Role of Circular RNA CCDC66 and Serine/Threonine Kinase-2 (AKT2) in the Pathogenesis of Crohn's Disease: A Potential Diagnostic Biomarker

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Abstract:

Background: Crohn's disease (CD) is a long-term inflammatory disease of the bowel characterized by recurring environmental influences, including gut microbiota. While non-coding RNAs and intracellular kinases are implicated in inflammatory processes, their specific roles in CD remain unclear.

Objectives: This study explored the involvement of circular RNA coiled-coil domain-containing 66 (circCCDC66) and serine/threonine kinase (AKT2) as potential biomarkers in CD pathogenesis.

Patients and methods: A Cross-sectional case-control study involving 50 participants: 34 CD patients (17 active and 17 inactive) and 16 healthy controls. Disease activity was tested with the Simple Endoscopic Score for Crohn's Disease (SES-CD) and the Crohn's Disease Activity Index (CDAI). Plasma circCCDC66 levels were assessed by quantitative real-time PCR (qRT-PCR), while serum AKT2 concentrations were tested using ELISA.

Results: CircCCDC66 expression was markedly higher in patients with CD than in control subjects, with no significant difference between active and inactive disease stages. AKT2 levels were elevated in CD; however, this increase did not reach statistical significance. Receiver operating characteristic (ROC) analysis indicated that circCCDC66 had a sensitivity of 79.41% for diagnosing CD, but it was unable to distinguish disease activity. A positive correlation was identified between circCCDC66 and Fc in patients with active CD ($p = 0.034$, $r_s = 0.517$).

Conclusion: These findings imply that circCCDC66 may contribute to CD pathology and hold potential as a non-invasive diagnostic marker, especially during remission. However, neither circCCDC66 nor AKT2 effectively distinguished disease activity. Further research is necessary to clarify their functional roles in CD.

Keywords: CD; circCCDC66; Protein Kinase B; Autophagy; Inflammation.

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Introduction

Crohn's disease (CD) is a chronic inflammatory condition affecting the gastrointestinal tract (GIT), often involving the terminal ileum and colon. It features transmural inflammation, granulomas, and relapses, leading to complications like fistulas, strictures, and bowel obstructions. The increasing global prevalence burdens healthcare systems, primarily due to related complications such as CRC **(Buie et al., 2023)**. Across the Arab world, the annual incidence of CD is approximately 1.46 per 100,000 people **(Mosli et al., 2021)**. Egypt has seen an increase in CD cases over the past twenty years, driven by various factors such as high consumption of fast food, reduced dietary fiber intake, psychological stress, and environmental pollution. **(Kamel et al., 2021)**.

The development of CD is a multifactorial process, including genetic predispositions, the intestinal immune response, autophagy defects, environmental influences, and dysbiosis **(Noble et al., 2023)**. Autophagy is a highly conserved and dynamic mechanism through which cells sequester and degrade specific components within lysosomes. **(Gómez-Virgilio et al., 2022)** Numerous studies show that autophagy is essential for intestinal epithelial cell function. Its impairment disrupts the mucosal barrier, hampers antimicrobial peptide release, bacterial clearance, and worsens inflammation. Abnormal autophagy links to diseases like inflammatory bowel disease (IBD) **(Tran et al., 2023)**, and cancers, including CRC. **(Ma et al., 2023)** Currently, diagnostic methods for CD rely on invasive techniques, such as endoscopy and histopathology, as well as non-specific biomarkers, including fecal calprotectin (Fc) and C-reactive protein (CRP).**(Alejandro and Cristina, 2023)**

Calprotectin, a calcium-zinc-binding protein in neutrophils, appears in feces due to neutrophil migration during inflammation. It remains stable in stool for as long as a week at room temperature, making it an ideal biomarker for intestinal inflammation. Fc helps distinguish IBD from irritable bowel syndrome, and acts as a positive acute-phase protein and chemotactic factor that enhances cell receptor expression involved in neutrophil migration, adhesion, and phagocytosis **(Dajti et al., 2023)**.

Recent findings increasingly suggest a key role for non-coding RNAs (ncRNAs), such as long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and microRNAs (miRNAs), in regulating inflammatory and immune responses in autoimmune and chronic inflammatory diseases, such as CD. circRNAs, a relatively recently discovered class of ncRNAs, are distinguished by their covalently closed-loop structures, which lack free 5′ and 3′ ends and are highly conserved across species. Their role as miRNA sponges **(Zhu et al., 2024)**, scaffolds for RNA-binding proteins **(Das et al., 2021)**, and influencers of transcriptional processes **(Hwang et al., 2024)**, establishes them as important molecular regulators.

Given their unique features, circRNAs serve as excellent therapeutic targets and diagnostic biomarkers. They are more stable than linear RNAs due to their covalently closed-loop structure, making them resistant to exonucleases. Additional benefits include high sensitivity, accessibility from body fluids, tissues, exosomes, and consistent expression in normal and disease states. **(Yuan et al., 2023)**.

The roles of circRNAs in health and disease have been extensively studied, revealing their associations with autophagy, apoptosis, cell cycle progression, and proliferation **(Saleem et al., 2024)**. These ncRNAs have been shown to exert regulatory effects in various diseases, such as cancer **(DeSouza et al., 2024)**, and inflammatory diseases, where circRNA-protein interactions can modulate signaling pathways **(Lian et al., 2021)**. However, only a limited number of circRNAs have been identified as diagnostic biomarkers or therapeutic targets for IBD **(Lun et al., 2023)**.

A key circRNA, circular RNA of coiled-coil domain containing 66 (circCCDC66), has been identified as a novel oncogenic ncRNA upregulated in several malignancies,

including CRC. It influences tumour progression by regulating transcription, protein translation, alternative splicing, and epigenetic modifications **(Wang et al., 2023)**. This 468-nucleotide circRNA originates from exons 8 to 10 of the CCDC66 gene, located at 3p14.3 on the short arm of chromosome 3. circCCDC66 is abundantly expressed in the cytoplasm of eukaryotic cells and is found in numerous eukaryotic cell types **(GeneCards, 2025).**

Beyond its oncogenic roles, circCCDC66 is also implicated in non-cancerous conditions, including inflammatory diseases **(Zhang et al., 2022)**. Evidence suggests that circCCDC66 may promote CRC progression by upregulating serine/threonine kinase 2 (AKT2) through the sponging of miRNA-510-5p and modulation of autophagy **(Tu et al., 2020)**. Given that impaired autophagy is recognized as a contributing factor in IBD and contributes to the development of CD, **(Alula and Theiss, 2023)** plasma levels of circCCDC66 may serve as a promising non-invasive biomarker for diagnosing CD.

Serine/threonine kinases, recognized as protein kinase B (PKB or AKT), phosphorylate various substrates upon activation, thus regulating multiple cellular functions, including proliferation, cytoskeletal organization, survival, migration, and autophagy **(Cohen, 2013)**. AKT2, an isoform of these protein kinases, it plays a vital role in autophagy by phosphorylating and shutting down Unc-51-like autophagy activating kinase-1 (ULK1) **(Canaud et al., 2013)**, as well as by inhibiting the tuberous sclerosis complex 1 (TSC1), both of which are key regulators of autophagy and autophagosome formation. A deficiency in TSC1, observed in CD patients, results in increased activity of the mTORC1, *suppressing autophagy* **(Xie et al., 2020)**. Furthermore, AKT2 contributes to inflammatory processes by activating NF-κB and modulating the production of inflammatory cytokines and adhesion molecules **(Cianciulli et al., 2020)**.

