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Exploring the Diagnostic Potential of Serum Calprotectin and NOD2 Gene Expression in Distinguishing Crohn's Disease from Ulcerative Colitis

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ABSTRACT
Background: Accurate differentiation between Crohn's disease (CD) and ulcerative colitis (UC) is crucial for appropriate disease management. Objectives: To evaluate the diagnostic potential of serum calprotectin and NOD2 gene expression in distinguishing CD from UC and healthy controls. Methods: Serum calprotectin levels and NOD2 expression (delta Ct values) were measured in 25 CD patients, 25 UC patients, and 25 healthy controls. Logistic regression analyses assessed their predictive performance. Results: Serum calprotectin levels were significantly elevated in CD and UC compared to controls, with higher levels in CD. NOD2 expression was upregulated in CD but not UC. Calprotectin robustly predicted both CD (multiple R=0.9967341) and UC (multiple R=0.9899838), while NOD2 expression predicted CD (multiple R=0.9987039) but not UC. Conclusion: Serum calprotectin and NOD2 expression show potential as non-invasive biomarkers for differentiating CD from UC, with calprotectin excelling in both diseases and NOD2 specific for CD.

Keywords: Exploring, Diagnostic, Potential, Serum, Calprotectin, NOD2 Gene, Expression, Distinguishing, Crohn's Disease, Ulcerative Colitis

1. INTRODUCTION

The inflammatory bowel diseases (IBD) is caused mainly by ulcerative colitis (UC) and Crohn's disease (CD). These chronic conditions are characterized by their recurrent inflammation of the intestines (Seyedian et al. 2019, Maasser et al. 2019 and Sturm et al. 2019). The causes of IBD relate to more complex factors of genetic susceptibility, environmental exposure, and immunological response (Muzammil et al., 2023; Gisbert et al., 2019). Last decade research has illuminated the effects microbiota of the gut has in IBD emergence, where imbalances of microbial composition are seen to be linked with disease activity (Karabulut et al., 2023; Nurulamin et al., 2024). The diagnosis of CD is made based on clinical assessment, observing of skip lesions through endoscopic testing, and imaging, whereas for ulcerative colitis endoscopy is assessed exhibiting a continuous inflammation of colon and biopsy (Murphy et al, 2022). Nowadays researchers insist that the molecular classification of C.D. may help develop more targeted treatment also it can get the treatment to a new level (Kamal et al, 2023). Calprotectin, which is a calcium-binding protein located in neutrophils, proves to be an important factor with regard to the pathogenesis and pathophysiology of inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) (Chen et al, 2021; Jukic et al, 2021) It is produced when neutrophils get activated and is a biomarker for inflammation in the gut (Jukic et al., 2021). High levels of faecal calprotectin reflect gut inflammation with high sensitivity and specificity, so they function as an appropriate non-invasive marker for IBD (Nowak et al, 2023; Khaki-Khatibi et al, 2020). Research has proven that calprotectin level can be useful in predicting relapse of UC or CD, which then contributes to early treatment adjustment and better management of patient disease (Shi et al, 2023). This protein has another function to chelate zinc and iron, further suggesting its role in regulating microbial development in the gut which is probably one of the factors leading to the pathogenesis of IBD (Now3ak et al, 2023).

