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# ORIGINAL RESEARCH—CLINICAL

## Mathematical Models Including microRNA Levels of Mesenteric Adipose Tissue May Predict Postoperative Relapse in Crohn's Disease Patients

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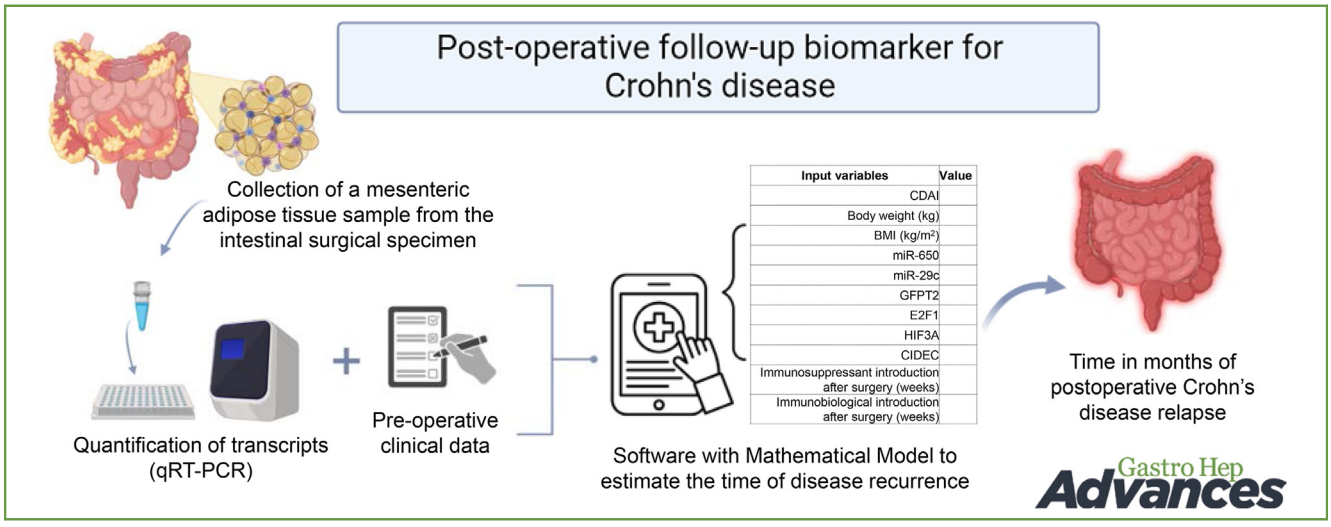


**BACKGROUND AND AIMS:** Recent evidence suggests that the mesenteric adipose tissue (MAT) near the affected intestine may play a role in Crohn's disease (CD) pathophysiology. Modulation of several transcripts has already been identified in the MAT of CD in the literature. Therefore, our aim was to validate the microRNA (miRNA) transcript levels and their target genes in the MAT of active CD patients and correlate them with clinical and epidemiological data. **METHODS:** Samples from the MAT of surgical specimens from 25 active CD patients were obtained. The control group comprised fifteen patients who underwent surgery for other diseases, except inflammatory bowel diseases. Transcriptional levels of miRNA and their target genes were assessed by quantitative real-time polymerase chain reaction. The correlation between transcripts and clinical characteristics was obtained using multiple linear regression. The mathematical models (M) underwent a statistical filter to ensure robustness and reliability ($P$ value $< .05$; adjusted R-squared (R-2)$> .99$; correct predictions of more than 60%). **RESULTS:** miRNA-650 and miRNA-29c were upregulated in the MAT of CD compared to the control group ($P < .0001$ and $P = .0032$, respectively), besides presenting decreased levels of their target genes. Two were target genes of the miRNA-650: glutamine-fructose-6-phosphate transaminase 2 ($P = .012$) and aldehyde dehydrogenase 4 family ($P = .0035$); and 4 were targets of the miRNA-29c: cell death-inducing

***Abbreviations used in this paper:*** **ALDH4A1, aldehyde dehydrogenase 4 family; BMI, body mass index; CD, Crohn's disease; CDAI, Crohn's disease activity index; cDNA, complementary DNA; CIDEC, cell death-inducing DFFA-like effector c; CTR, control; E2F1, E2F transcription factor-1; GFPT2, glutamine-fructose-6-phosphate transaminase 2; HIF3A, hypoxia-inducible factor 3 subunit alpha; IBD, inflammatory bowel disease; ING4, growth factor inhibitor 4; MAT, mesenteric adipose tissue; miRNA, microRNA; MRI, magnetic resonance imaging; PDK4, pyruvate dehydrogenase kinase 4; qRT-PCR, quantitative real-time polymerase chain reaction; RNA-seq, RNA sequencing; UC, ulcerative colitis.**

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DFFA-like effector c ($P = .001$), E2F transcription factor-1 ($P = .007$), hypoxia-inducible factor 3 subunit alpha ($P = .0029$), and pyruvate dehydrogenase kinase 4 ($P = .0054$). We found 2 M with statistical strength and robustness. The performance test identified one model with 100% accuracy for predicting the month of recurrence and determining patients with less risk of early relapse after surgery. **CONCLUSION:** We demonstrate that miRNA-650 and miRNA-29c and some of their target genes, besides clinical and epidemiological variables, may be useful in a model to predict when disease relapse may occur in CD patients who underwent surgery. These findings constitute a potential tool to guide postoperative clinical management.

*Keywords:* Inflammatory Bowel Disease; Recurrence; Surgery; Follow-Up; Biomarker

## Introduction

Crohn's disease (CD) is an inflammatory bowel disease (IBD), and its diagnosis is drawn from a combination of clinical, biological, and morphological characteristics.[1] The disease is usually marked by chronic transmural granulomatous inflammation with lesions in the gastrointestinal tract, mainly the ileum and colon.[2] Another relevant feature of CD is an increase in the size of the mesenteric adipose tissue (MAT) and of the mesenteric fat wrapping surrounding the inflamed bowel.[2] This alteration emerges from the mesentery, which often becomes thick and rigid, partially covers the circumference of the intestinal loop, and which can affect the small and large intestines.[3,4] This hypertrophic mesenteric fat is also named "creeping fat." It is associated with a complex and challenging course in CD, which may include the need for abdominal surgery.[5] Several studies have correlated the alterations in this tissue with mucosal involvement, clinical activity (Crohn's disease activity index [CDAI]), hypertrophy of the intestinal muscle layer, fibrosis, transmural inflammation, severe stricture and histological macrophage, and lymphocyte perivascular infiltration.[4–6] Moreover, visceral adiposity has been correlated with an increased risk of disease-related complications, including endoscopic recurrence postsurgery.[5,7] According to Coffey et al,[4] a fat wrapping greater than 50% allows us to predict increased reoperation rates.

