# Ferroptosis in the colon epithelial cells as a therapeutic target for ulcerative colitis

横手, 章人

# Ferroptosis in the colon epithelial cells as a therapeutic target for ulcerative colitis

Ferroptosis for colitis

Akihito Yokote [1], Noriyuki Imazu [1], Junji Umeno [1], Keisuke Kawasaki [1], Shin Fujioka [2], Yuta Fuyuno [1], Yuichi Matsuno [1], Tomohiko Moriyama [3], Kohta Miyawaki [4], Koichi Akashi [4], Takanari Kitazono [1], and Takehiro Torisu [1, *]

[1] Kyushu University, Department of Medicine and Clinical Science, Graduate School of Medical Science, Fukuoka 812-8582, Japan

[2] Kyushu University Hospital, Department of Endoscopic Diagnostics and Therapeutics, Fukuoka 812-8582, Japan

[3] Kyushu University Hospital, International Medical Department, Fukuoka 812-8582, Japan

[4] Kyushu University, Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Fukuoka 812-8582, Japan.

* Corresponding author: Torisu Takehiro (torisu.takehiro.437@m.kyushu-u.ac.jp)

Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

Telephone: +81-92-642-5261; Fax: +81-92-642-5273

E-mail: torisu.takehiro.437@m.kyushu-u.ac.jp

## Word count of the manuscript

5712 words

## Author contributions

Akihito Yokote, Noriyuki Imazu and Takehiro Torisu conceived the idea of the study. Akihito Yokote, and Takehiro Torisu were involved in the data analysis. Junji Umeno, Keisuke Kawasaki, Shin Fujioka, Yuta Fuyuno, Yuichi

Matsuno, Tomohiko Moriyama, and Takehiro Torisu collected the clinical data and contributed to data interpretation. Akihito Yokote, Kohta Miyawaki, Koichi Akashi, Takanari Kitazono, and Takehiro Torisu contributed to drafting of the manuscript and critical revision. All authors revised the manuscript, approved the manuscript to be published, and agree to be accountable for all aspects of the work.

**Abstract**

Background

Ferroptosis, a type of programmed cell death triggered by oxidative stress, was suspected to play a role in ulcerative colitis. Indigo naturalis is highly effective against ulcerative colitis, but its mechanism is unclear. This study found that indigo naturalis treatment suppressed ferroptosis.

Methods

We analyzed 770 mRNA expressions of patients with ulcerative colitis. Suppression of ferroptosis by indigo naturalis treatment was shown using a cell death assay. Malondialdehyde levels and reactive oxygen species were analyzed in CaCo-2 cells treated with indigo naturalis. Glutathione metabolism was shown by metabolomic analysis. Extraction of the ingredients indigo naturalis from the rectal mucosa was performed using liquid chromatograph - mass spectrometry.

Results

Gene expression profiling showed that indigo naturalis treatment increased antioxidant genes in the mucosa of patients with ulcerative colitis. In vitro analysis showed that nuclear factor erythroid2-related factor2 related antioxidant gene expression was upregulated by indigo naturalis. Indigo naturalis treatment rendered cells resistant to ferroptosis. Metabolomic analysis suggested that an increase in reduced glutathione by indigo naturalis. The protein expression of CYP1A1 and GPX4 was increased in the rectum by treatment with indigo naturalis. The main ingredients of indigo naturalis,

indirubin and indigo inhibited ferroptosis. Indirubin was detected in the rectal mucosa of patients with ulcerative colitis who were treated with indigo naturalis.

Conclusions

Suppression of ferroptosis by indigo naturalis in the intestinal epithelium could be therapeutic target for ulcerative colitis. The main active ingredient of indigo naturalis may be indirubin.

241 words

**Keywords**

Ferroptosis, Indigo naturalis, Ulcerative colitis, Glutathione, NRF2

**Abbreviation**

AHR; Aryl Hydrocarbon Receptor

CYP1A1; Cytochrome P450 1a1

Cys; Cysteine

DMSO; Dimethyl sulfoxide

FICZ; 6-Formylindolo[3,2-b]carbazole

GABA; Gamma-aminobutyric acid

γ-Glu-Cys; Gamma-glutamylcysteine

GCL; Glutamate-cysteine ligase

GCLC; Glutamate-cysteine ligase catalytic subunit

GCLM; Glutamate-cysteine ligase modifier subunit

GPX4; Glutathione peroxidase 4

GSH; Reduced glutathione

GSSG; Glutathione disulfide

GSTP1; Pi-class glutathione S-transferase

Gln; Glutamine

Glu; Glutamic acid

Gly; Glycine

HDAC2; histone deacetylase 2

$H_2O_2$; Hydrogen peroxide

HO-1; Heme oxygenase-1

IN; Indigo naturalis

LC/MS; Liquid chromatography - mass spectrometry

MDA; Malondialdehyde

NADPH; Nicotinamide adenine dinucleotide phosphate

NRF2; Nuclear factor erythroid2-related factor2

PRDX2; Peroxiredoxin 2

ROS; Reactive oxygen species

PCR; Polymerase chain reaction

SELENBP1; Selenium binding protein 1

SOD1; Superoxide dismutase 1

SOD2; Superoxide dismutase 2

SRXN; Sulfiredoxin 1

Ser; Serine

TCDD; Tetrachlorodibenzo-para-dioxin

TNF; Tumor necrosis factor

TXN; Thioredoxin 1

TXNR; Thioredoxin reductase 1

UC; Ulcerative colitis

## Introduction

Ulcerative colitis (UC) is characterized by chronic inflammation of the intestinal tract. The incidence and prevalence of UC are increasing with time around the world [1] [2].

Despite recent advancements in treatment options, a proportion of UC patients still present with refractory cases. One nonstandard alternative therapeutic approach is indigo naturalis, also known as Qingdai. The efficacy of indigo naturalis is supported by literature, including randomized controlled trials which demonstrated its effectiveness in UC patients [3] [4] [5] [6] [7]. Indigo naturalis has been found to be effective even in patients who have failed to respond to treatment with tumor necrosis factor (TNF) monoclonal antibodies [8] [9]. Indigo naturalis shows a high remission induction rate ranging from 69.6% to 89.2%, but some serious adverse effects, such as pulmonary hypertension [3] [10] [11], and the active ingredients are yet to be fully understood. Indigo naturalis has great potential for the treatment of UC by virtue of its ability to activate the Aryl Hydrocarbon Receptor (AHR) [12]. AHR ligands include from the potentially toxic tetrachlorodibenzo-para-dioxin (TCDD), to the in vivo ligand 6-Formylindolo[3,2-b]carbazole (FICZ) [13]. Still, not all ligands are effective for UC. Therefore, the mechanism by which indigo naturalis exerts its therapeutic effect on UC remains elusive.

The underlying causes of UC remain largely obscure, yet the accumulation of excessive reactive oxygen species (ROS) has been implicated in its pathogenesis [14] [15] [16]. Ferroptosis is a ROS-dependent unique programmed cell death linked to iron and lipid peroxidation accumulation [17].

The antioxidant system prevents ferroptosis, and reduced glutathione (GSH) and glutathione peroxidase 4 (GPX4) are essential in inhibiting ferroptosis [17]. Despite evidence of elevated levels of ferroptosis-related proteins in UC patients [18], little is known about whether ferroptosis plays a role in pathogenesis of UC.

This study demonstrates that part of the efficacy of indigo naturalis in treating UC is its ability to inhibit ferroptosis in intestinal epithelial cells through activation of nuclear factor erythroid2-related factor2 (NRF2) downstream of AHR. Our findings highlight the central role of indirubin as one of the major active ingredients responsible for this effect.

**Methods**

Patients' samples

In this study, biopsy specimens of the rectal epithelium from patients with UC who were treated with indigo naturalis were samples from a previous uncontrolled, open-label study [5], which was approved by the Clinical Trial Review Committee of Kyushu University Hospital (registration number: 27067). This previous study was registered at the University Hospital Medical Information Network Center (UMIN Clinical Trials Registry, UMIN 000022575). We specifically targeted the most inflamed rectal epithelium for endoscopic biopsies. If ulcers were present in the rectum, we sampled rectal epithelium from the margins of the ulcer to obtain glandular ducts, not necrotic tissue.

Specimens of the rectal mucosa from patients with UC who were treated with TNF monoclonal antibodies were also from a previous observational study, which was approved by the Clinical Ethics Review Committee of Kyushu University Hospital (registration number: 2021-312). We selected samples of each three patients with UC on treatment with indigo naturalis or TNF monoclonal antibodies. These patients were retrospectively selected, particularly those who had a favorable course of treatment one-year post-intervention. These biopsy specimens were taken before treatment and one year after treatment. All patients enrolled in these studies received and reviewed the study information, and then voluntarily provided written informed consent upon participation. They also consented to biopsy sample analysis at the participation in these studies. This analysis was reviewed and approved by the Ethics Review Committee of Kyushu University Hospital (registration number: 2020-313). Brief clinical information about patients who provided biopsy specimens is supported with Supplement table 3. In this study, we followed STROBE guidelines.