Given the incompletely clarified role of circCCDC66 in CD and its potential regulation of the autophagy-related protein AKT-2, this cross-sectional study was designed to evaluate the involvement of circCCDC66 and circulating AKT2 in CD pathogenesis, investigate their correlation with disease activity, and explore their potential as non-invasive biomarkers for CD diagnosis.

Patients and methods

Study design:

In pursuit of this goal, our study was designed as a cross-sectional observational study conducted at the Gastroenterology unit, Alexandria Main University Hospital over a one-year timeframe, between June 2023 to May 2024. The study aimed to evaluate the association between circular RNA (circRNA) expression levels and the occurrence of [Disease Name].

Subjects:

A total of 34 patients with CD who either attended the outpatient clinic for a scheduled colonoscopy or were admitted to the Gastroenterology unit were enrolled. Based on their disease activity levels, the CD patients were categorised into two subgroups: **Group Ia**, comprising 17 patients with active CD, and **Group Ib**, comprising 17 patients with inactive CD. A control group consisting of 16 healthy individuals' volunteers, matched for sex and age, was also included (**group II**).

The study was approved by the Institutional Ethics Committee of Alexandria Faculty of Medicine (IRB NO: 00012098, FWA NO: 00018699), and all participants provided written informed consent after a detailed explanation of the study procedures.

The study excluded patients and healthy volunteers who met any of these criteria: indeterminate colitis, GIT malignancy, recent abdominal surgery within the past three months, autoimmune diseases like rheumatoid arthritis, prior exposure to biological therapy, type 1 diabetes mellitus, coronary artery disease, pregnancy, chronic renal and liver diseases, or those who declined to participate.

Blood sampling:

Blood samples (5 mL) were collected from all participants via direct venous puncture into EDTA tubes. The tubes were centrifuged

at 1200×g for 10 minutes to isolate the plasma samples, which were then aspirated and divided into two aliquots. These aliquots were kept at -80 °C until required for biochemical analysis.

Methods:

A thorough history and physical examination were performed on all patients included in this study, including age, gender, smoking status, and GIT symptoms (abdominal pain, GIT bleeding, diarrhea, and weight loss). Clinical evaluation focused on signs of GIT diseases, including tenderness, palpable masses, organ enlargement, perianal fistula, and extraintestinal manifestations. The **Simple Endoscopic Score in Crohn's Disease** (SES-CD) **(Table 1) (Daperno et al., 2004)** was used for the diagnosis and assessment of CD activity. The Crohn's **Disease Activity Index (CDAI)** was also utilized to assess disease activity; a score of less than 150 indicates inactive disease (remission), and a score of 150 or greater indicates active disease. The CDAI considers factors like stomach discomfort, overall health status, complications, presence of an abdominal mass, anemia, and weight changes. A CDAI score <150 suggests asymptomatic remission, 150-200 indicates mild-to-moderate CD, 220-450 signifies moderate-to-severe CD, and > 450 represents severe-fulminant illness **(Thia et al., 2011)**.

Table 1: Crohn's disease simple endoscopic score results interpretation (Daperno et al 2004).

Items	**SES-CD Score**			
Variable	0	1	2	3
Ulceration	None	< 0.5 cm	0.5-2 cm	> 2 cm
Ulcerated Surface	None	< 10%	10 – 30%	> 30%
Inflamed Surface	None	< 50%	50 – 75%	> 75%
Stenosis	None	single, passable	multiple, passable	Can not be passed

Routine laboratory investigations:

Standard laboratory tests were conducted for all participants, including a complete blood picture, liver and renal test profile, CRP and ESR. Furthermore, a stool sample for Fc was also assessed.

Determination of plasma AKT2 protein by enzyme-linked immunosorbent assay

Plasma AKT2 concentrations were determined using a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit supplied by INNOVA BIOTECH CO., LTD. (Catalogue No. In-Hu4126, China). The assay was performed according to the manufacturer's instructions (**Hui et al., 2020**). The intra-assay coefficient of variation (CV) is < 10%, the inter-assay CV is < 12%, and the assay detection range is 16–1000 pg/ml.

Relative quantification of plasma level of circCCDC66 by real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR):

Total RNA was extracted from plasma samples using the Qiagen miRNeasy Mini Kit (Cat. No. 217004, Qiagen, Germany) based on the manufacturer's protocol. The purity and concentration of the isolated RNA were assessed by NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, USA). Complementary deoxyribonucleic acids (cDNAs) were subsequently produced using the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, USA, Cat. No. K1622). Each reverse transcription (RT) reaction comprised 11 µl of the isolated RNA sample and 9 µl 2× RT master mix, resulting in a final volume of 20 µl. The 9 µl of 2× RT master mix included 4 µl 5× RT reaction buffer, 2 µl deoxynucleotide triphosphate (dNTP) mix, 1 µl RevertAid M-MuLV reverse transcriptase, 1 µl RiboLock RNase Inhibitor, and 1 µl random hexamer primer. The reaction used a thermal cycler with this program: 5 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 70°C, then stopped. The cDNAs were kept at -20 °C until

used for RT-qPCR to measure circCCDC66 expression **(Li et al., 2020)**. RT-qPCR were performed using specific primers for the CCDC66 and for Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) (as endogenous control), sourced from Thermo Fisher Scientific Inc., Invitrogen (Website: http://www.thermoscientific.com/fermentas) (**Table 2**).

Table 2: Primer sets developed for RT-qPCR (El-Shendidi et al., 2022)

Primer	Sequence	Tm[a] (°C) (1M Na)	% GC	Concentration (pmol/µl)
CCDC66				
Forward primer	5'-ACCTACAACCGGAAGCCAG-3'	70	58	100
Reverse primer	5'-AGCAGTACTGTTTCCTGATGC-3'	69	48	100
GAPDH gene				
Forward primer	5'-GAAGGTGAAGGTCGGAGTCAAC-3'	73	55	100
Reverse primer	5'-CAGAGTTAAAAGCAGCCCTGGT-3'	71	50	100

a: Tm: melting temperature.

To ensure accurate pipetting of small volumes, a premix was prepared containing the Maxima SYBR Green qPCR master mix, ROX reference dye, and the appropriate primers, with volumes adjusted according to the total number of reactions. The volumes of these components per reaction are presented in **Table 3**. 7.5 µl of this premix was added to each PCR tube, followed by 2.5 µl of cDNA. All samples were tested in duplicate, and a no-template control was included in each experiment.

Table 3: Volumes of Maxima SYBR Green qPCR Master Mix and primers used for CCDC66 and GAPDH gene PCR per reaction

CCDC66 gene reaction
5 µL Maxima SYBR Green qPCR master mix (2X), no ROX.
0.5 µL CCDC66 gene forward primer (50 pmol).
0.5 µL CCDC66 gene reverse primer (50 pmol).
0.05 µL ROX solution
1.45 µL nuclease-free water
Total volume 7.5 µl
GAPDH gene reaction
5 µL Maxima SYBR Green qPCR master mix (2X), no ROX.
0.5 µL GAPDH gene forward primer (50 pmol).
0.5 µL GAPDH gene reverse primer (50 pmol).
0.05 µL ROX solution
1.45 µL nuclease-free water
Total volume 7.5 µL

RT-qPCR was performed using a protocol in which the annealing temperature was adjusted according to the melting temperature (Tm) of each primer set to optimize the PCR reaction (**Table 4**). Data analysis was conducted using StepOne™ Software v2.2. A melting curve analysis was performed to confirm the specificity and identity of the amplified PCR products. The relative quantification method ($RQ = 2^{-\Delta\Delta CT}$) was utilized to determine the fold change in CCDC66 mRNA expression levels between samples and the GAPDH standard control.

