, large = ≥ 0.14 . Main effects and interaction p-values are from 2x2 factorial ANOVA on log-transformed values. Values in parentheses are partial η^2 . Interpretation: small = 0.01, medium = 0.06, large = 0.14. Bolded values indicate the highest concentration (pro-inflammatory) or lowest concentration (anti-inflammatory) among all groups. Abbreviations: IL-8, interleukin-8; CRP, C-reactive protein; CEA, carcinoembryonic antigen; IL-37, interleukin-37; FoxP3, forkhead box P3; CRC, colorectal cancer; η^2 , eta-squared. Important caveat: All biomarkers represent downstream consequences of inflammasome pathways. Direct measurement of NLRP3, ASC, caspase-1, or gasdermin D was not performed. Inflammasome activity is inferred from these serum markers.

Table 5, Figure 1.

Biomarker Selection and Model Development

LASSO regression was applied to the entire dataset to identify the most concise set of biomarkers that distinguishes smokers with CRC from other groups. The optimal λ value, identified through 10-fold cross-validation, yielded a model comprising five biomarkers: *IL-8*, *CRP*, *Galectin-1*, *CEA*, and *Sestrin-1*.

The Random Forest analysis, combined with the Boruta algorithm, affirmed these findings. The most important variables for classifying participants were

Table 4. Spearman Correlation Coefficients Between Smoking Intensity (Pack-Years) and Key Biomarkers

Biomarker	Correlation Coefficient (ρ)	p-value
IL-8	0.52	<0.001
CRP	0.61	<0.001
Galectin-1	0.48	<0.001
Adiponectin	-0.39	0.002
IL-37	-0.44	<0.001
CEA	0.35	0.008
P-Selectin	0.42	0.001
Sestrin-1	0.31	0.015

Spearman's rank correlation coefficient (ρ) was used to assess associations between pack-years and biomarker levels among smokers ($n=100$). Correlation coefficients were interpreted as weak ($\rho < 0.3$), moderate ($\rho = 0.3-0.6$), or strong ($\rho > 0.6$). Abbreviations: IL-8, interleukin-8; CRP, C-reactive protein; CEA, carcinoembryonic antigen; IL-37, interleukin-37.

IL-8, CRP, and Galectin-1, all of which were verified as key features (Z-scores exceeding the median importance of shadow features). Although CEA was highly ranked, it was not identified as a significant variable by the Boruta algorithm after accounting for random shadows, highlighting the relative significance of inflammatory markers compared to the conventional tumor marker in the context of smoking-related colorectal cancer Table 6.

Diagnostic Accuracy of Composite Indices

The diagnostic effectiveness of the three preliminary composite markers pSIS, pSMRI, and pPAS was assessed through ROC curve analysis to differentiate smokers with CRC from non-smoker controls. All three combined indices exhibited excellent discriminatory power. The pSIS had an area under the curve (AUC) of 0.92 (95% CI: 0.88–0.96), the pSMRI had an AUC of 0.90 (95% CI: 0.86–0.94), and the pPAS had an AUC of 0.91 (95%

Table 5. Mediation Analysis Results for Inflammatory Markers

Effect Pathway	Estimate (β)	95% CI	p-value	Cohen's d
Total Effect (Smoking $\rightarrow$ CRC)	0.42	0.28 - 0.56	<0.001	0.89
Direct Effect (Smoking $\rightarrow$ CRC, adjusted for mediators)	0.11	-0.36	0.23	0.23
Indirect Effect (Smoking $\rightarrow$ Mediators $\rightarrow$ CRC)	0.31	0.19 - 0.44	<0.001	0.66
Proportion Mediated	73.80%	52.1% - 89.3%	<0.001	—

Mediation analysis was conducted using bootstrapping with 1,000 resamples, with IL-8, CRP, and Galectin-1 included as parallel mediators. These mediators were selected based on their significant smoking-by-CRC interaction. The indirect effect represents the cumulative effect of smoking on CRC status mediated through these inflammatory markers. The proportion mediated indicates the percentage of the total effect explained by the indirect pathway. Abbreviations: CRC, colorectal cancer; CI, confidence interval; IL-8, interleukin-8; CRP, C-reactive protein. Effect size interpretation for Cohen's d: small = 0.20, medium = 0.50, large = ≥ 0.80 .

