 doi:10.20944/preprints202204.0042.v1

*Communication*

# The Gut-*Ex-Vivo* System (GEVS) is a dynamic and versatile tool for the study of DNBS-induced IBD in BALB/C and C57BL/6 mice

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**Simple Summary:** IBD is considered a modern and western diet-related disease characterized by uncontrolled immune activation resulting in chronic bowel inflammation and tissue damage. Although the precise causes responsible for the onset of the disease are still elusive, it seems that both the environment, genetic predisposition and the dysregulation of the intestinal microbiota are actively involved. The development of a model to study the etiopathology of this disease characterized by an increasing incidence in the population is urgently needed. We have recently provided the evolution of an organ-on-chip system (Gut-Ex-Vivo System, GEVS) to model the IBD induced by the chemical molecule DNBS, in the colon of mice of the BALB / c strain. Here we have provided data demonstrating that the process can also be efficiently induced in mice of another strain, the C57BL / c which is usually less sensitive to this treatment, using our GEVS. Furthermore, we have shown that the system also summarizes other characteristics of human pathology, such as the induction of the two most represented cell death pathways responsible for the tissue damage characteristic of IBD.

**Abstract**

**Background:** IBD is a spectrum of pathologies characterized by dysregulated immune activation leading to uncontrolled response against intestinal, thus resulting in chronic gut inflammation and tissue damage. Due to its complexity, the molecular mechanisms responsible for disease onset and progression are still elusive, thus requiring intense research effort. In this context, the development of models recapitulating the etiopathology of IBD is critical.

**Methods:** Colon from C57BL/6 or BALB/c mice were cultivated in a gut-*ex-vivo* system (GEVS), exposed 5h to DNBS 1,5 or 2,5 mg/ml, and the main hallmarkers of IBD were evaluated.

**Results:** Gene expression analysis revealed a DNBS-induced: i) compromised Tight junction organization, responsible for tissue permeability dysregulation; induction of ER stress, and iii) tissue inflammation in colon of C57BL/6 mice. Moreover, the concomitant DNBS-induced apoptosis and ferroptosis pathways was evident in colon from both BALB/c and C57BL/6 mice.

 doi:10.20944/preprints202204.0042.v1

**Conclusions:** Overall, we have provided results demonstrating that GEVS is a consistent, reliable, and cost-effective system for modeling DNBS-induced IBD, useful for studying the onset and progression of human disease at the molecular level, while also reducing animal suffering.
**Keywords:** IBD; UC; UPR; *ex vivo* organ; DNBS; Gut-*Ex-Vivo* System; GEVS; ferroptosis; apoptosis

## 1. Introduction

Gut inflammation is a feature shared by several human diseases specifically affecting the gastrointestinal tract, such as Celiac Disease[1], Cystic Fibrosis[2] and Inflammatory Bowel Disease[3]. However, mounting evidence points to the role of inflammation in the pathogenesis of other human diseases, which apparently do not directly involve the gut, such as Parkinson's Disease[4] and Type 2 Diabetes[5]. Moreover, the role of microbiota in gut inflammation associated to the above-mentioned diseases is still unclear, deeply investigated and highly debated, with gut dysbiosis can potentially represent a new frontier in the clinical treatment of those patients.

In this scenario, the availability of tools to model the pathogenesis of such diseases is a critical point. Although tissues/cells directly derived from patients represents the best option for molecular studies, to test new potential therapeutic agents and design a personalized therapy, their availability and limited survival *in vitro* often represent the main limitations.

The most widely used models in these studies are 2D cell cultures, which are the most available, cost effective and easy to use models. However, they are characterized by several limitations due to the complete lack of tissue architecture and typical cell-cell and cell-ECM communication. Moreover, they usually involve the use of immortalized or transformed cells, which potentially impact on results[6]. An evolution of these models is represented by the 3D cell cultures, such as spheroids and organoids. While the first model can provide some characteristics of physiological conditions such as the 3D structure and the deposition of ECM, it also represents a too simple model compared to a tissue/organ condition, although cost effective and relatively easy to generate and manipulate[7]. On the other hand, organoids represent a consistent step forward in the generation of a model that closely represents a real tissue/organ[8]. However, organoids are difficult to generate, time consuming, expensive, and difficult to standardize[6]. Animal models are major source for studying human disease pathologies, with mice representing the most used species. They indubitably represent the best model when an animal model can be generated to recreate human pathology, the latter thus representing the main limitation. Of note, other limits are represented by obvious physiological and morphological differenced between human and mice. This is why not all diseases can be efficiently modeled in mice. In this context, several animal models and protocols have been developed to study and to mimic IBD, such as those using Dextran sulfate sodium (DSS)[9], 2,4,6-trinitrobenzenesulfonic acid (TNBS)[10] or dinitrobenzene sulfonic acid (DNBS)[11]. These models well recapitulate the pathogenesis of human IBD and are widely used, although they are not without animal suffering. Similarly to other animal models, they also have a number of disadvantages including the overall of experiments, and costs of animal purchase and maintenance. Moreover, animal models are intrinsically subjected to biological variations based on both genetic and nongenetic components. Indeed, even genetic littermates are not identical and can be differently affected by environmental conditions and social hierarchy. Altogether, these variations affect experimental results and their impact can be limited increasing the overall number of animals to include in each research group. Therefore, in a context of a continuous and increased demand in the reduction in the number of animals in scientific experimentation and reduction of animal suffering, the development of alternative models is required.