Cell lines

The human colon cancer cell line, CaCo-2 (RCB0988), was provided by RIKEN BRC through the National Bio-Resource Project of MEXT/AMED, Japan. Caco-2 cells were cultured at 37°C in high glucose Dubelcco's modified Eagle's medium containing 10% fetal bovine serum and 1% Penicillin-Streptomycin.

Reagents

Indigo naturalis was purchased from Seishin-shoyakudo (Tokyo, Japan). Indigo naturalis was dissolved in dimethyl sulfoxide (DMSO). If not stated, the final

concentration of indigo naturalis was 0.1 %. Indigo, indirubin, and isatin were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Isoindigo was purchased from Combi-blocks (San Diego, CA, USA). The final concentrations of reagents were as follows: indigo: 10 uM; indirubin: 500 nM; isoindigo: 500 nM; isatin: 500 nM as previously described reports [3] [19]. Erastin 2 and ferrostatin-1 were from Cayman Chemical (Ann Arbor, MI, USA), and hydrogen peroxide ($H_2O_2$) was from Nacalai Tesque (Kyoto, Japan). For ferroptosis analysis, 500 nM ferrostatin-1 and 10 uM erastin 2 were used.

N counter analysis

We extracted RNA from formalin-fixed paraffin-embedded samples using an RNA isolation kit (CELLDATA, Fremont, CA, America) after treatment with deparaffinization solution (CELLDATA). We assessed gene expression levels using 100 ng of total RNA by following the manufacturer's protocol (NanoString Technologies, Seattle, WA, USA). We used the nCounter® Autoimmune Profiling Panel (NanoString Technologies), which reads 770 mRNAs associated with inflammation. The nCounter analysis entails the specific hybridization of mRNA-targeted probes, followed by fluorescence detection from the probes without amplification, facilitated by the nCounter system instrument. The nCounter system can accurately quantify mRNA, even when derived from formalin-fixed, paraffin-embedded samples.

Heat map

We created a heatmap by standardizing total mRNA gene expression in each sample. The heat map included 183 genes with individual Z-scores > 0.1 and *P*-values > .1 for pre- and post-indigo naturalis treatment, pre- and post-TNF

monoclonal antibody treatment, and post-indigo naturalis treatment and TNF monoclonal antibody treatment. Heatmapper was used to create the heatmap [20].

Reactome pathway analysis

We analyzed the mRNA expression data from n counter analysis using the reactome pathway [21]. The selected genes in the analysis showed Z-scores > 0.1 for pre- and post-treatment with indigo naturalis and post-treatment with TNF monoclonal antibody and indigo naturalis. The genes were further selected if they showed *P* values <.05 for post-treatment of TNF monoclonal antibody and indigo naturalis.

Volcano plot

The mRNA expression levels in patients with UC at pre- and post-treatment with indigo naturalis, obtained using n counter analysis, are shown in a volcano plot. We corrected for the same total number of mRNAs in individuals. The volcano plot was generated for six patients with untreated UC and three patients after treatment with indigo naturalis. The figure was created by bioinfokit v2.0.8 library [22].

Western blotting

Reagents were added to logarithmically growing CaCo-2 cells and incubated for 48 hours. These cells were incubated for 6 hours for the nuclear fraction. Cell pellets had protein extracted with M-PER (Thermo Fisher Scientific, Waltham, MA, USA) or N-PER (Thermo Fisher Scientific). The primary antibodies used were as follows: anti-beta actin antibody (ab8227; Abcam, Cambridge, UK), anti-Cytochrome P450 1a1 (CYP1A1) antibody (13241-1-AP; Proteintech,

Rosemont, IL, USA) anti-histone deacetylase (HDAC2) antibody (ab75602; Abcam), anti-NRF2 antibody (ab137550; Abcam), anti-heme oxygenase-1 (HO-1) antibody (10701-1-AP; Proteintech), anti-thiredoxin reductase 1 (TXNR) antibody, (11117-1-AP; Proteintech), anti-glutamate-cysteine ligase modifier subunit (GCLM) antibody (14241-1-AP; Proteintech)

Real-time polymerase chain reaction

Reagents were added to logarithmically growing CaCo-2 cells, incubated for 12 hours, and collected as cell pellets. The cell pellets were purified for RNA using the Maxwell RSC simply RNA Tissue Kit (Promega, Madison, WI, USA). RNA concentrations were measured using Qubit Fluorometer and Qubit RNA BR Assay Kit (Thermo Fisher Scientific). Real-time polymerase chain reaction (PCR) was performed using TB Green Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Kusatsu, Japan) with the intercalator method, and the mRNA concentration ratio to the control was measured using the δδ method [23]. Real-time PCR analysis was performed using an Applied Biosystems QuantStudio 3 instrument (Thermo Fisher Scientific).

The primer sequences used were as follows: actin beta: forward, TGGCACCCAGCACAATGAA and reverse, CTAAGTCATAGTCCGCCTAGAAGCA; GCLM: forward, CACAGCGAGGAGGAGTTTCC and reverse, ATCCAGCTGTGCAACTCCAA; TXNR: forward, CCATTGCCTGGCATTTGGTA and reverse, AGCTTGCTTAGACCAGCACACAATA; HO-1: forward, TTGCCAGTGCCACCAAGTTC and reverse, TCAGCAGCTCCTGCAACTCC; superoxide dismutase 1 (SOD1): forward, CTGTACCAGTGCAGGTCCTC and

reverse, CCAAGTCTCCAACATGCCTCT; superoxide dismutase 2 (SOD2): forward, GCACTAGCATGTTGAGC and reverse, TTGATGTGAGGTTCCAGGGC; sulfiredoxin-1 (SRXN): forward, CTAAGACATTGTCCTGGCCCTCA and reverse, TCTGGAATGTCCTACCTGGCCTA.

Cell death assay

CaCo-2 cells were seeded in 96-well plates at a density of 1.0 x $10^4$ cells/$cm^2$ and incubated for 48 hours. The growth medium was replaced with the medium supplemented with DMSO, indigo naturalis, or reagents as a preincubation. Twenty-four hours later, the medium used in the preincubation was replaced with preincubated medium supplemented with DMSO or erastin 2. After another 24 hours of incubation in accordance with the manufacturer's protocol, the absorbance at 450 nm was measured using the viable cell counting reagent SF (Nacalai Tesque).

Observation of ROS using fluorescence

CaCo-2 cells were seeded in 96-well plates at a density of 2.0 x $10^4$ cells/$cm^2$ and incubated for 48 hours. The medium was replaced with DMSO, indigo naturalis, or reagents as a preincubation. Twenty-four hours later, the medium used in the preincubation was replaced with medium supplemented with DMSO or erastin 2. After another 24 hours of incubation, we prepared ROS Assay Kit -Highly Sensitive DCFH-DA- (Dojindo, Kumamoto, Japan) buffer and H342-Cellstain Hoechst 33342 solution (Dojindo) was added to the buffer at a ratio of 400:1. Observation of fluorescence was performed using a microscope (BZ-X700; Keyence, Tokyo, Japan) at a 20 x field of view. The relative fluorescence

intensity for ROS was calculated by dividing the 525-nm fluorescence intensity value of all pixels by the 460-nm fluorescence intensity value of all pixels.

MDA (malondialdehyde) assay

Preincubation of logarithmically growing CaCo-2 cells with DMSO, indigo naturalis, or reagents was performed for 24 hours. Erastin 2 was then added, and cell pellets were collected after 12 hours. After the cell pellets were collected, we determined fluorescence intensity using an MDA assay kit (Dojindo) in accordance with the manufacturer's protocol, using an excitation wavelength of 540 nm, and emission wavelength of 590 nm. The fluorescence intensity was corrected by protein content using a Protein Assay BCA Kit (Nacalai Tesque), and the MDA concentrations were determined.

Immunofluorescence staining

We utilized formalin-fixed paraffin-embedded biopsy specimens for our study. The primary antibodies employed were as follows: anti-CYP1A1 antibody (13241-1-AP; Proteintech), anti-GCLM antibody (14241-1-AP; Proteintech), and anti-GPX4 antibody (14432-1-AP; Proteintech). Each slide was examined using a microscope (BZ-X700; Keyence) at a 40 x field of view. Several glands were randomly selected, and their intestinal epithelium's average light intensity was analyzed. The differentiation in light intensity before and after treatment in the same patient was quantified as the differentiation of light intensity.

