

# Mitochondrial Dysfunction and Reduced TCA Cycle Metabolite Levels in Inflammatory Bowel Disease Patients

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**Introduction:** Inflammatory bowel disease (IBD) mainly includes ulcerative colitis (UC) and Crohn's disease (CD). These diseases are classified as chronic and recurrent inflammatory diseases affecting the digestive tract. An energy deficiency in intestinal cells is believed to be associated with IBD pathology.

**Methods:** Our study investigated the bioenergetic functionality of mitochondria using the plasma of patients with CD and UC by determining the concentration of intermediates of the tricarboxylic acid cycle (TCA), such as acetyl coenzyme A, succinate, fumarate,  $\alpha$ -ketoglutarate, NADH<sub>2</sub>, IDH<sub>2</sub>, Cytochrome C Oxidase, Cytochrome C Reductase, and ATP.

**Results:** Our results show an imbalance in mitochondrial homeostasis and bioenergetics, demonstrated by reduced activity of respiratory complexes and reduced production of TCA intermediates in the plasma of patients with CD and UC. In the group of patients with CD, treatment with corticosteroids had a significant positive effect, as significantly higher IDH<sub>2</sub> and succinate levels were found. Correlation analyses of mitochondrial functionality biomarkers with other blood markers revealed a significant relationship between CRP and ATP levels, with higher CRP significantly linked to lower ATP and a similar trend for succinate levels. Using the disease activity scale, we show that biomarkers such as IDH<sub>2</sub>,  $\alpha$ -ketoglutarate, and succinate levels are significantly lower in patients with higher disease activity.

**Conclusion:** We conclude that reduced metabolites and respiratory complexes associated with the TCA indicate mitochondrial bioenergetic failure in IBD patients. Besides, Krebs cycle metabolites can be a good marker of predisposition to the disease and the course of IBD. They can be easily determined in a blood sample taken from the patient. Pharmacological protection of mitochondria in individuals predisposed to IBD development and compensation for the changed function of mitochondria in persons with the developed disease may become a new approach to personalized therapies focused on restoring the proper activity of mitochondrial enzymes.

**Keywords:** inflammatory bowel disease, Krebs cycle, respiratory complexes

## Introduction

Inflammatory bowel disease (IBD) mainly includes ulcerative colitis (UC) and Crohn's disease (CD). These diseases are classified as chronic and recurrent inflammatory diseases affecting the digestive tract. Research by Lin et al indicates that the global incidence rate of IBD has been increasing from 1990 to 2021, and IBD will continue to be a major public health burden.<sup>1</sup> The exact cause of IBD remains unclear, but it is generally accepted that its etiopathology is multifactorial and includes genetic predisposition, dysregulated immune response, and microbial and environmental factors. Conventional methods of treating IBD include pharmacotherapy, aminosalicylates, immunomodulators, corticosteroids, biological treatment, and surgical resection if necessary.<sup>2</sup>

To diagnose and develop a clinical procedure, mainly endoscopic and histopathological examinations are used.<sup>3</sup> In patients with IBD, clinical symptoms often do not coincide with the endoscopic activity of the disease.<sup>4</sup> It is also difficult

to assess the risk of the disease, for example, in individuals genetically predisposed to the disease. Therefore, objective measurements to assess risk and monitor IBD disease activity continue to be considered.

IBD patients without reliable biomarkers, patients may face delays in receiving appropriate treatment, potentially leading to disease progression and complications such as strictures or fistulas in Crohn's disease or severe colitis in ulcerative colitis. Blood-based biomarkers could offer a less invasive and more cost-effective alternative, enabling clinicians to monitor disease progression and adjust treatments more proactively than endoscopy, which is invasive, costly, and impractical for frequent use. Moreover, IBD treatments vary in effectiveness among patients, and biomarkers could help stratify individuals based on their likely response to specific therapies, such as biologics or immunomodulators, reducing the reliance on trial-and-error approaches.<sup>5</sup>

Unfortunately, there is still a lack of simple biochemical markers that would help diagnose and determine the severity of the disease process.

Currently, very popular markers of inflammation are C-reactive protein and leukocyte count, although their sensitivity is low in IBD. In addition, fecal calprotectin, which has high sensitivity, is used to predict active disease.<sup>6</sup> However, metabolites in the TCA cycle may also be used as biomarkers of IBD in the future, and their sensitivity and specificity may be higher than other biomarkers.

Moreover, the pathogenesis of IBD is not fully understood. Therefore, research is conducted to determine the cause of the disease and develop an effective therapy.

Recently, research studies suggest that mitochondrial dysfunction is directly linked to chronic inflammation, including IBD. The resulting energy deficiency in intestinal cells is believed to be associated with both UC and CD pathology. Energy deficiency in enterocytes has been shown to impair nutrient absorption in the small intestine and water and electrolyte absorption in the colon. Energy deficiency in goblet cells leads to insufficient mucus production in the colon, while in Paneth cells, it affects stem cell homeostasis and AMP production, causing impaired epithelial cell turnover and dysbiosis in the small intestine.<sup>7</sup> Besides, immune cells such as macrophages and dendritic cells are activated, releasing pro-inflammatory cytokines and chemokines, which in turn induce the differentiation of T lymphocytes and the recruitment of inflammatory cells from the blood to the intestinal mucosa, creating inflammatory infiltrates and hypoxia.<sup>8</sup> Increased cellular metabolism and decreased oxygen supply are observed in the inflamed areas.

Mitochondria are at the center of Energy production. The mitochondrial electron transport chain (ETC) comprises five essential energy-acquisition complexes. Complex I NADH-ubiquinone oxidoreductase starts a series of reactions in the respiratory chain by oxidizing NADH and reducing ubiquinone in the mitochondrial membrane. The complex II enzyme succinate dehydrogenase is part of both the respiratory chain as the complex II enzyme and the Krebs cycle, in which it catalyzes the dehydrogenation reaction of succinate to produce fumarate. Complex II is the only one (compared to the other complexes) that is not an ion channel. It accepts electrons from its prosthetic group of the flavin adenine nucleotide and transfers them directly to coenzyme Q. Complex III, cytochrome c oxidoreductase, is an enzyme that catalyzes the transfer of electrons from ubiquinol to cytochrome c. Complex IV cytochrome c oxidase is the last transmembrane enzyme of the respiratory chain that uses electrons to reduce oxygen to water. Further, Complex V is an ATP synthase, where the proton gradient of ADP to ATP is used for conversion.<sup>9</sup>

Glycolysis and mitochondrial respiration are two primary energy-yielding pathways. In this process, glucose is converted into pyruvate in the glycolytic pathway.