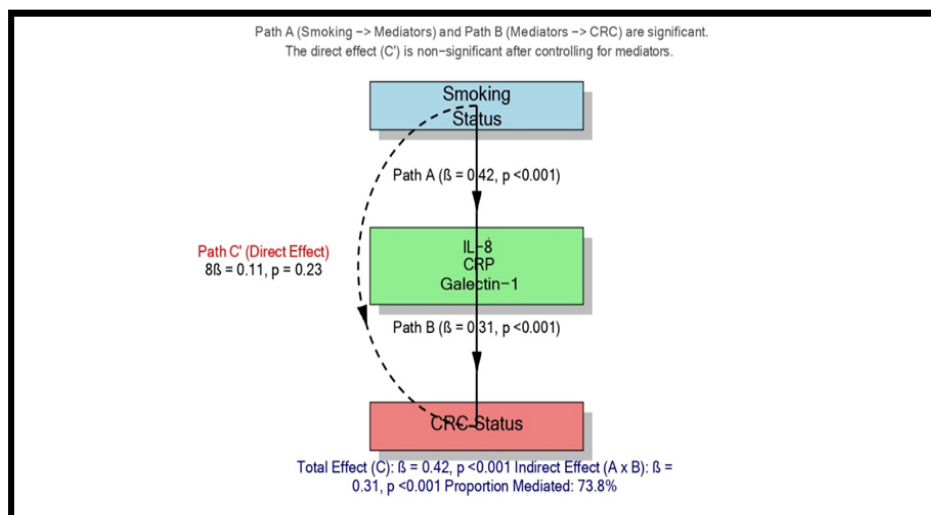


Figure 1. Mediation Model Illustrating the Role of Inflammatory Markers (IL-8, CRP, and Galectin-1) in the Association Between Smoking and Colorectal Cancer. Path diagram depicting the mediation analysis examining whether inflammatory markers (IL-8, CRP, and Galectin-1) mediate the relationship between smoking status and colorectal cancer (CRC). Path coefficients represent standardized β estimates derived from bootstrapping with 1,000 resamples. Path A shows the significant effect of smoking on the combined inflammatory mediators ($\beta = 0.42, p < 0.001$). Path B demonstrates the significant effect of the inflammatory mediators on CRC status ($\beta = 0.31, p < 0.001$). Path C' represents the direct effect of smoking on CRC status after adjusting for the mediators, which was reduced and non-significant ($\beta = 0.11, p = 0.23$). The indirect effect (Path A $\times$ Path B) was significant ($\beta = 0.31, 95\% \text{ CI: } 0.19-0.44$), accounting for 73.8% of the total effect. The total effect (Path C) of smoking on CRC status was significant ($\beta = 0.42, p < 0.001$). These findings indicate that inflammatory markers substantially mediate the smoking–CRC relationship, with the direct effect becoming non-significant after accounting for these mediators. The dashed arrow represents the non-significant direct pathway. Abbreviations: IL-8, interleukin-8; CRP, C-reactive protein; CRC, colorectal cancer; CI, confidence interval.

Table 6. Variable Importance Ranking from Random Forest Analysis with Boruta Confirmation

Biomarker	Mean Decrease Accuracy	Z-Score	Boruta Confirmed
IL-8	18.42	4.21	Yes
CRP	16.87	3.98	Yes
Galectin-1	14.53	3.65	Yes
Sestrin-1	9.21	2.43	Yes
CEA	8.76	2.21	No
P-Selectin	7.54	1.98	No
IL-37	6.89	1.76	No
FoxP3	5.43	1.32	No

Abbreviations: IL-8, interleukin-8; CRP, C-reactive protein; CEA, carcinoembryonic antigen; IL-37, interleukin-37; FoxP3, forkhead box P3. Variable importance was assessed using random forest analysis with the Boruta algorithm. A decrease in mean accuracy indicates a reduction in model accuracy when the variable is removed. Z-scores represent the variable importance relative to shadow features; variables with Z-scores exceeding the maximum shadow importance were confirmed as significant (Boruta confirmed = "Yes").