We recently described an evolution of an organ on chip model originally developed by Yissachar and colleagues[12], as a Gut-Ex-Vivo System (GEVS) to study both Celiac

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Disease[13] and IBD[3], providing evidences showing the consistency and reliability of the system, together with the reduced animal suffering and number. In particular, we demonstrated the ability of the system to model the pathogenesis of DNBS-induced IBD in colon from BALB/c mice.

Here we show that GEVS can also be used to model DNBS-induced IBD pathogenesis in colon from C57BL/6 mice and that both apoptotic and ferroptotic cell death pathways characteristic of the human pathology are recapitulated in both animal strains.

## 2. Materials and Methods

### *2.1 Reagents and Materials*

DNBS (2,4-Dinitrobenzenesulfonic acid hydrate; CAS Number: 698999-22-3), poly(dimethylsiloxane) (PDMS; Sylgard 184 Elastomer base) were from Merck; IMDM (Iscove's Modified Dulbecco's Medium), KnockOut serum replacement, B-27, N-2 supplements, L-glutamine, non-essential amino acids (NEAA) and HEPES were from GIBCO; Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Penicillin/Streptomycin were from Euroclone; TripleXtractor was from GRiSP; ExcelRT Reverse Transcriptase and Excel-Taq FAST qPCR SybrGreen were from SMOBIO; oligonucleotide based primers were from IDT.

### *2.2 Silicone-based device and colon culture*

Colons were freshly isolated from 13 days old BALB/c mice and cultivated in a silicone-based GEVS (Gut *Ex-Vivo* System), as previously described[3,13]. Briefly, each device consists of 6 parallel isolated chambers to allocate mouse colon (up to six). Colon lumens are infused with a complete medium, through a coordinated infusing-drying pump. Each chamber is filled with complete medium (outer medium) to maintain tissue viability, in a static condition. A lab hot plate is used to maintain a temperature of 37°C, during each experiment, while a mixture of 5% CO2 and 95% O2 is provided to the device from a compressed gas cylinder[3,12,13].

### *2.3 Colon cultures and treatments*

Each colon section was infused with serum-free tissue culture medium containing IMDM, supplemented with 20% KnockOut serum replacement (Gibco), 2% B-27 and 1% of N-2 supplements, 1% L-glutamine, 1% NEAA, 1% HEPES and stimulated with DNBS (1,5 – 2,5mg/ml).

The tissue culture medium was loaded into 5ml syringe and infused into the device input ports by a syringe pump (flow rate of 99ul/h)[3,13].

### *2.4 Quantitative PCR (qPCR)*

Total RNA was isolated by using TripleXtractor reagent and ExcelRT Reverse Transcriptase was used to produce cDNA, by using 2µg of total RNA. Quantitative PCR (qPCR) reactions were performed by using the Excel-Taq FAST qPCR SybrGreen and a CFX96 thermocycler (Bio-Rad). Primers sequences were designed by using the online IDT PrimerQuest Tool software (IDT; https://eu.idtdna.com/ Primerquest/Home/Index), and sequences reported below.

GAPDH mRNA level was used as an internal control, and comparative Ct method (ΔΔCt) was used for relative quantification of gene expression[14].

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| | |
|---|---|
| ATF4_F | GTTTAGAGCTAGGCAGTGAAG |
| ATF4_R | CCTTTACACATGGAGGGATTAG |
| ATF6_F | GATGGTGACAACCAGAAAGA |
| ATF6_R | TGGAGGTGGAGGCATATAA |
| XBP1_F | AGTCCGCAGCAGGTG |
| XBP1_R | GGTCCAACTTGTCCAGAATG |
| CLD-2_F | CCTCGCTGGCTTGTATTATC |
| CLD-2_R | AAAGACTCCACCCACTACA |
| CLD-15_F | GGGACCCTCCACATACTT |
| CLD-15_R | CATACTTGGTTCCAGCATACA |
| OCL_F | TCTTTGGAGGAAGCCTAAAC |
| OCL_R | CTGCTCTTGGGTCTGTATATC |
| IFNγ_F | CCACATCTATGCCACTTGAG |
| IFNγ_R | CTCTTCCTCATGGCTGTTTC |
| TNFα_F | CCTCCCTCTCATCAGTTCTAT |
| TNFα_R | ACTTGGTGGTTTGCTACG |
| IL-10_F | TGAATTCCCTGGGTGAGA |
| IL-10_R | CCACTGCCTTGCTCTTATT |
| BAX_F | GGTTGCCCTCTTCTACTTTG |
| BAX_R | AGTGTCCAGCCCATGAT |
| PUMA_F | GAGGGTCATGTACAATCTCTTC |
| PUMA_R | CTAGTTGGGCTCCATTTCTG |
| NOXA_F | CAGGAAGATCGGAGACAAAG |
| NOXA_R | CACACTCGTCCTTCAAGTC |
| CHAC1_F | TCACAGCACTGGCCTAT |
| CHAC1_R | CAAGGTTGTGACCAGAGAAG |
| PTGS2_F | GCCTGGTCTGATGATGTATG |
| PTGS2_R | GTCTGCTGGTTTGGAATAGT |
| ACSL4_F | TAAGCCCAXTTCAGACAAAC |
| ACSL4_R | GGCTACAGCATGGTCAAATA |
| TRF1_F | GGGCTATTGTAAGCGTGTAG |
| TRF1_R | CCTCTGTTTCCATGGTTTCT |
| GAPDH_F | TTCAACGGCACAGTCAAG |
| GAPDH_R | CCAGTAGACTCCACGACATA |

### *2.5 Statistical analysis*

Experiments were performed in triplicate and repeated at least three times, and statistical analysis was performed using GraphPad software (GraphPad Software; GraphPad Prism 6). Student's t test or ANOVA was used to determine statistical significance.