Interestingly, the glycolytic pathway significantly impacts the regulation and activation of the immune system and plays a significant role, especially in the development of IBD. Besides, glycolysis affects important immune cells such as neutrophils, macrophages, regulatory T cells, and dendritic cells. Any disturbances in this process can, therefore, contribute to the development of IBD, which is characterized by chronic intestinal inflammation.<sup>10</sup>

Pyruvate metabolism, in turn, is a key regulatory point of this glucose metabolism. The mitochondrial pyruvate dehydrogenase complex converts pyruvate to acetyl-CoA, which is then oxidized to the tricarboxylic acid cycle. Subsequently, the tricarboxylic acid cycle uses various substrates to generate reducing equivalents in the mitochondria, leading to ATP production via oxidative phosphorylation (OxPhos).<sup>11</sup> During the OxPhos reaction, mitochondria produce reactive oxygen species (ROS), which affect cell health and are the primary inflammatory mediators, activating the NLRP3 inflammasome.

The mitochondrial matrix contains, among others, enzymes involved in the tricarboxylic acid cycle (TCA), which produces not only NADH<sup>+</sup> and FADH<sub>2</sub><sup>+</sup>, which are the fuel for complex I and complex II in OxPhos, but also generate

intermediate products such as acetyl coenzyme A (acetyl-CoA),  $\alpha$ -ketoglutarate and succinate. These metabolic intermediates act as signaling molecules and control cellular responses.<sup>7</sup> IDH2 catalyzes the reaction of the transformation of isocitrate into  $\alpha$ -ketoglutarate to produce NADPH from NADP<sup>+</sup> in mitochondria. NADPH is an essential equivalent for a thioredoxin- and glutathione-dependent system. Thus, IDH2 is a necessary antioxidant enzyme to maintain intracellular redox homeostasis and eliminate damage caused by oxidative stress.<sup>8</sup>

$\alpha$ -Ketoglutaric acid is a central molecule of the TCA used as a precursor for glutamine and glutamate. Moreover, it is an important source of energy for intestinal cells, which is vital in protecting the intestinal mucosal barrier.<sup>12</sup> Furthermore, it influences epigenetic regulation through histone and DNA demethylation, impacting the transcription of genes involved in immune tolerance and inflammation.<sup>13</sup> Research by Wang et al showed that supplementation with 0.5% or 1% ornithine  $\alpha$ -ketoglutarate results in beneficial changes in intestinal microorganisms, increasing plasma amino acid levels and alleviating growth inhibition in pigs infected with *Escherichia coli*.<sup>12</sup>

Finally, succinate is produced and metabolized in mitochondria in the TCA cycle during the metabolic production of fats, carbohydrates, and proteins. It is an intermediate metabolite of the TCA cycle and is produced from  $\alpha$ -ketoglutarate (AKG) by 2-oxoglutarate dehydrogenase (OGDH) and succinyl-CoA synthetase (SCS).<sup>14</sup> Succinate acts as a signaling molecule that stabilizes hypoxia-inducible factor 1- $\alpha$ , promoting inflammatory cytokine production such as IL-1 $\beta$ , which exacerbates inflammation in IBD. Additionally, succinate can influence macrophage polarization toward a pro-inflammatory phenotype, which plays a critical role in the immune dysregulation seen in IBD.<sup>15</sup>

This study aims to decipher the bioenergetic functionality of mitochondria using the plasma of patients with CD and UC by determining the concentration of intermediates of the TCA, such as acetyl coenzyme A, succinate, fumarate,  $\alpha$ -ketoglutarate, NADH2, IDH2, Cytochrome C Oxidase, Cytochrome C Reductase, and ATP. As outlined above, the selected TCA cycle metabolites play important signaling roles that may contribute to the development of numerous diseases related to the response of both the innate and adaptive immune systems. Importantly, they may be necessary for developing IBD.<sup>16</sup> To date, there is no perfect biomarker for assessing the risk of IBD and monitoring the activity of the disease. Thus, investigating the reliability of these blood biomarkers (plasma) that are minimally invasive, easy to obtain, and most often used,<sup>5</sup> may be a significant step forward in biomarker development. In addition, our results may help understand the causes of mitochondrial bioenergetic failure in patients with IBD, making it possible to design further research and personalized therapies to regulate mitochondrial homeostasis.

## Materials and Methods

### Study Participants

In this study, a group of 138 individuals was enrolled: 72 IBD patients (51 with UC and 21 with CD) from the Department of Gastroenterology with Endoscopic Unit, University Clinical Hospital No. 4 in Lublin, and 66 healthy individuals. The Crohn's Disease Activity Index (CDAI), which combines the assessment of clinical outcomes, vital signs, and medical history, was used to assess CD activity. The Mayo scoring system (MDAI), which includes the assessment of the frequency of stools and rectal bleeding, was used to assess the activity of UC. The demographic and clinical data of study participants, including treatments, is shown in [Table 1](#).

The study protocol was approved by the Local Ethical Committee of the Faculty of Medicine at the Medical University, Lublin, number KE-0254/78/2021, and was in accordance with the Helsinki Declaration of 1975. All participants were informed about the aim and protocol of the study and gave written informed consent.

### Materials

ELISA kits for biomarker determination were purchased from MyBioSource (Acetyl Coenzyme A (Acetyl-CoA), Catalog # MBS9719208; NADH2-ELISA-kit, Catalog # MBS2602852), Assaygenie (Isocitrate dehydrogenase, Catalog # HUF101073), Cell Biolabs (Alpha-Ketoglutarate Assay Kit, Catalog # MET-5131), Sigma Aldrich (Succinate Colorimetric Assay Kit, Catalog # MAK184; Fumarate Assay Kit, Catalog # MAK060; Cytochrome c Oxidase Assay Kit, Catalog # CYTOCOX1-1KT; Cytochrome c Reductase (NADPH) Assay Kit, Catalog # CY0100-1KT), and Abcam (ATP Assay Kit, Catalog # ab83355). Unless stated otherwise, all materials and chemicals were supplied by Sigma Aldrich.

