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Immunological Evaluation of Certain Biomarkers in Crohn's Disease Patients Infected with *Yersinia Enterocolitica*

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ABSTRACT

Background: *Crohn's disease is a chronic autoimmune disorder producing irritation and swelling in several part of the gastrointestinal tract, greatest commonly disturbing the terminal ileum. The relationship among immune dysregulation and bacterial infection attitudes a important challenge for Crohn's disease patients. This study aimed to evaluate the immunological role with diagnostic efficacy of miR-10b, the macrophage marker CD68, and the innate cytokine IL-28B in Crohn's disease patients with concurrent infection by Yersinia enterocolitica.* ***Methodology:*** *A case-control study was directed involving 100 participants, equally divided into four groups(G-1,2,3,4) which included: Group1(Crohn's with Y.enterocolitica) patients , Group 2(Crohn's only) patients, Group 3(Y.enterocolitica only) patients , and Group 4(healthy controls). Infection was identified serologically, and disease severity was measured using the Crohn's Disease Activity Index(CDAI). Serum levels of CD68, IL-28B, and antibodies were evaluated using ELISA, while miR-10b expression was evaluated via RT-qPCR.* ***Results:*** *The results displayed a sharp and statistically significant increase in the serum levels of the 3 biomarkers in Group1(G1) patients. The relationship with other groups. A very strong positive correlation among the three biomarkers exclusively in G1 after act Correlation investigation; these biomarkers displayed a sharp positive correlation with the Clinical Disease Activity Index(CDAI). Multiple logistic regression analysis established that miR-10b is a strong self-determining predictor of microbially triggered relapse. The triple diagnostic panel verification the maximum diagnostic accuracy, this by ROC curve analysis.* ***Conclusion:*** *The study establishes a close immunological interaction between miR-10b, CD6, and IL-28B in cases Crohn's illness is connected with Y.enterocolitica infection. The developed triple serological panel serves as a highly accurate, non-invasive testing tool for characterizing immune dysfunction, assessing disease severity, and detecting the presence of bacteria in clinical cases.*

Keywords: Crohn's disease, Y. enterocolitica, miR-10b, CD68 and IL-28B.

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INTRODUCTION

Crohn's disease (CD) is an autoimmune disorder classified as a form of chronic inflammatory bowel disease rooted in immune system dysfunction. The disease is characterized by inflammation extending through the intestinal wall (transmural inflammation) and affects various regions of the digestive tract, particularly the area between the ileum and the colon (1).

While the precise causes of this disease remain unclear, key contributing factors include genetic predisposition, environmental and microbial triggers, and dysregulation of the innate immune response in host tissues; these factors can lead to a loss of immune tolerance toward the gut microbiome (2). In cases of bacterial intestinal infection such as that caused by *Y. enterocolitica* (a Gram-negative bacilli) environmental factors significantly trigger autoimmune disease

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activity, the pathogen is characterized by a high capacity to invade epithelial cells and colonize intestinal lymphoid tissues (3; 4).

Macrophages play a crucial role in the primary immune defense against pathogenic microbes within the digestive tract; the differentiation marker CD68 is used to assess macrophage infiltration and activation in inflamed tissues (5). Upon activation, the extracellular portion of the molecule is shed into the serum as soluble CD68, potentially serving as a serum biomarker that reflects the intensity of macrophage-driven inflammation and cellular immune response activity, thereby eliminating the need for invasive tissue biopsies in Crohn's disease patients (6). In response to microbial insults, metabolic byproducts, and bacterial components such as lipopolysaccharide (LPS), CD68-positive cells and mucosal cells at the site of injury secrete the cytokine IL-28B (also known as interferon-lambda 3 or IFN-λ3). This type of interferon plays a role in reinforcing intestinal barrier integrity and regulating defense mechanisms and antibacterial responses (7; 8). However, the continuous and uncontrolled secretion of IL-28B within the intestinal environment of Crohn's disease patients shifts the response from a protective state to a chronic, persistent inflammatory pathway that contributes to tissue and gastrointestinal tract damage (9).

Serum microRNA molecules are key regulators of the body's immune response; specifically, miR-10b acts as a crucial molecular regulator, with its serum levels rising carried within exosomes in direct response to inflammatory signals from activated macrophages (10). miR-10b functions by inhibiting anti-inflammatory pathways and regulatory genes such as (SOCS pathways); this disrupts the self-inhibitory mechanisms of CD68-positive cells and sustains cytokine responses such as that of IL-28B even after the microbial infection has been brought under control (11; 12). Evaluating this triad of parameters and their interplay in patient serum represents promising and significant research aimed at distinguishing between microbial infections and the severity of disease activity in Crohn's disease patients. Furthermore, a study has highlighted the need to establish serum-based immunological markers capable of detecting autoimmune diseases and their associated microbial infections (13).