Table 4: RT-qPCR cycler conditions for Applied Biosystems

Step	Time	Temperature
PCR Initial activation step	10 min	95°C
3–step cycling: (40 cycles)		
Denaturation	15 s	95°C
Annealing	30 s	65°C for CCDC66 65°C for GAPDH
Extension	30 s	72°C

Ethics approval: This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Faculty of Medicine, Alexandria University, Egypt (approval number: 0305388, IRB NO: 0007555-FWA 00018699). **Consent to participate:** Informed consent was obtained from all participants included in the study.

Statistical analysis:

All analyses were conducted using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA; released 2020). Quantitative variables were summarized by range, mean, standard deviation, median and Interquartile range. Quantitative Data were checked for completeness and outliers before analysis. The normality of continuous variables was assessed using the **Shapiro-Wilk test**. For normally distributed variables, the **one-way ANOVA test** was used to compare the three studied groups, followed by the **Post Hoc test (Tukey)** for pairwise comparison. The **student t-test** was used to compare the CD cases (total group I) and the control group (group II). On the other hand, for non-normally distributed quantitative data, the **Kruskal-Wallis's test** was used to compare three groups, followed by a Post Hoc test (Dunn's test for multiple comparisons) for pairwise comparisons. The **Mann-Whitney test** was used to compare the two groups: CD patients and the control group. **The Spearman coefficient** was used to correlate 2-ddct (CCDC66), a non-normally distributed variable, with different parameters, whereas the **Pearson coefficient** was used to correlate AKT2 (pg/ml) with ESR and SES-CD, which were normally distributed variables. The **receiver operating characteristic (ROC) curve** was used to assess the diagnostic performance of the markers. An area of more than 50% gives acceptable performance, and an area of about 100% is the best performance for the test. Significance of the results obtained was judged at the 5% level.

Results

Demographic data:

Regarding gender and age, no statistical significance was observed between the three studied groups (p = 0.160, 0.613, respectively), nor between the total CD patient group (group I) and the control group (group II) (p =0.328, 0.938, respectively). Females had a higher prevalence than males across all studied groups, with ratios of 0.89:1 in group Ia, 0.31:1 in group Ib, and 0.23:1 in group II. (**Table 5**)

Disease activity level in group 1a:

Based on SES-CD & CD-AI, seven patients (41.2%) in group Ia had mild activity, while 10 (58.8%) showed moderate activity. (**Table 5**)

Routine laboratory investigations:

Regarding **white blood cell (WBC) count**, the active CD patients (group Ia) exhibited a significantly higher count than the inactive group (p1 = 0.028) and the control group (p2 = 0.016)**.**

Erythrocyte sedimentation rate (ESR) levels were significantly elevated in the total CD group (I) than the healthy control group (II) (p 0< 0.001). Also, ESR levels were higher in the active group (Ia) compared to both the inactive group (Ib) and the control group (II) (p1 < 0.001, p2 < 0.001, respectively). Furthermore, the inactive group (Ib) also exhibited

significantly higher ESR levels (p3 = 0.001) compared to the control group (II).

Regarding ***CRP***, the total CD group (I) and both the active (Ia) and inactive (Ib) groups showed significantly higher levels than the control group (II) (p0 =0.007, p2 = 0.028, p3 = 0.012, respectively). However, no significant difference in CRP levels was observed between the active and inactive CD groups (p1 = 0.760). (**Table 5**)

Other clinical data were collected and shown in **Table 6.**

Table 5: Demographic data, disease activity level, laboratory data, faecal calprotectin, plasma AKT2, and CCDC66 relative expression levels in the studied groups

Variables	Total Group I (n = 34)	Group Ia (n = 17)	Group Ib (n = 17)	Group II (n = 16)	Test of Sig. (p)	Test of Sig. (p_0)
Sex						
Male	12 (35.3%)	8 (47.1%)	4 (23.5%)	3 (18.8%)	χ^2=3.659 p=0.160	χ^2=1.418 $^{FE}p_0$=0.328
Female	22 (64.7%)	9 (52.9%)	13 (76.5%)	13 (81.3%)		
Age (years)						
Min. – Max.	18.0 – 49.0	20.0 – 48.0	18.0 – 49.0	25.0 – 40.0	F=0.495 p=0.613	t=0.078 p_0=0.938
Mean ± SD.	31.65 ± 8.61	32.94 ± 8.91	30.35 ± 8.37	31.50 ± 4.68		
Median (IQR)	30.50 (24.0 – 37.0)	32.0(29.0 – 38.0)	28.0(24.0 – 35.0)	30.5(28.0 – 35.0)		
WBCs (×10³/uL)						
Min. – Max.	3.50 – 15.33	3.50 – 15.33	3.50 – 9.40	4.40 – 8.10	F=5.177* p=0.009*	t=1.710 p_0=0.094
Mean ± SD.	7.45 ± 2.86	8.51 ± 3.36	6.39 ± 1.78	6.17 ± 1.23		
Median (IQR)	7.15 (5.10 – 8.50)	7.50 (6.80 – 9.70)	6.60 (5.0 – 7.60)	6.10 (5.10 – 7.25)		
Sig. bet. grps.		**p_1**=0.028*, **p_2**=0.016*, **p_3**=0.960				
1st ESR (mm/h)						
Min. – Max.	8.0 – 69.0	18.0 – 69.0	8.0 – 30.0	4.0 – 7.0	F=45.766* p<0.001*	t=8.935* p_0<0.001*
Mean ± SD.	26.32 ± 13.61	34.76 ± 13.66	17.88 ± 6.63	5.31 ± 1.14		
Median (IQR)	23.0 (18.0 – 34.0)	34.0 (27.0 – 38.0)	18.0 (13.0 – 22.0)	5.0 (4.0 – 6.0)		
Sig. bet. grps.		**p_1**<0.001*, **p_2**<0.001*, **p_3**=0.001*				
CRP (mg/L)						
Min. – Max.	0.40 – 77.60	0.40 – 77.60	0.60 – 47.0	1.10 – 2.80	H=7.410* p=0.025*	U=142.00* p_0=0.007*
Mean ± SD.	10.40 ± 15.93	12.07 ± 19.66	8.73 ± 11.44	1.93 ± 0.55		
Median (IQR)	4.40 (1.90 – 12.0)	4.17 (2.0 – 11.0)	4.60 (1.90 – 12.0)	1.90 (1.45 – 2.35)		
Sig. bet. grps.		**p_1**=0.760, **p_2**=0.028*, **p_3**=0.012*				
Activity						
Mild		7 (41.2%)	–	–	–	–
Moderate		10 (58.8%)	–	–		
Calprotectin (mg/kg)						
Min. – Max.	12.0 – 542.0	20.0 –542.0	12.0 – 77.0	13.0 – 43.0		U=95.500*
Mean ± SD.	154.9 ± 182.8	269.1 ± 202.0	40.65 ± 19.95	26.44 ± 9.25		

