Original Article

From Gene Expression to Candidate Biomarkers in Ulcerative Colitis: Aquaporin-9 in Neutrophil Extracellular Traps Formation Pathways

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What's Known

- Ulcerative colitis (UC) involves chronic intestinal inflammation, where neutrophil extracellular traps (NETs) contribute to disease progression by disrupting the mucosal barrier. Excessive NET formation correlates with increased inflammation and disease severity. Despite their critical role, the detailed mechanisms driving NET formation and reliable tissue biomarkers in UC remain unclear.

What's New

- This study identifies *AQP9* as a highly upregulated gene in UC, validated in both new and treatment-resistant patients. *AQP9* shows strong diagnostic accuracy, distinguishing UC patients from healthy controls, and, along with *FPR1* and *FPR2*, may serve as key biomarkers and therapeutic targets in UC management.

Abstract

Background: Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD). Neutrophil extracellular traps (NETs) have been recognized as contributing to UC progression. This study aimed to identify key genes driving NET formation in UC and evaluate their potential as biomarkers.

Methods: Gene expression profiles from colon biopsies of UC patients and healthy controls were obtained from the GSE224758 dataset. Differentially expressed genes (DEGs) were identified using the GEO2R tool. Hub genes involved in NET formation, including *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, and *NCF2*, were validated through reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using colon biopsy samples from healthy controls (n=20), newly diagnosed UC (n=20), and treatment-resistant UC patients (n=20) collected between November 8, 2022, and February 2, 2025, at Tohid Hospital, Kurdistan University of Medical Sciences, Sanandaj, Iran. Data normality was assessed using the Shapiro–Wilk test; Kruskal–Wallis test and Dunn's *post hoc* test were applied for group comparisons. ROC analysis was applied to determine the potential of genes for the differentiation of healthy people from patients.

Results: RT-qPCR validation confirmed significantly increased expression of *AQP9* (P <0.0001), *FPR1*, and *FPR2* (P=0.03 and P=0.02) genes in patients with UC and those with refractory UC compared to healthy controls. Among these, *AQP9* exhibited the most significant differential expression, demonstrating high sensitivity and specificity in distinguishing newly diagnosed and treatment-resistant UC patients from healthy individuals (sensitivity 83.49% and 80.98%, and specificity 67.77% and 64.24%, respectively), indicating its potential as a diagnostic biomarker.

Conclusion: This study identifies NET-associated genes, particularly *AQP9*, *FPR1*, and *FPR2*, as candidate tissue biomarkers for UC, with *AQP9* exhibiting the strongest ability to distinguish patients from healthy controls. These findings support the utility of NET-related genes as tissue biomarkers for UC, while emphasizing that any therapeutic targeting of these molecules lies beyond the scope of the present work and will require additional functional and *in vivo* validation.

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Keywords ● Colitis, ulcerative ● Neutrophil extracellular traps ● *Aquaporin 9* ● Gene expression profiling

Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by recurring inflammation of the colon with periods of flare-ups and remission. It commonly affects individuals aged 30 to 40, with a rising global incidence and prevalence, including about 0.3% of the European population diagnosed with UC.[1, 2] Symptoms observed include diarrhea, presence of blood in stool, decreased appetite, abdominal discomfort, nausea, vomiting, and weight loss.[3] Disease severity varies from localized rectal inflammation to extensive colitis, and diagnosis involves clinical evaluation, laboratory tests, imaging, and colonoscopy. Treatment progresses through stages, including 5-aminosalicylic acid, glucocorticoids, azathioprine, and anti-Tumor Necrosis Factor (TNF) therapy. Refractory patients are defined by failure to respond to all therapeutic stages.[4]

The exact cause of inflammation in UC is unknown. Since the immune system mistakenly attacks the intestinal lining, causing inflammation, UC is considered an autoimmune disease. This inappropriate immune response may be triggered by intestinal bacteria or other environmental factors, such as diet, stress, and infections. Dysbiosis, or an imbalance in gut microbiota, might also contribute to colonic inflammation.[5, 6] The pathogenesis of UC is complex and involves genetic, environmental, immune dysregulation, and microbiome alterations, leading to the recruitment of various inflammatory cells, including neutrophils, dendritic cells, T cells, and B cells, to the intestine.[3]

Neutrophils play a critical role in UC by contributing to tissue damage through the release of reactive oxygen species, proteases, and pro-inflammatory cytokines.[7-9] An important neutrophil defense mechanism is NETosis, where neutrophils release neutrophil extracellular traps (NETs), web-like structures composed of DNA and antimicrobial proteins that trap pathogens.[10] While NETs help contain infections, excessive or dysregulated NET formation promotes inflammation and tissue injury.[11-13] Increased NET components have been found in inflamed mucosa, stool, and blood of UC patients, correlating with disease activity.[11] Previous studies have conducted transcriptomic and bioinformatic profiling of NET-associated genes in UC, identifying candidates such as *CXCR1* and *CXCR2* as potential diagnostic or prognostic biomarkers. These studies used methods including Mendelian randomization, machine learning, and co-expression network analyses to implicate NET-related genes in UC pathogenesis and response prediction.[14-16] However, the molecular mechanisms driving NET formation in UC remain poorly understood.