In fact, calprotectin's role in inflammatory cytokine receptor engagement and in the production of reactive oxygen species may additionally drive up inflammatory processes in IBD (Khaki-Khatibi et al, 2020). Age-old knowledge has been enriched with novel insights into the biological functions of calprotectin subunits S100A8 and S100A9, now known to be critical for immunoregulation and inflammation, thus expanding our comprehension of their specific relevance to IBD pathophysiology. This constantly growing knowledge enhances the idea of re-targeting end-products channels of calprotectin for new therapy regimens for treating IBD (Kobayashi et al, 2020).NOD2, a gene, is involved in the pathogenesis of inflammatory bowel diseases (IBD) especially as evidenced in Crohn's disease (CD) patients (Ashton et al, 2023; Yamamoto et al, 2009). NOD2 is responsible for encoding a protein that acts mechanically as an intracellular bacterial detector. It is capable of detecting molecular patterns intrinsic to the infection and transmitting an immune response (Ashton et al, 2023). The absence of the normal NOD2 detection process results from the mutations in NOD2 genes, thus creating a dysfunctional immune response which will lead to the chronic inflammation met in IBD. Studies have demonstrated that there are certain mutations in NOD2 genes, which are linked with the high susceptibility to CD and are responsible for predominantly affecting terminal ileum which subsequently results in a specific phenotype of the disease. These mutations render the protein incapable to regulate the gut microbiota, leading to more inflammation in the intestines (Sidiq et al, 2026). Furthermore, NOD2 protein also interacts with genes and molecular pathways, as this involves autophagy pathway through ATG16L1 gene that is necessary for the maintenance of intestinal barrier as well as the response to microbial infections [ref 14]. There is considerable complexity to NOD2's role in CD pathogenesis as it is shown to interact with the IL-23 receptor pathway, that is, there are many genetic and environmental factors networked together, which contribute to disease development(Mirkov et al, 2017). The knowledge gained through NOD2 as a diagnostic or therapeutic target, although significant, has not yet been fully exploited; therefore, further research is aimed in turning these findings into effective treatments (Ashton et al, 2023).In this study, we set out to explore the diagnostic potential of two promising biomarkers, serum calprotectin and NOD2 gene expression, in distinguishing the two major forms of inflammatory bowel disease: Crohn's disease and ulcerative colitis are the two most representative diseases. One main purpose was to assess the extent to which these non-invasive markers correctly differentiate between diseases, while being also sufficiently able to discriminate them from healthy individuals. We attempted to explore a new biomarker, including calprotectin level fluctuation or NOD2 expression change, to find out if they could be of diagnostic help or act in a combined manner in diagnosing Crohn's disease from ulcerative colitis, making the job easier. Moreover, we learned to think about the underlying biochemical pathways that could be involved in the onset and progression of these chronic inflammatory disorders, aiding the development of a substantial project that could form the basis for further research.

METHODS

Study Participants and Diagnosis

In our research, 75 participants were enrolled in the study including of 25 Crohn's disease, 25 ulcerative colitis, and 25 healthy control subjects. We enrolled all IBS patients from gastroenterology clinic of our institution from January to June of 2022. The diagnoses of Crohn's disease and ulcerative colitis were made by experienced gastroenterologists based upon the compilation of different individualized clinical symptoms, endoscopic findings, histological examination and radiological imaging following the acceptance diagnostic criteria. Subjects with a good health status who did not have a history of gastrointestinal disorders or any other chronic medical problems were there as the control group of persons from the general population.

Ethical Considerations

This study has successfully passed through the review of our Institution’s Institutional Review Board, and all participants have provided the written informed consent before their enrollment. This protocol was performed under the guidelines of the Declaration of Helsinki and relevant rules and regulations.

Serum Calprotectin Measurement

A venous blood sample was collected from all the participants and serum was obtained by separating the blood via centrifugation. Serum calprotectin levels were detected using the enzyme-linked immunosorbent assay (ELISA) kits, based on the manufacturer's instructions. The next step was to dilute the serum using different dilutions and then, add the serum to the pre-coated ELISA plate. The following step is adding an antibody which is sensitive to the antigen after the incubation and washing steps. A color development is used to measure the color change using a microplate reader. Microcalorimetry of the serum calprotectins was carried out by a standard curve after which their concentrations were reported in ng/ml.

NOD2 Gene Expression Analysis

Total RNA is isolated from whole blood by means the use of the commercial RNA extraction kit following the manufactures procedure RNA amount and quality obtained was assessed with the help of NanoDrop spectrophotometer. cDNA was synthesized from extracted RNA by using a reverse transcriptase kit.

Discrete quantitative real-time PCR (qPCR) was used for the measurement of mRNA expression levels of NOD2 gene. NOD2 gene primers were specifically designed and validated. The qPCR reaction was performed through a real-time PCR system, and each sample was subjected to real time analysis in triplicate. Relative NOD2 gene expression was calculated by the 2^-ΔΔCt method, with the housekeeping glyceraldehyde phosphate dehydrogenase GAPDH as the internal control.