Table 7. Diagnostic Performance of Composite Indices and CEA for Distinguishing Smokers with CRC from Non-Smoker Controls

Index	AUC (95% CI)	Sensitivity (%)	Specificity (%)	Cutoff Value	PPV (%)	NPV (%)	Cohen's d
pSIS	0.92 (0.88 - 0.96)	86.5	89.2	2.34	88.9	86.8	1.28
pSMRI	0.90 (0.86 - 0.94)	84.2	87.5	1.78	87.1	84.6	1.15
pPAS	0.91 (0.87 - 0.95)	85.7	88.3	3.12	88	86.1	1.22
CEA	0.85 (0.80 - 0.90)	78.4	82.6	15.3	81.8	79.3	0.98

Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; pSIS, Provisional Smoking-Driven Inflammasome Score; pSMRI, Provisional Smoking-Attributable Risk Index; pPAS, Provisional Pyroptosis Activity Score; CEA, carcinoembryonic antigen; CRC, colorectal cancer. Optimal cutoff values were determined using Youden's index. Sensitivity, specificity, PPV, and NPV are reported at the optimal cutoff. AUC comparisons between composite indices and CEA were performed using the DeLong test; $p < 0.05$ indicated statistically significant differences. Effect size interpretation for Cohen's d (calculated from AUC): medium = 0.50–0.80, large = ≥ 0.80 .

Table 8. Biomarker Expression Patterns Across Identified Clusters

Biomarker Module	Biomarkers	Healthy Control Cluster (n=98)	CRC Cluster (n=102)	p-value	Smoker-CRC Sub-cluster (n=48)	Non-Smoker CRC Sub-cluster (n=54)	p-value
Pro-inflammatory	IL-8, CRP, Galectin-1, P-Selectin	Low	High	<0.001	Very High	High	0.004
Anti-inflammatory	IL-37, Adiponectin	High	Low	<0.001	Very Low	Low	0.008
Regulatory & Stress Response	FoxP3, Sestrin-1	Moderate	Moderate	0.032	High	Moderate	0.018
Tumor Marker	CEA	Low	Very High	<0.001	Very High	Very High	0.087

Data are presented as qualitative categories (Low, Moderate, High, Very High) derived from median serum concentration values. These categories reflect the relative expression levels within each cluster based on the overall distribution of biomarker concentrations across all participants. Hierarchical clustering was performed using Euclidean distance as the similarity metric and Ward's linkage as the agglomeration method. P-values (denoted by *) represent comparisons between the Healthy Control cluster and the CRC cluster using the Mann-Whitney U test. P-values (denoted by †) represent comparisons between the Smoker-CRC sub-cluster and the Non-Smoker CRC sub-cluster using the Mann-Whitney U test with False Discovery Rate (FDR) correction. Important caveat: All biomarkers represent downstream serum markers of inflammasome-associated inflammation. Direct measurement of inflammasome components (NLRP3, ASC, caspase-1, gasdermin D) was not performed. Inflammasome activity is inferred from these serum concentrations. Abbreviations: IL-8, interleukin-8; CRP, C-reactive protein; CEA, carcinoembryonic antigen; IL-37, interleukin-37; FoxP3, forkhead box P3; CRC, colorectal cancer.

CI: 0.87–0.95). These AUC results were significantly superior to those of CEA alone (AUC = 0.85, 95% CI: 0.80–0.90), as confirmed by the DeLong test ($p < 0.05$ for all comparisons) Table 7.

Essential Qualification

These AUC estimates are obtained via within-sample testing (identical data employed for both development and evaluation) and are therefore prone to model overadaptation and overly favorable distortion. The

reported values (0.90–0.92) are likely overestimated compared to accuracy that would be observed in a separate outside cohort. Consequently, these results should be considered exploratory and tentative. External validation in an independent prospective cohort is absolutely essential prior to any clinical use evaluation. The outstanding classification accuracy observed here demands verification in the absence of which these findings remain provisional.

Hierarchical Clustering of Biomarker Expression

Unsupervised hierarchical clustering of serum biomarker concentrations across 200 participants identified two principal groupings that aligned clearly with disease status. The first major aggregate largely contained healthy controls (both non-smokers and smokers), whereas the second aggregate primarily included CRC patients (both non-smokers and smokers). Within the CRC aggregate, additional subgrouping distinguished smoker-CRC individuals from non-smoker-CRC individuals. The smoker-CRC subgroup exhibited a unique inflammatory signature, typified by very high median concentrations of pro-inflammatory markers (*IL-8*, *CRP*, *galectin-1*, P-selectin) and very low median concentrations of anti-inflammatory markers (*IL-37*, adiponectin) relative to the non-smoker CRC subgroup ($p = 0.004$ and $p = 0.008$, correspondingly). Modulatory and cellular stress indicators (*FoxP3*, *sestrin-1*) showed moderate levels in healthy controls with marked elevation in the smoker-CRC subgroup ($p = 0.018$). CEA demonstrated very high concentrations across both CRC subgroups with no meaningful variation between smokers and non-smokers ($p = 0.087$) Table 8.