The present study aims to evaluate three serum biomarkers CD68, IL-28B, and miR-10b in Crohn's disease patients who tested positive for *Y.enterocolitica* infection. It also seeks to investigate the relationship between these markers and the severity of Crohn's disease activity, with the goal of determining whether they reinforce the immunological understanding of the transition from acute microbial infection to a chronic disease state in patients with autoimmune conditions.

METHODOLOGY

1. Study Type and Sample Size Calculation:

a. **Study Type:** Cross-Sectional and case control study

b. **Sample Size:** To compare four groups, the sample size is designed by the Power statistical software (Version 3.1) established on the relevant scientific method. Significance level (α-Type I Error Rate);0.05 (suitable error rate of 5%). Statistical power (1-β); the test's capacity to distinguish true differences with(80–90)% likelihood. Expected Effect Size (f):Set at 0.30 to 0.35,based on earlier studies about : Crohn's disease; by the One-way ANOVA formula, the least obligatory sample size was 20-25 specimens\group.

2.Specimens : Collected from patients known to Al-Sadr Teaching Hospital in Najaf province through the period ranging since January 2023 -December 2024.

3.Study Population: The specimens divided into four groups:

a. **Group 1:** Crohn's disease patients infected with *Y.enterocolitica*.
b. **Group 2:** Crohn's disease patients only
c. **Group 3**: patients infected with *Y.enterocolitica* only
d. **Group 4:** Healthy controls.

4.Questionnaire : The questionnaire was include:

a. **Demographic data**: This including age,six, and residence.
b. Clinical Data : This includes 1. The location of the intestinal infection (Montreal classification) is recognized by the physician which includes: L1: The lower part of the small intestine, L2: The colon only, L3: The colon and small intestine together and L4: upper part of the gastrointestinal tract
2. **Crohn's Disease Activity Index (CDAI):** Remission activity (CDAI < 150), Mild to moderate activity (150-220), Moderate to severe activity (221-450), Very severe activity (CDAI > 450)
c. Is the patient taking any proton pump inhibitors, corticosteroids,NSAIDs,or biologic therapies?

5.Inclusion and Exclusion Criteria

a. **Inclusion Criteria:** The primary inclusion criterion is assignment one of the 3groups based on a physician confirmed diagnosis of Crohn's disease and serological validation (IgM/IgG) of a recent *Y. enterocolitica* infection.
b. **Exclusion Criteria :** Individuals were excluded based on the following: inflammatory or autoimmune diseases; presence of other microbial infections (bacterial, parasitic, or viral gastrointestinal); chronic diseases or malignancies; receipt of medical treatment within 4 weeks prior to specimens collection and breastfeeding or pregnancy

6. Control specimens : This collected from healthy individuals who were free from any disease and matched the patients in terms of age and six.

7. Specimens Collection and Processing

Five milliliters of venous blood were collected from all patients and control under sterile conditions,and the specimens was include **a.** EDTA tube For microRNA extraction and evaluated of miR-10b levels via quantitative reverse transcription-polymerase chain reaction(RT-qPCR). **b.** Gel tube which Serum was separated via centrifugation at 3000rpm for 10m., aliquoted, and stored at -80°C to measure levels of CD68,IL-28B and antibodies(IgM\IgG) against *Y.enterocolitica* using the enzyme-linked immunosorbent assay (ELISA)technique. The serum was distributed into Eppendorf tubes and stored at -80°C until immunological assays were accomplished, to avoid frequent freeze-thaw cycles.

8. Evaluation of immunological measurements:

a. Measurement of miR-10b expression levels

Sample: A 0.4 ml (400 µl) aliquot of separated serum was mixed with 600 µl of TRIzol™ Reagent; specimen were then stored at -20°C until use. **RNA Extraction**: Total RNA was extracted using TRIzol™ Reagent according to the manufacturer's instructions. **Concentration Measurement**: The concentration and purity of the extracted RNA were identified using a fluorometer(promega\USA). **cDNA Synthesis**: Reverse transcription of total RNA into complementary DNA (cDNA) was done in a total reaction volume of 20µl using the Script Reverse Transcriptase Kit(addbio\Korea)next the manufacturer's instructions. **Primer Sequences**: Primers for miR-10b which(F5ʾGGTTAATAAAGCCGCCATCC3ʾ , R5’CTTCTGGAGAGGGGAAAAGCTG3’ and the internal reference gene (housekeeping