Median (IQR)	52.50(37.0 – 208.0)	208.0(54.0– 457.0)	42.0 (25.0 – 51.0)	26.0(19.0 – 31.50)	**H**=23.840* p<0.001*	p_0<0.001*
Sig. bet. groups		**p_1** = 0.001*, **p_2** < 0.001*, **p_3** = 0.107				
AKT2 (pg/ml)						
Min. – Max.	1978.0 – 4329.0	1978.0 – 4029.0	2086.5 – 4329.0	1548.5 – 3590.0		
Mean ± SD.	3100.9 ± 529.0	3083.9 ± 544.3	3117.9 ± 529.4	2719.3 ± 525.2	**F**=2.804 p=0.071	t=2.385* p_0=0.021*
Median (IQR)	3105.8 (2831.5 – 3372.0)	2983.5 (2793.5 – 3362.0)	3191.0 (2853.0 – 3372.0)	2700.5 (2402.0 – 3053.3)		
2^{-ddct} (CCDC66)						
Min. – Max.	0.36 – 12.04	0.36 – 12.04	0.95 – 3.63	0.12 – 3.34		
Mean ± SD.	2.31 ± 2.12	2.63 ± 2.87	1.99 ± 0.89	1.14 ± 0.82		
Median (IQR)	1.64 (1.09 – 2.77)	1.68 (0.89 – 2.99)	1.61 (1.31 – 2.77)	0.89 (0.55 – 1.47)	**H**=8.191* p=0.017*	U=136.00* p_0=0.005*
Sig. bet. groups		**p_1** = 0.646, **p_2** = 0.025*, **p_3** = 0.008*				

*: significant; Group I: Total group I, Group Ia: Active Crohn's disease; Group Ib: Inactive Crohn's disease; Group II: Control group; IQR: Interquartile range; SD: Standard deviation; H: Kruskal-Wallis's test and a pairwise comparison between 2 groups was done using Post-Hoc Test (Dunn's multiple comparisons test); F: One way ANOVA test, Pairwise comparison between each 2- groups was done using Post Hoc Test (Tukey); χ^2: Chi square test, t: Student t-test, U: Mann Whitney test, p_1: Group Ia vs. Group Ib; p_2: Group Ia vs. Group II; p_3: Group Ib vs. Group II; p_0: Total Group I vs. Group II.

Table 6: Comparison between the three studied groups according to Hb, PLT, liver function tests, renal function tests, and 2nd ESR.

Variables	**Group Ia (n = 17)**	**Group Ib (n = 17)**	**Group II (n = 16)**	**F**	**p**
Hb (g/dl)					
Min. – Max.	8.40 – 14.0	9.40 – 13.90	10.66 – 15.0	6.284*	0.004*
Mean ± SD.	10.95 ± 1.57	12.23 ± 1.25	12.52 ± 1.25		
Sig. bet. grps.	**p_1**=0.023*, **p_2**=0.005*, **p_3**=0.812				
PLT (×10^3/uL)					
Min. – Max.	152.0 – 387.0	208.0 – 375.0	266.0 – 400.0	6.612*	0.003*
Mean ± SD.	260.4 ± 72.11	276.2 ± 42.70	325.9 ± 39.40		
Sig. bet. grps.	**p_1**=0.669, **p_2**=0.003*, **p_3**=0.029*				
Liver enzymes					
AST (U/L)				5.136*	0.010*
Min. – Max.	12.0 – 38.0	8.0 – 33.0	9.0 – 33.0		
Mean ± SD.	25.94 ± 7.82	17.88 ± 7.30	19.81 ± 7.80		
Sig. bet. grps.	**p_1**=0.010*, **p_2**=0.065, **p_3**=0.750				
ALT (U/L)					
Min. – Max.	9.0 – 32.0	10.0 – 36.0	11.0 – 35.0	0.469	0.629
Mean ± SD.	20.71 ± 8.27	23.29 ± 7.57	22.06 ± 7.51		
Albumin (g/dl)					
Min. – Max.	2.10 – 4.32	3.54 – 4.22	3.52 – 4.99	11.130*	<0.001*
Mean ± SD.	3.46 ± 0.60	3.84 ± 0.22	4.24 ± 0.51		
Sig. bet. grps.	**p_1**=0.065, **p_2**<0.001*, **p_3**=0.047*				

Renal function					
Urea (mg/dl)					
Min. – Max.	21.0 – 34.0	20.0 – 34.0	21.0 – 38.0	1.220	0.304
Mean ± SD.	27.0 ± 3.94	27.0 ± 4.53	29.25 ± 5.69		
Creatinine (mg/dl)					
Min. – Max.	0.60 – 1.10	0.60 – 1.10	0.60 – 1.10	0.188	0.829
Mean ± SD.	0.88 ± 0.17	0.88 ± 0.16	0.85 ± 0.15		
2nd ESR (mm/h)					
Min. – Max.	28.0 – 110.0	13.0 – 42.0	9.0 – 15.0	F 35.534*	<0.001*
Mean ± SD.	49.24 ± 20.77	26.29 ± 7.64	11.56 ± 2.03		
Sig. bet. grps.	**p_1**<0.001*, **p_2**<0.001*, **p_3** =0.006*				
Sig. bet. grps.	**p_1**=0.760, **p_2**=0.028*, **p_3**=0.012*				
Sig. bet. grps.	**p_1**=0.760, **p_2**=0.028*, **p_3**=0.012*				

SD: Standard deviation *: Statistically significant at $p \leq 0.05$

Group Ia: Active Crohn's disease Group Ib: Inactive Crohn's disease Group II: Control group

F: F for one-way ANOVA test. Pairwise comparison between each two groups was tested using a Post Hoc Test (Tukey)

H: H for Kruskal-Wallis test. Pairwise comparison between each two groups was done using Post Hoc Test (Dunn's multiple comparisons test)

p: p-value for comparing the three studied groups

p_1: p-value for comparing Group Ia vs Group Ib

p_2: p-value for comparing Group Ia vs Group II

p_3: p-value for comparing Group Ib vs Group II

Fecal Calprotectin:

Total CD patients had significantly higher Fc than the control group (group II) (p_0< 0.001). Moreover, active CD patients had higher Fc levels than inactive patients (p1=0.001) and controls (p2<0.001), but there was no difference between inactive patients and controls (p3=0.107). **(Table 5 and Fig. 1)**