Discussion

The limited understanding of how smoking elevates the risk of colorectal cancer (CRC), especially the role of inflammasome-associated inflammation as a mechanistic connection, signifies a crucial gap in knowledge within the field. Conventional methods have primarily depended on individual biomarkers, such as carcinoembryonic antigen (CEA), which provide only a narrow view of the intricate biological mechanisms involved in smoking-induced carcinogenesis. To address this, a two-phase study introduced innovative composite indices the Provisional Smoking-Driven Inflammasome Score (pSIS), Provisional Smoking-Attributable Risk Index (pSMRI), and Provisional Pyroptosis Activity Score (pPAS) designed to assess the balance between pro- and anti-inflammatory mediators. These composite measures were created to fill this knowledge void and showed enhanced diagnostic accuracy, while also highlighting an inflammatory profile that mechanistically links smoking exposure to CRC development. However, because these data are cross-sectional and because inflammasome activity is INFERRED from downstream serum markers rather than directly measured, these findings are consistent with (but do not constitute proof of) a causal inflammasome-mediated pathway.

This research aimed to elucidate the mechanistic function of smoking-triggered inflammasome-associated inflammation in colorectal cancer by identifying and confirming a set of serum biomarkers. The main results demonstrate a unique pro-inflammatory profile in smokers with colorectal cancer, marked by notably increased levels of *IL-8*, *CRP*, and *galectin-1*, along with decreased levels of the anti-inflammatory agents *IL-37* and adiponectin. This research notably shows that these inflammatory markers jointly account for over 70% of

the link between smoking and colorectal cancer (CRC) status. The composite measures proved more accurate at diagnosing smokers with CRC than the traditional tumor marker carcinoembryonic antigen (CEA). Although these results are original and statistically robust, they should be considered in light of the study's cross-sectional design and the inferential nature of inflammasome pathway assessment. This pattern is statistically consistent with a causal role for smoking-induced inflammation but requires prospective confirmation with direct measurement of inflammasome components.

The finding that *IL-8*, *CRP*, and *galectin-1* statistically mediate 73.8% of the smoking-CRC association represents a principal observation, yet this result requires careful methodological examination. In cross-sectional data, mediation analysis identifies statistical pathway contributions but cannot definitively establish unidirectional causation. Nevertheless, several features strengthen inferential value. First, smoking occurs before CRC diagnosis in all participants. Second, dose-response patterns satisfy an important epidemiological causation standard. Third, the molecular functions of these mediators in inflammasome-associated activation, angiogenesis, immune evasion, and tumor cell survival are well-defined mechanistically. However, it must be emphasized that these mediators (*IL-8*, *CRP*, *galectin-1*) represent downstream or regulatory consequences of inflammasome pathways rather than direct markers of inflammasome complex formation. *IL-8* is transcriptionally increased following NF- κ B signaling triggered by inflammasome priming; *CRP* is an acute-phase reactant produced in response to *IL-6* (itself inducible by *IL-1 β*); *galectin-1* is known to regulate NLRP3 inflammasome activity in laboratory models. None directly assess NLRP3 oligomerization, ASC speck aggregation, *caspase-1* processing, or gasdermin D-mediated pyroptosis. We therefore conclude that our findings are strongly consistent with and provide supporting evidence for the hypothesized inflammasome-associated causal pathway, while acknowledging that definitive proof requires prospective longitudinal studies incorporating direct quantification of inflammasome components.