In this study, we performed bioinformatic analysis of gene expression data from colonic biopsies of UC patients and healthy controls and identified DEGs involved in NET formation, including Fc Gamma Receptor IIIb (*FCGR3B*), Aquaporin-9 (*AQP9*), Formyl Peptide Receptor 1 (*FPR1*), Formyl Peptide Receptor 2 (*FPR2*), and Neutrophil cytosol factor 2 (*NCF2*). These five genes were specifically selected from the largest gene module identified through PPI network analysis of 429 upregulated DEGs from the GSE224758 dataset. Pathway enrichment analysis using DAVID and ShinyGO revealed significant enrichment of the NET formation pathway within this module, with these five genes representing the complete set annotated to NET formation by KEGG pathway analysis. FCGR3B is a receptor expressed on neutrophils that induces NET formation through cross-linking with specific monoclonal antibodies. AQP9 regulates neutrophil migration and hyperactivation, promoting NET formation, with its distinct expression pattern highlighting biomarker potential. FPR1 and FPR2, G protein-coupled receptors, activate neutrophils and NETosis by recognizing formylated peptides from bacteria and damage signals, with roles demonstrated in multiple inflammatory diseases. NCF2, an NADPH oxidase subunit, generates ROS essential for NETosis and has been associated with NET formation in inflammatory conditions.[17-25]

By validating the expression of these key genes in new and treatment-resistant UC patients, we aim to elucidate their role in UC pathogenesis and assess their potential as tissue biomarkers for improved diagnosis and therapeutic targeting. Our findings may provide novel insights into the contribution of NETs in UC and suggest new avenues for managing difficult-to-treat cases.

Materials and Methods

RNA Sequencing (RNA-seq) Data Resources and Identification of DEGs

This study employed a two-pronged approach, combining a bioinformatic analysis of publicly available RNA-seq data with subsequent experimental validation using patient-derived samples in a cross-sectional study design. RNA-seq data from the GSE224758 dataset, gene expression profiling of colon biopsies

from UC patients and healthy volunteers, was obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) to identify DEGs. The GEO2R online tool (http://www.ncbi.nlm.nih.gov/geo/geo2r/) (NCBI, USA) was used to identify DEGs between UC and healthy control groups. This analysis employed the limma package for differential expression analysis, incorporating normalization methods such as quantile normalization to ensure comparability across samples by adjusting for technical variations. When addressing multiple testing correction, the Bonferroni correction was applied to control for false positives (supplementary file S1). DEGs were filtered using adjusted P value <0.01 and Log_2 fold change threshold=1, yielding 492 DEGs (429 upregulated, 63 downregulated) in supplementary file S2.

Protein-Protein Interaction Network and Clustering

For the DEGs that are upregulated in the UC group, a co-expression Protein-Protein Interaction (PPI) network was constructed using the STRING database (version 12.0; https://string-db.org, Switzerland) with a medium confidence score (0.4) (supplementary file S3). The network was exported and visualized using Cytoscape software (version 3.10.1; Cytoscape Consortium, USA) (supplementary file S4). Clustering and module identification were performed using the Clusterviz plugin with the Fast Agglomerate Algorithm based on Edge Clustering Coefficients (FAG-EC algorithm) and a complex size threshold ≥3, identifying 14 modules. The largest module contained 31 nodes and 83 edges.

Gene Ontology and Pathway Enrichment Analyses

Gene Ontology and KEGG pathway enrichment analysis were conducted for the genes in the largest module using the DAVID database (https://davidbioinformatics.nih.gov) (NCBI/NIAID, USA). Results for KEGG pathways were downloaded and visualized using ShinyGO 0.81 (version 0.81; South Dakota State University, USA). The NET formation pathway was significantly enriched with *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, and *NCF2* genes.

Patient Characteristics and Sample Collection

The study population and sample collection methodology here were based on our previous study.[26] In brief, colon biopsy samples were collected between November 8, 2022, and February 2, 2025, at Tohid Hospital (Kurdistan University of Medical Sciences, Sanandaj, Iran). Written informed consent was secured from every participant involved in the study. Three groups were included: healthy controls (n=20), newly diagnosed UC patients (n=20), and treatment-resistant UC patients (n=20). Healthy controls had suspected UC ruled out by colonoscopy and pathology. UC diagnosis followed clinical, laboratory, endoscopic (Mayo score ≥6), and pathological criteria by gastroenterology specialists. Treatment-resistant UC was defined as failure to respond to 5-aminosalicylic acid (5-ASA), glucocorticoids, azathioprine, and anti-TNF therapy. Exclusion criteria included diabetes, rheumatoid arthritis, irritable bowel syndrome (IBS), and immunodeficiency. Colon biopsy samples were immediately placed in RNAlater (Sigma-Aldrich, USA) and stored at -70 °C. The study protocol was thoroughly reviewed and approved by the Ethics Committee at Kurdistan University of Medical Sciences, under approval number IR.MUK.REC.1403.285.