**Table 1** Demographic and Clinical Characteristics of Study Participants. n, Number of Observations; t-Test for Independent Samples; One-Way ANOVA With Tukey Post Hoc for Multiple Comparisons. Disease Activity Is Presented as an Average CDAI Score for CD and an Average MDAI Score for UC

	CD	UC	Controls	P value
Number of observations	21	51	58	
Anthropometric data:				
Female/male	1.22	0.17	0.53	
Age, average [y]	39.7 ±15.6 SD	41.3 ±14.7 SD	34.1 ±11.6 SD	n.s.
Length of illness [mo]	8.98 ±6.2 SD	6.7 ±6.5 SD	–	0.1749
Disease activity:				
Exacerbation, n (%)	18 (85.7)	39 (76.5)	–	
CDAI/MDAI I	163 (1–305)	2.1 (1–3)	–	–
Blood parameters:				
CRP, average [mg/l]	17.2 ±12.4 SD	21.7 ±38.1 SD	–	0.5992
RBC, average [ $\times 10^{12}/l$ ]	4.2 ±0.6 SD	4.3 ±0.7 SD	–	0.5684
WBC, average [ $\times 10^9/l$ ]	6.7 ±3.1 SD	8.2 ±3.6 SD	–	0.0994
Albumin, average [g/dl]	3.8 ±0.5 SD	3.7 ±0.5 SD	–	0.4431
Treatment:				
Aminosalicylates, n (%)	18 (85.7)	41 (80.4)	–	
Biological, n (%)	3 (14.3%)	11 (21.6)	–	
Imuran Azathioprine, n (%)	8 (38.1%)	27 (53)	–	
Corticosteroids, n (%)	7 (33.3%)	18 (35.3)	–	
Surgical, n (%)	5 (23.8%)	6 (11.8)	–	

## Biological Samples

Blood samples were collected as whole blood in K-EDTA vacutainers 2.7 mL. Then, the blood samples were centrifuged for 10 minutes at 1500 x g using a refrigerated centrifuge. After centrifugation of the samples, the plasma was aspirated and transferred to a new clean Eppendorf tube. The samples were stored at  $-80^{\circ}\text{C}$  until the test was carried out.

## ELISA Analyses

The quantification of participants' biomarkers in the plasma was performed using ELISA assays according to the manufacturer's instructions.

## Statistics

Statistical analysis was performed using Graph Pad Prism Version 8.4.2 (464) (La Jolla, CA, USA). Correlation analysis was done by calculating R square and p values. Comparisons were performed by one-way ANOVA followed by Tukey post hoc analyses and incorporated adjustments for multiple testing, such as the Bonferroni correction. Pairwise comparisons were done by t-tests. Statistical tests were two-tailed with a significance level of  $\alpha \leq 0.05$ . Significances are stated with p values  $<0.05^*$ ;  $<0.01^{**}$ ;  $<0.001^{***}$ . Trends are indicated as  $\# 0.1 > p > 0.05$ . Results are shown as mean and SEM.

## Results

To understand whether the mitochondrial function is compromised in patients with CD and UC, we measured plasma concentrations of intermediates of the TCA, which can be used as biomarkers, namely acetyl coenzyme A, succinate, fumarate,  $\alpha$ -ketoglutarate, NADH2, IDH2, Cytochrome C Oxidase, Cytochrome C Reductase, and ATP. In the first set of analyses, we calculated the average biomarker concentration measured in 21 individuals with CD and 51 individuals with UC and compared the values to 66 healthy individuals (Figure 1A–I). Our results show that all selected biomarkers are significantly reduced in individuals with CD and UC. No significant difference was detected in any of the markers between CD and UC patients.

Next, we investigated whether the reduction in mitochondrial functionality biomarkers is linked to specific characteristics of patients, such as age, gender, duration of illness, severity, treatments, and other blood markers such as red and white blood cell count, C-reactive protein (CRP), or Albumin levels.

For patients with CD, we did not detect any effect of gender for any of the plasma biomarkers assessed (Supplementary Table S1). Besides, we did not detect any effect of age (Supplementary Figure S1A). However, the length of the illness significantly affected the levels of NADH2, with levels lower in patients with longer durations (Figure 2A, Supplementary Figure S1B). A similar but insignificant trend can be seen for AcetylCoA and IDH2 levels (Figure 2A). Exacerbation of the illness had no influence (Supplementary Table S1). However, only two patients had no exacerbation.

A correlation was found with the disease activity scale: IDH2,  $\alpha$ -ketoglutarate, and succinate levels are significantly lower in patients with higher disease activity. A similar but non-significant trend can be seen for AcetylCoA ( $p=0.0669$ ) and Cytochrome C Reductase ( $p=0.0687$ ) (Figure 2B, Supplementary Figure S1C).

Regarding treatment, we did not see significant differences in mitochondrial functionality biomarkers between patients taking Aminosalicylates, a biological treatment, Imuran (Supplementary Table S1). However, patients taking Corticosteroids had significantly higher plasma IDH2 and succinate levels (Figure 2C; Supplementary Table S1). Surgical treatment significantly affected NADH2 levels (Figure 2D; Supplementary Table S1).