Liquid chromatograph-mass spectrometry

We used a rectal mucosa biopsy (6.4 mg) from a patient who was treated with indigo naturalis for 8 weeks. We stored the sample at −80°C until measurement. We washed the sample with phosphate-buffered saline, used a

homogenizer under liquid nitrogen freezing, and when powdered, 100 µl of DMSO was added. We centrifuged the sample at 2,000 x g for 10 minutes, and the upper 94 µl of supernatant was collected. The above-mentioned extracts were measured using liquid chromatograph-mass spectrometry (LC/MS). The concentrations of indigo, indirubin, isoindigo, and isatin were determined. Quantification was performed using a Waters Acquity H-Class system (Waters, Milford, MA, USA) for preparative high performance liquid chromatography and waters, ACQUITY UPLC BEH C18 (2.1 mm × 50 mm inner diameter, 1.7 µm column) (Waters) for MS. Measurement data were analyzed with TargetLynx (Waters). The above-mentioned measurements were performed at Preppers (Hamamatsu, Japan).

Metabolomic analysis of CaCo-2 cells

CaCo-2 cells were spread in 10-cm dishes at a density of 2.0 x $10^4$ cells/$cm^2$ and incubated for 24 hours. Indigo naturalis or DMSO was then added to the medium and incubated for another 24 hours. Erastin 2 or DMSO was added to the medium and incubated for 12 hours. After culturing CaCo-2 cells, the medium was removed and washed twice with mannitol. Methanol solution was then added and stirred. Ultrapure water containing 10 µM of internal standard (Human Metabolome Technologies, Tsuruoka, Japan) was added, stirred, and centrifuged (2,300 × g, 4°C, 5 min). The filtrate dissolved in ultrapure water again for measurement. Samples were measured using capillary electrophoresis time-of-flight mass spectrometry by an Agilent CE-TOFMS system (Agilent Technologies, Santa Clara, CA, USA), in cation and anion

mode. In this study, analysis was performed on substances registered in the HMT Metabolite Library.

Statistical analyses

Values are shown as the mean ± standard error of the mean. Statistical analyses were conducted using JMP 14 software (SAS Institute, Cary, NC, USA).

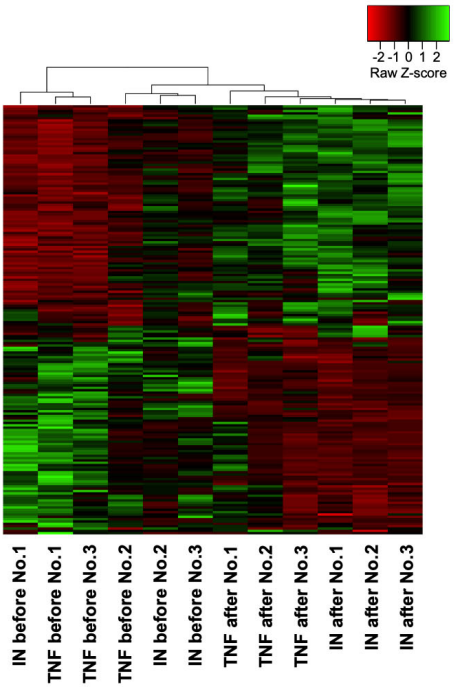
## Results

### Treatment with indigo naturalis results in a different gene expression pattern from TNF monoclonal antibody treatment.

Superficial biopsy tissue of rectal mucosal mRNA expression in patients with UC was analyzed to determine whether indigo naturalis is involved in specific gene expression. Our findings indicate, as anticipated, a marked variation in mRNA expression pre- and post-treatment, regardless of the intervention. The alterations in mRNA expression following treatment resulted in a significant grouping of six patients, with those treated with indigo naturalis exhibiting even closer clustering than those treated with TNF monoclonal antibodies. The mRNA expression of the six patients pre-treatment demonstrated a different clustering pattern, implying that the administration of indigo naturalis and TNF monoclonal antibodies may have elicited dissimilar mRNA expression changes. It is noteworthy that all patients were in remission from UC at the time of the post-treatment sample collection (Figure 1A, Supplement table 1). Subsequently, we analyzed the genes that demonstrated a propensity for

differential expression in response to the mode of treatment. The expression of genes corresponding to oxidative stress during treatment with indigo naturalis was the most significant in pathway analysis (Figure 1B). We observed changes in gene expression before and after treatment with indigonaturalis in a volcano plot. mRNA levels of antioxidant genes associated with NRF2 tended to be upregulated. The antioxidant genes that were measurable in this study are as follows: glutamate-cysteine ligase catalytic subunit (GCLC), thioredoxin (TXN), peroxiredoxin 2 (PRDX2), selenium binding protein1 (SELENBP1), and pi-class glutathione S-transferase (GSTP1) (Figure 1C) [24].

A




B

| Reactome Pathway | *P-Value* |
|---|---|
| Detoxification of Reactive Oxygen Species | 0.00031 |
| Cellular response to chemical stress | 0.0082 |
| Cellular response to stress | 0.016 |
| Cellular response to stimuli | 0.017 |
| Generic Transcription Pathway | 0.23 |

C

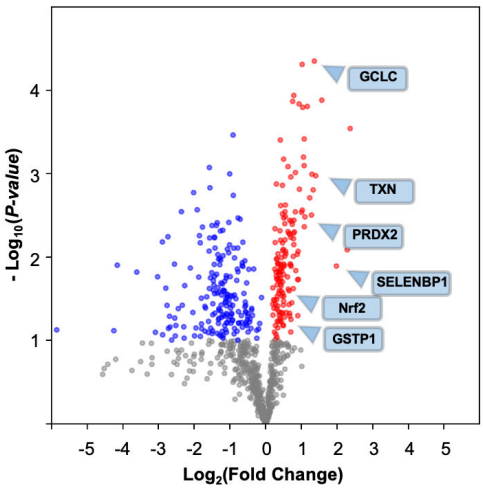



**Fig.1 Comparison of mRNA expression between indigo naturalis and TNF monoclonal antibody treatment.**

A. Clustering analysis in individual gene expression of pre- and post-treatment with indigo naturalis and pre- and post-treatment with TNF monoclonal antibody. No1, No2, and No3 correspond to three patients each treated with

indigo naturalis and TNF monoclonal antibody. Biopsies were taken from the rectal superficial mucosa, and post-treatment specimens were obtained from patients who have achieved full remission of UC.

B. Top five pathways shown by reactome pathway analysis.

C. Volcano plot of mRNA expression in patients with UC pre- and post-treatment with indigo naturalis. GCLC, TXN, PRDX2, SELENBP1, and GSTP1 are glutathione thioredoxin antioxidant system-related genes that are regulated by NRF2.

IN; Indigo naturalis, GCLC; Glutamate-cysteine ligase catalytic subunit, TXN; Thioredoxin 1, PRDX2; Peroxiredoxin 2, SELENBP1; Selenium binding protein 1, NRF2; Nuclear factor erythroid2-related factor2, GSTP1; Pi-class glutathione S-transferase

**Indigo naturalis promotes the nuclear translocation of NRF2 in CaCo-2 cells and the expression of a group of antioxidant genes.**

We speculate that indigo naturalis drives antioxidant genes. Indigo naturalis is known as an AHR ligand, and certain AHR ligands modulate NRF2 [25-29]. NRF2 is a transcriptional regulator known as a master switch of antioxidant genes, including the glutathione-thioredoxin antioxidant system [30]. NRF2 is usually in the cytoplasm, and upon activation translocate into the nucleus for transcriptional regulation. In CaCo-2 cells, indigo naturalis induces CYP1A1, a target gene of AHR, and promoted nuclear translocation of NRF2 (Figure 2A). Then, we evaluated the expression of GCLM and TXNR, which play critical role

in the glutathione-thioredoxin antioxidant system [31] among NRF2 target genes. Indigo naturalis upregulated gene expression of GCLM and TXNR at the mRNA levels (Figure 2B). Indigo naturalis also upregulated gene expression of HO-1 and SRXN at the mRNA levels. These genes are associated with ROS [32] [33]. We also found that indigo naturalis increased protein levels of GCLM, TXNR, and HO-1 (Figure 2C).