RNA Extraction and Synthesis of cDNA

RNA extraction was performed using the FavorPrep™ Total RNA Mini Kit (Cat. No. FABRK 000-Mini, Favorgen Biotech, Taiwan) following the manufacturer's instructions. RNA quality was assessed (A260/280 1.8-2.0), and the purified RNA was stored at -70 °C until further analysis. Complementary DNA (cDNA) was synthesized from 1 µg RNA using the YT4500 cDNA Synthesis Kit (Yekta Tajhiz Azma, Iran) according to the standard protocol. The synthesized cDNA was diluted 1:5 and stored at -20 °C.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

For the RT-qPCR assay, primers were designed using Gene Runner software (Hastings Software, USA), as listed in table 1. Each 20 µL reaction mixture contained 10 µL of YTA SYBR Green qPCR Mastermix 2X (Cat. No. YT2551; Yekta Tajhiz Azma, Iran), 1 µL each of forward and reverse primers (10 µM), 2 µL of cDNA template, and 6 µL of nuclease-free water. The thermal cycling conditions were carried out according to the manufacturer's protocol after preparing the reaction mixture.

To confirm primer specificity and optimize reaction conditions, a temperature gradient PCR was conducted for each primer pair targeting *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, *NCF2*, and the reference gene β-ACTB. PCR products were analyzed on 2% agarose gels to verify the presence of single amplicons of expected sizes (table 1 and supplementary figure 1S). Standard curves generated from 5-point, 10-fold serial cDNA dilutions confirmed amplification efficiencies (E) of 90-110% for all primers.

Table 1: Specific primers to assess the genes involved in NETs formation and internal control

Primer	Accession codes	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Amplicon Size (bp*)
β-ACTB	NM_001101	AGATCATTGCTCCTCCTGAG	AGTCATAGTCCGCCTAGAAG	159
FCGR3B	NM_001244753 NM_000570 NM_001271035 NM_001271036 NM_001271037	TCCTGGAGCCTCAATGGTAC	TGGCAGCGTCAATGAAGTAG	153
AQP9	NM_020980 NM_001320635 NM_001320636	AAGCTATTCTCAGTCGAGGAC	TCATCCGTCCAAAGAGACAC	156
FPR1	NM_001193306 NM_002029	TGCCAGTTATCATTCGTGTG	AATGATGAACCGGATGATGC	152
FPR2	NM_001462 NM_001005738	TCTTTCACGGCCACATTACC	TTGATGTCCACCACGATGTG	107
NCF2	NM_000433 NM_001127651 NM_001190794 NM_001190789 NM_001410895	TCATGTTCAACGGGCAGAAG	CTTTGGAACTAGGAGGAGCTG	135

*bp: Base pair

Melt curve analysis (65-95 °C) verified product specificity (supplementary figures 2S-7S). All reactions were performed in technical duplicates. Relative gene expression data were analyzed using the $2^{-\Delta CT}$ [$2^{-(CT\ target\ gene - CT\ reference\ gene)}$] method.[27]

Statistical Analysis

The normality of the data distribution was evaluated using the Shapiro–Wilk test. For comparisons among more than two groups, the Kruskal–Wallis test was applied, followed by Dunn's *post hoc* test with multiple-comparison correction. ROC curves were generated in GraphPad Prism (version 7.0; GraphPad Software, USA), and the optimal cutoff was determined using the Youden index. Data visualization was conducted with GraphPad Prism. Statistical significance was considered at P values less than 0.05, with non-significant results indicated as "ns".

Results

Number of Participants

A total of 83 individuals were assessed for eligibility for the experimental validation phase of the study. Of these, 23 were excluded for not meeting the inclusion criteria, as 15 patients had a concurrent diagnosis of IBS, and eight patients had insufficient biopsy material. The final analysis included a total of 60 participants, comprising 20 healthy controls, 20 newly diagnosed UC patients, and 20 treatment-resistant UC patients.

Identification of DEGs in UC

The expression and distribution of all DEGs with an adjusted P value of less than 0.01 and Log_2 fold change threshold =1 are illustrated in a volcano plot (figure 1). We identified 492 DEGs, which included 429 upregulated and 63 downregulated genes.