The MAT presents an autophagy pathway defect in CD and it promotes inflammatory and fibrotic processes in the inflamed bowel.[8–12] An expansion and infiltration of the intestinal tissue with innate and adaptive immune inflammatory cells, including neutrophils, macrophages, dendritic cells, natural killer T cells, and innate lymphoid cells take place.[13] Moreover, T-, B-lymphocytes, and plasma cells are activated, potentially constituting a memory immune reservoir and supporting antigen-driven immune responses.[14] The activation of these cells increases intestinal mucosa cytokine levels, such as tumor necrosis factor, interferon $\gamma$, interleukin 1$\beta$, IL6, and IL23, as well as T-helper 17 cell pathway cytokines.[13]

A complete transcriptional analysis RNA sequencing (RNA-seq) conducted by Silva et al[14] identified the modulation of 2 microRNAs (miRNAs) in the MAT of CD patients (miRNA-650 and miRNA-29c). Altered miRNA expression has been associated with numerous diseases.[15–17] As far as IBD is concerned, few specific miRNA profiles have been described in the literature, but they may be involved in predicting susceptibility, disease activity, disease progression, complications, and response to therapy in those patients.[17–21] Moreover, identifying the miRNAs involved with CD and their target genes may provide additional information on CD pathogenesis and establish biomarkers for disease follow-up.[19]

Some studies have focused on predictive models for the clinical course or early onset complications leading to severe CD.[22–24] However, no studies provide a mathematical model showing the prognosis after surgery. With this purpose, we aimed to validate the expression of miRNAs and their target genes in the MAT affected by CD compared to a control (CTR) group. In addition, our study aimed to develop and validate a novel prediction prognosis model for postoperative relapse, including transcriptional, clinical, and epidemiological data of CD patients who underwent surgery.

## Patients and Method

### Sample Collection

This study included 40 patients operated on at the Clinical Hospital of the University of Campinas (Unicamp) by the Colorectal Surgery Unit from May 2014 to February 2018. The clinical and epidemiological characteristics of the patients were obtained (Table 1).

The CD group was composed of 25 patients with the ileal disease. They were classified according to Montreal's clinical classification[25] (Table 1). All CD patients included in the study were on anti-tumor necrosis factor therapy and were operated upon because they failed secondarily to respond to treatment and developed disease complications such as obstruction and abscess/fistula. The CTR group comprised 15 patients who underwent surgery on the left colon for non-IBDs and presented normal distal ileum.

The MAT fragments were collected near the ileum from the surgical specimens for the CD group. A MAT biopsy was collected near the ileum during surgery for the CTR group samples. The fresh samples were included in vials with RNAlater and stored at $-80$ °C immediately.

After surgery, all patients were followed up at the IBD outpatient clinic at the Gastrocenter-Unicamp, a reference center for IBD treatment in Campinas, São Paulo state, Brazil. Postoperative patient follow-up data were collected from the medical records (Table 1). Colonoscopy examination and imaging tests, such as magnetic resonance imaging (MRI) or computed tomography scan, were used as criteria to determine postoperative relapse.

**Table 1.** Clinical and Demographic Characteristics of Patients Included in the Study

| Clinical and demographic variables | CD group | CTR group | *P* value |
|---|---|---|---|
| Sex | | | .6028[2] |
| Female | 15 (60%) | 10 (66.7%) | |
| Male | 10 (40%) | 5 (33.3%) | |
| Total | 25 | 15 | |
| Age (y) | 30 (20–70) | 56 (42–61) | .0130 |
| Disease duration (y) | 6 (1–30) | - | |
| Age at diagnosis[a] | | - | |
| A1 | 2 (8%) | | |
| A2 | 18 (72%) | | |
| A3 | 5 (20%) | | |
| Total | 25 | | |
| Location[a] | | - | |
| L1 | 9 (36%) | | |
| L2 | 0 | | |
| L3 | 16 (64%) | | |
| L4 | 0 | | |
| Total | 25 | | |
| Behavior[a] | | - | |
| B1 | 0 | | |
| B2 | 14 (56%) | | |
| B3 | 11 (44%) | | |
| Total | 25 | | |
| Perianal disease[a] | | - | |
| Yes | 23 (92%) | | |
| No | 2 (8%) | | |
| Total | 25 | | |
| Immunosuppressant | | - | |
| Yes | 14 (56%) | | |
| No | 11 (44%) | | |
| Total | 25 | | |
| Anti-TNF-$\alpha$ therapy | | - | |
| Yes | 25 (100%) | | |
| No | 0 (0.0%) | | |
| Total | 25 | | |
| CDAI | 305 (162–609) | - | |
| Presence of ulcers (yes/no) | 25/0[b] | 0/15[c] | |
| Body weight (kg) | 56 (43.4–79) | 65.5 (49–107) | |
| Body mass index (kg/m$^2$) | 21.6 (14.7–30.5) | 27.4 (21.2–36.1) | |
| Follow-up (mo) | 45.5 (24–68) | - | |
| Relapse[d] | | | |
| Yes | 13 (54.2%) | - | |
| No | 11 (45.8%) | | |
| Time from the surgery to relapse (mo) | 16 (6–41) | - | |
| Immunosuppressant use (postop.) | | | |
| Yes | 22 (91.7%) | - | |
| No | 2 (8.3%) | - | |
| Time to the immunosuppressant introduction after surgery (wk) | 5.5 (1–53) | - | |
| Biological agent use (postop.) | | | |
| Yes | 20 (83.3%) | - | |
| No | 4 (16.7%) | - | |
| Time to biological therapy introduction after surgery (wk) | 5.5 (3–65) | - | |

The numerical variables were described as median [min, max] and the categorical variables as absolute frequencies.
TNF, tumor necrosis factor.
[a]Montreal classification.[25]
[b]The presence of ulcers in the ileum was assessed by macroscopic and microscopic histological examination of the surgical sample.
[c]Presence of ulcers in the ileum assessed by colonoscopy.
[d]One patient was excluded from this analysis because it was impossible to evaluate relapse. She had sepsis complications after surgery due to a fungal infection in the central nervous system and died a few weeks after surgery.

### Biological Validation of miRNAs and Their Target Genes

***In silico* analysis.** A total of 40 patients were enrolled for biological validation of the transcripts; 25 patients for the CD group and 15 patients without IBD for the CTR group. According to Silva et al,[14] miRNA-650 and miRNA-29c were the only miRNAs modulated on MAT of CD. Thus, we conducted an *in silico* analysis to identify the predicted target gene of those miRNAs. We used miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), a free-use miRNA database, through 12 different algorithms (miRWalk, miRDB, PITA, MicroT4, miRMap, RNA22, miRanda, miRNAMap, RNAhybrid, miRBridge, PICTAR2, and TargetScan). As an exclusion criterion, only the targets provided by TargetScan (http://targetscan.org/) and at least 5 more of the evaluated algorithms were considered.

After this initial screening, the target genes were input into the DAVID platform (https://david.ncifcrf.gov/) to select the enriched signaling pathways with the target genes. With this, it was possible to filter the metabolic pathways and miRNA-650 and miRNA-29c target genes that have greater importance and were modulated in CD, according to RNAseq.[14]

**RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction.** For the biological validation, a quantitative real-time polymerase chain reaction (qRT-PCR) analysis of miRNAs and target genes, selected by *in silico* study, was performed.