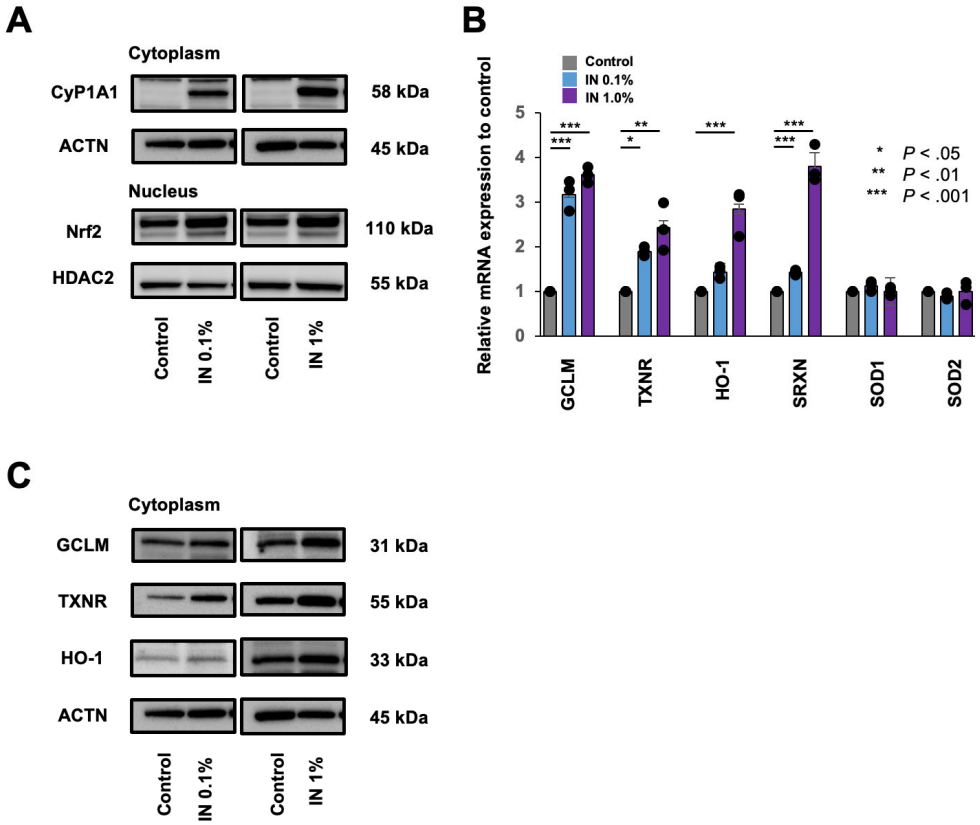



**Fig.2 Indigo naturalis upregulates NRF2 nuclear translocation and expression of a group of antioxidant genes.**

A. Western blot analysis of indigo naturalis treatment in CaCo-2 cells. Indigo naturalis induces CYP1A1, a target gene of AHR (48 hours), and promotes

nuclear translocation of NRF2 (6 hours). The blot represents one of three independent experiments.

B. Real-time-PCR analysis of GCLM, TXNR, SRNX, HO-1, SOD1, and SOD2 (known to be regulated by NRF2) mRNAs levels after indigo naturalis treatment in CaCo-2 cells. SOD1 and SOD2 mRNA expression was not upregulated (n = 3 independent experiments, each containing 3 to 4 biological replicates). Tukey–Kramer's multiple comparison test was used for the analysis.

C. Western blot analysis of HO-1, TXNR, and GCLM protein levels after indigo naturalis treatment in CaCo-2 cells (48hours). This blot represents one of three independent experiments.

IN; Indigo naturalis

**Indigo naturalis inhibits erastin 2-induced ferroptosis in CaCo-2 cells.**

We next examined the cytoprotective effect of increased antioxidant by indigo naturalis. In CaCo-2 cells, indigo naturalis did not affect cell death in $H_2O_2$ treatment (Figure 3A). We confirmed cell death by erastin 2, which is an inducer of ferroptosis inhibiting cystine uptake, and cell death was rescued by ferrostatin-1, which is a specific inhibitor of ferroptosis in CaCo-2 cells (Figure 3B). Notably, Indigo naturalis treatment reduced erastin 2 induced cell death (Figure 3C). Concentrations of MDA, which is a marker of lipid peroxide [34], were elevated by erastin 2 treatment, but reduced by indigo naturalis treatment in CaCo-2 cells (Figure 3D). Fluorescence microscopy showed that erastin 2-induced ROS accumulation was suppressed with indigo naturalis treatment

(Figure 3E, F). These results suggest that indigo naturalis hinders erastin 2-induced ferroptosis in CaCo-2 cells.

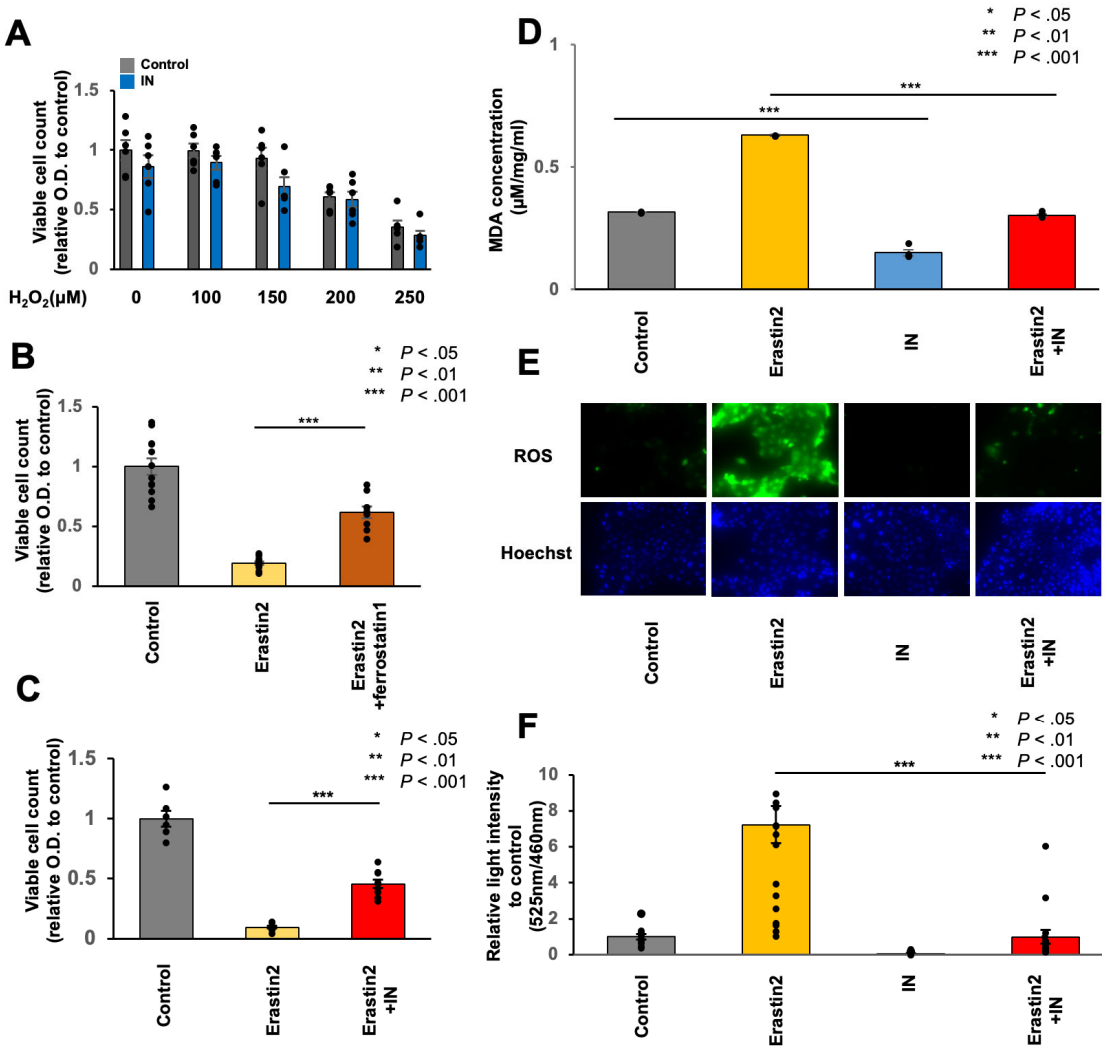



**Fig.3 Indigo naturalis inhibits erastin 2-induced ferroptosis in CaCo-2 cells.**

A. Relative number of viable cells treated with $H_2O_2$ and indigo naturalis in CaCo-2 cells. There were no significant differences in any of the $H_2O_2$ concentrations between the two groups (n = 6 independent experiments).

B. Relative number of viable cells treated with erastin 2 and ferrostatin-1 in CaCo-2 cells. Ferrostatin-1 is a specific inhibitor of ferroptosis (n = 6 independent experiments).

C. Relative number of viable CaCo-2 cells treated with indigo naturalis or with erastin 2 plus indigo naturalis (n = 6 independent experiments).

D. MDA concentrations with erastin 2 and indigo naturalis treatment in CaCo-2 cells (n = 4 independent experiments).

E. Representative fluorescence observations for intracellular ROS in CaCo-2 cells treated with indigo naturalis and erastin 2.

F. Relative fluorescence intensity ratio of ROS (n = ≥ 10 independent experiments).

Tukey–Kramer's multiple comparison test was used for all analyses.

IN; Indigo naturalis

**Indigo naturalis treatment increases GSH concentrations in CaCo-2 cells.**

Glutathione metabolism regulates ROS, and GSH is the most important factor in the inhibition of ferroptosis. GSH reduces oxidative stress and GSH changes to the oxidized form glutathione disulfide (GSSG). Therefore, we examined glutathione metabolism induced by treatment with indigo naturalis using metabolomic analysis. Under normal conditions, indigo naturalis increased GSH concentrations in CaCo-2 cells by 1.4 times higher than those treated with controls. GSSG concentrations were unchanged with indigo naturalis treatment (Figure 4A, Supplement table 2). Cystine is converted to cysteine (Cys), an

essential component of GSH. Under ferroptosis conditions, as expected for erastin 2 action, Cys and γ-glutamylcysteine (γ-Glu-Cys) were below detection limits. Indigo naturalis increased GSH concentrations by 5.2 times higher than those treated with controls, and GSSG also increased GSH concentrations by 1.6 times. These results suggested that indigo naturalis hindered GSH depletion (Figure 4B, Supplement table 2).