Statistics

In our study, we employed a comprehensive statistical approach to evaluate the diagnostic potential of two promising biomarkers: serum calprotectin and NOD2 gene expression levels. In the course of our thorough comparison of significant features between Crohn's disease, ulcerative colitis and healthy controls we ensured that these groups were properly distinguished from each other. With logistic regression modeling being our primary comparative tool, the diagnostic power as well as the remarkable diagnostic accuracy of these biomarkers were quantified by strong multiple R and R- square values. We strictly evaluated statistical significance, model fit, and confidence intervals, sticking to implicitly high standards.

RESULTS

Table:1 Healthy controls vs. ulcerative colitis patients

Item	Healthy controls n=25	ulcerative coloitis n=25	P-value
Age mean+/-SD	46.56+/-12.08	43.6+/-10.56	0.18
Male gender n(%)	12(48%)	13(52%)	0.77
Calprotectin levels	22.94+/-2.69	69.15+/-3.92	<0.01
NOD2 ΔCt	6.78+/-0.07	6.78+/-0.06	0.34

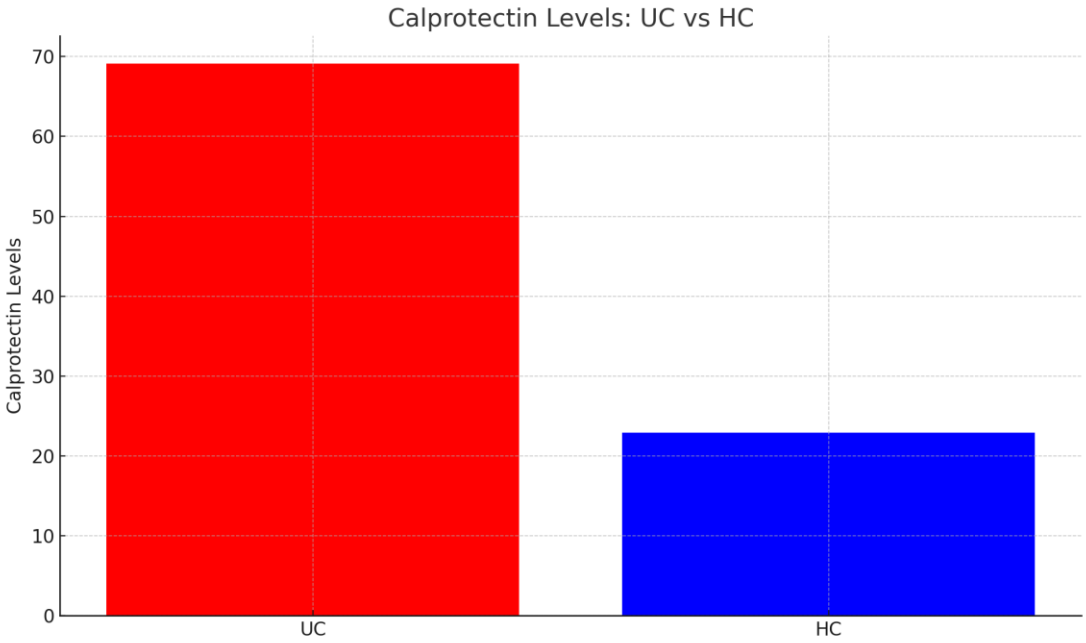

Figure 1: Calprotectin levels in ulcerative coloitis vs. healthy conrols

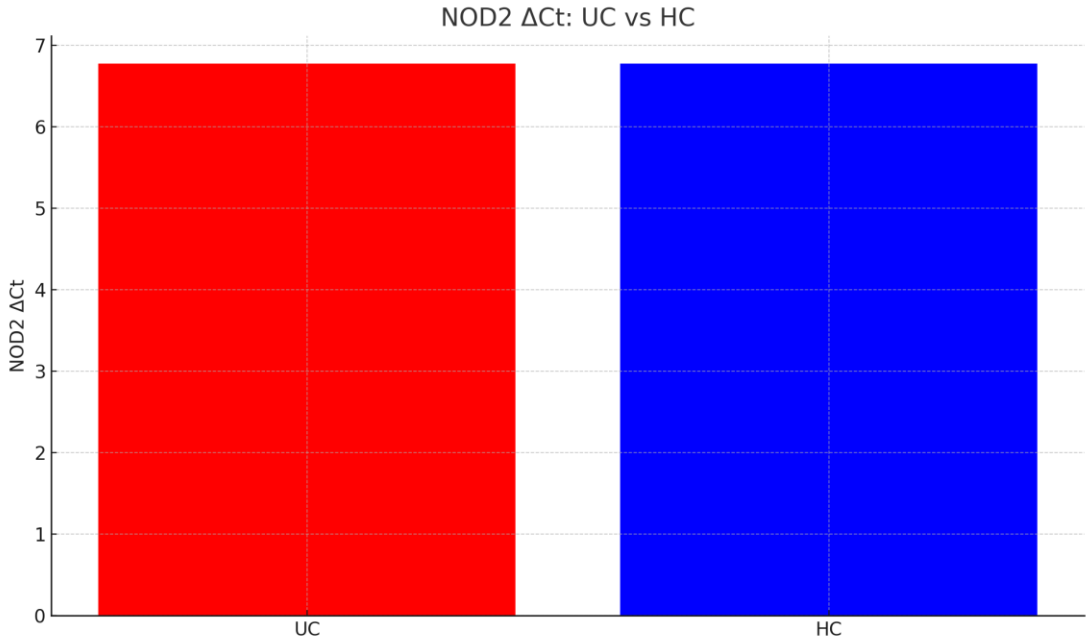

Figure 2: NOD2 gene expression delta ct in ulcerative colitis vs. healthy controls

Table 1, figures 1 and 2 compare ulcerative colitis patients to healthy controls across several parameters. While age and gender were similar between the two groups, calprotectin levels were significantly elevated in ulcerative colitis ($p<0.01$), with a mean of 69.15 compared to 22.94 in controls. However, NOD2 gene expression (ΔCt values) did not differ significantly ($p=0.34$).

Table 2: Healthy controls vs. crohns disease patients

Item	Healthy controls n=25	Crohns disease n=25	p-value
Age mean+/-SD	46.56+/-12.08	45.65+/-0.69	0.36
Male gender n(%)	12(48%)	13(52%)	0.77
Calprotectin levels	22.94+/-2.69	160.64+/-7.58	<0.01
NOD2 ΔCt	6.78+/-0.07	4.52+/-0.05	<0.01