Total RNA and the pool of small RNAs (including miRNAs) were extracted from the fresh-frozen MAT samples (CD and CTR groups) using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Cat No./ID: 74,104) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were determined using UV spectrophotometry at 260 nm of the Nanodrop 2000 (Thermo Fisher Scientific, Epsom, Surrey, UK). cDNA synthesis was done using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative PCR reactions were performed on the ABI 7500 FAST platform using the TaqMan system (Applied Biosystems). The specific primers for each miRNA and target genes used were: miRNA 650 (TM:001603), glutamine-fructose-6-phosphate transaminase 2 (GFPT2) (Hs01049570), aldehyde dehydrogenase 4 family (ALDH4A1) (Hs01013142), miRNA 29c (TM:000587), E2F transcription factor-1 (E2F1) (Hs00153451), hypoxia-inducible factor 3 subunit alpha (HIF3A) (Hs00541709), cell death-inducing DFFA-like effector c (CIDEC) (Hs01032998), and pyruvate dehydrogenase kinase 4 (PDK4) (Hs01037712).

The transcriptional levels of the target genes were normalized using glyceraldehyde 3-phosphate dehydrogenase (4325792) as the endogenous control gene or U6snRNA (RT:001973) for miRNAs. Gene expression was then determined using fold change, obtained by the delta-delta Ct method.

**Statistical analysis for the qRT-PCR data.** All data were analyzed and reported using median values. The Grubbs test (https://www.graphpad.com/quickcalcs/grubbs1/) was used to identify the outliers. The nonparametric Mann–Whitney U test was performed between the groups to verify significant differences in the gene expressions. A $P$ value less than .05 was considered significant. All the analysis was performed using GraphPad Prism version 8.0.

### Mathematical Models for CD Relapse's Prognosis after Intestinal Resection

Only CD patients were enrolled in this analysis. We performed a multiple linear regression analysis with data input from clinical and epidemiological CD patients and values of miRNAs and their target gene levels modulated in MAT of these patients. For each patient, metrics belonging to 14 factors were obtained: use of immunosuppressant, CDAI, body weight, body mass index (BMI), time to the introduction of immunosuppressants and immunobiological therapy after surgery, and miRNA-650, GFPT2, ALDH4A1, miRNA-29c, E2F1, HIF3A, CIDEC, and PDK4 transcriptional levels.

The models were generated from a subgroup of 24 patients. Only one patient who died weeks after surgery was excluded from this analysis. We verify how these 14 factors can be related to a patient's relapse through mathematical models. The output of the models was expressed in months, which estimated how many months after surgery the patient will relapse.

All generated models went through 2 statistical filters to ensure reliability and robustness. For this proposal, we used a first statistical filter with a $P$ value $< .05$ for each variable included in the model and an adjusted R-squared (R-2) $>0.99$ for the model result. Aiming to find the most assertive model, we compared the results of the statistically more robust models with the patients' real-time relapse. A second statistical filter was applied to select only the models that showed better performance and assertiveness, so that several correct predictions were above 60%. The evaluation of recurrence was carried out during outpatient follow-up using endoscopy and imaging tests, most often MRI. All mathematical model analysis was conducted on Python, a programming language for software development, data science, and machine learning.

### Ethical Considerations

This study was conducted by the Declaration of Helsinki at a single tertiary referral center and approved by the University of Campinas Ethics Committee (CAAE #78145517.0.0000.5404). All participants read and signed a written informed consent form for study participation.

## Results

### Biological Validation of miRNA 650 and miRNA 29c

The qRT-PCR analysis showed a significant increase in the miRNA-650 transcriptional levels on MAT samples from the CD group compared to the CTR ($P < .0001$) (Figure 1A).

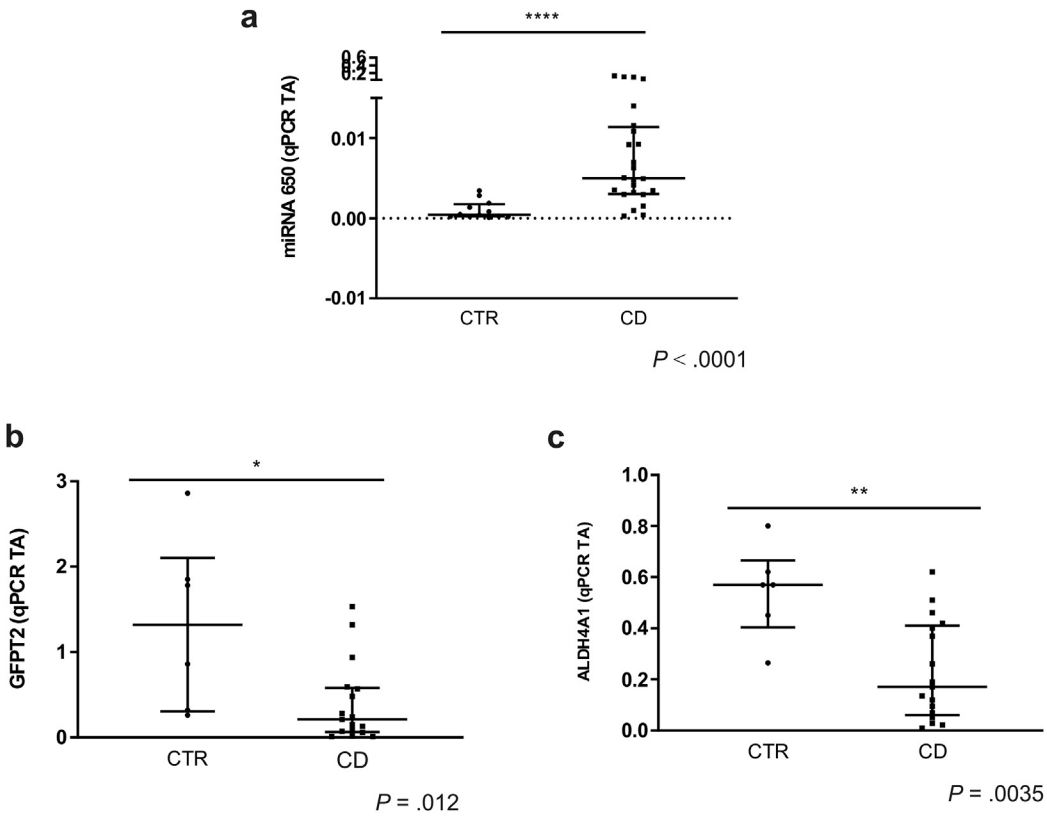


**Figure 1.** Transcriptional analysis of microRNA 650 and its target genes in the mesenteric adipose tissue of Crohn's disease and control groups. (A) mRNA transcriptional level of miRNA-650 was significantly increased in the CD group compared to the CTR group. The transcriptional levels were normalized using U6snRNA as the endogenous control gene. n = 24 CD patients and 12 controls. (B and C) mRNA transcriptional levels of miRNA-650 target genes (GFPT2 and ALDH4A1, respectively) were significantly decreased in the CD group compared to the CTR group. The transcriptional levels of the target genes were normalized using GAPDH as the endogenous control gene. n = 25 CD patients and 8 controls for GFPT2 analysis, and n = 25 CD patients and 8 controls for ALDH4A1 analysis. Transcriptional levels as determined by qPCR are shown as a transcript amount (TA), *$P < .05$, **$P < .001$, ****$P < .0001$ (nonparametric Mann–Whitney U test). mRNA, RNA messenger.