These results also indicated that indigo naturalis induced a group of antioxidant genes related to NRF2. Indigo naturalis reduced the accumulation of erastin 2-induced ROS and regulated glutathione metabolism, which in turn suppressed ferroptosis.

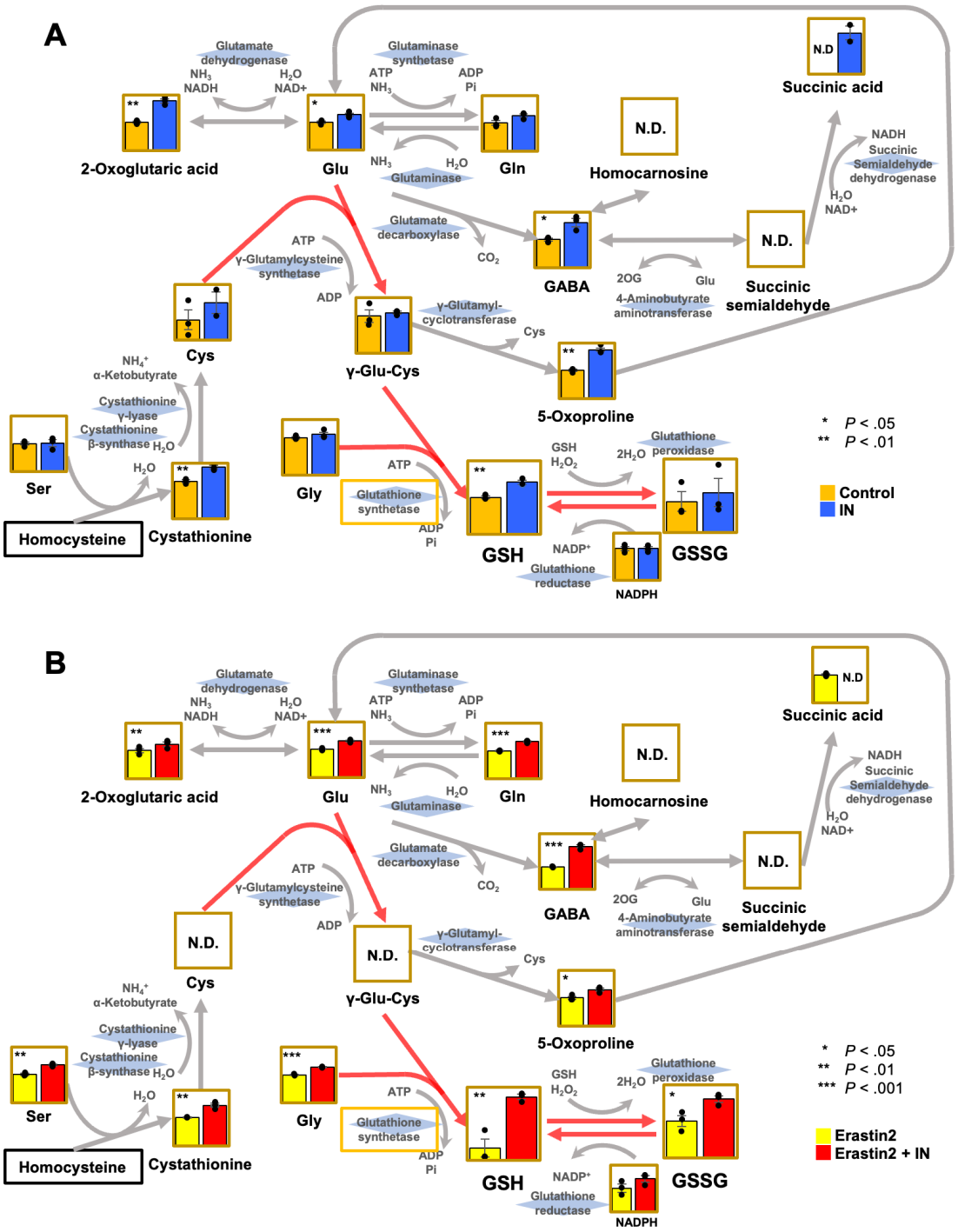



**Fig.4 Indigo naturalis treatment upregulates GSH expression in CaCo-2 cells.**

A. Metabolomic analysis in control and indigo naturalis-treated CaCo-2 cells. A metabolome pathway diagram of GSH and GSSG is shown (n = 3). The

analysis revealed an elevation in the concentration of GSH upon stimulation with indigo naturalis. The results of the experiment did not demonstrate a corresponding increase in the levels of Cys or γ-Glu-Cys, a component involved in the synthesis of GSH. Student's t-test was used for each comparison.

B. Metabolomic analysis in control and indigo naturalis treated CaCo-2 cells under erastin 2 stimulation. A metabolome pathway diagram of GSH and GSSG is shown (n = 3). Erastin 2 impedes cystine uptake; cystine is a dimer of two Cys molecules. The presence of Cys and γ-Glu-Cys was not observed upon treatment with erastin 2. Nevertheless, simultaneous treatment with indigo naturalis sustained GSH levels relative to erastin 2 treatment alone. Student's t-test was used for each comparison.

Pathways important in the synthesis of GSH are highlighted with red arrows. The metabolome was measured in three independent samples each. Items that were below the detection limit two or more times out of three samples are indicated as not detectable (N.D.) in the metabolome pathway diagram.

IN; Indigo naturalis, γ-Glu-Cys; Gamma-glutamylcysteine, Glu; Glutamic acid, Gln; Glutamine, GSH; Reduced glutathione, GSSG; Glutathione disulfide, GABA; Gamma-aminobutyric acid, Ser; Serine, Gly; Glycine, Cys; Cysteine, NADPH; Nicotinamide adenine dinucleotide phosphate

**Indigo naturalis treatment increases expression of CYP1A1 and GPX4 in patients with UC.**

We analyzed the expression of proteins associated with ferroptosis in patients with UC treated with indigo naturalis. For comparison, we evaluated samples from patients treated with TNF monoclonal antibody. Immunofluorescent staining was performed for CYP1A1, which is induced by AHR, and for GCLM and GPX4, which play an important role in reduction of oxidized lipids. The UC patients treated with indigo naturalis showed increased protein expression of CYP1A1 in rectal mucosa. The change in GCLM expression was not significant, but GPX4 expression was increased in the UC patients treated with indigo naturalis. Patients who experienced remission through TNF monoclonal antibodies did not change the expression levels of these proteins. The increased expression of CYP1A1 and GPX4 suggested that indigo naturalis stimulates the intestinal epithelium, which is more resistant to ferroptosis.

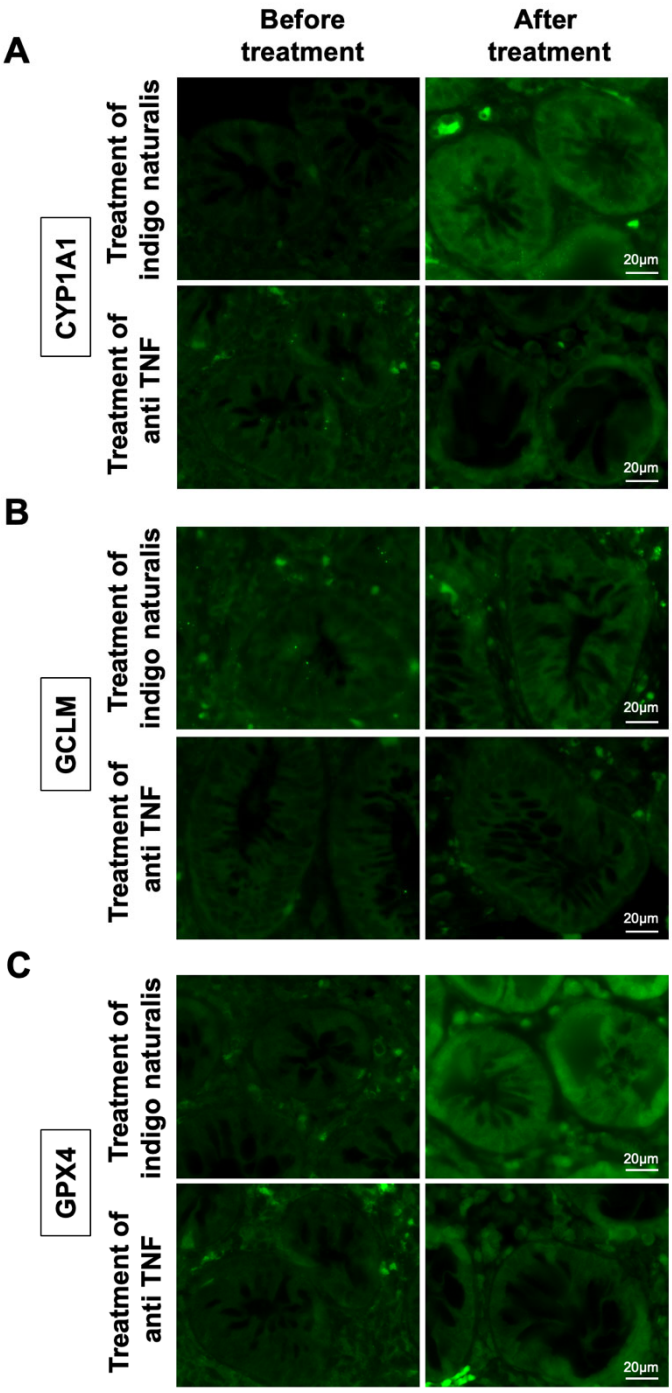



**Fig.5 Immunofluorescence staining showed indigo naturalis treatment upregulates CYP1A1 and GPX4 expression in patient with UC.**