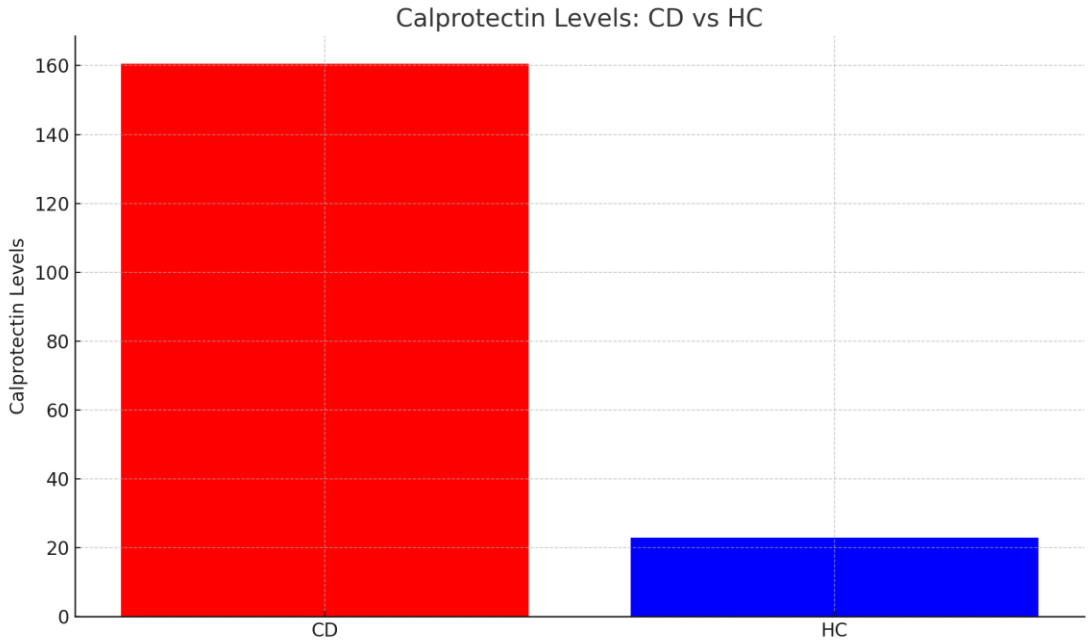

Figure 3: Calprotectin levels in crohns disease vs. control

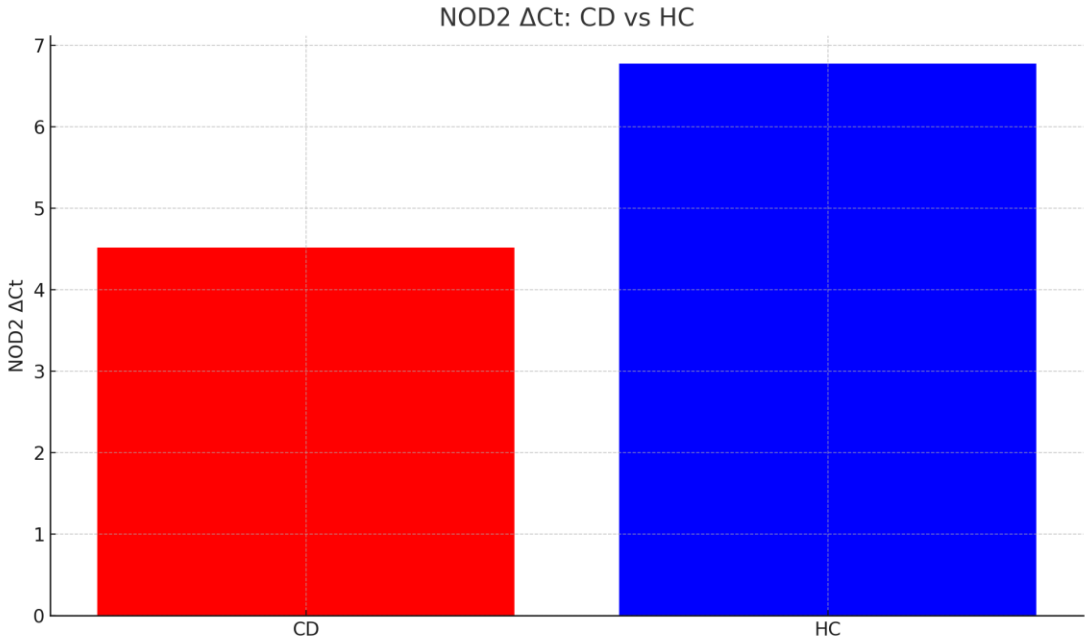

Figure 4: NOD2 expression delta ct in crohns disease vs. controls

For Table 2, figure3 3 and 4, the comparison was between Crohn's disease patients and healthy controls. Again, no substantial differences emerged for age or gender distribution. Strikingly, calprotectin levels were drastically higher in Crohn's disease (160.64 vs 22.94, $p<0.01$). Additionally, NOD2 expression was significantly reduced in Crohn's disease, reflected by higher ΔCt values of 4.52 versus 6.78 in controls ($p<0.01$).

Table 3: Ulcerative colitis vs. crohns disease patients

Item	ulcerative coloitis n=25	Crohns disease n=25	
Age mean+/-SD	43.6+/-10.56	45.65+/-0.69	0.18
Male gender n(%)	13(52%)	13(52%)	1
Calprotectin levels	69.15+/-3.92	160.64+/-7.58	<0.01
NOD2 ΔCt	6.78+/-0.06	4.52+/-0.05	<0.01