gene U6) which F5' CTCGCTTCGGCAGCACA3' , R5'AACGCTTCACGAATTTGCGT3', These manufactured by (Macrogen\Korea). **Quantitative Real-Time PCR** (qRT-PCR) Amplification was achieved by the Cycler Real-Time PCR system (Bioer LinearGene\Chine), with the next thermal cycling program: Initial denaturation: 95°C for3minutes (1cycle). Denaturation and Annealing: 95°C\20seconds, followed by (60°C\30seconds) repeated for45cycles. Melt curve: Gradient from 60-95°Cover (30minutes). **Gene Expression Calculation**:Relative gene expression alteration (fold change)was calculated by the $^{2^{-\Delta\Delta Ct}}$method, normalizing expression in contrast to the housekeeping reference gene(U6). ΔCt = Ct(target gene(miR-10b)−Ct(Housekeeping gene(U6). ΔΔCt = ΔCt(Patient) − ΔCt(Control).

c. Determination of Serum CD68 Levels

Cluster of differentiation CD68 levels was evaluated by CD68ELISA Kit (Cusabio\chani).**Principle:** Sandwich ELISA for the quantitative measurement of CD68 level. **Preparation:** Dilute the wash buffer(1x),dilute Biotin-antibody and HRP-avidin (1:100); prepare the standard series (1000 to 15.6 pg/ml). **Procedure**: Add 100µl of specimen and standards,Incubate for 2hours\37°C. Aspirate liquid and add 100µl of Biotin-antibody,Incubate for 1hour\37C° then Wash 3 times. Afer them Add 100 µl of HRP-avidin,Incubate for 1 hour\37C°then Wash 5times. Also Add 90µl of TMB, Incubate for (15–30m.) in the dark (37°C) then Add 50µl of Stop Solution, after them measure optical density at 450nm. **Results**: Concentrations are calculated via plotting a standard curve by the 4-PL equation through Curve Expert software.

c. Determination of Serum IL-28B Concentration

Serum IL-28B concentrations are quantitatively evaluted by a Sandwich-ELISA kit (Elabscience\China) according to:**Assay Principle:** The enzyme catalyzes the alteration of the substrate into a blue colored product, which turns yellow upon the addition of a stop solution. The wash buffer (25×) was diluted by distilled water, the reference standard was reconstituted also serially diluted(1:2) to create a concentration range of (0–1000pg/mL). The biotinylated antibody and the enzyme conjugate were diluted at a 1:100ratio. **Procedure:** 100µL of standards and specimen were added to the wells and incubated at 37C°\90m. . add of 100 µL biotinylated antibody, then by incubation at 37C°\60 m.; 3times washed of the plate then add 100µl of the enzyme conjugate(HRP) and incubate at 25C\30m., then 5 times wash of the plate then add 90 µl of the substrate and incubate in the dark for 15m., then add 50µl of the stop solution. **Reading results:** Measure absorbance at a wavelength of 450nm by an ELISA reader, then calculate concentrations depended on the standard curve.

9. Statistical Analysis

Data were analyzed by SPSS at version 26 also GraphPad Prism at version9 software; the Shapiro-Wilk test was employed, data were presented as(M±SD)which mean ± standard deviation. A one-way ANOVA followed through Tukey'stest was used to compare the four groups(G1-4) regarding quantitative data. The chi-square (χ^2) test was used for categorical variables (age,six, residence, and Montreal classification). Groups Correlation were identified by (r) defined as the Pearson correlation coefficient. ROC curve analysis was achieved to calculate AUC defined as area under the curve, specificity, and sensitivity and alongside multiple logistic regression to determine aOR defined as adjusted odds ratios.

Results were measured statistically significant at P < 0.05 and highly significant at P < 0.001.

RESULTS

1. Distribution of Demographic characteristic of Crohn's disease patients

Crohn's disease happen in the young adult age group (15–35 years), representative the first peak of diagnosis; there is no significant difference in age among the groups at P =0.095. the male-to-female ratio is close in number which 14males to 11females detected in most groups as G1 also G3, this isn't statistically significant. The distribution displays a slight, expected rise in the proportion of rural residents in G1 and G3 which the two bacterial positive groups compared to the other groups but non-significant difference at P-value=0.063. This shown in Table 1.