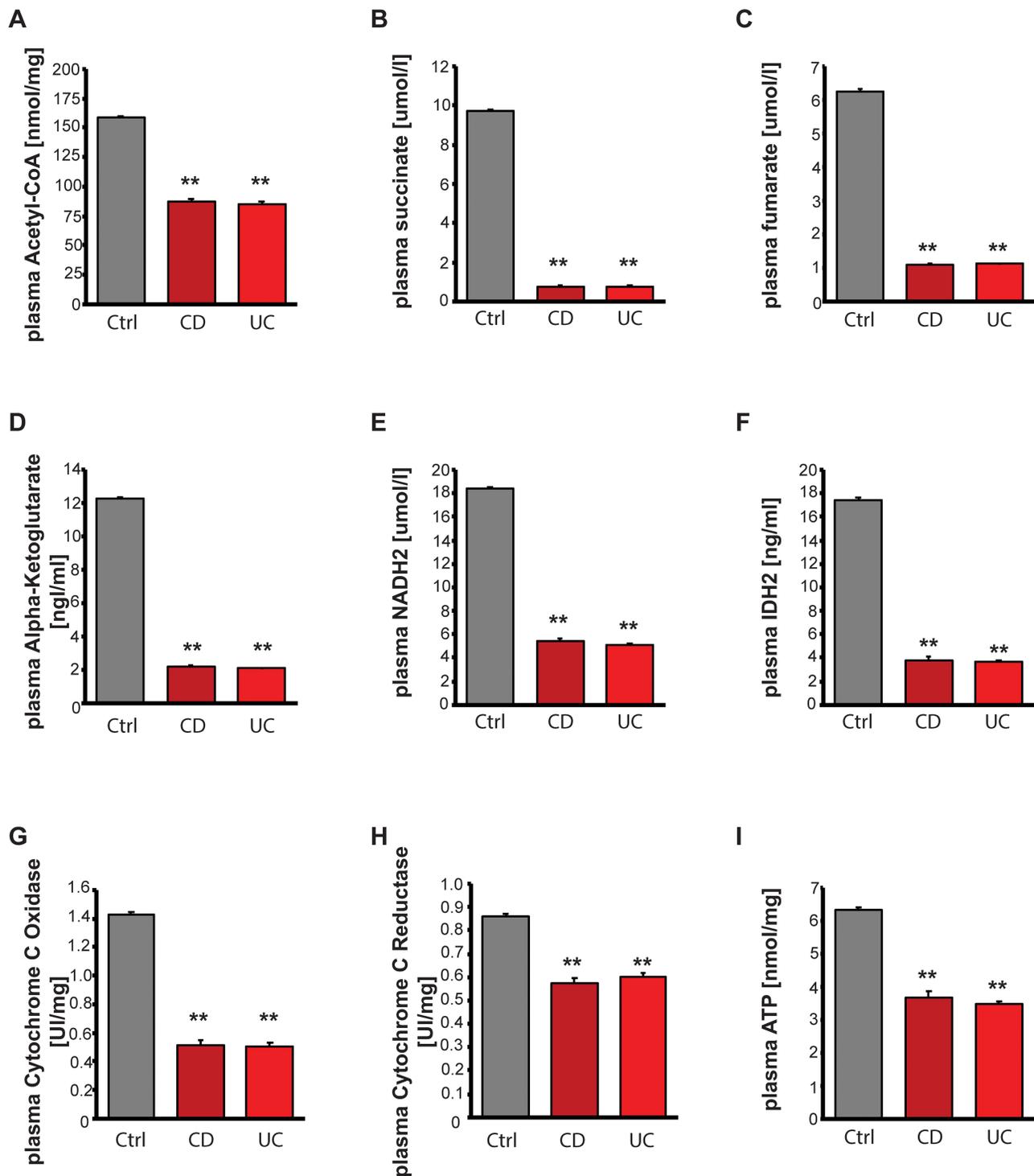
Correlation analyses of mitochondrial functionality biomarkers with other blood markers revealed a significant relationship between CRP and ATP levels, with higher CRP significantly linked to lower ATP and a similar trend for succinate levels (Figure 2E; Supplementary Figure S1D). While we did not detect a correlation between any of the biomarkers and Albumin levels, as well as White blood cell (WBC) counts (Supplementary Figure S2A and B), a significant correlation was found between red blood cell (RBC) counts and fumarate levels—the more RBC, the higher the fumarate (Figure 2F; Supplementary Figure S2C).

For patients with UC, as for those with CD, we did not detect an effect of gender for any of the plasma biomarkers assessed (Supplementary Table S2). However, here, we did detect an effect of age on the levels of IDH2 as a trend ( $p=0.0798$ ) and a significant effect on NADH2 levels (Figure 3A; Supplementary Figure S3A). The length of the illness significantly affected the levels of fumarate, with levels lower in patients with shorter duration (Figure 3B; Supplementary Figure S3B). Exacerbation of the illness had no influence (Supplementary Table S2), and no correlation was found with the disease activity scale (Supplementary Figure S3C).

Regarding treatment, we did not see significant differences in mitochondrial functionality biomarkers between patients taking Aminosalicylates, Imuran, or Corticosteroids (Supplementary Table S2). However, patients taking biological treatments had significantly lower plasma NADH2 levels (Figure 3C; Supplementary Table S2). Surgical treatment did not affect the biomarker levels assessed (Supplementary Table S2).

Correlation analyses of mitochondrial functionality biomarkers with other blood markers revealed a significant relationship between CRP and succinate, as well as NADH2 levels, with higher CRP being significantly linked to lower succinate and NADH2 (Figure 3D; Supplementary Figure S4A).