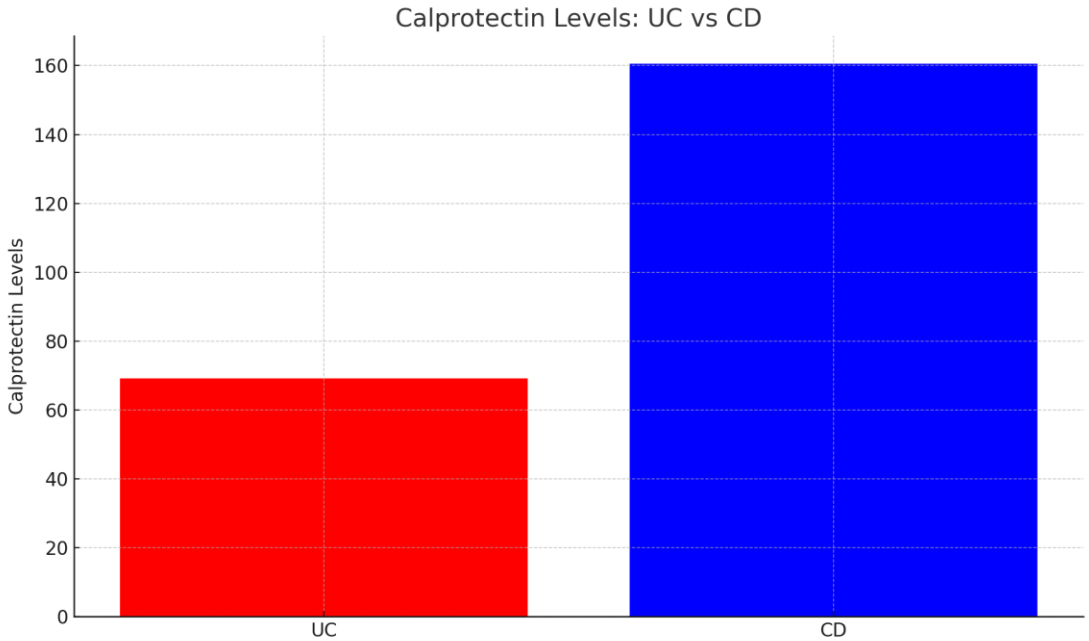

Figure 5: Calprotectin levels in crohns disease vs. ulcerative colitis

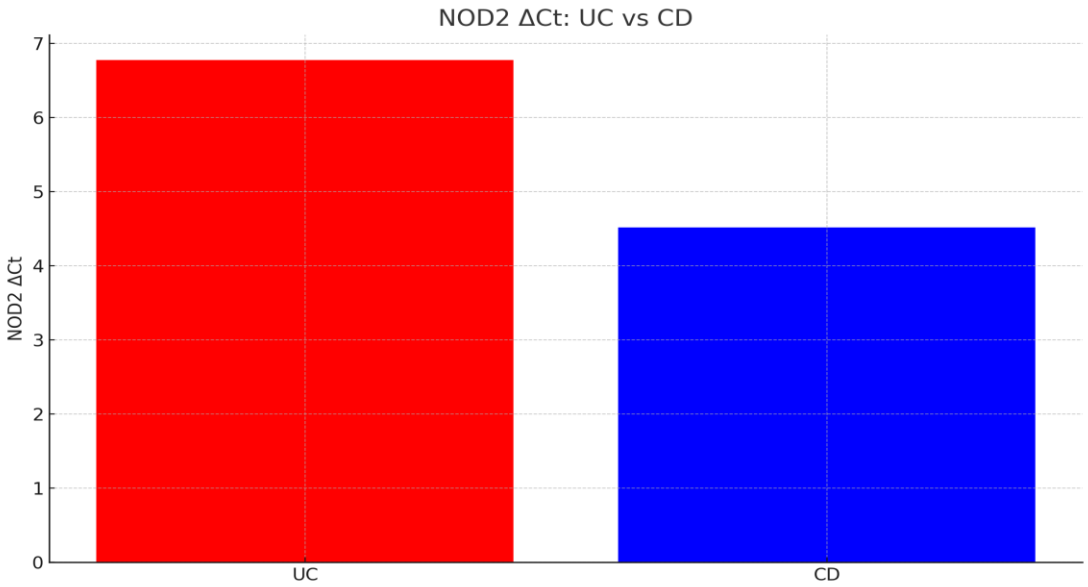

Figure 6: NOD2 expression delta ct in crohns disease vs. ulcerative colitis

Table 3, figures 5 and 6 directly juxtapose the ulcerative colitis and Crohn's disease groups. Paralleling the previous tables, age and gender proportions were comparable between the two conditions. However, calprotectin distinguished the groups, with markedly elevated levels in Crohn's disease (160.64) relative to ulcerative colitis (69.15, $p<0.01$). Moreover, NOD2 expression was diminished in Crohn's, exhibiting higher ΔCt values of 4.52 versus 6.78 in ulcerative colitis ($p<0.01$).