Table 1: Distribution of Demographic characteristic of Crohn's disease patients.

Demographic variables		G1 N=25	G2 N=25	G3 N=25	G4 N=25	P-value
Age (Years)	**Mean±SD**	33.4±9.8	32.8±8.5	31.5±7.3	32.2±6.9	0.095NS
	15-35 years	16(64%)	15(60%)	15(60%)	15(60%)	
	36-55 years	9(36%)	10(40%)	10(40%)	10(60%)	
Sex	**Female**	11(44%)	12(48%)	11(44%)	12(48%)	0.08 NS
	Male	14(56%)	13(52%)	14(56%)	13(52%)	
Residents	**Rural**	16(64%)	14(56%)	15(60%)	13(52%)	0.063NS
	Urban	9(36%)	11(44%)	10(40%)	12(48%)	

**NS=non-significant, G1(Crohn's disease patients and Yersinia enterocolitica), G2(patients with Crohn's disease only), G3(patients with Y. enterocolitica only), G4(Control). N=number.*

2. Distribution of Crohn's disease patients according to the Crohn's Disease Activity Index (CDAI):

Disease severity in Crohn's disease patients in G1 also G2 revealed a statistically significant difference in disease activity levels depended on *Y. enterocolitica* infection. Group 1 which give a greater mean CDAI score of 328.6± 3.3 compared to 208.7 ± 8.2 in Group 2, was significant difference at P < 0.001. In Group 1, cases were disseminated as follows: 60% which moderate-severe activity and 20% represented very severe activity. the common in Group 2 which 60% represented mild to moderate activity. This is illustrated in Table 2.

Table 2: Distribution patients according to the Crohn's Disease Activity Index.

CDAI	G1 No.(%)	G2 No.(%)	Total	P-value
Mean±SD	328.6± 3.3	208.7 ± 8.2	-------	
Remission (CDAI < 150)	-------	--------	--------	
Mild to moderate activity (150-220)	5(20%)	15(60%)	20(40%)	
Moderate to severe activity (221-450),	15(60%)	7(28%)	22(44%)	
Very severe activity (CDAI > 450)	5(20%)	3(12%)	8(16%)	
Total of all Crohn's Disease			**50(100%)**	0.001**

CDAI : Crohn's Disease Activity Index, G1(Crohn's disease and Yersinia enterocolitica patients), G2(patients with Crohn's disease aonly), No.(number), % (percentages) , **(Highly significant).

3. Distribution of Crohn's disease patients according to the anatomical site of intestinal infection (Montreal Classification):

The intestinal infection sites in Crohn's disease patients as Groups1+2 discovered a significant difference at P = 0.023 related by the occurrence of *Y. enterocolitica* infection. The common of cases obtainable in L1, this include the lower part of the small intestine,terminal ileum at 52% and the colon besides small intestine together which L3 at 40%, significance that ileal involvement for 90% of cases in G1. While patients G2 displayed a higher balanced dissemination: colonic only which L2 at 40%, L1 and L3 at (28%) for each them. this shown in table 3.

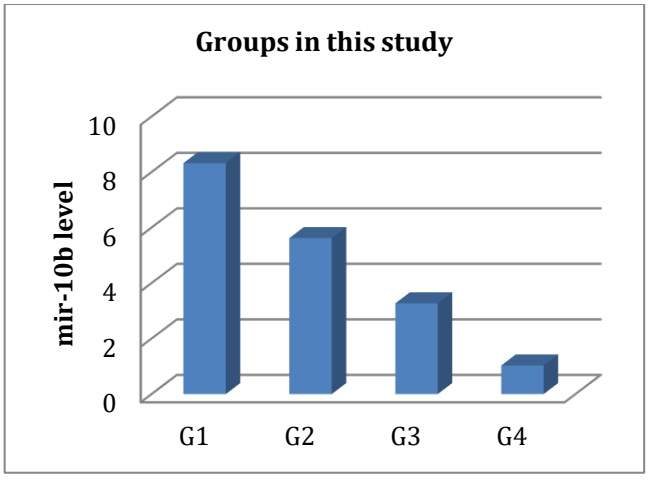
Table 3: Distribution patients according to the anatomical site of intestinal infection.