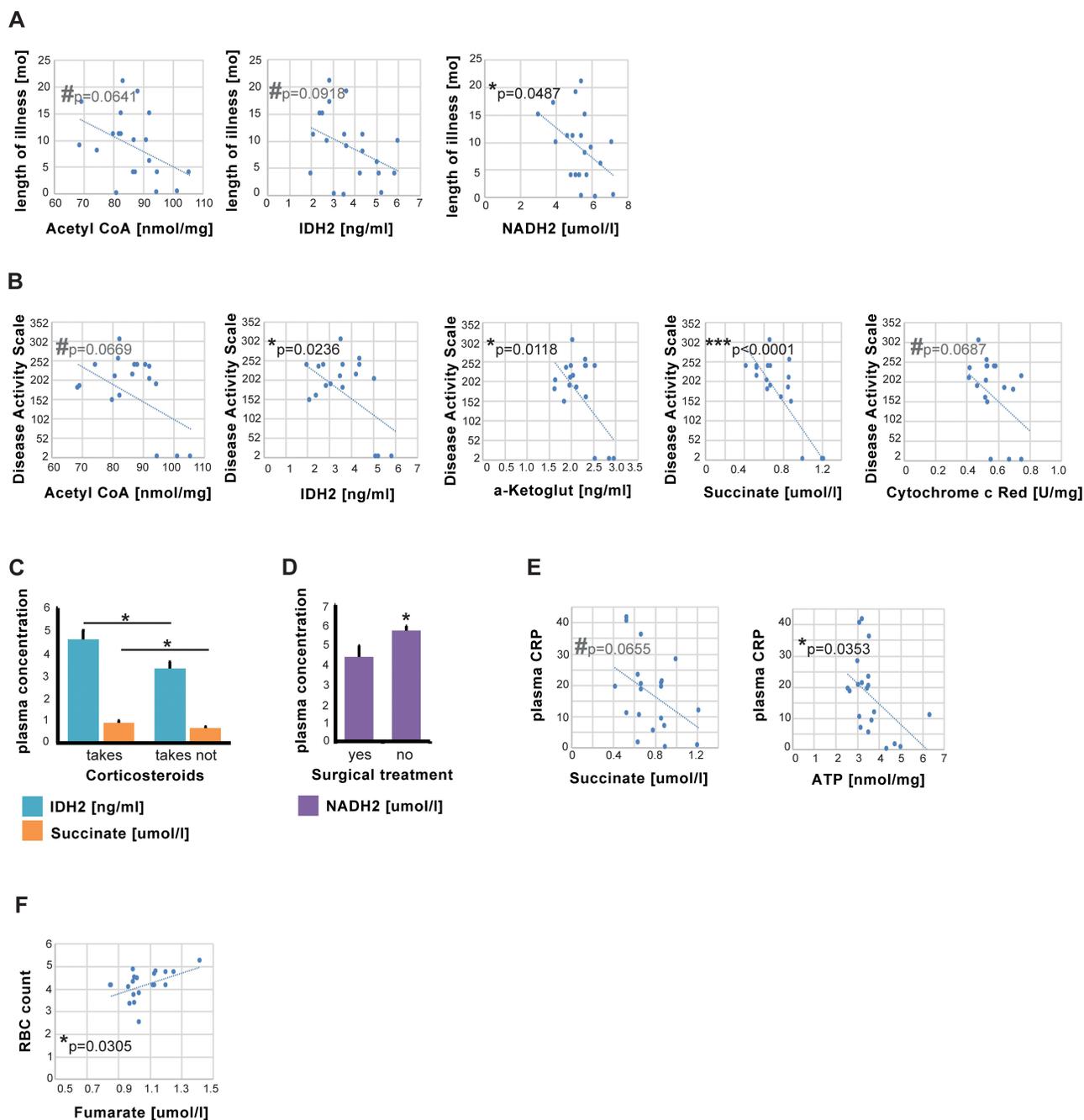
Albumin levels significantly correlated with IDH2,  $\alpha$ -ketoglutarate, succinate, and NADH2 levels. The more Albumin, the lower the biomarker levels (Figure 3E; Supplementary Figure S4B). WBC was significantly correlated with AcetylCoA and Cytochrome C Oxidase levels, with higher WBC found in UC patients with lower biomarker levels (Figure 3F; Supplementary Figure S4C). RBC counts correlated significantly with IDH2,  $\alpha$ -ketoglutarate, and NADH2 levels, with higher RBC found in UC patients with lower biomarker levels (Figure 3G; Supplementary Figure S4D).



**Figure 1** Plasma concentrations of (A) acetyl coenzyme A, (B) succinate, (C) fumarate, (D)  $\alpha$ -ketoglutarate, (E) NADH2, (F) IDH2, (G) Cytochrome C Oxidase, (H) Cytochrome C Reductase, and (I) ATP. (A–I) One-way ANOVA ( $p < 0.001$ ); Tukey post-hoc test: Ctrl vs CD:  $p < 0.01$ ; Ctrl vs UC:  $p < 0.01$ ; UC vs CD  $p = n.s.$  ( $n = 66$  (Ctrl); 21 (CD); 51 (UC)). A significant decrease was observed in UC and CD patients compared to healthy controls for all mitochondrial function biomarkers. Significances are indicated with \*\* for  $p$  values  $< 0.01$ .