A p-value of equal to or less than 0.05 was considered significant. mRNA expression levels were represented as 'fold change over control', r.l. relative levels. Histograms represent mean ± SD; **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns non-significant.

## 3. Results

We recently demonstrated the chance of using an organ *ex vivo* system (Gut-*Ex-Vivo* System or GEVS) in parallel or in alternative to animal models to study the onset and progression of human diseases characterized by gut inflammation, such as Celiac Disease or IBD [3,13]. Indeed, in the case of IBD we designed a method consisting in the isolation of colon from BALB/c mice, and cultivation in our GEVS in presence of DNBS (Fig.1).

We demonstrated that 5h of exposure to DNBS is sufficient to: i) deregulate colon permeability through Tight Junction (TJ) proteins expression deregulation, ii) induce an ER stress response, iii) up-regulate pro-inflammatory cytokines and down-regulate anti-

 doi:10.20944/preprints202204.0042.v1

inflammatory cytokines, resulting in inflammation and tissue damage, all typical Ulcerative Colitis (UC) signs[3].

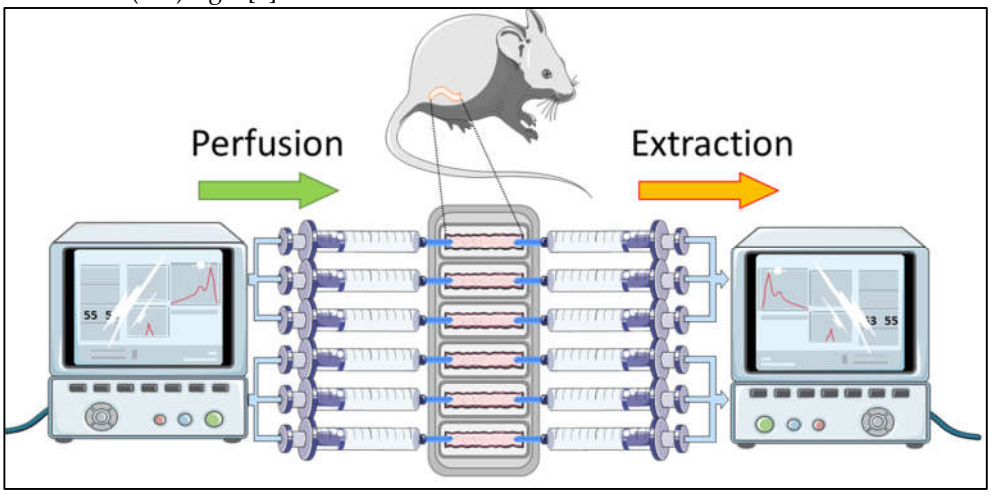


**Figure 1. Gut-Ex-Vivo System – GEVS – and IBD induction.** Schematic representation of a GEVS, in which mouse colon are cultivated in a dynamic condition and stimulated 5h with DNBS, to stimulate the onset of UC [Gagliardi, Biology 2021].

We, therefore, decided to expand the use of our GEVS to study the DNBS-induced UC in colon derived from the C57BL/6 mouse strain, notoriously less sensitive compared to the widely used BALB/c strain. We also evaluated the ability of our system and method to recapitulate the two main cell death pathways known to be responsible for epithelial cell demise upon UC onset/progression, such as apoptosis[15] and the recently described ferroptosis[16].

*3.1. DNBS triggers UC in the colon from C57BL/6 mice by using a Gut-Ex-Vivo System (GEVS)*

Colon freshly explanted from C57BL/6 mice were cultivated in a Gut-Ex-Vivo System as previously described[3]. UC was induced by exposing organs to 0, 1,5 or 2,5 mg/ml of DNBS, in the infusing medium, for 5 hours. Tissue barrier efficiency was evaluated by measuring the expression of TJ proteins. Data reported in figure 2 clearly show an imbalanced expression of these factors, as evidenced by down-regulated occludin (Fig.2B) and up-regulated expression of both claudin-2 (Fig.2C) and claudin-15 (Fig.2D), in a dose-dependent manner[1,3,13], thus indicating a compromised barrier function. These data are perfectly overlapping those previously obtained by using colon from the BALB/c mouse strain[3].

Next, we evaluated the ability of DNBS to stimulate the ER stress response, as previously evidenced in colon from BALB/c mice, in the same experimental conditions[3]. To this aim, the expression of the well-characterized ER stress markers ATF4, ATF6 and XBP1s were evaluated at mRNA level. Data shown in figure 3A indicate a DNBS-dependent induction of ER stress, in a dose-dependent manner.