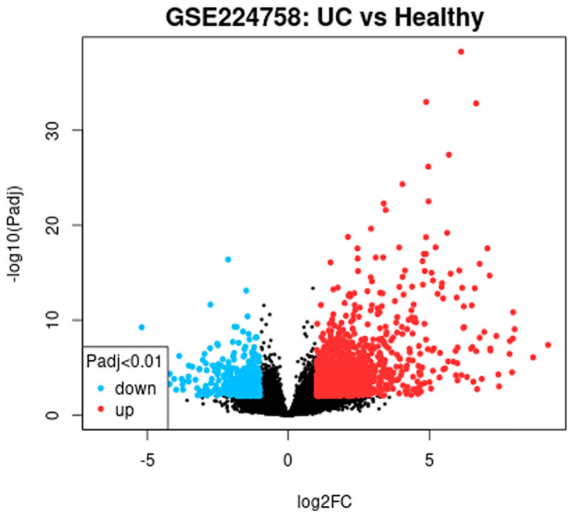

Figure 1: Volcano plot analysis of the GSE224758 dataset reveals 492 DEGs between UC patients and healthy controls. Red dots represent 429 significantly upregulated genes (adj. P<0.01, Log2FC ≥1), while blue dots indicate 63 downregulated genes (adj. P<0.01, Log2FC ≤-1). Black dots show non-significant genes. DEGs: Differentially expressed genes; UC: Ulcerative colitis.

PPI Network and Clustering

A co-expression PPI network for DEGs upregulated in UC compared to healthy controls was constructed using the STRING database. Clustering was performed using Cytoscape software with the Clusterviz plugin, revealing 14 modules (clusters). The largest module included 31 nodes and 83 edges, as shown in figure 2.