Anatomical site of intestinal infection (Montreal Classification	G1 No.(%)	G2 No.(%)	Total	P-value
L1	13(52%)	7(28%)	20(40%)	
L2	2(8%)	10(40%)	12(24%)	
L3	10(40%)	7(28 %)	17(34%)	
L4	0(0%)	1(4%)	1(2%)	
Total	25(100%)	25(100%)	50(100%)	0.023*

*L1: The lower part of the small intestine, L2: The colon only, L3: The colon and small intestine together and L4: The upper part of the gastrointestinal tract. G1(Crohn's disease and Yersinia enterocolitica)patients , G2(Crohn's disease only)patients. No.(number), % (percentages) , *(statistically significant at P < 0.05).*

4. Results of measuring the three immunological biomarkers across the study groups:

Analysis exposed greatly significant differences at p< 0.001 in the levels of all studied biomarkers (miR-10b, CD68, and IL-28B) amongst the four groups (G1-4) This displayed in Figure 1. G1 noted the highest miR-10blevel (8.32 ± 0.05), followed by G2 (5.62 ± 0.02), and then G3 (3.27 ± 0.08) while healthy control (1.03 ± 0.03). The values CD68 levels were 92.22 ± 0.58 ng/mL in G1, 73.15 ± 0.66 ng/mL in G2, 51.30 ± 0.80 ng/mL in G3, and 19.25 ± 0.35 ng/mL in the healthy group. IL-28B levels peaked in G1 which 942.80 ± 2.12 pg/mL, whereas G3 (Yersinia infection only) showed a significantly higher concentration (801.90 ± 1.60 pg/mL) compared to G2 which (Crohn's only: 608.3 ±3.42pg/mL), highlighting the direct role of the bacteria in stimulating the innate cytokine response while healthy group control (22.13 ± 1.3 pg/mL).



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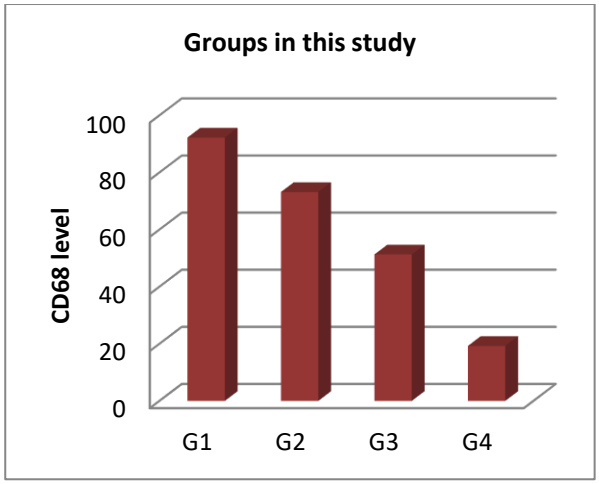

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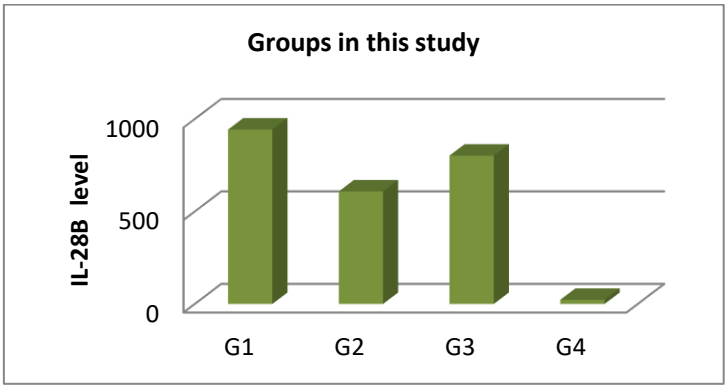

C

Figure 1: Measurement the immunological biomarkers (miR-10b, CD68 and IL-28B in four the study groups. *G1(patients with Crohn's disease and Yersinia enterocolitica), G2(patients with Crohn's disease aonly), G3(patients with Y.enterocolitica only), G4 (Control). A: miR-10b expression level , B: CD68 level and C: IL-28B level .*

5. Correlation among the three biomarkers across patient groups:

To evaluate the relationship between miR-10b(serum microRNA), CD68(the cellular marker), and IL-28B(innate cytokin), a personal correlation analysis which(r) was showed in the three patient groups(G1-3). The results established the strength of the correlations, depending upon the pathological context and the presence of the bacterial inducement , this shown in table 4 . Analysis discovered a very strong in G1,with highly significant positive correlation amongst all serum biomarkers; the correlation between miR-10b and CD68 was r=+0.94 at P < 0.001, and between miR-10b and IL-28B was =+0.77 at p < 0.001, while the correlation between CD68 and IL-28B reached the maximum level r=+0.89 at < 0.001 . This pronounced correlative relationship is identified to a synergistic design and a positive feedback loop triggered through bacteria and perpetuated by the epigenetic abnormalities related with Crohn's disease.