Finally, the expression of the pro-inflammatory cytokines IFNγ and TNFα together with the anti-inflammatory IL-10 were evaluated at mRNA level, in the same experimental conditions. As reported in figure 3B, a clear dose-dependent up-regulation of pro-inflammatory and down-regulation of anti-inflammatory cytokines were evident in tissues exposed to DNBS, compared to untreated controls.

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Collectively, these data are similar to those previously obtained stimulating BALB/c derived colon tissues with DNBS, in the same experimental conditions[3].

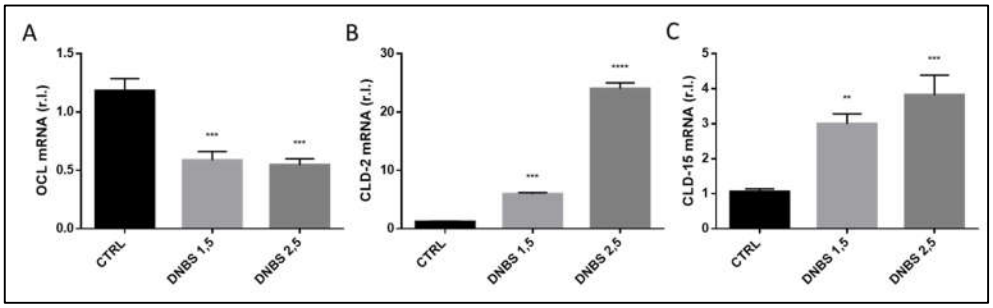


**Figure 2. DNBS-induced impaired tissue permeability.** Colon from C57BL/6 were cultivated 5h in a GEVS and untreated (CTRL) or treated with the indicated concentrations of DNBS and tissue permeability was evaluated by measuring the mRNA levels of Tight Junction (TJ) components Occludin (OCL, A), Claudin-2 (CLD2, B) or Claudin-15 (CLD15, C), by qPCR. Histograms represent mean ± SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01.

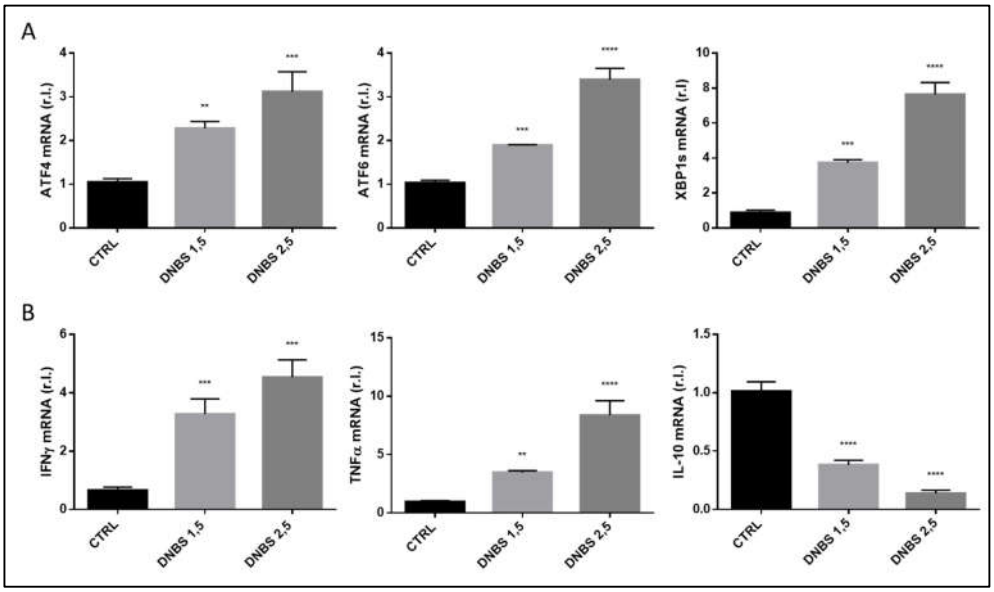


**Figure 3. ER Stress and inflammation.** Colon from C57BL/6 were cultivated 5h in a GEVS and untreated (CTRL) or treated with the indicated concentrations of DNBS and the expression levels of the ER stress markers ATF4, ATF6 or XBP1s were evaluated by qPCR. The upregulation of the pro-inflammatory cytokines IFNγ and TNFα or the anti-inflammatory cytokine IL-10 were evaluated in the same experimental condition of A, by qPCR. Histograms represent mean ± SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01.

*3.2 Apoptotic and Ferroptotic cell death modalities associated to DNBS-induced UC can be studied by using a GEVS, in colon from both C57BL/6 and BALB/c mice*

Ulcerative Colitis is characterized by progressive colonic epithelial cell demise due to consistent cell death induction, mainly through the induction of the apoptotic process[17]. To verify the induction of apoptosis in colon cultivated in our Gut-*Ex-Vivo* System and exposed to DNBS (5h), we evaluated the expression of key pro-apoptotic markers such as BAX, PUMA and NOXA[18–20], at mRNA level. Data reported in figure 4 show a clear induction of the apoptotic markers described above, in a dose-dependent manner, and perfectly overlapping in the two mouse strains.