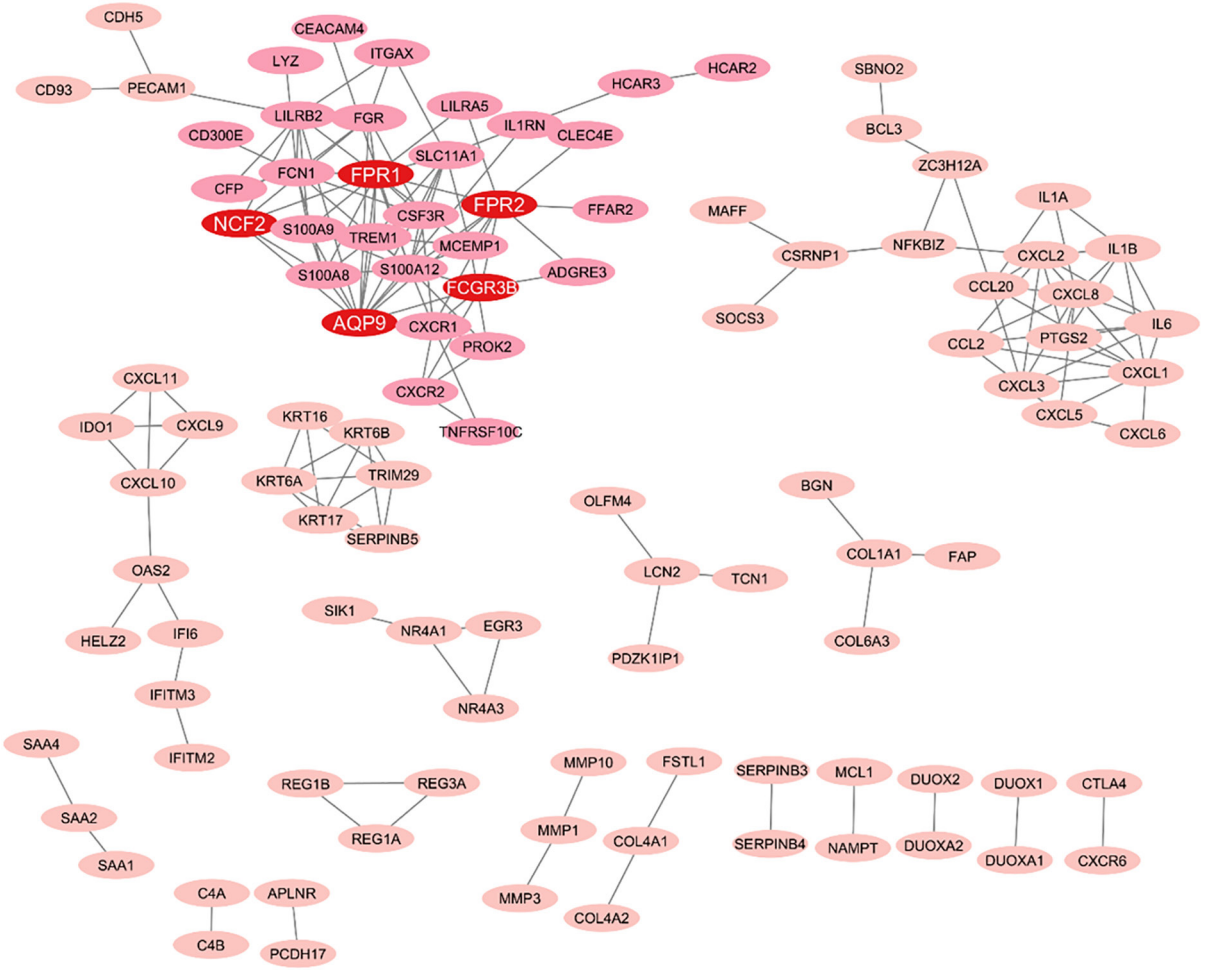

Figure 2: Co-expression PPI network analysis of UC-upregulated DEGs (STRING v. 12.0, confidence ≥0.4) uncovers the largest cluster (31 nodes/83 edges) via Cytoscape 3.10.1 Clusterviz. Node size represents degree (max=14), and edge thickness reflects STRING score (0.4–0.999, average hub confidence 0.59–0.63). In the largest module, red nodes highlight the five key hub genes (*FPR1*, *AQP9*, *FPR2*, *FCGR3B*, *NCF2*), whereas light pink nodes indicate their interacting partners within the module. FPR1 (degree=14, Betweenness=0.147, Closeness=0.559, Stress=484), *AQP9* (12, 0.218, 0.550, 644), *FPR2* (10, 0.179, 0.485, 488), *FCGR3B*/*NCF2* (degree=6). PPI: Protein-Protein Interaction; DEGs: Differentially expressed genes; UC: Ulcerative colitis; *FPR1:* Formyl Peptide Receptor 1; AQP9: Aquaporin-9; FP*R2:* Formyl Peptide Receptor 2; *FCGR3B*: Gamma Receptor IIIb; *NCF2*: Neutrophil cytosol factor 2

Pathway Enrichment Analyses of Upregulated Genes

Pathway enrichment analyses for the largest gene module indicate that UC is associated with abnormal immune function. More specifically, genes involved in the NET formation pathway were significantly upregulated (figure 3). The study also identified five genes involved in the NET formation pathway: *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, and *NCF2*.

RT-qPCR Results for the Five Top Genes in the NET Pathway

RT-qPCR validation confirmed the differential expression of NET-associated genes among the study groups. *AQP9* expression was markedly elevated in both new UC cases and treatment-resistant patients. Compared with healthy controls, *AQP9* increased approximately 9.7-fold in new cases and 19.3-fold in resistant patients (Control median: 4.17×10^{-5}, New Case: 3.86×10^{-4}, Resistance: 7.88×10^{-4}). These increases were statistically significant ($P<0.0001$ for both comparisons). *FPR1* and *FPR2* also showed significant upregulation, with ~4–5-fold higher expression in UC groups relative to controls ($P=0.03$ and $P=0.02$). In contrast, *NCF2* and *FCGR3B* did not demonstrate statistically significant changes across groups (figure 4). Receiver operating characteristic (ROC) curve analysis was performed to evaluate the ability of AQP9 expression to discriminate UC patients from healthy controls. As shown in figure 5, comparison between healthy controls and newly diagnosed UC patients demonstrated excellent discriminatory performance, with an area under the curve (AUC) of 0.9382 (SE=0.04109; 95% confidence interval [CI]: 0.8577–1.000; $P<0.0001$). At the optimal cutoff determined using the Youden index, sensitivity was 83.49%, and specificity was 67.77%.