A. Representative immunofluorescence staining images of CYP1A1. The observed mean enhancement in fluorescence intensity after the indigo naturalis treatment was 8.6, contrasting with 0.0 after the TNF monoclonal antibodies treatment. (P = 0.02)
B. Representative immunofluorescence staining images of GCLM. The observed mean enhancement in fluorescence intensity after the indigo naturalis treatment was 4.8, contrasting with -1.7 after the TNF monoclonal antibodies treatment. (P = 0.12)
C. Representative immunofluorescence staining images of GPX4. The observed mean enhancement in fluorescence intensity after the indigo naturalis treatment was 11.4, contrasting with 1.0 after the TNF monoclonal antibodies treatment. (P = 0.02)

For detailed clinical data of the patients and differentiation of light intensity, please refer to Supplement table 3.

**Indigo and indirubin inhibit erastin 2-induced ferroptosis in CaCo-2 cells.**

The main ingredients of indigo naturalis were indigo, indirubin, isoindigo, and isatin. We analyzed the ability of AHR activation, the translocation of NRF2 and the downstream of antioxidant genes in these substrates. We found that indigo and indirubin is the AHR ligands and translocated NRF2 into the nucleus (Figure 6A). Real-time-PCR showed that indigo and indirubin upregulated mRNA expression of GCLM, HO-1, TXNR, and SRXN, which are target genes of NRF2 (Figure 6B). Additionally, the expression of these antioxidant proteins

was increased with indigo and indirubin treatment (Figure 6C). We next analyzed whether each ingredient of indigo naturalis inhibits ferroptosis. Indigo and indirubin suppressed cell death by erastin 2-induced ferroptosis (Figure 6D) and MDA accumulation (Figure 6E), and it also reduced ROS (Figure 6F and 6G). These results suggested that indigo and indirubin were cytoprotective against ferroptosis.

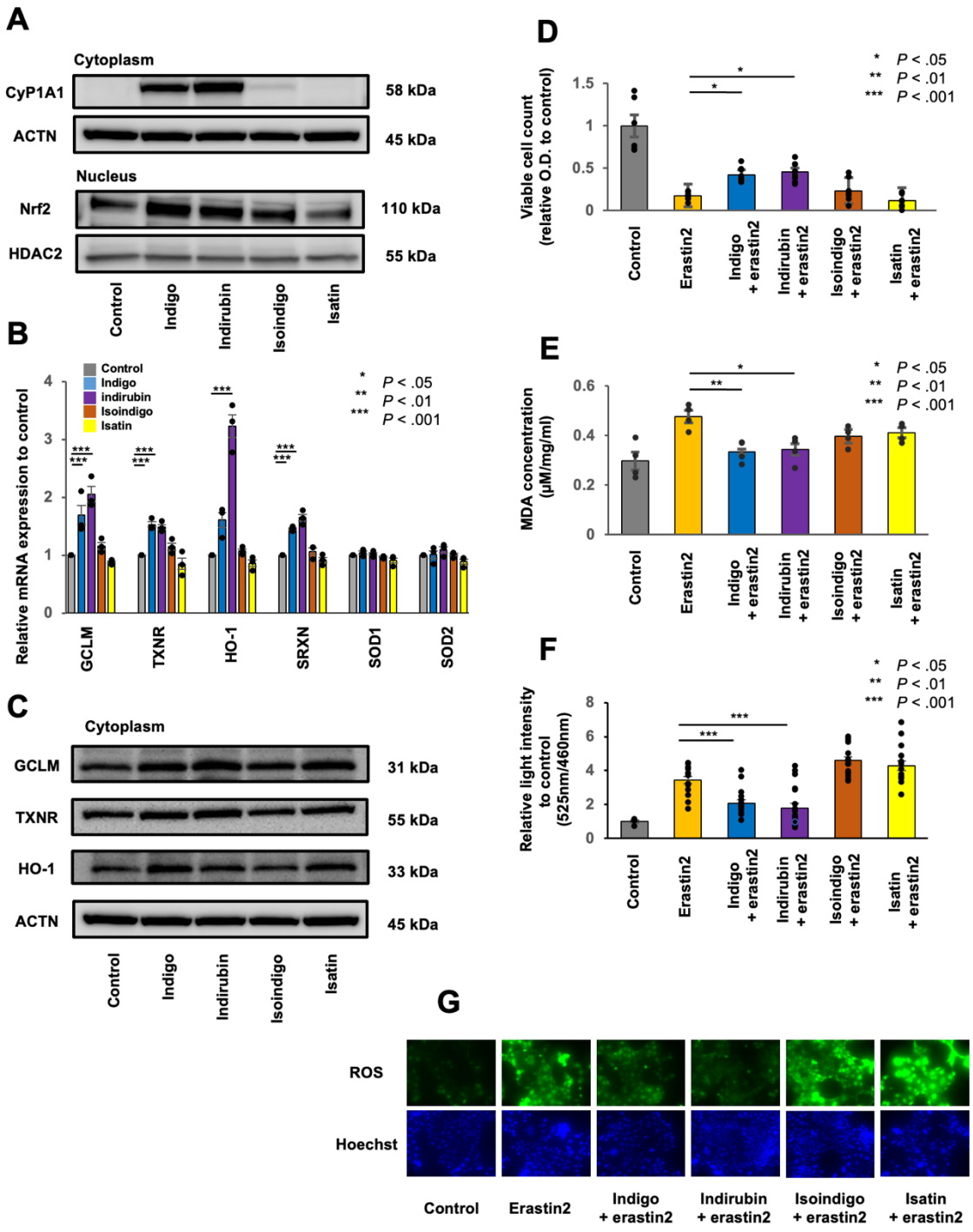



**Fig.6 Indigo and indirubin promote the nuclear translocation of NRF2 and the expression of a group of antioxidant genes in CaCo-2 cells.**

A. Western blot analysis of indigo naturalis ingredients in CaCo-2 cells. Indigo and indirubin induces CYP1A1, a target gene of AHR (48 hours), and showed

nuclear translocation of NRF2 (6 hours). The blot represents one of three independent experiments.

B. Real-time-PCR analysis of mRNA levels in CaCo-2 cells treated with indigo naturalis ingredients. GCLM, TXNR, HO-1, SRNX, SOD1, and SOD2 are known to be regulated by NRF2. Indigo and indirubin upregulated GCLM, TXNR, HO-1, and SRNX. The mRNA expression levels of SOD1 and SOD2 were unchanged (n = 3 independent experiments, each containing 3 to 4 biological replicates).

C. Western blot analysis of antioxidant protein levels after treatment of indigo naturalis ingredients in CaCo-2 cells. Indigo and indirubin increased antioxidant protein expression of HO-1, TXNR, and GCLM (48 hours). The blot represents one of three independent experiments.

D. Relative number of viable CaCo-2 cells treated with erastin 2 and indigo naturalis ingredients (n = 6 independent experiments).

E. MDA concentrations in CaCo-2 cells treated with erastin 2 and indigo naturalis ingredients (n = 4 independent experiments).

F. Relative fluorescence intensity ratio of ROS (n = ≥ 10 independent experiments).

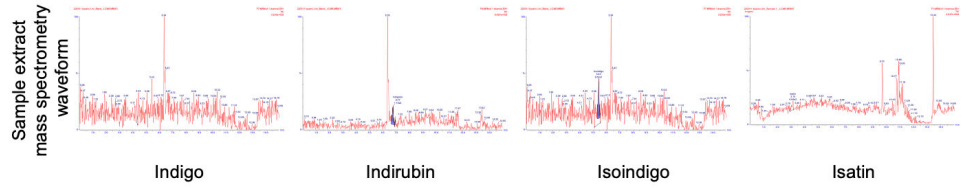
G. Representative fluorescence observations of intracellular ROS in CaCo-2 cells treated with indigo naturalis ingredients and erastin 2.