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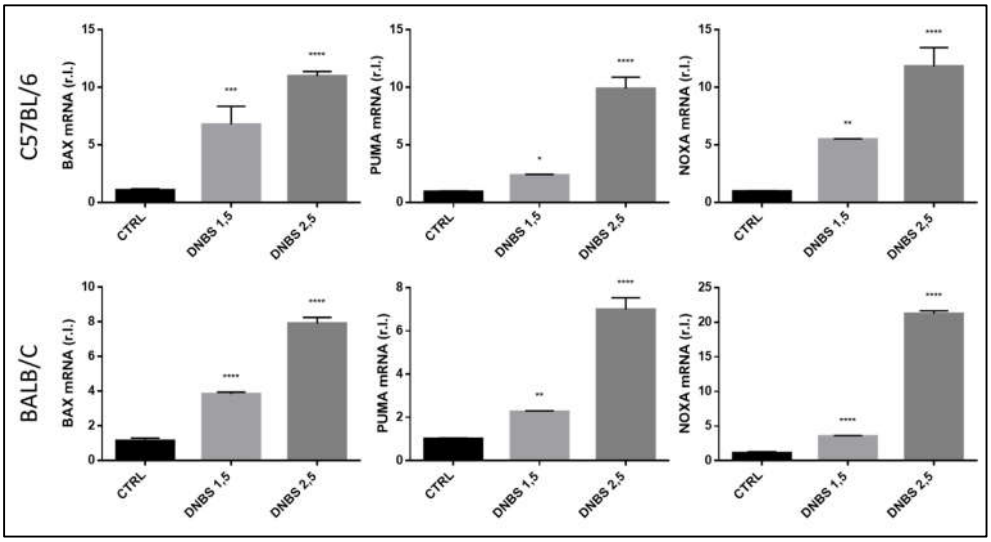


**Figure 4. Apoptosis induction.** Colon from C57BL/6 or BALB/c mice were cultivated 5h in a GEVS and untreated (CTRL) or treated with the indicated concentrations of DNBS and the expression levels of BAX and the two BH3-only proteins PUMA and NOXA, well-known pro-apoptotic markers, were evaluated by qPCR. Histograms represent mean ± SD of triplicate sample; **** p<0.0001; *** p<0.001; ** p<0.01.

Furthermore, it has been proposed that a recently described new form of cell death called ferroptosis may contribute to the demise of epithelial cells associated with UC[16]. To verify the involvement of this cell death signaling pathway in colon exposed to DNBS in our GEVS, we evaluated the expression of key ferroptotic markers. To this aim, we observed a dose-dependent up-regulation of the early ferroptotic markers such as CHAC1[21,22] and PTGS2[23], together with the downstream markers ACSL4[24] and TRF1[25], in tissues from both C57BL/6 (Fig.5, upper panels) and BALB/c (Fig.5, bottom panels) mice, with no major differences.

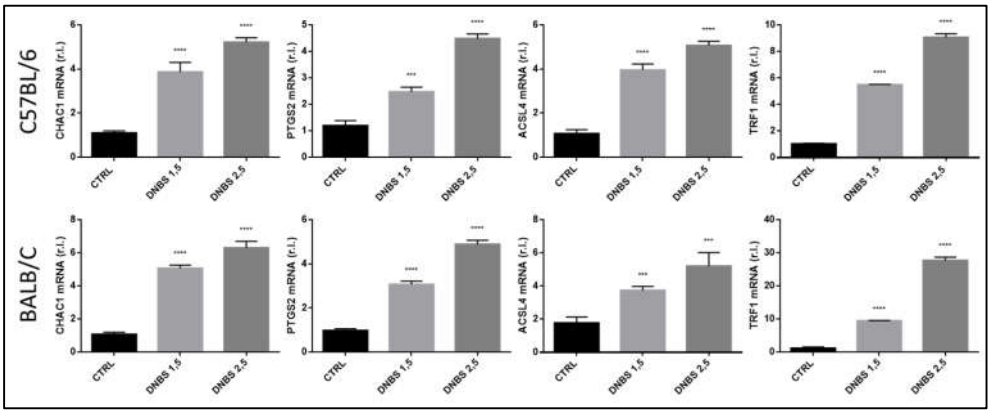


**Figure 5. Ferroptosis induction.** Colon from C57BL/6 (upper panels) or BALB/c (bottom panels) mice were cultivated 5h in a GEVS and untreated (CTRL) or treated with the indicated concentrations of DNBS and the expression levels of the well-known ferroptotic markers CHAC1, PTGS2, ACSL4 and TRF1 were evaluated by qPCR. Histograms represent mean ± SD of triplicate sample; **** p<0.0001; *** p<0.001.

## 4. Discussion

IBD is characterized by gut inflammation, tissue dysregulation and damage, representing a complex multifactorial systemic disease with both genetic and environmental components being involved in the onset and progression[26]. Due to its complexity, its etiology at molecular level and mechanisms are still elusive and under deep investigation.

 doi:10.20944/preprints202204.0042.v1

In this context, although biopsies directly derived by affected individuals remains the gold standard for research, also potentially ensuring a personalized clinical intervention, the very low amount of sample together with their limited viability require the development of alternative approaches. Although cell lines are widely used, they are intrinsically limited since they completely lack the complexity and physiology of tissues. Of note, several animal models have been described and are currently used in this field of research, such as mice exposed to DSS or DNBS, which well recapitulate the onset of the disease. However, animals are expensive and experiments require time. Moreover, the above-mentioned models are not without suffering for animals.