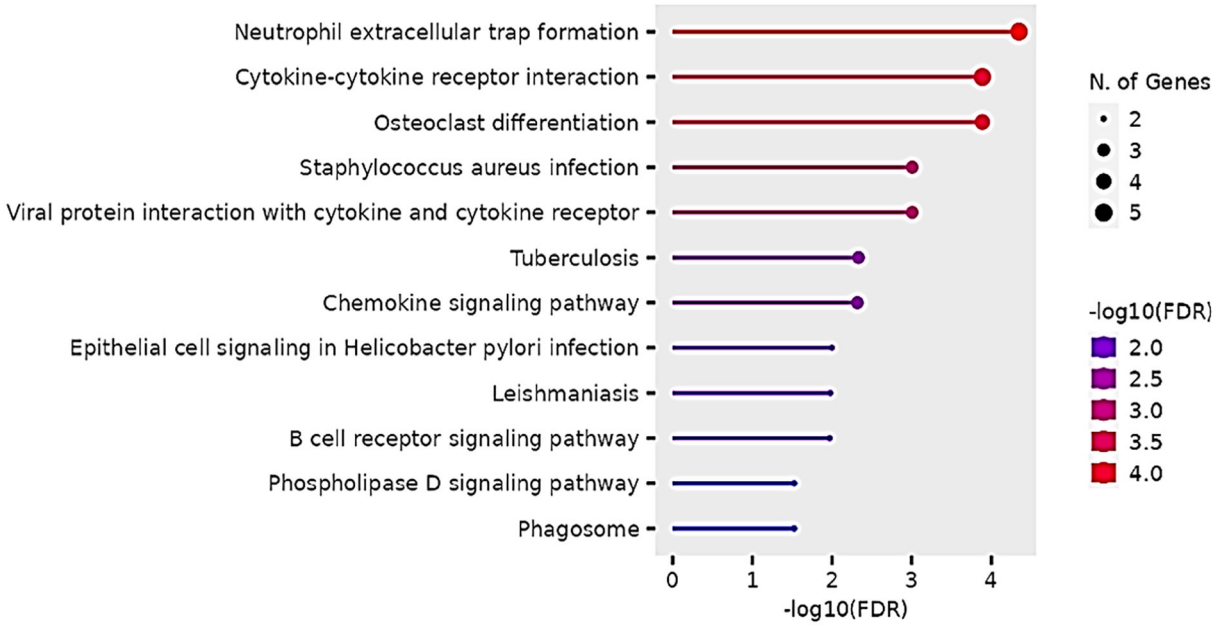

Figure 3: Pathway enrichment analysis of the largest gene module reveals significant enrichment of genes involved in the NETs formation pathway. Key genes highlighted include *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, and *NCF2*. *FCGR3B*: Gamma Receptor IIIb; *AQP9*: Aquaporin-9; *FPR1*: Formyl Peptide Receptor 1; *FPR2*: Formyl Peptide Receptor 2; *NCF2*: Neutrophil cytosol factor 2

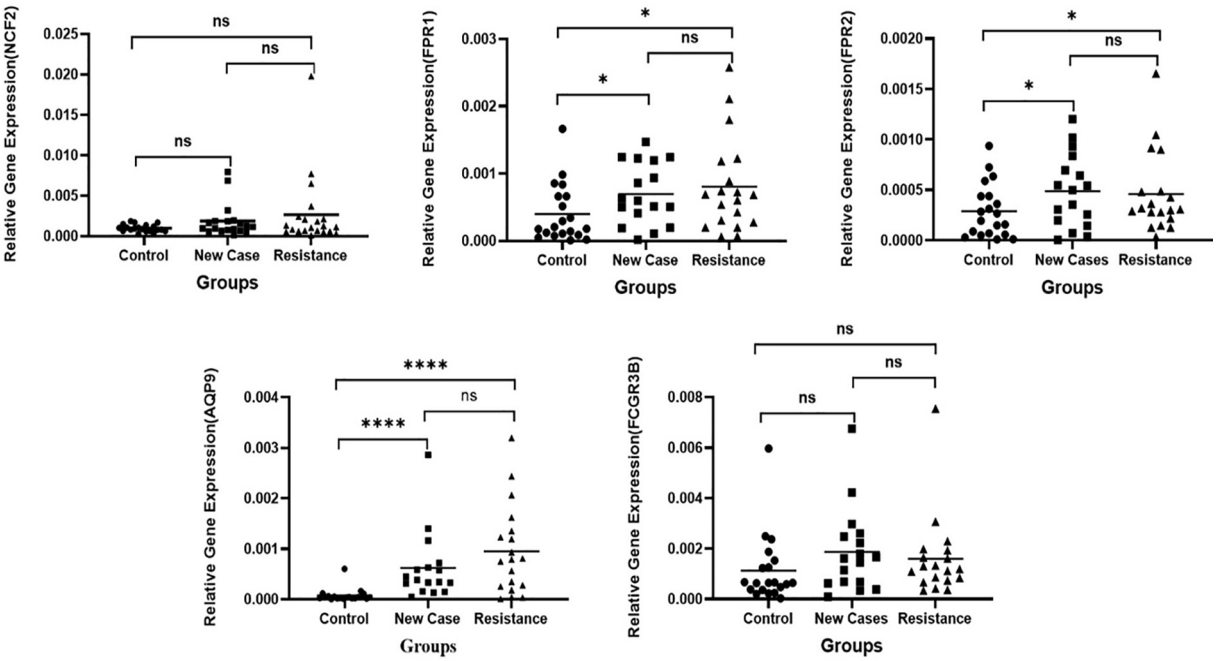

Figure 4: Results of RT-qPCR analysis are shown, which was performed to quantify expression of five NET-related genes (*NCF2, FPR1, FPR2, AQP9, and FCGR3B*) in healthy controls (n=20), new UC cases (n=20), and treatment-resistant UC patients (n=20). Statistical analysis was performed using the Kruskal–Wallis test for overall group differences, followed by Dunn's *post hoc* test for pairwise comparisons. *NCF2*: No significant differences were observed among the three groups (Kruskal–Wallis P=0.47; all pairwise comparisons ns). *FPR1*: Expression was significantly increased in new UC cases compared with controls, while control vs. resistant and new case vs. resistant comparisons were non-significant. *FPR2*: Both new UC cases and resistant patients showed significantly higher expression relative to controls (P=0.03 and P=0.02, respectively), whereas new case vs. resistant comparisons were non-significant. *AQP9*: Expression was markedly elevated in both UC groups relative to controls (Kruskal–Wallis P<0.0001). Pairwise Mann–Whitney tests showed strong significance for control vs. new case (P<0.0001) and control vs. resistant (P<0.0001), while new case vs. resistant remained non-significant. Median values correspond to approximately 9–10-fold (new case) and 19–20-fold (resistant) increases compared with controls. *FCGR3B*: Expression trended higher in UC groups but did not reach statistical significance (Kruskal–Wallis P=0.08); all pairwise comparisons ns. Data are presented as individual values with horizontal bars indicating median±interquartile range. Significance levels are indicated in the figure as: ns: not significant; *P<0.05, ****P<0.0001. RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; NET: Neutrophil Extracellular Traps; UC: Ulcerative colitis; *NCF2*: Neutrophil cytosol factor 2; *FPR1:* Formyl Peptide Receptor 1; FP*R2:* Formyl Peptide Receptor 2; *AQP9:* Aquaporin-9*; FCGR3B:* Gamma Receptor IIIb