Tukey–Kramer's multiple comparison test was used for all analyses.

**Only indirubin and isoindigo are detected in the rectal mucosa of patients treated with indigo naturalis.**

Indigo was not detected by LC/MS of rectal mucosa extract, and only indirubin and isoindigo were detected (Figure 7A, 7B). This finding suggested that indigo was poorly absorbed in the intestinal epithelium, and that the inhibitory effect of indigo naturalis on ferroptosis was due to indirubin.

**A**




**B**

| | Concentration of sample extract (nM) | Estimated intramucosal concentration (nM) |
|---|---|---|
| Indigo | 0 | 0 |
| Indirubin | 6.8 | 99.9 |
| Isoindigo | 3.9 | 59.1 |
| Isatin | 0 | 0 |

**Fig.7 Indirubin and isoindigo are detected in the rectal mucosa of patients treated with indigo naturalis.**

A. LC/MS waveforms for indigo naturalis ingredients in the sample extract (blue).

B. Concentrations of indigo naturalis ingredients in the sample and intraepithelial mucosa as estimated by mass spectrometry.

## Discussion

Our study suggests that indigo naturalis and its ingredient, indirubin, inhibit ferroptosis in the intestinal epithelium.

Ferroptosis, a type of programmed cell death related to oxidative stress, is exacerbated in the context of ulcerative colitis tissue [18]. This particular type of cell death has been linked to conditions such as Alzheimer's disease [35], amyotrophic lateral sclerosis [36], and psoriasis [37]. ROS has been identified as a key factor in the pathogenic factor of UC [14] [15] [16]. Ferroptosis accumulates lipid peroxidation, which is dependent on both iron and ROS levels [17]. Elevated iron concentrations increase the susceptibility to ferroptosis and a high iron diet may present a risk factor for the development of UC [38]. Suppressing ROS levels through the utilization of iron chelator has been proposed as a possible therapeutic strategy for UC [39]. These results suggest that ferroptosis plays a role in the pathogenesis of UC.

Several clinical studies have reported the effectiveness of indigo naturalis in treating UC [3-7], but the detailed mechanism has remained elusive. The impact of indigo naturalis is known to be mediated by AHR. AHR has been identified as one of the genetic loci associated with susceptibility to UC through association analysis [40]. AHR serves as a transcriptional regulator. The downstream genes influenced by each AHR ligand exhibit diversity, and it is crucial to decipher the cytoprotective consequences of the numerous downstream signaling alterations in UC [41]. Indigo naturalis has been observed to induce the production of interleukin-22 [12] and recruit regulatory T cells in the gut through AHR [42]. AHR activation has been documented to cause induction and differentiation of regulatory T cells and inflammatory Th17 cells [43-45]. These observations imply that the effectiveness of indigo naturalis in treating UC is not solely attributed to the immunomodulatory properties of AHR. Given the reported

capacity of AHR ligands to regulate intestinal tight junctions [28] and oxidant stress [46], we focused on their protective effects on intestinal epithelial cells. NRF2, a master regulator of antioxidant activity, is subject to regulation and induction through the AHR [25-29] [47] [48]. In our study, indigo naturalis upregulated the glutathione and thioredoxin system through the activation of NRF2, which are involved in scavenging cellular ROS [31] [49]. The glutathione and thioredoxin systems are strongly associated with the regulation of ferroptosis [50] [51]. Among NRF2-related ROS scavenger, GSH is considered to regulate ferroptosis directly by preventing the oxidation of polyunsaturated fatty acids peroxide [52] [53]. In vitro, high levels of arachidonic acid, n-6 polyunsaturated fatty acids, increases the susceptibility to ferroptosis [53]. Generally, a Western diet and arachidonic acid is a possible risk factor for UC [54-56]. The important aspect of our study is that indigo naturalis did not prevent hydrogen peroxide-induced cell death but prevented ferroptosis-induced cell death. Patients with UC have decreased plasma cystine and cysteine levels, which are required for glutathione synthesis, and decreased GSH levels in intestinal epithelial cells [57]. The relationship between nutritional changes and ferroptosis in patients with UC needs further investigation. Our study showed that indigo naturalis is implicated in not only oxidative stress but in the ferroptosis pathway, and ferroptosis would play a role in the genesis of UC.

We examined the active ingredients in indigo naturalis in a CaCo-2 cells model. Indigo and indirubin were responsible for the antioxidant and ferroptosis inhibitory effects, while isoindigo and isatin had no effect. Suppression of ferroptosis was also observed only with indigo and indirubin. Indigo was not

detected in the LC/MS analysis of the rectal mucosa in patients taking indigo naturalis. In a dextran sodium sulfate-induced mouse colitis model, oral administration of indirubin rescued the mice from death, while oral administration of indigo did not [58]. This would be attribute the low solubility and minimal transferability of indigo into the body, as opposed to the solubility of indirubin in alcohols, oils, and fats [59].

This study has some limitations, including the small sample size of human biopsies obtained. Due to technical difficulties, human biopsies were obtained from patients who were particularly well treated rather than from the entire group of patients in the previous prospective comparative study. In previous clinical trials, it has been observed that around 20-30% of patients did not respond adequately to indigo naturalis treatment [3] [10] [11]. The Genome-Wide Association Study has revealed that UC is a complex disease influenced by multiple risk factors, such as epithelial barriers, innate immunity, antigen presentation, and immune tolerance [40]. Our observed outcomes may come from a specific type of UC that exhibits enhanced sensitivity to signal modulation by indigo naturalis. Also, our report did not address immunization, the effect on lymphocytes of the newly discovered mechanism by which indigo naturalis suppresses ferroptosis. While our study is focused on epithelial cells, it is worth noting that indigo naturalis has been shown to activate AHR in hematopoietic cells. AHR ligands play a role in immune cell differentiation [43-45]. Indigo naturalis explicitly induces FOX3+Treg cells and the production of interleukin-22 [12, 42]. Previous study has demonstrated that the GPX4-dependent neutralization of lipid peroxides protects regulatory T cells from

ferroptosis, thereby sustaining their activation and function in immune regulation [60]. While inhibiting ferroptosis of immune cells by indigo naturalis requires further investigation, our findings demonstrate that indigo naturalis exerts its influence not only on the immune system but also on the intestinal epithelium. Currently, guidelines for the treatment of moderate to severe UC include TNF monoclonal antibodies, anti-integrins, Janus kinase inhibitors and immunomodulators [61], but these therapies occasionally can be challenging to use due to the risk of immunosuppression or resistance to treatment. The suppression of ferroptosis in the intestinal epithelium may be a new therapeutic target for UC and further research is needed.

## Disclosures

The authors declare that they have no conflict of interest.

## Grant support

This work was supported by JSPS KAKENHI grants '[Grant-in-Aid for Scientific Research (C) JP21K06783 and Grant-in-Aid for Young Scientists (B) JP22K15668]'.

## Acknowledgments

We thank Ellen Knapp, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript. We appreciate the technical assistance from The

Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences.

**Supplementary Material (Data Transparency Statement)**

The raw data of mRNA levels from patients with ulcerative colitis performed by n counter analysis system and metabolomic analysis of CaCo-2 treated with erastin 2 and indigo naturalis are provided as Supplement table 1 and table 2. Brief clinical information about patients who provided biopsy specimens is supported with Supplement table 3.

## References

1. Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;142:46-54.e42; quiz e30.
2. Barnes EL, Loftus EV, Jr., Kappelman MD. Effects of Race and Ethnicity on Diagnosis and Management of Inflammatory Bowel Diseases. Gastroenterology. 2021;160:677-89.
3. Naganuma M, Sugimoto S, Mitsuyama K, et al. Efficacy of Indigo Naturalis in a Multicenter Randomized Controlled Trial of Patients With Ulcerative Colitis. Gastroenterology. 2018;154:935-47.
4. Uchiyama K, Takami S, Suzuki H, et al. Efficacy and safety of short-term therapy with indigo naturalis for ulcerative colitis: An investigator-initiated multicenter double-blind clinical trial. PLoS One. 2020;15:e0241337.
5. Matsuno Y, Torisu T, Umeno J, et al. One-year clinical efficacy and safety of indigo naturalis for active ulcerative colitis: a real-world prospective study. Intest Res. 2022;20:260-8.
6. Sugimoto S, Naganuma M, Kiyohara H, et al. Clinical Efficacy and Safety of Oral Qing-Dai in Patients with Ulcerative Colitis: A Single-Center Open-Label Prospective Study. Digestion. 2016;93:193-201.
7. Matsuno Y, Hirano A, Torisu T, et al. Short-term and long-term outcomes of indigo naturalis treatment for inflammatory bowel disease. J Gastroenterol Hepatol. 2020;35:412-7.
8. Saiki JP, Andreasson JO, Grimes KV, et al. Treatment-refractory ulcerative colitis responsive to indigo naturalis. BMJ Open Gastroenterol. 2021;8.
9. Naganuma M, Sugimoto S, Fukuda T, et al. Indigo naturalis is effective even in treatment-refractory patients with ulcerative colitis: a post hoc analysis from the INDIGO study. J Gastroenterol. 2020;55:169-80.
10. Hiraide T, Teratani T, Uemura S, et al. Pulmonary Arterial Hypertension Caused by AhR Signal Activation Protecting against Colitis. Am J Respir Crit Care Med. 2021;203:385-8.