We recently described an organ *ex vivo* culture of colon from 13 days-old mice which can reproduce both morphological and molecular features of IBD induced in mice exposed to DNBS. In particular, we reported that colon from BALB/c mice can efficiently be stimulated for 5h with a range of concentrations of DNBS, ranging from 0,5 to 2,5 mg/ml, to induce the main hallmarkers of IBD, such as decreased tissue permeability and altered morphology, induction of pro-inflammatory cytokines, and down-regulation of IL-10, in a dose-dependent manner. Interestingly, we also found the induction of ER stress.

Here we reported the induction of IBD in colon from 13 days-old mice from a different strain, the C57BL/6. This is important because this strain has been described to be a bit resistant to DNBS stimulation *in vivo*, thus requiring a higher dose of drug[27,28]. Indeed, we found overlapping induction of main IBD markers in both C57BL/6 and BALB/c mice, by using DNBS at both 1,5 or 2,5 mg/ml. Moreover, we confirmed the induction of ER stress, a pro-inflammatory and stress-mediated signaling pathway, recently described as potentially involved in the etiology of IBD[29,30]. Although the precise role is still elusive, its Janus-faced activities, in sustaining cell survival and inducing cell demise through apoptosis induction[18,31], might represent a new potential therapeutic target. Further studies are therefore required to unveil the role of ER stress in the pathogenesis of IBD. In this context, the organ *ex vivo* system (GEVS) we provided represents a useful tool to explore this hypothesis at molecular level, together with the opportunity to test molecules/probiotics to inhibit inflammation and to restore physiological tissue conditions.

Indeed, cell demise responsible for gut tissue dysfunction and damage associated to prolonged inflammation of gut tissues upon IBD onset and progression has been reported to mainly relay on apoptosis execution [17]. We previously confirmed the induction of apoptosis in BALB/c colon exposed to DNBS by using our GEVS at both morphological and molecular levels [3]. Here we provide data confirming these results in BALB/c mice-derived colon at molecular level, as evidenced by dose-dependent up-regulation of pro-apoptotic factors such as BAX, PUMA and NOXA. Importantly, we have provided data showing that the same proapoptotic pathway is also induced in C57BL/c in the same experimental condition, reinforcing the concept related to the involvement of apoptosis in IBD, and also providing evidence of versatility and reliability of our organ *ex vivo* culture model.

The involvement of a new form of cell death, ferroptosis, has also been described to take part in the pathogenesis of IBD, very recently[16]. This is a non-apoptotic cell death modality which rely on the generation and intracellular accumulation of lipid peroxides, resulting in cell death[22]. Our results, obtained by using the gut-*ex-vivo* system – GEVS – confirmed the involvement of this new form of cell death in colon from both BALB/c and C57BL/6 mice exposed to DNBS, in a dose-dependent manner. Importantly, the magnitude of ferroptosis-related expression of markers is perfectly overlapping in the two mice strain-derived tissues, thus: i) reinforcing the conclusion that GEVS can be used to also study this aspect of IBD, in both animals, at molecular level, and ii) sustaining the active involvement of this new form of cell death in the pathogenesis of IBD.

Finally, collectively our results also clearly indicate a strong consistency of results obtained by our organ *ex vivo* culture system (GEVS), in terms of variability of results from different animals, an aspect that is not negligible when working with animals and which has a significant impact on the definition of number within each group of animals during the experimental design. Indeed, our approach allows to consistently reduce the total

number of animals to include in a study, thus overall resulting in: i) reduced experimental costs, ii) reduced length of experiments, and iii) reduced/no animal suffering. This last consideration is becoming of great interest in view of the increasing demand, by the scientific community, of constant reduction in numbers and animal suffering.

**Author Contributions:** Conceptualization, M.C.; methodology, R.M., M.G. N.C; formal analysis, M.C., R.M., M.G., V.S., E.P.; data curation, M.C.; writing—original draft preparation, M.C.; writing—review and editing, M.C.; supervision, M.C.; project administration, M.C.; funding acquisition, M.C. All authors have read and agreed to the published version of the manuscript."

**Funding:** This work was supported by the Italian Ministry of Education, University and Research (MIUR) program "Departments of Excellence 2018-2022", FOHN Project—Department of Health Sciences, Università del Piemonte Orientale. The support of FAR 2019 (Progetti di Ateneo), the EU grant "PREMUROSA" (ID#860462), "ExcellMater" (ID #952033) H2020 projects, and AGING Project – Department of Excellence – DIMET are also acknowledged. E.P. was supported by H2020 EU grant (PREMUROSA).

**Institutional Review Board Statement:** All procedures were approved by the local Ethics Committee for Animal Welfare (IACUC No 178/219-PR) and conformed to the European Community regulations for animal use in research (2010/63 UE).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data is contained within the article.