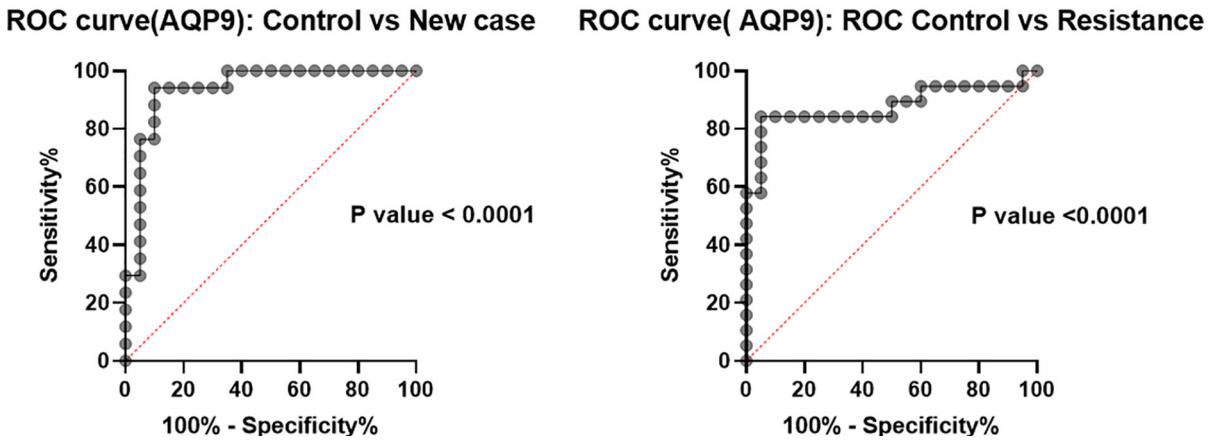

Figure 5: ROC curve analysis of *AQP9* expression demonstrates its ability to discriminate UC patients from healthy controls. ROC analysis comparing newly diagnosed UC patients versus controls showed an AUC of 0.9382 (SE=0.04109; 95% CI: 0.8577-1.000; P<0.0001). ROC analysis comparing treatment-resistant UC patients versus controls yielded an AUC of 0.8789 (SE=0.06258; 95% CI: 0.7563-1.000; P<0.0001). Sensitivity and specificity values were calculated at the optimal cutoff determined using the Youden index in GraphPad Prism. These results indicate that AQP9 exhibits strong discriminatory performance as a candidate tissue biomarker for UC. ROC: Receiver operating characteristic; AUC: area under the curve; UC: Ulcerative colitis; *AQP9*: Aquaporin-9; SE: Standard error

Consistently, ROC analysis comparing healthy controls and treatment-resistant UC patients showed strong discrimination, with an AUC of 0.8789 (SE=0.06258; 95% CI: 0.7563–1.000; P<0.0001). At the optimal cutoff, sensitivity was 80.98%, and specificity was 64.24%. Together, these findings indicate that *AQP9* expression exhibits high discriminatory accuracy for distinguishing UC patients from healthy individuals.

Discussion

This study set out to determine the key genes of NET formation in IBD patients compared to healthy individuals. Bioinformatic analysis led us to focus on five genes, including *FCGR3B*, *AQP9*, *FPR1*, *FPR2*, and *NCF2*. Our findings indicated that the *AQP9* gene was not only significantly upregulated in the new cases and the resistant patients compared with the control group, but it also showed a candidate tissue biomarker in UC patients.

AQP9 is an aquaglyceroporin expressed in immune cells, including neutrophils, where it contributes to water and solute transport processes that are important for inflammatory responses. Recent work in sepsis indicates that dysregulated aquaporin function, including AQP9, can modulate leukocyte recruitment and inflammatory injury, suggesting that altered AQP9 activity may influence neutrophil-driven tissue damage in severe inflammation. Moreover, integrative transcriptomic analyses have shown that *AQP9* expression correlates with neutrophil infiltration and other immune signatures in diverse diseases, supporting its role as an immune-related gene, although direct mechanistic evidence linking *AQP9* to NETosis in UC is still limited and warrants further investigation.[28, 29]