According to the *in silico* analysis, we identified 25 predicted target genes for miRNA-650 among the 226 genes that showed a decrease in MAT according to the study of RNA-seq.[14] These 25 target genes were inserted in the DAVID platform to analyze enriched metabolic pathways. Two predicted miRNA-650 target genes were identified acting in a single pathway related to the metabolism of the alanine, aspartate, and glutamate amino acids: ALDH4A1 and GFPT2.

The biological validation by qRT-PCR of these target genes confirmed, in an independent cohort, the findings of the previously published RNA-seq.[14] Transcriptional levels of the GFPT2 and ALDH4A1 genes in the MAT of the CD group decreased when compared to the CTR group ($P < .05$), which confirms the modulation of these target genes by the miRNA-650 in the MAT of the CD group when compared to the CTR group (Figure 1B and C).

Unlike what was predicted by RNA-seq,[14] the biological validation of miRNA-29c showed a significant increase in transcriptional levels of these miRNA in MAT samples from the CD group compared to the CTR group ($P < .05$) (Figure 2A).

*In silico* analysis identified 28 predicted target genes for miRNA-29c among the 226 decreased genes in the MAT of CD patients.[24] According to the metabolic pathway enrichment analysis, 2 cancer-related pathways in which more than one of these genes acted together were enriched. Among the genes affecting these 2 pathways, the E2F1 was selected for its involvement in the cell cycle and proliferation. In addition, 3 other genes were selected that act on different metabolic pathways related to CD pathophysiology, such as fibrosis, apoptosis, and energetic metabolism. These genes were HIF3A, PDK4, and CIDEC. The HIF3A, PDK4, E2F1, and CIDEC transcriptional levels were significantly decreased in the MAT of CD patients compared to the controls, according to the biological validation by qRT-PCR ($P < .05$) (Figure 2B–E). These findings confirmed the modulation of the miRNA-29c target genes in the MAT of the CD group when compared to the CTR group.

## *Enriched Signaling Pathways of miRNAs with Their Target Genes*

Metabolic pathway enrichment analysis identified GFPT2 and ALDH4A1 genes in the amino acid metabolism pathway: alanine, aspartate, and glutamate (Figure 3A).

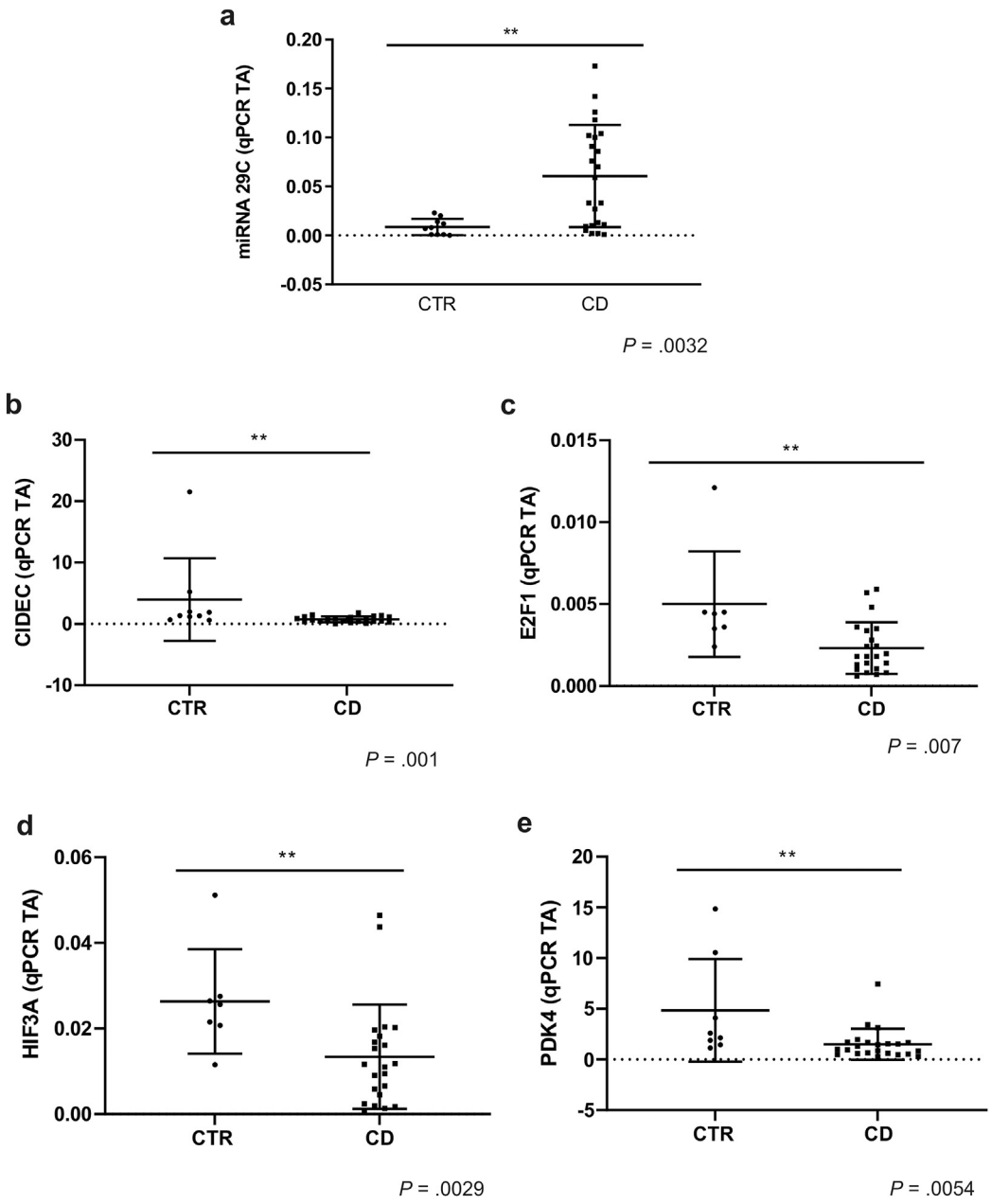


**Figure 2.** Transcriptional analysis of microRNA 29c and its target genes in the mesenteric adipose tissue of Crohn's disease and control groups. (A) mRNA transcriptional level of miRNA-29c was significantly increased in the CD group compared to the CTR group. The transcriptional levels were normalized using U6snRNA as the endogenous control gene. n = 25 CD patients and 12 controls. (B–E) mRNA transcriptional levels of miRNA-29c target genes (CIDEC, E2F1, HIF3A, and PDK4, respectively) were significantly decreased in the CD group compared to the CTR group. The transcriptional levels of the target genes were normalized using GAPDH as the endogenous control gene. n = 24 CD patients and 7 controls for CIDEC analysis, n = 24 CD patients and 7 controls for E2F1 analysis, n = 24 CD patients and 7 controls for HIF3A analysis, and n = 24 CD patients and 7 controls for PDK4 analysis. Transcriptional levels as determined by qPCR are shown as a transcript amount (TA), $^{*}P < .05$, $^{**}P < .001$, $^{***}P < .0001$ (nonparametric Mann–Whitney U test). mRNA, RNA messenger.