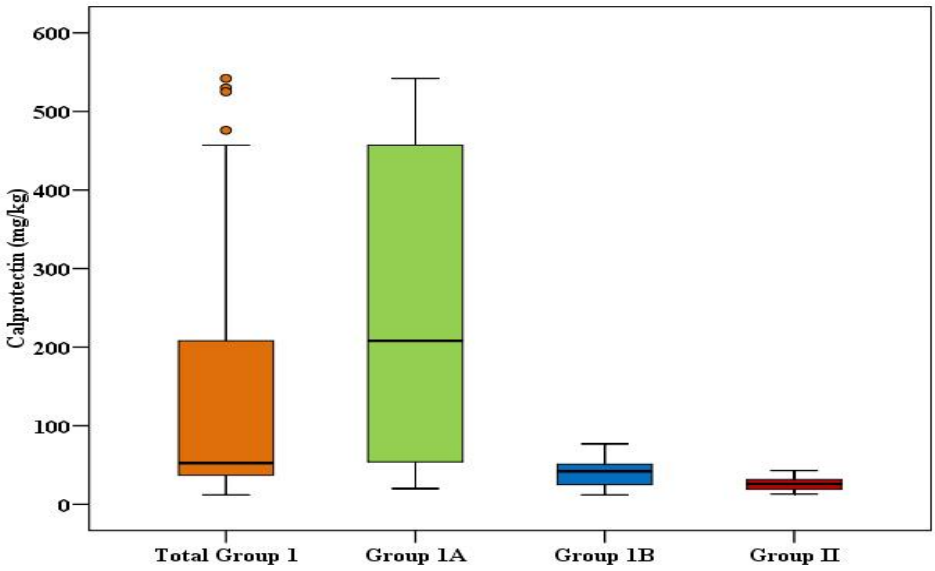

Fig. 1: Calprotectin (mg/kg) means box-blot levels in the studied groups

Plasma AKT2 When mean plasma AKT2 levels were compared among the three studied groups, no statistically significant difference was

found (p=0.071). However, they were significantly higher when comparing the total CD group (group I) and healthy volunteers (group II). (p0=0.021) **(Table 5 and Fig. 2).**

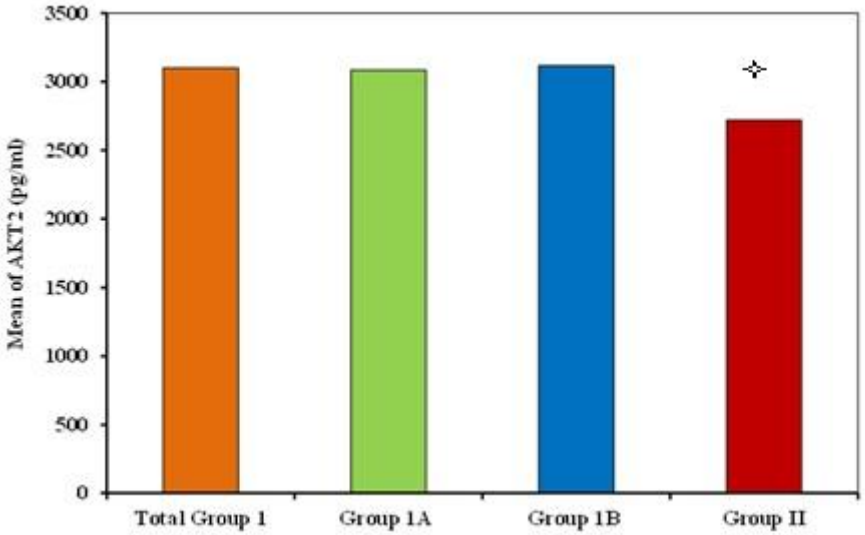

Fig. 2: AKT2 (pg/ml) means levels in the studied groups

Relative expression of circCCDC66 mRNAs

There was a notable increase in circCCDC66 expression in both active and inactive CD patients (groups Ia and Ib) compared to the control group (p2 = 0.025, p3 = 0.008, respectively). Furthermore, total CD patients (group I) exhibited higher levels of circCCDC66 than the volunteer group (group II) (p0=0.005). However, there was no statistical significance when the active group Ia was compared to the inactive group Ib (p = 0.646) **(Table 5 and Fig. 3).**

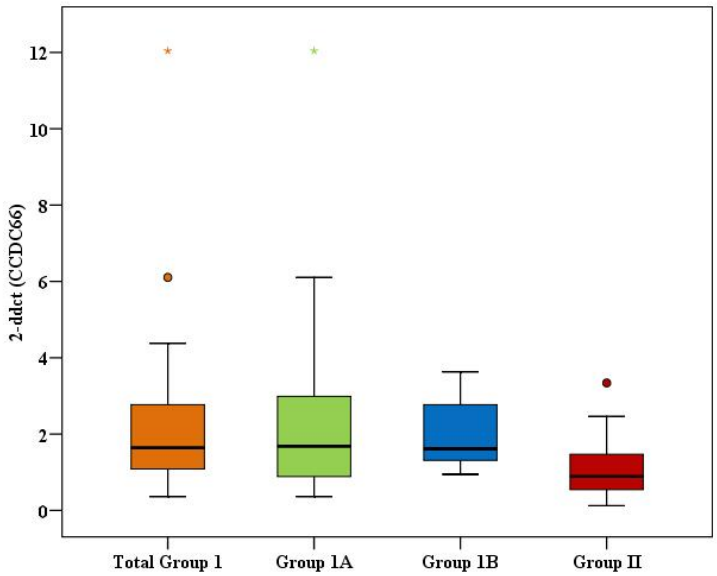

Fig. 3: CCDC66 2-ΔΔct box-blot in the studied groups

Receiver operating characteristic curve (ROC) analysis of fecal calprotectin, plasma circCCDC66, and plasma AKT2:

To discriminate CD patients from controls, **Fc** exhibited the highest significant specificity (81.25%) and a sensitivity of 76.47% in identifying CD, with an area

under the curve (**AUC) of 0.824**, a cutoff of >33 mg/kg, PPV of 89.7%, and NPV of 61.9% ($p < 0.001$). Plasma **circCCDC66** showed promise for distinguishing between CD patients (groups Ia + Ib) and healthy controls, yielding an **AUC of 0.750**, a cutoff of >1.014 $\mathbf{2^{-\Delta\Delta ct}}$, a PPV of 81.8%, and NPV of 58.8% ($p = 0.005$). This marker exhibited the highest sensitivity (79.41%) in correctly identifying individuals with CD, while its specificity was 62.50%.

Plasma **AKT2** also displayed potential in distinguishing between CD patients (groups Ia + Ib) and the control group, achieving an **AUC of 0.706**, a cutoff of >2853 pg/ml, PPV of 78.6%, and NPV of 45.5% ($p = 0.020$), with a sensitivity of 64.71%, and a specificity of 62.50% for CD detection. **(Table 7, Fig. 4).**

Table 7: Diagnostic performance for different laboratory parameters to discriminate CD patients (group Ia and Ib) from the control (group II)

Variables	AUC	p-value	95% CI	Cut off#	Sensitivity %	Specificity %	PPV %	NPV %
Calprotectin (mg/kg)	0.824	*<0.001**	0.712 – 0.937	>33	76.47	*81.25*	89.7	61.9
AKT2 (pg/ml)	0.706	*0.020**	0.550 – 0.862	>2853	64.71	62.50	78.6	45.5
$2^{-\Delta\Delta ct}$ (CCDC66)	0.750	*0.005**	0.606 – 0.894	>1.014 #	*79.41*	62.50	81.8	58.8

*: significant; AUC: Area Under a Curve; p-value: Probability value; CI: confidence intervals; NPV: negative predictive value; PPV: positive predictive value; # Cut off was chosen following Youden index.