Results from the second group discovered a moderate positive correlation between miR-10b and CD68 was r=+0.72 at P = 0.032 , as well as positive correlations among CD68 and IL-28B (r=+0.63, P = 0.021) and between miR-10b and IL-28B (r=+0.56 at P = 0.035). This pattern reveals the chronic inflammatory response representative of Crohn's disease in the absence of a direct bacterial trigger. Whereas results from the Group3 displayed a strong positive correlation only between the CD68 biomarker and cytokine IL-28B (r=+0.75 at P <0.001) but no significant correlation was detected between miR-10b and CD68 which r=+0.48 at p=0.088 however IL-28B and miR-10b which r=+0.27 at p=0.246 .

Table 4: Correlation among biomarkers in this study.

Groups	Parameters		miR-10b	CD68	IL-28B
A: Group 1 (patients with Crohn's disease and Yersinia enterocolitica)	miR-10b	r	1	+0.94 ***	+0.77 ***
		p	-----	< 0.001	< 0.001
	CD68	r	+0.94 ***	1	+0.89***
		p	< 0.001	------	< 0.001
	IL-28B	r	+0.77 ***	+0.89***	1
		p	< 0.001	< 0.001	------
B: Group 2	miR-10b	r	1	+0.72*	+0.56*
		p	---------	0.032	0.035

Groups	Parameters		miR-10b	CD68	IL-28B
(patients with Crohn's disease aonly)	CD68	r	+0.72*	1	+0.62*
		p	0.032	--------	0.021
	IL-28B	r	+0.56*	+0.62*	1
		p	0.035	0.021	------
C: Group 3 (patients with Y.enterocolitica only)	miR-10b	r	1	-0.48	-0.27
		p	-------	0.088	0.246
	CD68	r	-0.48	1	+0.75***
		p	0.089	------	< 0.001
	IL-28B	r	-0.27	+0.75***	1
		p	0.246	< 0.001	------

6. ROC Curve Analysis:

The results demonstrated that serum miR-10b showed the maximum diagnostic accurateness as a single biomarker (AUC=0.889 at P=0.01) at a cut-off value > 4.92, with a specificity of 88% and a sensitivity of 86%.Then the biomarker CD68 (AUC=0.835 at P=0.013 with cut-off >69.57 and the cytokine IL-28B (AUC=0.773 at P=0.002), This shown in table 5. Combining the 3 biomarkers into a triple diagnostic panel significantly improved diagnostic performance, resulting in an AUC of 0.981 at P< 0.001) and increasing sensitivity which 96% and specificity to 97%. These findings confirm the effectiveness of this joint serum marker panel as a extremely accurate, non-invasive tool for identifying the autoimmune-related dysfunctions and changes related with Crohn's disease, particularly in cases of simultaneous *Y. enterocolitica* infection.

Table 5 : Receiver Operating Characteristic curve analysis Biomarker in this study.

Biomarker	AUC	Cut-off	Specificity	Sensitivity	P-value
miR-10b	0.889	>4.92	88%	86%	0.01*
CD68	0.835	>69.57	82%	81%	0.013*
IL-28B	0.773	>121.9	77%	76%	0.002**
Bi-panal (miR-10b and CD68)	0.926	----	92%	91%	< 0.001**
Triple panal (miR-10b , CD68 and IL-28B)	0.981	----	97%	96%	< 0.001**

*ROC = Receiver Operating Characteristic curve; AUC = Area Under the Curve, ** High significance at the 0.001 level.*

7. Multivariable Logistic Regression Analysis:

Multivariable logistic regression analysis revealed that raised levels of miR-10b (aOR=3.23 at P =0.001) and CD68 (aOR=1.19 at P = 0.008) were strong, independent predictors of *Y.enterocolitica* triggered numerous relapses in Crohn's disease patients, even after altering for demographic and clinical variables such as age and disease location, this shown in table 6.

Table 6: Logistic Regression Analysis of Biomarker with infection location and age.