These 2 targets of the miRNA-650 showed reduced transcriptional levels in the MAT of CD patients compared to the control, which can cause an alteration in the production of glutamate and affect endogenous glucose balance.

Distinctly, the analysis performed with miRNA-29c target genes identified cancer-related enriched metabolic pathways. The E2F1 gene, validated in this study, is present in this metabolic pathway. This gene acts in 2 distinct metabolic processes in cancer: (I) as a transcriptional factor stimulating cell progression and (II) directly in blocking cell differentiation (Figure 3B). The other genes selected for validation act individually in different metabolic pathways. However, all of them may be related to CD pathophysiology (Figure 4).[26–37]

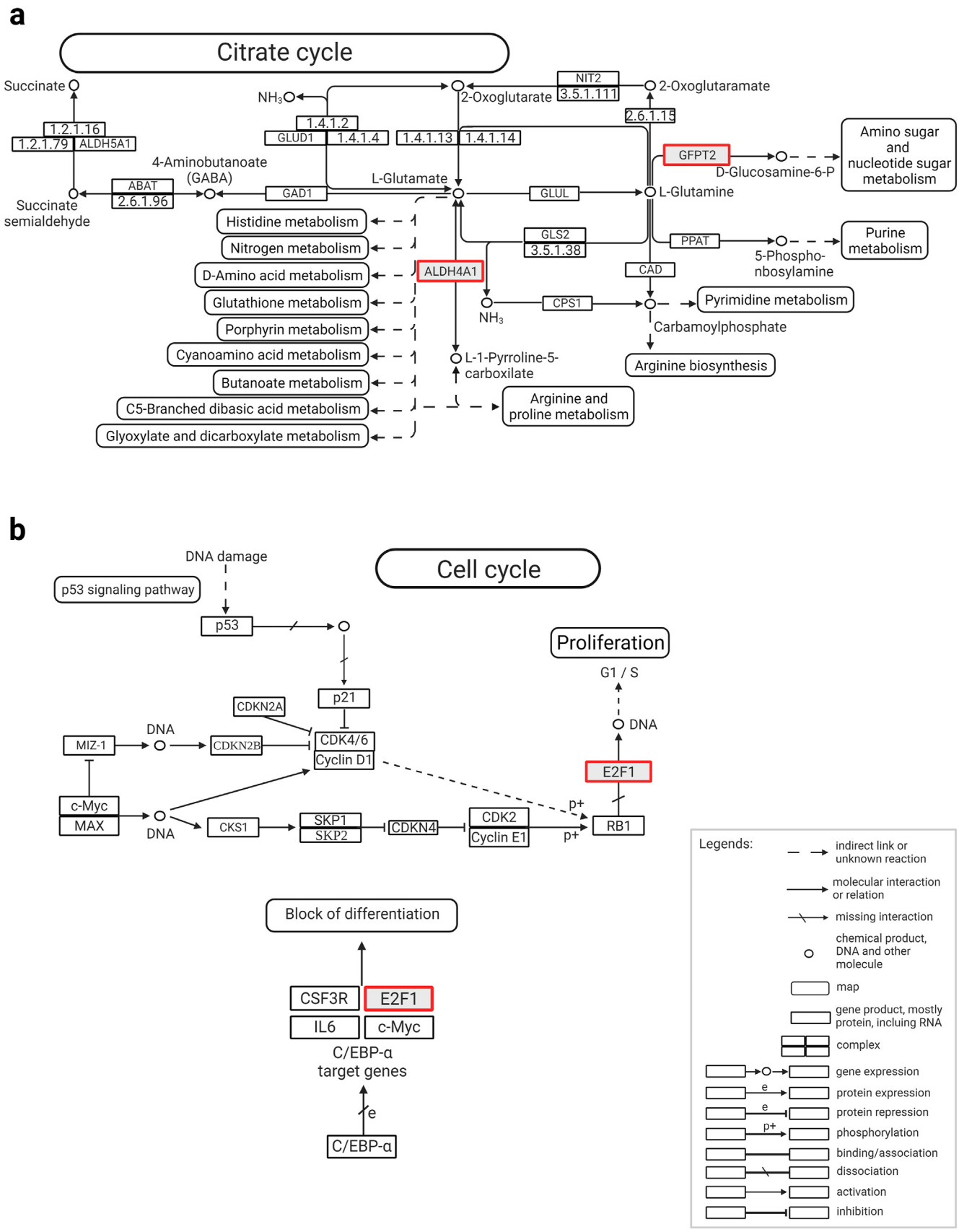


**Figure 3.** Metabolic pathways of the microRNAs and their target genes. (A) Alanine, aspartate, and glutamate metabolism pathway with the role of microRNA 650 target genes, GFPT2 and ALDH4A1, which were downregulated in mesenteric adipose tissue (MAT) in Crohn's disease (CD). (B) Cancer-related metabolic pathway with the role of microRNA 29c target gene, E2F1, which was downregulated in mesenteric adipose tissue (MAT) in Crohn's disease (CD). Figures created with BioRender.com.

**Box 1. Target genes of miR-650 downregulated in the mesentery of Crohn's disease**

- **GFPT2** (glutamine-fructose-6-phosphate transaminase 2): encodes the Glutaminefructose-6-phosphate transferase 2 enzyme, responsible for converting L-glutamine + D-fructose 6-phosphate into L-glutamate + D-glucosamine 6-phosphate. According to Zhang et al.[26] this enzyme limits the biosynthesis rate of hexosamine, a glucose precursor amino acid in the metabolism of glycogenic amino acids. It increases endogenous glucose, which might be involved in regulating inflammation in CD.

- **ALDH4A1** (L-glutamate gamma-semialdehyde dehydrogenase): encodes Delta1-pyrroline-5-carboxylate (P5C) dehydrogenase enzyme. This enzyme acts on the proline degradation pathway and catalyzes the $NAD^+$-dependent conversion of P5C to the neurotransmitter glutamate.[27] When at low levels, it leads to a plasma accumulation of proline (hyperprolinemia). This enzyme can be found in the urine of patients with hyperprolinemia.[28] Besides its importance in glutamate metabolism, ALDH4A1 acts in DNA repair and cell survival by attenuating oxidative stress.[27] Yoon et al.[29] showed that reduced ALDH4A1 expression in tumor cells increases susceptibility to p53-mediated apoptosis.