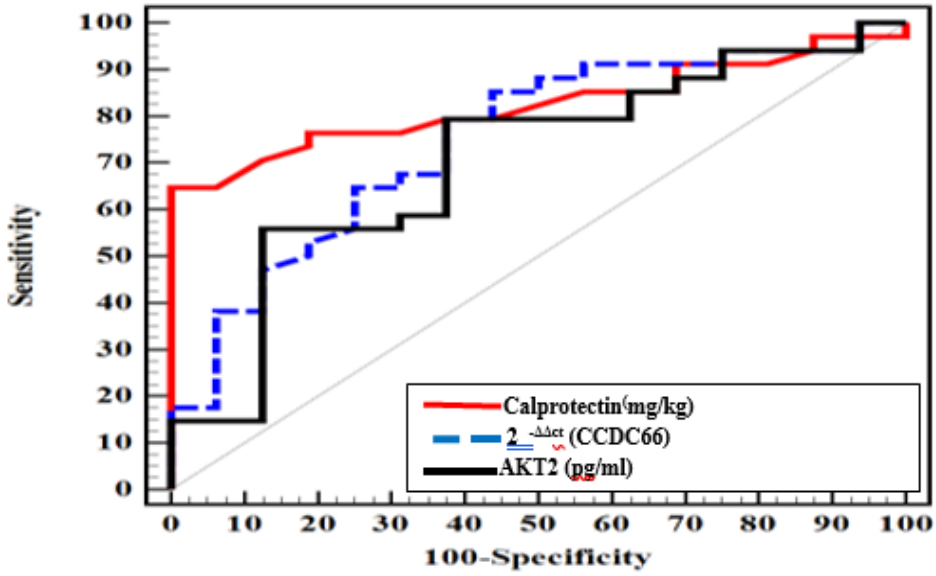

Fig. 4: Diagnostic performance for different laboratory parameters to discriminate CD patients (group Ia + Ib) from the control (group II)

ROC curve analysis showed that **Fc** effectively differentiated active (group Ia) from inactive CD patients (group Ib), with an AUC of 0.856, a cutoff of >51 mg/kg, a PPV of 76.5%, a NPV of 76.5%, a sensitivity of 76.47%, and a specificity of 76.47% ($p<0.001$). Plasma **AKT2** and **circCCDC66** did not distinguish between groups, with AUCs of 0.562 and 0.519; cutoffs of ≤ 3108.5 pg/ml and ≤ 1.66 $2^{-\Delta\Delta ct}$; PPVs of 55.6 and 44.4; and NPVs of 56.2 and 43.7. (**Table 8 and Fig. 5).**

Table 8: Diagnostic performance for different laboratory parameters to discriminate active (group Ia) from inactive CD (group Ib)

Variables	AUC	p-value	95% CI	Cut off#	Sensitivity %	Specificity %	PPV %	NPV %
Calprotectin (mg/kg)	0.856	*<0.001**	0.726 – 0.987	> 51	76.47	76.47	76.5	76.5
AKT2 (pg/ml)	0.562	0.535	0.362 – 0.763	≤ 3108.5	58.82	52.94	55.6	56.2
$2^{-\Delta\Delta ct}$ (CCDC66)	0.519	0.850	0.314 – 0.724	≤1.66	47.06	41.18	44.4	43.7

*: significant; AUC: Area Under a Curve; p-value: Probability value; CI: confidence intervals; NPV: negative predictive value; PPV: positive predictive value; # Cut off was chosen following Youden index.

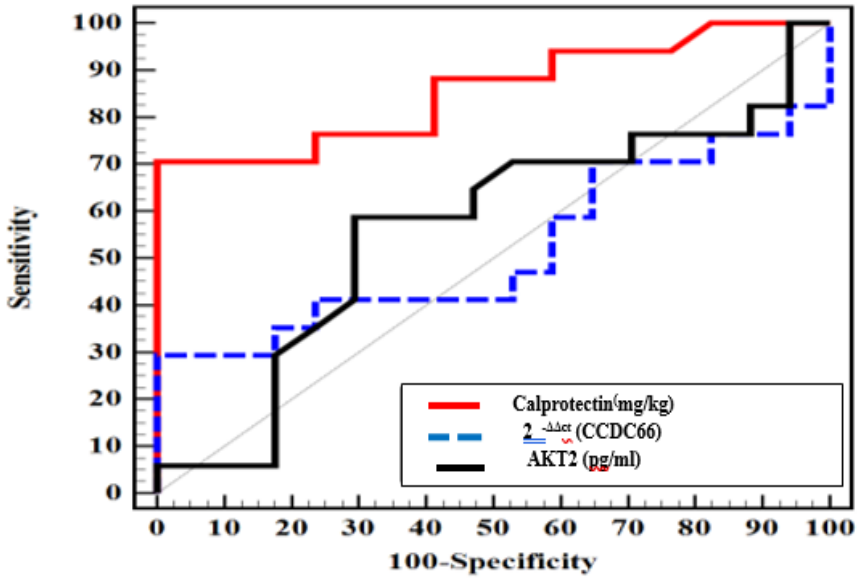

Fig. 5: ROC curve for different parameters to discriminate active CD patients (group Ia) from inactive CD (group Ib)

ROC curve analysis was performed to differentiate inactive CD patients in remission from healthy controls (group Ib vs. group II). **Fc** showed 64.71% sensitivity, 62.50% specificity, an AUC of 0.700, and a cutoff >28 mg/kg, PPV of 64.7%, NPV of 62.5% (p = 0.049). **Plasma AKT2** had the highest sensitivity (70.59%) and specificity (87.50%), with an AUC of 0.739, a cutoff >3067 pg/ml, PPV of 85.7%, and NPV of 73.7% (p = 0.019). **Plasma circCCDC66** had 76.47% sensitivity, 68.75% specificity, and an AUC of 0.798 with a cutoff >1.206 $2^{-\Delta\Delta ct}$, PPV of 72.2%, and NPV of 73.3% (p = 0.004). **(Table 9 and Fig. 6).**

Correlation analysis:

When we conducted correlation analysis, no significant correlations were found between circCCDC66 or AKT2 and any other parameters examined in all CD patients (group I). Meanwhile, when the correlation was tested in the active CD patients (group Ia), the only significant positive correlation was found between plasma circCCDC66 expression and Fc (p = 0.034) **(Tables 10 & 11, and Fig. 7)**

Table 9: Diagnostic performance for different parameters to discriminate CD patients in remission (group Ib) from the control group

Variables	AUC	p-value	95% CI	Cut off#	Sensitivity %	Specificity %	PPV %	NPV %
Calprotectin (mg/kg)	0.700	0.049*	0.515 – 0.885	>28	64.71	62.50	64.7	62.5
AKT2 (pg/ml)	0.739	0.019*	0.557 – 0.921	>3067	70.59	***87.50***	85.7	73.7
$2^{-\Delta\Delta ct}$ (CCDC66)	0.798	0.004*	0.641 – 0.955	>1.206	76.47	68.75	72.2	73.3

*: significant; AUC: Area Under a Curve; p-value: Probability value; CI: confidence intervals; NPV: negative predictive value; PPV: positive predictive value; # Cut off was chosen following Youden index.