Biomarkers	Crude OR	Adjusted OR	CI 95%	p-value
miR-10b	3.56	3.23	1.96-5.27	0.001**
CD68	1.23	1.19	1.26-1.13	0.003**
IL-28B	1.16	1.14	1.17-1.12	0.021**
Infection location	1.12	1.01	1.14-1.01	0.623(NS)
Age (years)	2.21	1.86	3.59-0.99	0.783(NS)

***High significance at the 0.001 level, NS= non-significant.*

8. Clinical Correlation Between the Three Biomarker and Clinical Disease Activity Index

Correlation analysis discovered a strong, significant positive relationship between the 3 studied serum biomarkers and the Clinical Disease Activity Index (CDAI), reinforcing the clinical dependability of this serum panel as a non-invasive alternative for measuring disease action and severity, this shown in table 7.

Table 7*:* Clinical Correlation Between Biomarker and Clinical Disease Activity Index.

Biomarkers	Personal correlation(r) with CDAI	p-value
miR-10b	+0.92	0.001**
CD68	+0.89	0.006**
IL-28B	+0.71	0.002**

** *High significance at the 0.001 level.*

DISCUSSION

The results showed no statistically significant differences regarding age, six, or place of residence among the four groups. Studies indicate that the onset of Crohn's disease most commonly occurs during the second and third decades of life (14; 15). As age advances, physiological changes occur that affect the levels of serum biomarkers and cytokines (16). Furthermore, studies confirm that Crohn's disease affects males and females at approximately equal rates in most geographical regions, as the disease is linked to the individual's immune system rather than the region itself (17).

Estrogen and the menstrual cycle significantly and directly influence macrophage responses and the production of cytokines associated with innate immunity (18); these subtle sex-related differences are linked to the pathological environment and bacterial infection. *Y.enterocolitica* is a food- and waterborne pathogen that is prevalent in rural areas, largely due to the consumption of unpasteurized milk and undercooked animal products (19; 20). Exposure to this environmental bacterium in rural settings can modulate intestinal mucosal immune responses, thereby reinforcing the link between bacterial infection and autoinflammation in genetically predisposed individuals (2).

The results indicate that intestinal lesions are concentrated in the terminal ileum (L1), with cases of dual involvement affecting both the ileum and the colon (L3); furthermore, in patients with colonic disease (L2), *Y. enterocolitica* demonstrated a potent histological effect characterized by adhesion to and invasion of the ileal mucosa, a region rich in M cells and Peyer's patches (21).

Assessment of disease severity revealed statistically significant differences at $P<0.001$ between the two patient groups: the majority of patients in the G1 fell into the moderate-to-severe and severe categories, whereas the majority in the G2 fell into the mild-to-moderate category. The presence of *Y. enterocolitica* acts as a second acute trigger consistent with the Second Hit Hypothesis that interacts with the underlying autoimmune background of Crohn's disease patients (14). This dual stimulation leads to the overexpression of miR-10b and the inhibition of negative regulatory genes such as SOCS1; this prevents the resolution of inflammation, resulting in persistent infiltration of CD68-positive cells and high production of inflammatory cytokines manifesting directly as more severe clinical symptoms and an raised Crohn's Disease Activity Index (CDAI) score (22; 23).

The results revealed a sharp increase in the levels of all three markers in the serum of G1 patients compared to the other groups. This notable rise in miR-10b levels aligns with other studies indicating that this microRNA molecule plays a pivotal role in regulating innate and adaptive immune-inflammatory pathways within the digestive tract by inhibiting negative regulatory genes such as SOCS1(23;24). Raised CD68 levels indicate increased macrophage infiltration and activation within the ileal mucosa, as CD68 serves as a biomarker reflecting the magnitude of the tissue macrophage response in inflammatory bowel diseases (25).The raised IL-28B level highlights its role as a crucial cellular defense mechanism protecting intestinal epithelial cells upon exposure to invasive gut bacteria (26).

Correlation analysis revealed a very strong, significant positive correlation across all groups and for all three parameters. In G1 , a positive feedback loop is triggered by *Y. enterocolitica* components (such as Yop proteins and lipopolysaccharide [LPS]) interacting with pattern recognition receptors (PRRs) on the membranes of infiltrating macrophages (CD68+). This initiates a dual response: the components acutely activate macrophages thereby increasing serum CD68 levels and stimulating high production of the cytokine IL-28B as an acute defense mechanism against the microbe (26) and also induce the overexpression of miR-10b within serum-derived vesicles (24).

There is a moderate positive correlation among the three parameters in G2, where macrophage(CD68) infiltration is gradually stimulated by endogenous inflammatory cytokines coinciding with regulated miR-10b expression and moderate IL-28B secretion thereby protecting the mucosal lining without reaching the cytokine storm stage (27).