**Box 2. Target genes of miR-29c downregulated in the mesentery of Crohn's disease**

- **E2F1** (E2F transcription factor 1): the E2F family controls the cell cycle besides acting in tumor suppressor proteins. E2F1 plays a role in the cell cycle and promotes cell proliferation from the G1 phase to the S phase by induction of genes required for DNA synthesis.[30] According to recent research, these genes have been highly upregulated in late-stage tumors promoting cancer invasion and metastasis.[31] The deregulated activation by overexpression, gene amplification, and post-translational modification induces unrestrained cell cycle progression and cell proliferation, establishing the oncogenic role of E2F1.[31] However, this gene is also responsible for inducing apoptosis under some cell pathways by p53-dependent or -independent mechanisms: (1) E2F1 activates p14/p19 that inhibits p53 degradation inducing apoptosis; (2) E2F1 regulates genes by increasing the activity of p73, CASP-3, CASP-7, which also induces apoptosis and playing function as a tumor suppressor.[32] The decrease in E2F1 levels was related to decreased apoptosis by upstream regulation in the p53 pathway, and to decrease cell-cycle arrest in CD progression from dysplasia to cancer.[33] Until now, there is no consensus on whether E2F1 functions as an oncogene or a tumor suppressor.[32]

- **HIF3A** (Hypoxia Inducible Factor 3 Subunit Alpha): encodes alpha-3 subunit protein, one of several alpha/beta-subunit heterodimeric that act as transcription factors regulating adaptive responses to hypoxia. These factors containing this subunit are negative regulators of hypoxia-inducible gene expression. HIF1A and -2A play critical roles in cellular and systemic adaptation to hypoxia, but the function and regulation of HIF3A in the HIF pathway still need to be better understood.[34] The HIF3A gene was evaluated in human biopsies of non-inflamed colons from patients with UC and normal controls.[35] A significant decrease in the level of this protein was demonstrated in the non-inflamed mucosa of UC patients compared to the normal mucosa of healthy individuals.

- **PDK4** (Pyruvate Dehydrogenase Kinase 4): a member of the protein kinase family PDK/BCKDK is responsible for encoding a mitochondrial protein with a histidine kinase domain. It is located in the mitochondria matrix, thus contributing to glucose metabolism regulation, regulated by insulin, glucocorticoids, and retinoic acid. A recent trial observed that expression of PDK4 increased during colitis development in a dextran sulfate sodium (DSS)-induced colitis model and evaluated the effect of PDK4 deletion on T-cell activation *in vitro*.[36] They identified that PDK4 silencing improved murine colitis by inhibiting T-cell activation and reducing calcium transfer from the endoplasmic reticulum to mitochondria, demonstrating that this gene may be a potential therapeutic target for IBD.

- **CIDEC** (Cell death-inducing DFFA-like effector C): encodes a protein that acts in adipocytes promoting lipid droplet formation and may mediate apoptosis. This gene is regulated by insulin. Its expressionpositively correlates with insulin sensitivity and may contribute to insulin-resistant diabetes. This gene was associated with a positive functional interaction with genes related to mitochondrial biogenesis in human adipose tissue, demonstrating to be more expressed in subcutaneous adipose tissue samples than in visceral adipose tissue. The reduction in CIDEC expression associated with obesity may reflect lipid storage, altered adipose tissue function, and oxidative capacity, especially visceral fat deposit.[37]

**Figure 4.** Metabolic functions of the downregulated target genes of miRNA-650 and miRNA-29c in mesenteric adipose tissue of Crohn's disease patients confirmed by qPCR analysis. Figure created with BioRender.com.

**Table 2.** Performance of the 2 Multiple Linear Regression Models on the Patients' Data set Who Did or Did Not Relapse

| Patient | Time for relapse[a] (mo) | Model 1 | Model 2 |
|---|---|---|---|
| 1 | 32 | 32.0 | 32.0 |
| 2 | Nr | −80.4 | 38.3 |
| 3 | Nr | 216.0 | 34.4 |
| 4 | 33 | 33.0 | 33.0 |
| 5 | 41 | 41.0 | 41.0 |
| 6 | Nr | −172.9 | 46.5 |
| 7 | 30 | 30.0 | 30.0 |
| 8 | Nr | 56.9 | 32.5 |
| 9 | Nr | −28.1 | 16.4 |
| 10 | Nr | - | - |
| 11 | Nr | 120.1 | −74.6 |
| 12 | 13 | 13.0 | 13.0 |
| 13 | 9 | 9.0 | 9.0 |
| 14 | Nr | 67.3 | 54.5 |
| 15 | Nr | −160.3 | 30.3 |
| 16 | Nr | - | - |
| 17 | 22 | 22.0 | 22.0 |
| 18 | 16 | 16.0 | 16.0 |
| 19 | 11 | - | 247.0 |
| 20 | 9 | 9.0 | 9.0 |
| 21 | 11 | 11.0 | 11.0 |
| 22 | 6 | 6.0 | 6.0 |
| 23 | 21 | 21.0 | 21.0 |
| 24 | Nr | 127.8 | 27.7 |

Nr, not relapse.
[a]According to clinical data obtained from medical records in the patient's follow-up.

### *Prognosis Mathematical Models*

Patient follow-up ranged from a minimum of 24 to a maximum of 68 months. Of the 25 patients, 24 were included in the models, and 54,2% (n = 13) had endoscopic recurrence (Table 1). Only 2 patients (8.3%) did not have immunosuppressants reintroduced after surgery, and 4 did not return to therapy with immunobiological (16.7%). The postoperative management in our outpatient clinic was conducted according to the recommendations of international guidelines.[38]

A multiple linear regression analysis generated 32,767 mathematical models (M). After we applied the first statistical filter, 9 models showed statistical strength and robustness to predict the time to CD relapse within months after the intestinal resection. According to the performance test, after applying the second statistical filter, we reached 2 models with greater than 60% accuracy in predicting how many months the recurrence occurred in patients who relapsed (Supplementary Information).

The performance test showed that these 2 models were 100% accurate in predicting the time in months of patients' relapse (Table 2). However, M1 also presented this precision in patients who did not relapse, differently from M2. For patients who had not yet relapsed, M1 had values much higher than the follow-up time or negative values for relapse (which we interpret as a low risk of developing recurrence). This model presents the following input variables: CDAI, body weight, BMI, miRNA-650, GFPT2, miRNA-29c, E2F1, HIF3A, CIDEC transcriptional levels, the number of postsurgery weeks the immunosuppressant was introduced, and the number of postsurgery weeks the biological agent was introduced. The technology (software) developed for this model was registered under the number BR512023000506-1 at the National Institute of Industrial Property from Brazil by the innovation agency from the University of Campinas (Unicamp) on March 3, 2023.

Detailed information on the construction of the mathematical models is compiled in the Supplementary materials (Figures A1 and A2).