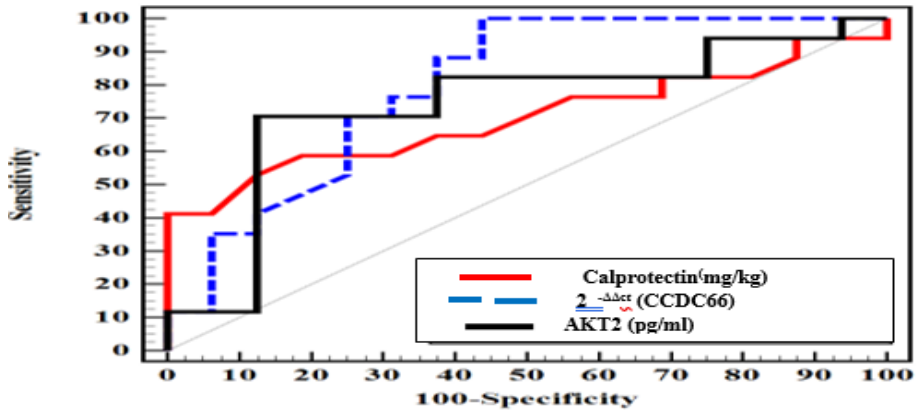

Fig. 6: ROC curve for different laboratory parameters to discriminate CD patients in remission (group 1b) from the control group

Table 10: Correlation between 2^{-ddct} (CCDC66) and AKT2 with different parameters in patients Group (group Ia + group Ib) (n = 34)

Variables	2^{-ddct} (CCDC66)		AKT2 (pg/ml)	
	r_s	p	r_s	p
AKT2 (pg/ml)	-0.096	0.590	-	-
Calprotectin (mg/kg)	0.262	0.134	-0.212	0.230
Time of diagnosis (years)	0.139	0.434	-0.012	0.947
CRP (mg/L)	-0.055	0.756	-0.034	0.849
1st ESR (mm/h)	0.219	0.214	-0.029#	0.873
CDAI	0.043	0.811	-0.188	0.288
SES-CD	0.133	0.453	-0.074#	0.679

r_s: Spearman coefficient; #: Pearson coefficient

Table 11: Correlation between $2^{-\Delta\Delta ct}$ (CCDC66) and AKT2 with different parameters in active CD patients (Group Ia)

Variables	2^{-ddct} (CCDC66)		AKT2 (pg/ml)	
	r_s	p	r_s	p
AKT2 (pg/ml)	0.039	0.881		
Calprotectin (mg/kg)	0.517	**0.034***	-0.047	0.859
Time of diagnosis (years)	0.349	0.170	0.051	0.846
CRP (mg/L)	0.114	0.663	0.000	1.000
1st ESR (mm/h)	0.361	0.155	0.104#	0.690
CDAI	-0.069	0.793	-0.136	0.602
SES-CD	0.347	0.173	-0.084#	0.748

*: significant; r_s: Spearman coefficient; #: Pearson coefficient.

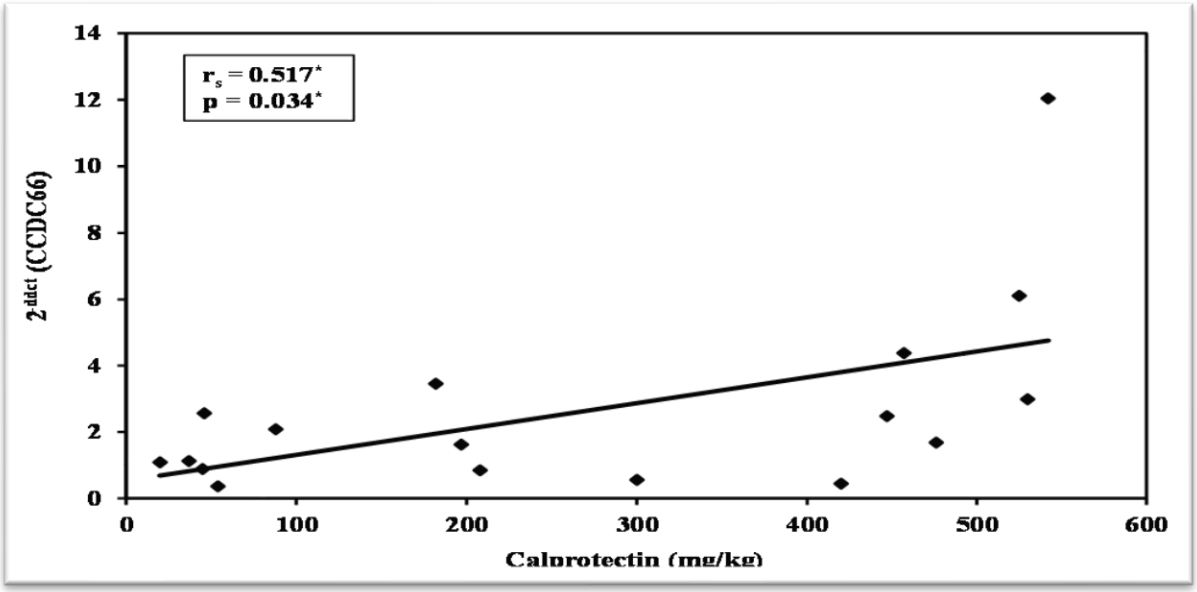

Fig. 7: Correlation between fecal calprotectin (mg/kg) with 2-ΔΔct (CCDC66) in active CD patients (group Ia)

Discussion:

Circular RNAs (circRNAs) have been linked to various diseases, including chronic inflammatory conditions and cancers **(Lun et al., 2023)**. Among these circRNAs is circCCDC66, which is hypothesized to be involved in CD pathogenesis by regulating the autophagy-related serine/threonine kinase 2 (AKT-2) **(Tu et al., 2020)**. The current study was carried out to understand the roles of plasma circCCDC66 and AKT2 in CD pathogenesis and their potential as diagnostic biomarkers.

In this study, total CD patients had significantly higher Fc than the control group (II). Moreover, we found that Fc levels were considerably higher in the active CD group (Ia) compared to the inactive (Ib) and control (II) groups. Notably, Fc levels did not differ significantly between the inactive CD group and the control group. Interestingly, in contrast to our findings, **Penna et al. (2020)**, reported Fc levels that were considerably higher in patients in remission than in the control group.

Interestingly, we found that AKT2 levels were significantly higher in total CD patients (group I) than in healthy controls (group II). (p0=0.021). Similarly, **Yin et al. (2021)** observed a notable rise in AKT2 levels in CD patients using the Western blotting technique.

In the current study, there was a notable increase in circCCDC66 expression in total CD patients (group I) compared to the volunteer group (group II). Furthermore, an increase in circCCDC66 expression was observed in both active and inactive CD patients (groups Ia and Ib) compared to the control group. However, there was no statistically significant difference in circCCDC66 expression between active and inactive CD groups. Therefore, while circCCDC66 may contribute to the inflammatory nature of CD, it does not seem to correlate with disease activity. To our knowledge, this is the first investigation of circCCDC66 expression in CD, although it has been studied in other inflammatory conditions, for instance, **Zhang et al. (2022)** found that high levels of circCCDC66 might regulate chondrocyte apoptosis and proliferation in osteoarthritis. When circular RNAs sequester microRNAs, downstream gene expression is upregulated **(Singh et al., 2024)**. CircCCDC66 sponges anti-inflammatory miRNAs, including miR-370 **(Mo et al., 2022)**, which can increase inflammation by targeting toll-like receptor 4 (TLR4). Many cells, including GIT epithelial

cells, express TLRs that recruit neutrophils and macrophages to inflammatory sites, releasing proinflammatory cytokines **(Tian et al., 2018)**.