In general, *AQP* genes are responsible for water channels, playing an essential role in maintaining water homeostasis in various tissues. They are strongly associated with IBD progression. Escudero and colleagues revealed that Aquaporin 8 (*AQP8*), as part of this family, was downregulated in collagenous colitis, while its expression was reversed upon corticosteroid therapy.[30] Consistently, Ricanek and colleagues showed that the expression of Aquaporin 3 (*AQP3*), 7, and 8 was reduced in IBD patients.[31] Moreover, Bing Yu and others showed that *AQP9* expression was suppressed in Crohn's disease (CD), with a high diagnostic value.[32] By contrast, Minhoa Yu and colleagues showed that in a refractory model of CD, *AQP9* expression was increased constantly. They demonstrated that anti-TNF therapy was more effective when complemented with inhibiting AQP9, whereby blocking AQP9 decreased inflamed macrophage functions and cytokine expression, especially IL-23 and IL-1β.[33] These findings, together with our data in UC, suggest that *AQP* expression patterns in IBD are highly context-dependent and tightly regulated to preserve intestinal homeostasis, and that disruption of this balance may contribute to disease onset and progression. Our results support the potential of AQP9 for distinguishing UC patients from healthy individuals, while also underscoring the need for mechanistic studies to clarify how modulation of *AQP9* expression could be exploited therapeutically in UC.

FPR1 and *FPR2*, which play critical roles in neutrophil chemotaxis and chronic inflammation, were other genes involved in NET formation that were upregulated in patient groups. McAllister and others discovered that FPR1 drives neutrophilic inflammation, leading to the development of IBD.[34] Furthermore, FPR1 strongly contributes to the progression of CD by promoting macrophage M1 polarization.[35] Similarly, FPR2 is highly expressed on neutrophils, where it drives their recruitment to inflamed colonic tissue. The interaction of FPR2 with damage-associated molecular patterns (DAMPs) and with pathogen-associated molecular patterns (PAMPs) leads to neutrophil activation and NET formation. Implantation of NETs in the intestinal mucosa of IBD patients, with continued accumulation, results in functional epithelial barrier disruption, immune cell recruitment, and sustained inflammation.[36] This concept is also supported by our findings, considering the upregulation of *FPR2* in UC, which could contribute to exaggerated neutrophil-driven tissue damage. NET degradation defects have been implicated in IBD; therefore, *FPR2*-mediated NET may have a significant role in disease worsening. Collectively, it seems that co-expression of *FPR1* and *FPR2* causes IBD exacerbation through sustained hyperactivation of NET formation and macrophage M1 polarization. Therefore, targeting FPR1 and FPR2 could be a novel or complementary approach in the therapy for IBD.

NCF2 and *FCGR3B* were other candidate genes in the current study, and we did not find meaningful changes in their expression in the studied groups. There is little evidence to support NCF2's contribution to IBD development. Muise and colleagues reported that a missense variant in *NCF2* might be associated with the onset of IBD.[25] There is also a weak association between *FCGR3B* and IBD, although the allele copy number of *FCGR3B* may have a significant association with UC susceptibility.[37] We suggest that NET formation is key to promoting both UC and CD, as supported by strong evidence, and *AQP9*, *FPR1*, and *FPR2* are critical in its formation. In the present study, we demonstrated that these three genes were upregulated in UC patients, suggesting these genes ensure the maintenance of activation of NET formation. We suggest inhibiting these genes as a complementary treatment alongside standard medical approaches for UC patients.

Several limitations must be acknowledged in this study. Firstly, smoking status, active infections, and medication use were not fully controlled, as these factors may affect mucosal gene expression profiles and potentially confound biomarker discovery. Although patients with immune deficiencies were excluded, detailed records of smoking history and concurrent medications were not systematically collected due to constraints related to the sample size. Secondly, this cross-sectional design allows for the establishment of associations but does not prove causality between NET gene expression and the pathogenesis of UC. Thirdly, the relatively small sample size (n=60) limits the statistical power for subgroup analyses, particularly when comparing new and refractory UC patients. Finally, functional assays to confirm direct NET formation by the upregulated genes were not conducted. Future studies should involve larger cohorts, prospective designs, matched controls for confounding variables, and the validation of functional assays.

Conclusion

AQP9, FPR1, and FPR2 are candidate tissue biomarkers for UC, with AQP9 demonstrating excellent discriminatory performance. Further multi-center validation studies are necessary to confirm their clinical utility and establish their role in UC diagnosis.

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Authors' Contribution

H.A: Conceptualization, Funding acquisition, conducted experimental stages, data collection, and drafting; S.F: Conceptualization, study design, bioinformatics analyses, writing the final report, and drafting; M.M: Statistical analysis, manuscript writing and editing; A.A, A.J, and M.R.R: Sample collection, experimental stages, and reviewing the manuscript; B.N, as a pathologist, and F.S, as a gastroenterologist: Patient identification and editing the final draft of the manuscript. All authors have thoroughly reviewed and approved the final manuscript and agree to take full responsibility for all aspects of the work, ensuring that any questions concerning the accuracy or integrity of any part of the work are properly addressed.

Declaration of AI

The authors declare that no AI tools were used in the preparation of this manuscript.

Conflict of Interest: None declared.

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