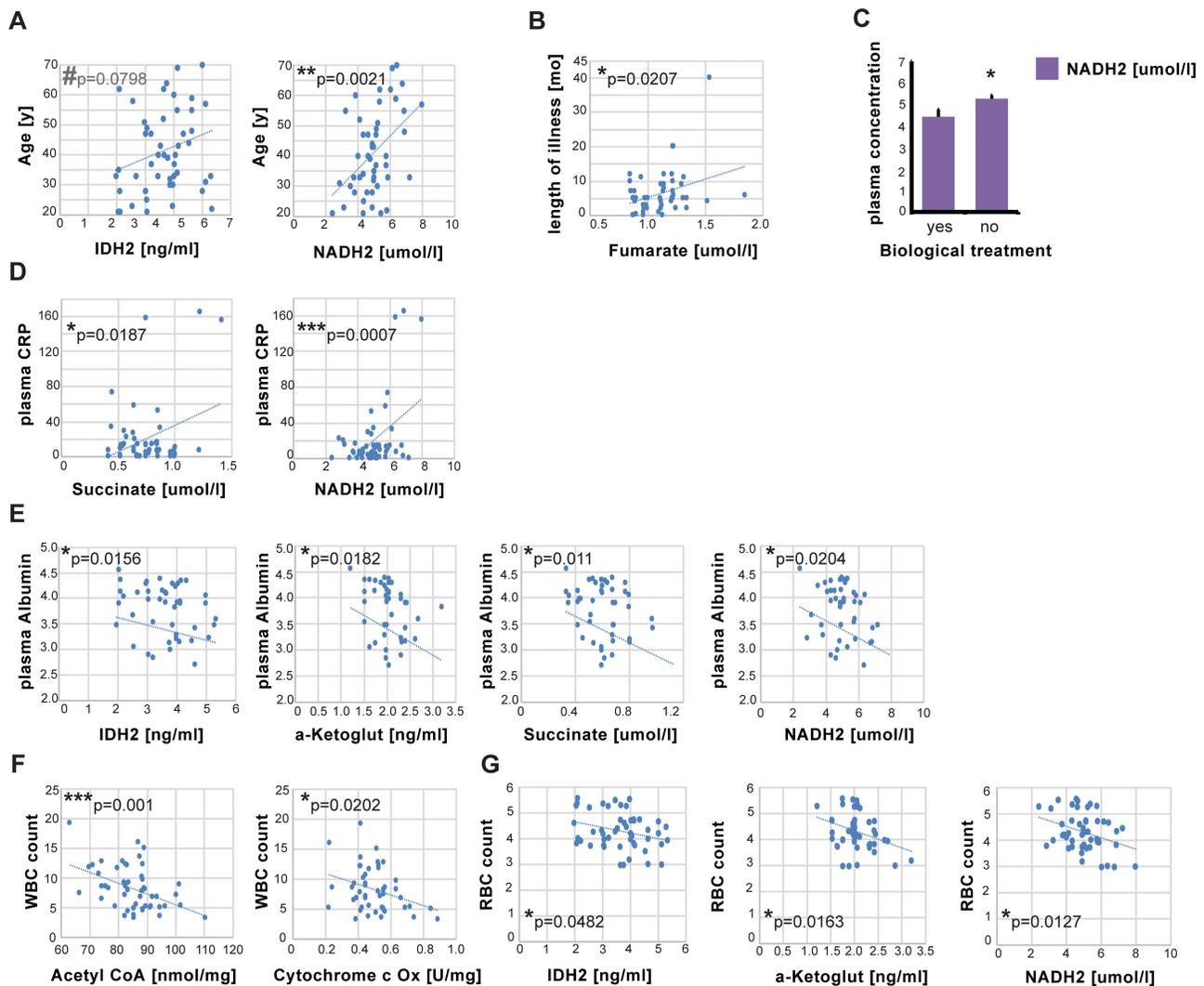
## Discussion

Here, we show an imbalance in mitochondrial homeostasis and bioenergetics, demonstrated by reduced activity of respiratory complexes and reduced production of TCA intermediates in the plasma of patients with CD and UC.



**Figure 2** Correlations analysis using clinical data from CD patients. **(A)** A trend was detected for the correlation between the length of illness and Acetyl CoA and IDH2 plasma levels. NADH2 concentrations significantly correlate with the length of illness ( $p=0.0487$ ): The longer the duration of illness, the lower the NADH2 levels are. **(B)** A trend was detected for the correlation of the disease severity and Acetyl CoA and Cytochrome C Reductase plasma levels. IDH2 ( $p=0.0236$ ),  $\alpha$ -ketoglutarate ( $p=0.0118$ ), and succinate ( $p<0.0001$ ) concentrations significantly correlated with the disease severity: The more severe the disease, the lower the IDH2,  $\alpha$ -ketoglutarate, and succinate levels. **(C)** CD patients taking Corticosteroids had significantly higher IDH2 ( $p=0.0217$ ) and succinate ( $p=0.0181$ ) plasma levels compared to patients who did not take them (*t*-test). **(D)** CD patients who had surgical treatment had significantly lower NADH2 ( $p=0.0205$ ) plasma levels compared to patients without surgical treatment (*t*-test). **(E)** A trend was detected for the correlation between plasma CRP levels and succinate levels. ATP concentrations significantly correlated with plasma CRP levels ( $p=0.0353$ ): The higher the plasma CRP, the lower the ATP levels. **(F)** Fumarate concentrations significantly correlated with red blood cell (RBC) numbers ( $p=0.0305$ ): The lower the RBC, the lower the fumarate levels. **(A–F)**  $n=20$ . Significances are indicated with \* for  $p$  values  $<0.05$ ; and \*\*\*  $<0.001$ . A trend ( $0.05 < p < 0.10$ ) is indicated by #.

Upon intestinal inflammation, the architecture of mitochondria in intestinal epithelial cells is dysmorphic, with the destruction of the cristae, which leads to increased apoptosis. Changes in mitochondrial morphology and bioenergetics promote metabolic changes in the glycolytic pathway and decrease antioxidant signaling.<sup>17</sup> In line with this, in this study,



**Figure 3** Correlations analysis using clinical data from UC patients. **(A)** A trend was detected for the correlation of the patient's age and IDH2 plasma levels. NADH2 concentrations significantly correlated with the patient's age ( $p=0.0021$ ): The younger the patient, the lower the NADH2 levels are. **(B)** Fumarate concentrations significantly correlate with the length of illness ( $p=0.0207$ ): The shorter the duration, the lower the fumarate levels. **(C)** UC patients taking biological treatments had significantly lower NADH2 ( $p=0.0185$ ) plasma levels than patients who did not take those (*t*-test). **(D)** Succinate ( $p=0.0187$ ) and NADH2 ( $p=0.0007$ ) concentrations significantly correlated with plasma CRP levels: The lower the plasma CRP, the lower the succinate and NADH2 levels. **(E)** IDH2 ( $p=0.0156$ ),  $\alpha$ -ketoglutarate ( $p=0.0182$ ), succinate ( $p=0.011$ ), and NADH2 ( $p=0.0204$ ) concentrations significantly correlated with plasma Albumin levels: The higher the Albumin, the lower the IDH2,  $\alpha$ -ketoglutarate, succinate, and NADH2 levels. **(F)** Acetyl CoA ( $p=0.001$ ), Cytochrome c oxidase ( $p=0.0202$ ), IDH2 ( $p=0.0482$ ),  $\alpha$ -ketoglutarate ( $p=0.0163$ ), and NADH2 ( $p=0.0127$ ) concentrations significantly correlated with white blood cell (WBC) numbers: The higher the WBC, the lower Acetyl CoA, Cytochrome c oxidase, IDH2,  $\alpha$ -ketoglutarate, and NADH2 levels. **(G)** RBC counts correlated significantly with IDH2 ( $p=0.0482$ ),  $\alpha$ -ketoglutarate ( $p=0.0163$ ), and NADH2 levels ( $p=0.0127$ ), with higher RBC found in UC patients with lower biomarker levels. **(A–F)**  $n=50$ . Significances are indicated with \* for  $p$  values  $<0.05$ ; \*\*  $<0.01$ ; and \*\*\*  $<0.001$ . A trend ( $0.05 < p < 0.10$ ) is indicated with #.

compared to controls, reduced levels of molecules associated with the TCA cycle were observed in the plasma of patients with UC and CD, suggesting that TCA-related molecules are closely linked to the pathogenesis of UC and CD.