## Discussion

Recent studies have focused on the role of the mesentery in several diseases.[39] Specific tissue differences were already identified in CD patients MAT and intestinal mucosa by evaluating the transcription signaling pathways and cytokines expressions in these tissues.[40] A study by Silva et al[14] in 2020 was the first to employ RNA-seq analysis to assess transcriptome changes across the MAT genome-wide in patients with CD and controls. This made it possible to identify the signaling process of different molecular pathways activated in CD.

Silva et al[14] presented a list of 425 upregulated genes and 226 downregulated genes in the MAT associated with CD compared to the controls without IBD. Only 2 miRNAs were present in these lists: miRNA-650, the highest expressed gene in the MAT associated with CD (FC = 23,45), and miRNA-29c, downregulated (FC = −1,82).[14] Our study confirmed the significant upregulation of miRNA-650 in the MAT of the CD group compared to the control, validating the RNA-seq analysis. However, unlike what was predicted by RNA-seq,[14] the qRT-PCR analysis showed a significant increase in miRNA-29c transcriptional levels in MAT samples from the CD group. Other similar studies that performed validation of microarray analyses by qRT-PCR also found different results from what was expected according to the transcriptomic platforms, showing the relevance of biological validation in this context.[41,42]

Studies have demonstrated the miRNA modulation in CD and IBD.[17–21] Different miRNA profiles are expressed in active ulcerative colitis (UC), Crohn's ileitis, and Crohn's colitis.[18] They also vary according to the sample used (epithelial cells, peripheral blood, or endoscopic biopsy) and the storage method (fresh-frozen tissue or formalin-fixed, paraffin-embedded).[18,19] Although this is the first work to validate the modulation of miRNA-650 and miRNA-29c in the MAT of CD and to correlate with clinical and epidemiological data, few studies mentioned alterations in the expression of these miRNAs in the intestinal mucosa of IBD patients.

Fisher and Lin[17] reviewed miRNA expression patterns involved in IBD, focusing on their use as biomarkers to classify and prognosticate disease severity. Among other miRNAs, the miRNA-650 showed an increase in the inflamed mucosal tissue of the UC patients compared to the non-inflamed area. MiRNA-29c, on the other hand, was increased in the colonic mucosa of CD, both in active and inactive disease, compared to a healthy control group.

Classically, the miRNAs act at a post-transcriptional level by inhibiting the translation of their target genes through RNA messenger degradation and, more recently, they have been believed to exert nuclear functions and act at the promoter level to inhibit the translation of the target genes.[43] Considering the modulation of miRNA-650 and miRNA-29c in our findings, the qRT-PCR confirmed the downregulation of their target genes. The decrease in transcriptional levels of GFPT2, ALDH4A1, HIF3A, PDK4, E2F1, and CIDEC was confirmed by the biological validation in samples of MAT of CD patients who underwent surgery. The biological function of these genes differentially expressed in the MAT of CD patients is shown in Figure 4. Those genes are involved in several pathways that may be explored as potential new insights into CD etiology.

The miRNA-650 and miRNA-29c target genes evaluated in the present study act on some critical metabolic pathways affected in CD, such as cellular energy metabolism, cell differentiation, and apoptosis pathways. Given the complexity of transcriptional analyses, knowledge about the action mechanism that the modulation of these genes directly or indirectly presents in CD still needs to be fully understood.

Besides in CD, miRNA-650 is also modulated in patients with UC, demonstrating its importance in the pathogenesis disease, in which it is overexpressed in the inflamed mucosa.[17] In UC, miRNA-650 downregulates the expression of the target gene NLRP6, a member of the Nod-like receptor family, that acts to maintain intestinal homeostasis by regulating gut microbiome, epithelial repair, and proliferation.[44] The overexpression of miRNA-650 in UC leads to a modulation of genes related to the apoptosis regulation of the epithelial cells.[44]

Although miRNA-650 modulation occurs in IBD, the main findings are associated with neoplastic pathways.[45–48] Another downstream target for miRNA-650 is the growth factor inhibitor 4 (ING4) gene.[48] ING4 is a member of a novel tumor suppressor gene family and is related to increasing the function of p53 in genetic transcription, apoptosis, and DNA repair. According to Zhang and collaborators,[48] miRNA-650 likely plays an essential role in the tumorigenicity of human gastric cancer and is a promising molecular target for gastric cancer therapy.

Furthermore, research suggests that miRNA-650 acts as a regulator of pro-inflammatory cytokine production. Indeed, research shows that the negative regulation of ING4 by miRNA-650 is related to controlling IL6, IL8, and nuclear factor kappa B.[45,46,48] Overexpression of miRNA-650 leads to negative regulation of ING4 in osteosarcoma cells, where it also increases the transcriptional activity of nuclear factor kappa B, increasing IL6 production, induced by interleukin 1.[46]

The miRNA-29 family has a role in regulating the adaptive immune system. Nonetheless, the family has 3 members besides miRNA-29c; miRNA-29a, miRNA-29b-1, and miRNA-29b-2.[49] Despite similar sequences, they have a nucleotide difference and subcellular localization, as miRNA-29a is mainly cytoplasmic, while miRNA-29b and miRNA-29c are in the nucleus. This slight difference is enough to present a different function in gene expression. The miRNA-29 family is highly expressed in T cells and B cells. It is known that miRNA-29a and miRNA-29b act in thymic function regulation, the mature T cells polarization, and B cells oncogenesis.[49] However, more is needed to know about the miRNA-29c. Therefore, the relative expression of every subset of this miRNA still needs to be better elucidated. This miRNA family has up to 6000 predicted targets overlapping between the different members. According to Liston and collaborators,[49] only about 50 predicted targets have been experimentally validated.

The highly conserved all round evolution of miRNAs makes them highly stable molecular structures.[17] This makes it possible to use them as biomarkers or therapeutic targets. In this way, identifying the miRNAs involved with CD and their target genes may provide additional information about CD pathogenesis and establish essential biomarkers for disease monitoring, prognosis, and follow-up.

## Prognosis Mathematical Models

Surprisingly, even with the advanced use of immunobiological therapy, the number of patients requiring surgery is substantial.[50] Many patients still require surgery for refractory disease or disease-related complications.[50] The mathematical models we have developed may represent a new era in postsurgical patient management, where more accurate decision-making can be defined based on the patient's increased risk of early relapse.