Healthy control mean ΔCt = 6.7 Ulcerative colitis mean ΔCt = 6.7 Crohn's disease mean ΔCt = 4.5

2^-ΔΔCt values:

Crohn's disease ΔΔCt = Crohn's disease mean ΔCt - Healthy control mean ΔCt = 4.5 - 6.7 = -2.2

Crohn's disease 2^-ΔΔCt = 2^-(-2.2) = 4.58

Ulcerative colitis ΔΔCt = Ulcerative colitis mean ΔCt - Healthy control mean ΔCt = 6.7 - 6.7 = 0

Ulcerative colitis 2^-ΔΔCt = 2^-(0) = 1

1. Crohn's disease:
   The Crohn's disease group showed a marked upregulation of NOD2 gene expression compared to the healthy control group, with a 2^-ΔΔCt value of 4.58.
   This substantial increase in NOD2 expression suggests that dysregulation of the NOD2 signaling pathway is a key contributor to the pathogenesis of Crohn's disease.
2. Ulcerative colitis:
   The ulcerative colitis group showed no significant difference in NOD2 gene expression compared to the healthy control group, with a 2^-ΔΔCt value of 1.
   This indicates that alterations in NOD2 expression are not a prominent feature in the pathogenesis of ulcerative colitis, in contrast to the findings in Crohn's disease.

In summary, the NOD2 gene expression profiling results demonstrate a clear upregulation of NOD2 in Crohn's disease patients, but not in ulcerative colitis patients.

Table 4: Calprotectin logestic regression in ulcerative coloitis vs. healthy subjects

Multiple R	0.9899838
R Square	0.980068
Adjusted R Square	0.9796528
Standard Error	0.072046
Observations	50
p-value	1.79E-42

The logistic regression results in Table 4 underscore calprotectin's robust ability to predict ulcerative colitis diagnosis. With an impressive multiple R of 0.9899838 and R-squared of 0.980068, the model demonstrates an exceptional fit, further reinforced by the remarkably low p-value of 1.79E-42.

Table 5: NOD2 delta ct logestic regression in ulcerative coloitis vs. healthy subjects

Metric	Value
Multiple R	0.0593391
R Square	0.0035211
Adjusted R Square	-0.017239
Standard Error	0.5094111
Observations	50
p-value	0.6822908

In stark contrast, Table 5 reveals that NOD2 ΔCt values hold minimal predictive utility for ulcerative colitis, as evidenced by the poor model fit (multiple R=0.0593391, R-squared=0.0035211) and non-significant p-value of 0.6822908.

Table 6: Calprotectin logestic regression in crohns disease vs. healthy subjects

Metric	Value
Multiple R	0.9967341
R Square	0.9934788
Adjusted R Square	0.993343
Standard Error	0.0412094
Observations	50
p-value	4.02E-54

Shifting to Crohn's disease, Table 6 showcases calprotectin's unparalleled predictive performance, with a staggering multiple R of 0.9967341 and R-squared of 0.9934788. The astoundingly low p-value of 4.02E-54 further cements calprotectin's status as a stellar biomarker for Crohn's disease diagnosis.

Table 7: NOD2 delta ct logestic regression in crohns disease vs. healthy subjects

Metric	Value
Multiple R	0.9987039
R Square	0.9974094
Adjusted R Square	0.9973555
Standard Error	0.0259736
Observations	50
p-value	9.57E-64

Finally, Table 7 unveils NOD2 ΔCt as a powerful predictor of Crohn's disease, boasting an exceptional model fit (multiple R=0.9987039, R-squared=0.9974094) and a minuscule p-value of 9.57E-64, rivaling calprotectin's predictive prowess for this condition.