Furthermore, the sequestration of miR-129-5p **(Cai et al., 2023)** leads to upregulation of Mitogen-Activated Protein Kinase 1 (MAPK1), a key protein in inflammation, **(Chang et al., 2021)** promoting inflammasome expression and pro-inflammatory cytokines secretion. (**Mahmoud et al., 2021**) Additionally, MAPK1 can activate the nuclear factor kappa-B (NF-κB) pathway, a key regulator of inflammation that also influences intestinal microbiota **(Wen et al., 2024)**.

Additionally, the sponging of miR-338-3p can promote inflammation by upregulating the expression of the IL-6 receptor and signal transducer and activator of transcription 1 (STAT1), thereby increasing transcription of inflammatory mediators, such as TNF-α. **(Liu et al., 2023)** Therefore, the elevated levels of circCCDC66 observed in CD patients may suggest its role in inflammatory processes. This circular RNA could indirectly promote inflammation by acting as a sponge for the previously mentioned anti-inflammatory miRNAs.

ROC analysis showed that **Fc** has the highest specificity (81.25%) for accurately excluding individuals without CD. Additionally, Fc proved effective as a CD activity marker, showing the highest sensitivity and specificity (both 76.47%). In contrast, plasma circCCDC66 and plasma AKT2 were ineffective in distinguishing between active and inactive CD patients. This might be because Fc, measured in feces, is more sensitive to GIT diseases than plasma markers. Previous studies have also highlighted the superior diagnostic performance of Fc for assessing disease activity in CD. **(Dajti et al., 2023)** Fecal (FC) showed the lowest sensitivity and specificity for distinguishing patients with CD in remission from healthy individuals.

An interesting finding from the ROC analysis of diagnostic performance was the potential of plasma circCCDC66 as a non-invasive diagnostic biomarker for CD, with the highest sensitivity (79.41%) for distinguishing CD patients from healthy controls.

A notable observation was that AKT2 displayed the highest specificity (87.5%) in discriminating patients in remission (group Ib) from healthy controls, while plasma circCCDC66 exhibited the highest sensitivity (76.47%) in this distinction. To our knowledge, no prior studies have assessed the sensitivity and specificity of AKT2 and circCCDC66 as biomarkers for CD using ROC curve analysis.

While we found no significant correlation between circCCDC66 or AKT2 levels and the different parameters in the total CD patients' group (I), we observed a significant positive correlation between plasma circCCDC66 expression and F in the active CD group (Ia). This association may be explained by circCCDC66's ability to upregulate inflammatory markers, such as TNF-α, which has been shown to induce calprotectin production in monocytes (**Zhang et al., 2022**), thereby reinforcing a link between circCCDC66 and intestinal inflammation in CD.

Conclusion

Plasma circCCDC66 is essential in the pathogenesis of CD. It may also function as a valuable noninvasive diagnostic biomarker for CD. Simultaneous measurement of plasma circCCDC66 and plasma AKT2 can differentiate patients with CD in remission from healthy individuals. Nonetheless, unlike Fc, circ CCDC66 nor AKT2 is considered a key marker of CD activity.

Limitations and recommendations:

Due to a targeted one-year recruitment focusing on specific patients undergoing endoscopy or admitted to internal medicine, with strict criteria and meticulous data collection, this study had a smaller sample size. Consistent effects suggest that the preliminary findings warrant more research on circCCDC66 and AKT2 in CD. Larger, multi-centre studies will validate these results,

requiring around 29 participants per group for 80% power. In-vitro and in-vivo experiments are also needed to clarify the circCCDC66/miRNA/AKT2 pathway and its role in CD's inflammatory mechanisms. Ultimately, examining the therapeutic modulation of these markers could support the development of targeted treatments for IBD.

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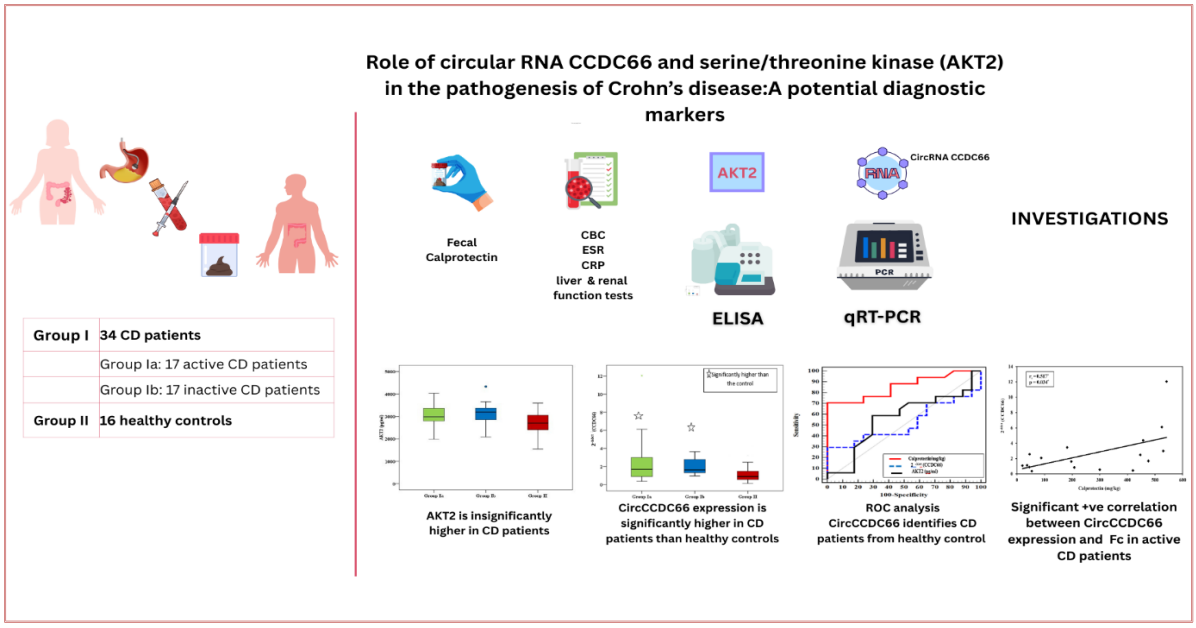
List of abbreviations

AKT2: Serine/threonine kinase-2.
CD: Crohn's disease.
circCCDC66: circular RNA containing the coiled-coil domain 66.
CDAI: Crohn's Disease Activity Index
SES-CD: Simple Endoscopic Score for Crohn's Disease.
Fc: Fecal Calprotectin
ncRNA: non-coding RNA.
miRNA: microRNA.
lncRNA: long non-coding RNA.
CRC: Colorectal cancer.
GIT: Gastrointestinal tract.
IBD: Inflammatory bowel diseases.
mTORC1: mammalian target of rapamycin complex 1.
TSC1: tuberous sclerosis complex.
ULK-1: Unc-51-like autophagy activating kinase-1
CRP: C-reactive protein
CBC: Complete blood count
ESR: erythrocyte sedimentation rate.
ALT: Alanine transaminase.
AST: Aspartate transaminase.
PCR: polymerase chain reaction.
qRT-PCR: Quantitative real-time reverse transcriptase polymerase chain reaction.
GAPDH: glyceraldehyde-3-phosphate dehydrogenase.



Graphic abstract