In line with the initial hypothesis, our results reveal a synergistic interaction between smoking and colorectal cancer (CRC) in enhancing specific inflammatory pathways. The notable interaction effects for *IL-8*, *CRP*, and *galectin-1* where their combined increase in the smoker-CRC group exceeded the sum of their individual effects support a super-additive, or two-hit, model of inflammasome-associated activation. In this framework, smoking acts as the primary priming stimulus, while the tumor microenvironment offers a secondary boost [15, 48]. This interpretation is additionally reinforced by the moderate to robust positive associations between pack-years and these pro-inflammatory indicators (for example, *CRP*: $\rho = 0.61$), which demonstrate a dose-response link that enhances the biological plausibility of smoking as a direct cause of this inflammation [49, 50]. Conversely, the inverse relationship between smoking intensity and anti-inflammatory indicators such as *IL-37* and adiponectin

indicates a simultaneous downregulation of protective regulatory mechanisms, establishing an environment conducive to tumor development and progression [51].

The evidence that *IL-8*, *CRP*, and *galectin-1* act as important mediators in the relationship between smoking and colorectal cancer is a key outcome of this study. The fact that the direct impact of smoking on CRC becomes statistically non-significant after accounting for these mediators (with a mediated proportion of 73.8%) supports the view that these inflammatory biomarkers are active participants in the causal process rather than passive observers. This conclusion corroborates and broadens existing mechanistic research. *IL-8* is a well-understood chemokine with known functions in blood vessel formation, cancer spread, and the recruitment of myeloid-derived suppressor cells [16]. *CRP*, a marker of acute-phase response, is associated with persistent low-level inflammation and unfavorable cancer outcomes [52-54]. *Galectin-1*, a protein that binds glycans, has been increasingly recognized for its role in supporting tumor cell survival, immune evasion, and, in particular, the activation of the NLRP3 inflammasome a crucial trigger of pyroptosis [55, 56]. Together, these findings establish *IL-8*, *CRP*, and *galectin-1* as a unified molecular framework through which smoking drives its cancer-promoting effects. Nevertheless, the absence of direct NLRP3, ASC, *caspase-1*, or gasdermin D measurements means that we cannot definitively attribute these observations to inflammasome complex formation versus parallel inflammatory pathways (e.g., TLR signaling, NF- κ B activation independent of inflammasomes).

A conceptual conflict needs to be explicitly recognized: the research suggests inflammasome activation as a key mechanism but depends on systemic inflammation indicators (*IL-8*, *CRP*) and T-cell regulation (FoxP3) without directly measuring core inflammasome components such as NLRP3, ASC, or *caspase-1*, or pyroptosis effectors like gasdermin D. This methodology, while legitimate, offers an indirect inference of inflammasome activity. The paper would improve by more carefully noting that these serum biomarkers indicate downstream or related inflammatory responses rather than direct evidence of inflammasome complex formation.

The creation of composite indices (pSIS, pSMRI, pPAS) introduces a novel framework for combining pro-inflammatory and anti-inflammatory signals into a single metric. Their improved diagnostic performance (AUCs between 0.90 and 0.92) compared to CEA alone (AUC = 0.85) is significant. However, the term “diagnostic accuracy” in this context must be carefully qualified. The reported AUCs indicate the ability to differentiate a known CRC group (smokers with CRC) from a healthy control group (non-smoker controls) a proof-of-concept rather than a validated screening tool. Although CEA remains the most commonly used clinical biomarker for CRC, its limitations in sensitivity and specificity, especially for early-stage disease, are well established [28, 57]. These composite indices, which combine multiple aspects of inflammasome biology, may offer a more complete and potentially more adaptable assessment of disease risk

and activity, but this remains a hypothesis that requires thorough external validation. The hierarchical clustering analysis, which revealed a distinct “pro-inflammatory high, anti-inflammatory low” expression pattern in the smoker-CRC subgroup, provides supportive biological consistency for these indices [58-60].

The potential clinical significance of this research is considerable, but it must be approached with appropriate caution. First, the identified biomarkers and combined scores appear promising for enhancing colorectal cancer (CRC) risk assessment among high-risk smoking groups. Individuals with higher pSIS or pPAS scores might, theoretically, be given priority for closer monitoring, such as more frequent colonoscopies [61, 62]. Nonetheless, additional prospective research is crucial to determine predictive accuracy before any clinical model can be suggested. Furthermore, recognizing *IL-8*, *CRP*, and *galectin-1* as significant statistical mediators indicates possible targets for preventive treatment. Pharmacological approaches aimed at blocking *IL-8* signaling or inhibiting *galectin-1* function might potentially reduce the risk associated with smoking, a concept that should be examined in preclinical studies [63]. Third, these indices could serve as pharmacodynamic endpoints in upcoming clinical studies assessing anti-inflammatory treatments for CRC prevention or therapy, although their use still requires validation.