Research confirms that the mucosa of the large intestine in the group of patients with UC is in a state of energy deficit and is characterized by a low level of adenosine triphosphate (ATP).<sup>9</sup> The results of the study by Hsieh et al show that ultrastructural abnormalities of mitochondria occur even before global changes in colonocytes in the colonic mucosa of patients with UC and long before the onset of mucosal inflammation. Among others, the expression of mitochondrial ATP synthase (ATP5B), a protein responsible for the production of ATP from ADP, is reduced.<sup>18</sup> Our study also observed a significant reduction in ATP in the group of IBD patients.

Furthermore, it was shown that damage to mitochondria is an event preceding the development of inflammation in patients with UC. The reason may be increased ROS production in epithelial cell mitochondria in UC patients.<sup>19,20</sup> Therefore, preventive measures using antioxidant agents in the group of people genetically predisposed to autoimmune diseases seem reasonable to

prevent the development of IBD. For example, Batjargal et al showed that herbal mixtures used in Mongolian traditional medicine activate cell defense mechanisms through anti-inflammatory and antioxidant effects. The action mentioned above was mainly attributed to phenolic components in the mixtures<sup>21</sup> and may be relevant for IBD management.

A study by Scoville et al found significant reductions in TCA intermediates, including citrate, aconitate,  $\alpha$ -ketoglutarate, succinate, fumarate, and malate in plasma samples of CD patients compared to control and UC patients. Additionally,  $\beta$ -hydroxybutyrate, synthesized from excess acetyl-CoA, was significantly reduced in CD patients compared to healthy controls and UC patients.<sup>22</sup> Besides, in mitochondria isolated from the colonic mucosa of patients with UC, it was shown that the respiratory chain II, III, and IV mitochondrial complexes were significantly reduced by approximately 50 to 60% compared to mitochondria isolated from the control mucosa of healthy people, suggesting the involvement of mitochondrial dysfunction in the pathogenesis of UC.<sup>7,19</sup> Our studies also confirmed this, as the data shows a reduction in Krebs cycle intermediates in the plasma of IBD patients. Significantly lower concentrations of Cytochrome C Oxidase and Cytochrome C Reductase levels were observed among the patients with CD and UC.

Furthermore, studies confirm that acetyl-CoA concentration in various cellular or pericellular compartments correlates with the severity of pathological conditions. For example, low acetyl-CoA levels play a key role in the pantothenate kinase-related neurodegenerative disease (PKAN). Thus, measuring acetyl-CoA concentration in biological fluids can help monitor the progression of diseases related to acetyl-CoA metabolism and assess the effectiveness of possible new therapies. Although acetyl-CoA concentration in different organs can vary greatly, whole blood levels can represent the accumulated acetyl-CoA in the body. Moreover, blood can be easily collected for clinical trials or therapy monitoring.<sup>23</sup>

These observations linking Pantothenate regeneration to increased CoA-dependent cell function might also explain the importance of a local Pantothenate supply for colonic homeostasis. Indeed, dietary Pantothenate deficiency alters the intestinal barrier function in the carp. Furthermore, combined supplementation of vitamins B and D in humans mitigates the symptoms associated with IBD. These results suggest that colonocytes might be particularly sensitive to CoA depletion. Indeed, these cells use the butyrate produced by microbiota for energy production through fatty acid  $\beta$ -oxidation and regulation of stem cell renewal.<sup>24</sup> In our study, significantly lower Acetyl CoA levels were found in the plasma of IBD patients.

Besides, it has been shown that (IDH2), a mitochondrial NADP<sup>+</sup>-dependent isocitrate dehydrogenase, is involved in catalyzing the conversion of isocitrate to  $\alpha$ -ketoglutarate while producing NADPH from NADP<sup>+</sup> in mitochondria. NADPH is an essential factor involved in detoxification, similar to the thioredoxin and glutathione systems. Therefore, IDH2 is a critical antioxidant enzyme in regulating redox status and reducing oxidative stress damage.<sup>25</sup> Our results show that patients with IBD had significantly lower NADH2 and IDH2 levels. In the group of patients with CD, treatment with corticosteroids had a significant positive effect, as significantly higher IDH2 and succinate levels were found in patients taking corticosteroids compared to those not.

Glucocorticoids are strong anti-inflammatory and immunomodulatory agents used in moderate to severe IBD, but their use is limited by several important adverse drug effects.<sup>26</sup> Additionally, corticosteroids have not been proven effective in maintaining remission in IBD and thus should not be used for this purpose.<sup>27</sup> Corticosteroids inhibit the synthesis of pro-inflammatory proteins. In IBD patients treated with corticosteroids, mononuclear cells, and colon epithelium cells down-regulate the production of nuclear factor kappa-B and many pro-inflammatory cytokines compared to untreated patients.<sup>28</sup> However, Ding et al showed that glucocorticoids do not have a uniform immunosuppressive effect. Their impact on inflammatory reactions depends partly on the dose and the length of administration. Excess endogenous and exogenous glucocorticoids can even potentially induce inflammatory reactions in the organism.<sup>29</sup> Therefore, to avoid potential adverse effects, it is necessary to administer corticosteroids reasonably in IBD patients.<sup>27</sup> The results of our research can be explained by the short-term use of corticosteroids, which led to the inhibition of the inflammatory process in patients with CD and, thus, an increase in the level of IDH2 and the level of succinate. In line with this, We have also detected a positive correlation between blood inflammation markers and the TCA cycle. In the group of patients with CD, we observed a correlation with CRP: the higher the CRP, the lower the ATP, and there was a similar trend for succinate levels. Acute inflammation mainly affects cells with mitochondrial impairments, as during inflammation, the intestinal mucosa cells require large amounts of energy to repair the damaged mucosa. Previous reports have shown that active inflammation in cells leads to significantly increased energy expenditure, leading to significant losses of energy and protein.<sup>30</sup>

In addition, studies of the transcriptional profiles of the colonic mucosa of patients with a first diagnosis of IBD without treatment showed that the inflamed tissue shows an overrepresentation of genes involved in inflammation and an underrepresentation of genes involved in mitochondrial respiration, both in CD and UC. This confirms the vital role of mitochondria in IBD development,<sup>31</sup> which can also be confirmed by our research in which we showed the correlation of several mitochondrial function biomarkers with the disease activity scale in CD patients. For example, IDH2,  $\alpha$ -ketoglutarate, and succinate levels are significantly lower in patients with higher disease activity.