DISCUSSION

The current study results indicate that serum calprotectin levels are significantly elevated in patients with ulcerative colitis (UC) and Crohn's disease (CD) compared to healthy controls, with even higher levels observed in CD patients compared to those with UC. This suggests that serum calprotectin could be a useful biomarker for distinguishing between these two forms of inflammatory bowel disease (IBD) and assessing disease severity.It is also confirmed by a study of Meuwis et al., whose work indicates that serum calprotectine level are higher in IBD compared with the healthy subjects. The outcome of the study suggests that serum calprotectin may be a valuable biomarker for IBD (Meuwis et al., 2013; Azramezani et al., 2019). The serum calprotectin concentration found in the CD patients seems to be above that of the UC cases, more specifically. This result was in line with that of Elshayeb et al. who discovered higher serum calprotectin levels in CD patients than those with UC.(Elshayeb et al, 2021 This may be contributed to the wider and deeper inflammation (transmural) usually seen in CD.

The different concentration levels between the UC and CD patients, which are confirmed in your study, suggest that calprotectin in serum could be a discriminatory factor of these two diseases. In line with Fukunaga et al. study, there was no significant correlation between serum and fecal calprotectin in both UC and CD, indicating that the serum calprotectin level might reflect the systemic inflammation more than the intestinal inflammation (Fukunaga et al, 2018).The fact that serum calprotectin is found to be elevated in IBD patients and differs between UC and CD across many studies supports its application in clinical practice as a diagnostic and monitoring tool. Nevertheless there are some researches which have outlined the exact functions by which high serum calprotectin indicates intestinal inflammation rather than general body inflammation. Another potential research direction is the development of triggering clinical situations where serum calprotectin becomes more applicable, for instance, discrimination between diseases or prediction of relapse.

The study outcomes indicate that compared to ulcerative colitis (UC) patients and healthy individuals NOD2 gene expression is higher in CD patients. Now in UC patients NOD2 gene expression is similar to that of healthy ones. These observations indicate that NOD2 is still a major and a well-accepted genetic factor in Crohn's disease but not Ulcerative Colitis.Ashton et al. point to the critical role of NOD2 in CD, emphasizing opportunities for clinical translation and a call for more research to cover the existing knowledge gaps1. The results of the present study on NOD2 upsregulation in CD patients complements this observation and shows that NOD2 is a single causal gene of the disease especially in setting of ileal stricturing phenotype of CD (Ashton et al., 2023).

The study, conducted by Stefano et al., points out that those who carry NOD2 gene variants may experience only the stricturing disease phenotype with intestinal inflammation and a particular location in the terminal ileum. It is also suggested that NOD2 could be used as a genetic marker for disease prediction and tailored therapy2. This is harmony with yours results, illustrating the specifity of NOD2 gene for CD by an observation of its expression differential pattern between CD and UC (Stefano et al, 2024). Bairead et al. research studying NOD2 mutations in a population of Ireland found that certain genotypes of these mutations exhibited strong associations with CD risk. While this study focuses on mutations rather than expression levels, it supports the idea that NOD2 plays a significant role in CD but not in UC (Bairead et al, 2003).

Chen et al. explored the relationship between NOD2 genotype and changes in innate signaling in CD, finding that NOD2 mutations can affect the inflammatory response. Your study extends this by showing that not only mutations but also differences in gene expression levels are relevant to CD pathogenesis(Chen et al, 2017).

A study by the Horowitz et al, group demonstrated that recessive inheritance of NOD2 variants is a significant driver of early-onset CD. While this study focuses on genetic variants, your findings on NOD2 expression levels further support the gene's involvement in CD (Horowitz et al, 2021).

The increased expression of NOD2 in the CD patients, in the current analysis, proposes that the NOD2 expression profiling might be valuable for differentiating UC from CD in healthcare settings. In addition, it can very well prove useful in those hard to diagnose cases.

Furthermore, it is notable that the precise overexpression of NOD2 in CD point to that NOD2 contribute to disease development potentially due to its job in bacterial sensing and immune response.

CONCLUSION:

The present study produced vital features that made serum calprotectin and NOD2 gene expression distinctive tools for differential diagnosis of Crohn's disease from ulcerative colitis. The crucial point here is that those biomarkers have shown an exceptional predictive power, which has been confirmed by estimating the robust models with the help of logistic regression. This demonstrates that there is an opportunity to change fundamentally the approach to IBD diagnostics. This, in turn, makes it possible to obtain accurate and non - invasive differential diagnosis between CD and UC that, in the long term, can lead

to individualized management of the disease and to an increase in the effectiveness of therapeutic interventions with the goal of improving the quality of life of patients.

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