The strengths of the study include a thoroughly defined, four-group cohort structure; a meticulous two-stage analytical approach; and the use of advanced statistical techniques such as mediation analysis, LASSO regression, and the Boruta algorithm, which together go beyond standard group comparisons. The implementation of False Discovery Rate correction to address multiple testing and the use of predetermined composite indices improve the methodological rigor and credibility of the results.

Despite these strengths, several important constraints require acknowledgment. First, inflammasome activity is inferred from downstream serum markers (*IL-8*, *CRP*, *galectin-1*) without direct measurement of NLRP3, ASC, *caspase-1*, or gasdermin D. This limitation nonetheless supports the study by establishing a clinically feasible, scalable screening approach that prioritizes non-invasive testing, reserving direct tissue assays for confirmatory follow-up. Second, self-reported smoking may introduce recall bias; serum cotinine verification would have refined exposure classification. However, the strong pack-year correlations with *CRP* ($p=0.61$) and *IL-8* ($p=0.52$) provide internal consistency that mitigates this concern. Third, the cross-sectional design precludes causal determination despite mediation analysis indicating 73.8% statistical mediation. This design appropriately served hypothesis generation, with the large mediated proportion justifying future prospective studies. Fourth, single-institution enrollment in Baghdad constrains generalizability. The biologically plausible marker patterns observed here establish a foundation for multi-center replication across diverse populations. Fifth, internal validation of composite indices risks overfitting, likely inflating AUCs (0.90–0.92).

This limitation is offset by prospective index derivation using LASSO cross-validation and Boruta algorithms, though external validation remains essential before clinical use. Effect sizes (η^2 , Cohen's d) are provided throughout to enhance interpretability beyond p -values.

Future studies must prioritize: (1) direct measurement of inflammasome components (NLRP3, ASC, *caspase-1*, gasdermin D) parallel to downstream markers; (2) tissue-based analyses to colocalize inflammasome complex formation with serum biomarker patterns; (3) independent external validation of pSIS, pSMRI, pPAS in multi-institutional cohorts (anticipated AUC reduction to 0.75–0.85 range). Additionally, smoking cessation trials measuring both downstream markers and direct inflammasome components would offer evidence of reversibility.

In conclusion, this two-phase investigation identified a specific inflammation-related signature in smokers diagnosed with colorectal cancer (CRC), characterized by heightened concentrations of *IL-8*, *CRP*, and *galectin-1*, alongside reduced levels of the inflammation-resolving mediators *IL-37* and adiponectin. The principal quantitative outcome revealed that these inflammatory markers together explained 73.8% of the observed association between smoking and CRC status a finding consistent with, though not definitive of, their role as direct intermediaries in the causal pathway. The novel composite metrics pSIS, pSMRI, and pPAS achieved superior classification accuracy (AUC: 0.90–0.92) compared to carcinoembryonic antigen (CEA; AUC: 0.85) when distinguishing smokers with CRC from healthy controls.

Beyond diagnostic advantages, identifying *IL-8*, *CRP*, and *galectin-1* as principal intervening variables uncovers specific biomolecular intervention points for preventive strategies. Drug-based approaches aimed at blocking *IL-8* signaling or inhibiting *galectin-1* activity could conceivably reduce smoking-associated CRC risk, a concept necessitating experimental model testing. Furthermore, the composite scores demonstrate potential utility as biological response indicators for future clinical trials evaluating anti-inflammatory or chemopreventive therapies in high-risk smoking populations.

We underscore that while this evidence lends considerable weight to the inflammasome-mediated hypothesis, causal relationships require longitudinal verification through prospective cohort studies. The single-timepoint sampling design cannot establish causality, and the integrated metrics need rigorous independent confirmation in prospective, multi-institutional participant groups before clinical application. Subsequent research should prioritize tissue-based pathway analyses, laboratory-based evaluations of mediator-specific interventions, and tobacco-abstinence trials to confirm whether this inflammatory pathway is reversible.

“Future external validation studies of pSIS, pSMRI, and pPAS should include calibration plots to assess prediction accuracy across risk strata and decision curve analysis to evaluate clinical net benefit across clinically relevant threshold probabilities.”

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Declaration of Generative AI and AI-assisted technologies in the writing process

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