The sample number and follow-up time of patients did not allow us to have adequate presuppositions for using other statistical analyses, such as Cox regression. However, the follow-up time range was long enough (up to 68 months) to apply a linear regression. Although it is a simpler analysis, this allowed us to obtain a robust mathematical model with the database that we had available to estimate the time to CD recurrence. The M1 model can constitute a prognostic tool for evaluating the patient's relapse in the postoperative follow-up (Figure 5). In these cases, CDAI, body weight, and BMI data can be obtained at the moment of the surgery, and biopsies can be taken from the surgical specimen. Afterward, qRT-PCR of miRNA-650 and miRNA-29c and their target genes GFPT2, E2F1, HIF3A, and CIDEC can be performed. All this information is entered into the model, together with the prediction of the reintroduction use of immunosuppressive and immunobiological therapy. An estimate of how many months each patient will relapse will be obtained. If the result is a relapse in a few

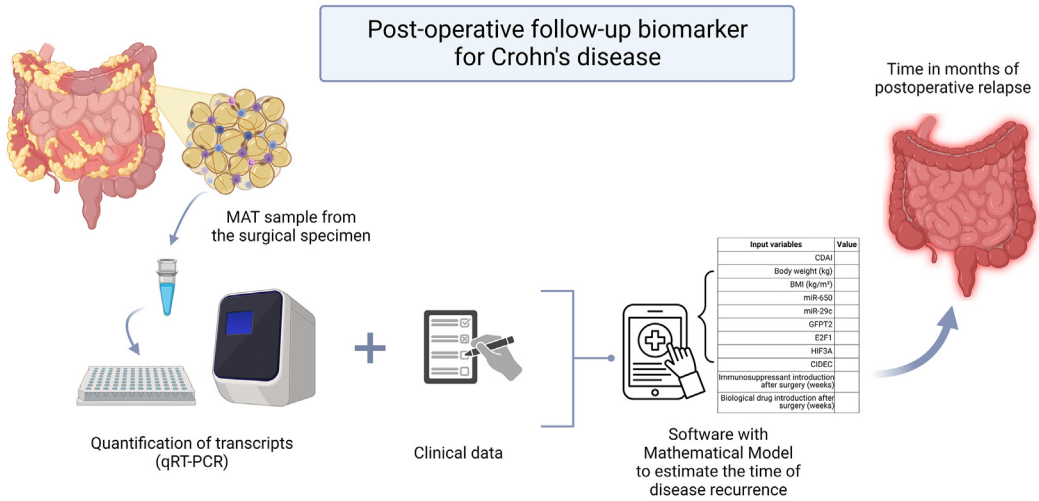


**Figure 5.** The mathematical model generated through clinical and transcriptional variables may predict Crohn's disease relapse in the postoperative follow-up. miR, microRNA; target genes (GFPT2, E2F1, HIF3A, CIDEC). Figure created with BioRender.com.

months, reintroducing the drugs, optimizing doses, and nutritional care can be considered, seeking to increase the months until a possible recurrence.

Two possible limitations of our study are the limited sample size and the fact that there was only one center where the samples were obtained. However, our patients were carefully selected, and even in that small cohort, the obtained statistical results were strong. So far, this is the first study that correlates clinical characteristics of the CD patients and modulated genes in the MAT of CD surgical specimens with postsurgical endoscopic or radiological relapse of the disease.

A recent review showed that prognostic research in this disease was heterogeneous, and only some biomarkers were investigated sufficiently.[51] They found evidence of predictive potential for 5 clinical biomarkers (montreal behavior, age, disease duration, disease location, and smoking), one genetic (NOD2), and 2 serological (anti-*Saccharomyces cerevisiae* antibodies and anti-CBir1). All these mentioned clinical biomarkers were tested as input variables in our models. However, they did not show statistical significance.

Althoff et al[5] also developed a multiple linear regression model that defines a patient at risk of disease-related complications in CD. They based their model on clinical data and MRI. Creeping fat identified by MRI was independently associated with some adverse outcomes, such as disabling course, bowel damage, and surgery, with an odds ratio of 3.5 each and a $P$ value $<.05$.[5] In fact, visceral adiposity is an independent risk factor for the recurrence of CD after surgery,[7] confirming the relevance of studying molecular aspects of MAT from CD patients.

Coffey and collaborators[4] observed that including the mesentery in ileocolic resections for CD reduces surgery recurrence. The authors developed a mesenteric disease activity index based on surgical specimen evaluation. This index evaluates the mesentery alteration in the surgical specimen: a bigger fat wrapping of the MAT in the intestinal circumference presents a higher severity of CD.[4] This index can be created for other protocols to improve the monitoring and management of the disease and even guide surgical procedures. However, this procedure may carry complications and must still be established in clinical practice. As the mesentery presents creeping fat, bleeding associated with this wider surgical resection is one of the possible complications, requiring accurate surgical technique.[4]

In contrast, Kono and collaborators[52] have shown the results with a new anastomotic technique for CD patients, where the mesentery of the excised segment is preserved. And yet, this technique presents a significantly lower endoscopic and surgical recurrence rate. Assuming that the mesentery is the driver of inflammation in CD, one possibility could be the existence of 2 phenotypes of the disease: one mediated by the mesentery and the other independent from the mesentery that still need to be identified and classified.[53] Another hypothesis is related to the distinct cellular responses and immune mediators in the mesentery to the same external stimulus.[53]

Lipopolysaccharide, present in the gram-negative bacteria cell membrane, linked to Toll-like receptor 4, generates intestinal inflammation by producing pro-inflammatory cytokines.[54] The excess of saturated fat and the Western diet pattern enhance the dysbiosis and leak gut, increasing the activation of this pathway by lipopolysaccharide and worsening systemic inflammation.[55] However, diet can play a whole function in modulating miRNA expression. Biersack[56] published a review of phenolic and terpenoid dietary factors and natural products as noncoding RNA/miRNA modulators for improved cancer therapy and prevention. According to the author, miRNA-29c can be upregulated by glyceollins, a represent further natural isoflavonoids biosynthetically derived from soy isoflavones which exhibited interesting

anticancer properties.[56] There are no studies with dietary factors modulating miRNAs in IBD. Furthermore, other environmental factors, such as exposome, still needed to be clarified to understand IBD outcomes.[57] Therefore, besides prognosis biomarkers, as we showed in the present study, further investigation could be considered with the miRNA-650 and miRNA-29c as potential therapeutic targets in CD in the future.

## Conclusion

This study deals with some advances, as it was the first report that confirmed overexpression of the miRNAs modulated in the MAT of CD patients (miRNA-650 and miRNA-29c) and the downregulation of their target genes. Moreover, we developed a mathematical model that can predict the time for relapse in the postoperative follow-up of CD patients. Our results of biological validation showed the importance of exploring laboratory techniques to prove *in silico* findings. The transcriptional levels of miRNA-650 and miRNA-29c obtained by qRT-PCR validation presented themselves as one of the variables of a potential tool to predict relapse in CD patients who have undergone intestinal resection.

The M1 model can be helpful to the medical team, who will be able to define the best decision after surgical resection, considering the risk of early relapse or not. Although the test of these models in other centers is still needed, these findings may constitute a potential tool to guide postoperative clinical management in the future, mapping the higher risk patients.

## Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2023.08.020.

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**Ethical Statement:**
This study was conducted by the Declaration of Helsinki at a single tertiary referral center and approved by the University of Campinas Ethics Committee. The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

**Data Transparency Statement:**
All relevant data supporting the findings of this study are available within the paper.

**Reporting Guidelines:**
Helsinki Declaration.