Our results also show a significant correlation between UC patients' albumin levels and IDH2,  $\alpha$ -ketoglutarate, succinate, and NADH2 levels. The results of our analysis may indicate the effects of albumin as an antioxidant and protein protecting against chronic inflammation. Albumin is the main component of plasma proteins responsible for approximately 80% of the antioxidant effect of thiol in the body.<sup>32</sup> In an experimental study of liver inflammation, the protective effect of albumin on mitochondria against TNF $\alpha$  damage was demonstrated. In the presence of albumin, liver cells showed decreased production of mitochondrial ROS and fatty acid  $\beta$ -oxidation, which was explained by restoring the breakpoint between isocitrate and  $\alpha$ -ketoglutarate in the TCA. These findings demonstrate that albumin is necessary to protect hepatocyte cells from mitochondrial oxidative stress.<sup>33</sup>

The role of oxidative stress in IBD is unquestionable, and the implementation of antioxidant therapies seems rational. However, IBD patients show significant heterogeneity in response to treatment, inhibiting the formation of reactive oxygen species. In the case of 5-aminosalicylic acid (5-ASA), commonly used in the treatment of IBD, which exerts its therapeutic effects mainly through antioxidant mechanisms, it has been shown that the effects of the drug are not the same in all patients. Therefore, it should not be assumed that all patients' treatment with antioxidants will provide clear health benefits. Antioxidant therapies in IBD should be selected individually for each patient, considering the pathological overproduction of selected reactive species and careful monitoring of redox signaling.<sup>34</sup>

Importantly, cell biosynthesis of thiol compounds (eg, glutathione, lipoic acid, albumin, coenzyme A) decreases with age.<sup>35</sup> CD patients showed a correlation between the length of illness and mitochondrial function biomarkers: the length of the illness significantly affected the levels of NADH2, with levels lower in patients with longer durations. A similar but insignificant trend can be seen for Acetyl CoA and IDH2 levels. No correlation was found between the length of illness and other biomarker levels ([Supplementary figure S1B](#)). For patients with UC, we detected an effect of age on the levels of IDH2 as a trend and a significant effect on NADH2 levels. Thus, energy metabolism, age, and length of illness are closely related. Studies of animal models and people confirm that ATP levels fall linearly with age and, thus, length of illness. A critical relationship was also observed, where the bioenergetic failure of the cell is often associated with such variables as cumulative DNA damage, stem cell exhaustion, oxidative stress, and inflammation. Understanding these mechanisms can help prevent many diseases associated with lowering ATP in cells, for example, by using adequately adapted preventive measures.<sup>36</sup>

The correlation between CD patients and RBC is also interesting: the higher the RBC, the higher the fumarate levels. Carboxylic acid intermediates of the Krebs cycle, such as succinyl-CoA, are involved in erythropoiesis by driving heme synthesis. Additionally, small molecule dicarboxylates in this pathway (eg, fumarate) maintain erythropoiesis through the mechanism of stabilizing hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) after exposure to hypoxia, which is otherwise degraded by hydroxylation by  $\alpha$ -ketoglutarate-dependent prolyl hydroxylases.<sup>37,38</sup> Therefore, our results suggest a coordinated regulation that occurs in mitochondria: We demonstrate that mitochondrial metabolites influence erythropoiesis and may regulate specific aspects of the systemic inflammatory response in the intestinal epithelium. Therefore, the development of effective drugs affecting the interactions of Krebs cycle intermediates is a promising area of new therapeutic opportunities in IBD.

Finally, our data shows the effect of surgical treatment on CD patients: patients with surgical treatment have significantly lower NADH2 levels. A similar situation occurred in UC patients taking biological treatments with significantly lower plasma NADH2 levels. The indication of surgical treatment and biological treatments is usually the final solution when conservative treatment is ineffective. Surgical treatment is necessary when there is an urgent situation that threatens the patient's life or the development of cancer.<sup>39</sup> Exacerbating the disease process (biological treatments), the condition after major surgery, which involves a lengthy recovery process, and the presence of severe forms of IBD in those undergoing surgery and requiring biological treatments may be reflected by the particularly low NADH2 levels in that group of patients.

While the study's cross-sectional design is suitable for an initial exploration of biomarker differences between IBD patients and healthy controls, this design limits the ability to establish causation or observe changes in biomarkers over time. To that end, longitudinal studies or interventional designs should be performed to determine how the identified biomarkers change in

a patient in response to treatment or disease progression, thereby providing more substantial evidence for their clinical utility. Another limitation is that the study uses participants from a single clinical center. Genetic diversity, dietary habits, or environmental exposures could influence TCA cycle metabolite levels. Multi-center studies should be performed in the future to account for variations in biomarker levels that may arise from differences in population characteristics.

## Conclusions

Our research confirms reduced metabolites and respiratory complexes associated with the TCA, leading to mitochondrial bioenergetic failure in IBD patients. Therefore, Krebs cycle metabolites can be a good marker of predisposition to the disease and the course of IBD. They can be easily determined in a blood sample taken from the patient. Pharmacological protection of mitochondria in individuals predisposed to IBD development and compensation of the changed function of mitochondria in persons with developed disease may become a new approach to developing personalized therapies focused on restoring the proper activity of mitochondrial enzymes.

## Ethical Approval and Consent to Participate

The samples used in this study were isolated specifically for our research. Ethical approval and informed consent from the patients were obtained prior to the isolation and use of patient samples.

All patients were recruited following written informed consent. The protocol was approved by the Local Ethical Committee of the Faculty of Medicine at the Medical University, Lublin, number KE-0254/78/2021.

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## Disclosure

The authors report no conflicts of interest in this work.

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