**Conflicts of Interest:** "The authors declare no conflict of interest."

1. Ferrari, E.; Monzani, R.; Saverio, V.; Gagliardi, M.; Pańczyszyn, E.; Raia, V.; Villella, V.R.; Bona, G.; Pane, M.; Amoruso, A.; et al. Probiotics Supplements Reduce ER Stress and Gut Inflammation Associated with Gliadin Intake in a Mouse Model of Gluten Sensitivity. *Nutrients* **2021**, *13*, doi:10.3390/NU13041221.
2. Tam, R.Y.; van Dorst, J.M.; McKay, I.; Coffey, M.; Ooi, C.Y. Intestinal Inflammation and Alterations in the Gut Microbiota in Cystic Fibrosis: A Review of the Current Evidence, Pathophysiology and Future Directions. *J Clin Med* **2022**, *11*, doi:10.3390/JCM11030649.
3. Gagliardi, M.; Monzani, R.; Clemente, N.; Fusaro, L.; Saverio, V.; Grieco, G.; Pańczyszyn, E.; Yissachar, N.; Boccafoschi, F.; Corazzari, M. A Gut-Ex-Vivo System to Study Gut Inflammation Associated to Inflammatory Bowel Disease (IBD). *Biology (Basel)* **2021**, *10*, doi:10.3390/BIOLOGY10070605.
4. Li, Y.; Chen, Y.; Jiang, L.; Zhang, J.; Tong, X.; Chen, D.; Le, W. Intestinal Inflammation and Parkinson's Disease. *Aging Dis* **2021**, *12*, 2052–2068, doi:10.14336/AD.2021.0418.
5. Bessac, A.; Cani, P.D.; Meunier, E.; Dietrich, G.; Knauf, C. Inflammation and Gut-Brain Axis During Type 2 Diabetes: Focus on the Crosstalk Between Intestinal Immune Cells and Enteric Nervous System. *Front Neurosci* **2018**, *12*, doi:10.3389/FNINS.2018.00725.
6. Jensen, C.; Teng, Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci* **2020**, *7*, doi:10.3389/FMOLB.2020.00033.
7. Decarli, M.C.; Amaral, R.; Santos, D.P. dos; Tofani, L.B.; Katayama, E.; Rezende, R.A.; Silva, J.V.L. da; Swiech, K.; Suazo, C.A.T.; Mota, C.; et al. Cell Spheroids as a Versatile Research Platform: Formation Mechanisms, High Throughput Production, Characterization and Applications. *Biofabrication* **2021**, *13*, doi:10.1088/1758-5090/ABE6F2.
8. Kakni, P.; Truckenmüller, R.; Habibović, P.; Giselbrecht, S. Challenges to, and Prospects for, Reverse Engineering the Gastrointestinal Tract Using Organoids. *Trends Biotechnol* **2022**, doi:10.1016/J.TIBTECH.2022.01.006.

9. Eichele, D.D.; Kharbanda, K.K. Dextran Sodium Sulfate Colitis Murine Model: An Indispensable Tool for Advancing Our Understanding of Inflammatory Bowel Diseases Pathogenesis. *World J Gastroenterol* **2017**, *23*, 6016–6029, doi:10.3748/WJG.V23.I33.6016.
10. Antoniou, E.; Margonis, G.A.; Angelou, A.; Pikouli, A.; Argiri, P.; Karavokyros, I.; Papalois, A.; Pikoulis, E. The TNBS-Induced Colitis Animal Model: An Overview. *Ann Med Surg (Lond)* **2016**, *11*, 9–15, doi:10.1016/J.AMSU.2016.07.019.
11. Barone, M.; Chain, F.; Sokol, H.; Brigidi, P.; Bermúdez-Humarán, L.G.; Langella, P.; Martín, R. A Versatile New Model of Chemically Induced Chronic Colitis Using an Outbred Murine Strain. *Front Microbiol* **2018**, *9*, doi:10.3389/FMICB.2018.00565.
12. Yissachar, N.; Zhou, Y.; Ung, L.; Lai, N.Y.; Mohan, J.F.; Ehrlicher, A.; Weitz, D.A.; Kasper, D.L.; Chiu, I.M.; Mathis, D.; et al. An Intestinal Organ Culture System Uncovers a Role for the Nervous System in Microbe-Immune Crosstalk. *Cell* **2017**, *168*, 1135-1148.e12, doi:10.1016/J.CELL.2017.02.009.
13. Gagliardi, M.; Clemente, N.; Monzani, R.; Fusaro, L.; Ferrari, E.; Saverio, V.; Grieco, G.; Pańczyszyn, E.; Carton, F.; Santoro, C.; et al. Gut-Ex-Vivo System as a Model to Study Gluten Response in Celiac Disease. *Cell Death Discov* **2021**, *7*, 45, doi:10.1038/s41420-021-00430-2.
14. Giglio, P.; Gagliardi, M.; Tumino, N.; Antunes, F.; Smaili, S.; Cotella, D.; Santoro, C.; Bernardini, R.; Mattei, M.; Piacentini, M.; et al. PKR and GCN2 Stress Kinases Promote an ER Stress-Independent EIF2$\alpha$ Phosphorylation Responsible for Calreticulin Exposure in Melanoma Cells. *OncoImmunology* **2018**, doi:10.1080/2162402X.2018.1466765.
15. Wan, Y.; Yang, L.; Jiang, S.; Qian, D.; Duan, J. Excessive Apoptosis in Ulcerative Colitis: Crosstalk Between Apoptosis, ROS, ER Stress, and Intestinal Homeostasis. *Inflamm Bowel Dis* **2021**, doi:10.1093/IBD/IZAB277.
16. Xu, M.; Tao, J.; Yang, Y.; Tan, S.; Liu, H.; Jiang, J.; Zheng, F.; Wu, B. Ferroptosis Involves in Intestinal Epithelial Cell Death in Ulcerative Colitis. *Cell Death Dis* **2020**, *11*, doi:10.1038/S41419-020-2299-1.
17. Nunes, T.; Bernardazzi, C.; De Souza, H.S. Cell Death and Inflammatory Bowel Diseases: Apoptosis, Necrosis, and Autophagy in the Intestinal Epithelium. *Biomed Res Int* **2014**, *2014*, doi:10.1155/2014/218493.
18. Pagliarini, V.; Giglio, P.; Bernardoni, P.; Zio, D.D.; Fimia, G.M.; Piacentini, M.; Corazzari, M. Downregulation of E2F1 during ER Stress Is Required to Induce Apoptosis. *Journal of Cell Science* **2015**, *128*, doi:10.1242/jcs.164103.
19. Finucane, D.M.; Bossy-Wetzel, E.; Waterhouse, N.J.; Cotter, T.G.; Green, D.R. Bax-Induced Caspase Activation and Apoptosis via Cytochrome c Release from Mitochondria Is Inhibitable by Bcl-XL. *J Biol Chem* **1999**, *274*, 2225–2233, doi:10.1074/JBC.274.4.2225.
20. Qiu, W.; Wu, B.; Wang, X.; Buchanan, M.E.; Regueiro, M.D.; Hartman, D.J.; Schoen, R.E.; Yu, J.; Zhang, L. PUMA-Mediated Intestinal Epithelial Apoptosis Contributes to Ulcerative Colitis in Humans and Mice. *J Clin Invest* **2011**, *121*, 1722–1732, doi:10.1172/JCI42917.
21. Gagliardi, M.; Cotella, D.; Santoro, C.; Corà, D.; NA, B.; Piacentini, M.; M., C. Aldo-Keto Reductases Protect Metastatic Melanoma from ER Stress-Independent Ferroptosis. *Cell Death Dis.* **2019**, *10*, doi:10.1038/s41419-019-2143-7.
22. Gagliardi, M.; Saverio, V.; Monzani, R.; Ferrari, E.; Piacentini, M.; Corazzari, M. Ferroptosis: A New Unexpected Chance to Treat Metastatic Melanoma? *Cell Cycle* **2020**, *19*, 2411–2425, doi:10.1080/15384101.2020.1806426.
23. WS, Y.; KJ, K.; Gaschler MM, et al. Peroxidation of Polyunsaturated Fatty Acids by Lipoxygenases Drives Ferroptosis. *Proc Natl Acad Sci U S A.* **2016**, *113*, E4966-75, doi:10.1073/pnas.1603244113.
24. Doll, S.; Proneth, B.; Tyurina, Y.Y.; Panzilius, E.; Kobayashi, S.; Ingold, I.; Irmler, M.; Beckers, J.; Aichler, M.; Walch, A.; et al. ACSL4 Dictates Ferroptosis Sensitivity by Shaping Cellular Lipid Composition. *Nature Chemical Biology* **2017**, *13*, 91–98, doi:10.1038/nchembio.2239.