11. Naganuma M, Sugimoto S, Suzuki H, et al. Adverse events in patients with ulcerative colitis treated with indigo naturalis: a Japanese nationwide survey. J Gastroenterol. 2019;54:891-6.
12. Kawai S, Iijima H, Shinzaki S, et al. Indigo Naturalis ameliorates murine dextran sodium sulfate-induced colitis via aryl hydrocarbon receptor activation. J Gastroenterol. 2017;52:904-19.
13. Bock KW. From TCDD-mediated toxicity to searches of physiologic AHR functions. Biochem Pharmacol. 2018;155:419-24.
14. Jena G, Trivedi PP, Sandala B. Oxidative stress in ulcerative colitis: an old concept but a new concern. Free Radic Res. 2012;46:1339-45.
15. Wang Z, Li S, Cao Y, et al. Oxidative Stress and Carbonyl Lesions in Ulcerative Colitis and Associated Colorectal Cancer. Oxid Med Cell Longev. 2016;2016:9875298.
16. Wan Y, Yang L, Jiang S, et al. Excessive Apoptosis in Ulcerative Colitis: Crosstalk Between Apoptosis, ROS, ER Stress, and Intestinal Homeostasis. Inflamm Bowel Dis. 2022;28:639-48.
17. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012;149:1060-72.
18. Xu M, Tao J, Yang Y, et al. Ferroptosis involves in intestinal epithelial cell death in ulcerative colitis. Cell Death Dis. 2020;11:86.
19. Qi-Yue Y, Ting Z, Ya-Nan H, et al. From natural dye to herbal medicine: a systematic review of chemical constituents, pharmacological effects and clinical applications of indigo naturalis. Chin Med. 2020;15:127.
20. Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016;44:W147-53.
21. Gillespie M, Jassal B, Stephan R, et al. The reactome pathway knowledgebase 2022. Nucleic Acids Res. 2022;50:D687-d92.
22. Bedre R. reneshbedre/bioinfokit: Bioinformatics data analysis and visualization toolkit. Zenodo. 2020.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8.
24. Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol. 2013;53:401-26.
25. Wakabayashi N, Slocum SL, Skoko JJ, et al. When NRF2 talks, who's listening? Antioxid Redox Signal. 2010;13:1649-63.
26. Shaw P, Chattopadhyay A. Nrf2-ARE signaling in cellular protection: Mechanism of action and the regulatory mechanisms. J Cell Physiol. 2020;235:3119-30.
27. Yeager RL, Reisman SA, Aleksunes LM, et al. Introducing the "TCDD-inducible AhR-Nrf2 gene battery". Toxicol Sci. 2009;111:238-46.
28. Singh R, Chandrashekharappa S, Bodduluri SR, et al. Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. Nat Commun. 2019;10:89.
29. Tocmo R, Le B, Heun A, et al. Prenylated xanthones from mangosteen (Garcinia mangostana) activate the AhR and Nrf2 pathways and protect intestinal barrier integrity in HT-29 cells. Free Radic Biol Med. 2021;163:102-15.
30. Tonelli C, Chio IIC, Tuveson DA. Transcriptional Regulation by Nrf2. Antioxid Redox Signal. 2018;29:1727-45.
31. Lu J, Holmgren A. The thioredoxin antioxidant system. Free Radic Biol Med. 2014;66:75-87.
32. Loboda A, Damulewicz M, Pyza E, et al. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol Life Sci. 2016;73:3221-47.
33. Ramesh A, Varghese SS, Doraiswamy J, et al. Role of sulfiredoxin in systemic diseases influenced by oxidative stress. Redox Biol. 2014;2:1023-8.
34. Stockwell BR. Ferroptosis turns 10: Emerging mechanisms, physiological functions, and therapeutic applications. Cell. 2022;185:2401-21.
35. Stockwell BR, Friedmann Angeli JP, Bayir H, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. Cell. 2017;171:273-85.
36. Chen L, Na R, Danae McLane K, et al. Overexpression of ferroptosis defense enzyme

Gpx4 retards motor neuron disease of SOD1G93A mice. Sci Rep. 2021;11:12890.
37. Shou Y, Yang L, Yang Y, et al. Inhibition of keratinocyte ferroptosis suppresses psoriatic inflammation. Cell Death Dis. 2021;12:1009.
38. Kobayashi Y, Ohfuji S, Kondo K, et al. Association between dietary iron and zinc intake and development of ulcerative colitis: A case-control study in Japan. J Gastroenterol Hepatol. 2019;34:1703-10.
39. Millar AD, Rampton DS, Blake DR. Effects of iron and iron chelation in vitro on mucosal oxidant activity in ulcerative colitis. Aliment Pharmacol Ther. 2000;14:1163-8.
40. Liu JZ, van Sommeren S, Huang H, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet. 2015;47:979-86.
41. Safe S, Jayaraman A, Chapkin RS. Ah receptor ligands and their impacts on gut resilience: structure-activity effects. Crit Rev Toxicol. 2020;50:463-73.
42. Yoshimatsu Y, Sujino T, Miyamoto K, et al. Aryl hydrocarbon receptor signals in epithelial cells govern the recruitment and location of Helios(+) Tregs in the gut. Cell Rep. 2022;39:110773.
43. Rouse M, Singh NP, Nagarkatti PS, et al. Indoles mitigate the development of experimental autoimmune encephalomyelitis by induction of reciprocal differentiation of regulatory T cells and Th17 cells. Br J Pharmacol. 2013;169:1305-21.
44. Singh NP, Singh UP, Singh B, et al. Activation of aryl hydrocarbon receptor (AhR) leads to reciprocal epigenetic regulation of FoxP3 and IL-17 expression and amelioration of experimental colitis. PLoS One. 2011;6:e23522.
45. Quintana FJ, Basso AS, Iglesias AH, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature. 2008;453:65-71.
46. Denison MS, Faber SC. And Now for Something Completely Different: Diversity in Ligand-Dependent Activation of Ah Receptor Responses. Curr Opin Toxicol. 2017;2:124-31.
47. Miao W, Hu L, Scrivens PJ, et al. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. J Biol Chem. 2005;280:20340-8.
48. Lebwohl MG, Stein Gold L, Strober B, et al. Phase 3 Trials of Tapinarof Cream for Plaque Psoriasis. N Engl J Med. 2021;385:2219-29.
49. Lillig CH, Berndt C, Holmgren A. Glutaredoxin systems. Biochim Biophys Acta. 2008;1780:1304-17.
50. Forcina GC, Dixon SJ. GPX4 at the Crossroads of Lipid Homeostasis and Ferroptosis. Proteomics. 2019;19:e1800311.
51. Dodson M, Castro-Portuguez R, Zhang DD. NRF2 plays a critical role in mitigating lipid peroxidation and ferroptosis. Redox Biol. 2019;23:101107.
52. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. Trends Cell Biol. 2016;26:165-76.
53. Kagan VE, Mao G, Qu F, et al. Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. Nat Chem Biol. 2017;13:81-90.
54. Schreiner P, Martinho-Grueber M, Studerus D, et al. Nutrition in Inflammatory Bowel Disease. Digestion. 2020;101 Suppl 1:120-35.
55. de Silva PS, Olsen A, Christensen J, et al. An association between dietary arachidonic acid, measured in adipose tissue, and ulcerative colitis. Gastroenterology. 2010;139:1912-7.
56. Tjonneland A, Overvad K, Bergmann MM, et al. Linoleic acid, a dietary n-6 polyunsaturated fatty acid, and the aetiology of ulcerative colitis: a nested case-control study within a European prospective cohort study. Gut. 2009;58:1606-11.
57. Sido B, Hack V, Hochlehnert A, et al. Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. Gut. 1998;42:485-92.
58. Ozawa K, Mori D, Hatanaka A, et al. Comparison of the anti-colitis activities of Qing Dai/Indigo Naturalis constituents in mice. J Pharmacol Sci. 2020;142:148-56.
59. Stasiak N, Kukuła-Koch W, Głowniak K. Modern industrial and pharmacological applications of indigo dye and its derivatives--a review. Acta Pol Pharm. 2014;71:215-21.
60. Xu C, Sun S, Johnson T, et al. The glutathione peroxidase Gpx4 prevents lipid

peroxidation and ferroptosis to sustain Treg cell activation and suppression of antitumor immunity. Cell Rep. 2021;35:109235.
61. Feuerstein JD, Isaacs KL, Schneider Y, et al. AGA Clinical Practice Guidelines on the Management of Moderate to Severe Ulcerative Colitis. Gastroenterology. 2020;158:1450-61.