The presence of a strong positive correlation exclusively between CD68 and IL-28B alongside the complete absence of any statistically significant association involving miR-10b in G3suggests a functional dissociation in the acute bacterial response among individuals with an intact immune system. In this context, CD68+ cells (activated by the bacteria) effectively secrete IL-28B to control Yersinia bacteria. Meanwhile, miR-10b expression remains normal due to immune system integrity, thereby preventing regulatory dysfunction; this allows SOCS proteins to terminate the inflammatory response and facilitate complete recovery following the clearance of the infection (21).These findings confirm that miR-10b dysregulation and its direct association with cytokines are not caused by the bacterial infection itself, but rather serve as an immunological marker of the autoimmune dysfunction associated with Crohn's disease.

Receiver Operating Characteristic (ROC) curve analysis demonstrated that miR-10b yielded the largest area under the curve as an independent biomarker. Serum microRNAs are characterized by high stability and a superior ability to detect abnormalities in inflamed tissues compared to conventional cytokines (23; 24). Next is the CD68 biomarker, which serves as an indicator of the magnitude of the cellular response and the extent of cell infiltration into intestinal tissues(25;26).Although IL-28B recorded a lower value compared to the aforementioned markers, it demonstrated unique efficacy in directly distinguishing between Crohn's disease cases with and without bacterial infection, making it a sensitive indicator for detecting microbial infection.

Diagnostic capability is maximized when all these criteria are combined into a (triple-test panel).This marked improvement in diagnostic accuracy stems from the functional and biological synergy among the panel's components: CD68 measures macrophage infiltration density, IL-28B determines the

severity of the acute innate cytokine response to bacteria, and miR-10b reveals the dysfunction driving persistent autoimmune inflammation (22). This integration reduces false-positive and false-negative results to 5% or less.These findings align with modern global medical and laboratory guidelines advocating for multi-marker serological test panels as a non-invasive alternative that minimizes the need for complex endoscopic procedures to diagnose and monitor Crohn's disease activity (27; 28).

Multivariate logistic regression analysis revealed that miR-10b overexpression is a very strong independent predictor of bacterial infection-associated immune dysfunction in Crohn's disease, followed via the macrophage marker CD68. Raised levels of these markers are not merely secondary consequences of demographic factors or lesion location; rather, they represent independent risk factors driving disease progression. These findings align with other studies confirming that altered serum microRNA levels reflect significant structural changes in the immune pathways of intestinal epithelial cells, making them early, accurate indicators of acute inflammation prior to the onset of overt clinical symptoms (22; 24).

A strong statistically significant positive correlation exists between the three serum markers and the Crohn's Disease Activity Index (CDAI), with miR-10b exhibiting the highest correlation coefficient. Overproduction of miR-10b leads to the inhibition of negative regulatory genes (such as SOCS1) and exacerbates tissue damage; this is directly reflected in the increased severity of clinical symptoms such as diarrhea, abdominal cramps, and weight loss as measured by the CDAI (23). Evaluating this panel of three serum markers not only ensures high diagnostic accuracy but also enables the use of a non-invasive serum marker that reflects disease activity more effectively than traditional methods (27).

CONCLUSIONS

1. There is an immunological synergy between miR-10b overexpression, tissue macrophage activation (CD68), and the innate immune response (IL-28B) in cases where Crohn's disease is associated with *Y.enterocolitica* infection; this demonstrates the existence of a positive feedback loop that exacerbates the severity of the inflammatory flare-up.
2. Serum miR-10b levels serve as an accurate independent biomarker for distinguishing between an endogenous inflammatory flare-up of Crohn's disease and a transient bacterial infection in healthy individuals.
3. This triple diagnostic panal offers excellent diagnostic accuracy, making it a comprehensive serological option that minimizes the likelihood of erroneous results.
4. Serum levels of these three markers show a strong positive correlation with the Crohn's Disease Activity Index (CDAI), serving as a non-invasive serological indicator of disease severity.

RECOMMENDATIONS

1. Utilize a "triple serological panel" as a rapid, non-invasive screening test in clinical laboratories and hospitals to facilitate the early differentiation between bacterial infections and exacerbations associated with Crohn's disease.
2. Conduct periodic serological screening for *Y. enterocolitica* in all Crohn's disease patients, particularly those with disease localized to the ileum.
3. Direct future pharmacological research toward developing therapies that target the inhibition of miR-10b overexpression.
4. Conduct prospective longitudinal studies with larger sample sizes to monitor changes in these serological immune markers which serve as predictive biomarkers both before and after treatment.

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