25. Feng, H.; Schorpp, K.; Jin, J.; Yozwiak, C.E.; Hoffstrom, B.G.; Decker, A.M.; Rajbhandari, P.; Stokes, M.E.; Bender, H.G.; Csuka, J.M.; et al. Transferrin Receptor Is a Specific Ferroptosis Marker. *Cell Rep* **2020**, *30*, 3411-3423.e7, doi:10.1016/J.CELREP.2020.02.049.
26. Mentella, M.C.; Scaldaferri, F.; Pizzoferrato, M.; Gasbarrini, A.; Miggiano, G.A.D. Nutrition, IBD and Gut Microbiota: A Review. *Nutrients* **2020**, *12*, doi:10.3390/NU12040944.
27. Leung, G.; Petri, B.; Reyes, J.L.; Wang, A.; Iannuzzi, J.; McKay, D.M. Cryopreserved Interleukin-4-Treated Macrophages Attenuate Murine Colitis in an Integrin B7 - Dependent Manner. *Mol Med* **2016**, *21*, 924–936, doi:10.2119/MOLMED.2015.00193.
28. Reardon, C.; Wang, A.; McKay, D.M. Transient Local Depletion of Foxp3+ Regulatory T Cells during Recovery from Colitis via Fas/Fas Ligand-Induced Death. *J Immunol* **2008**, *180*, 8316–8326, doi:10.4049/JIMMUNOL.180.12.8316.
29. Cao, S.S. Epithelial ER Stress in Crohn's Disease and Ulcerative Colitis. *Inflamm Bowel Dis* **2016**, *22*, 984–993, doi:10.1097/MIB.0000000000000660.
30. Ma, X.; Dai, Z.; Sun, K.; Zhang, Y.; Chen, J.; Yang, Y.; Tso, P.; Wu, G.; Wu, Z. Intestinal Epithelial Cell Endoplasmic Reticulum Stress and Inflammatory Bowel Disease Pathogenesis: An Update Review. *Front Immunol* **2017**, *8*, doi:10.3389/FIMMU.2017.01271.
31. Corazzari, M.; Gagliardi, M.; Fimia, G.M.; Piacentini, M. Endoplasmic Reticulum Stress, Unfolded Protein Response, and Cancer Cell Fate. *Frontiers in Oncology* **2017**, *7*, doi:10.3389/fonc.2017.00078.