Journal Pre-proof

Identification of potential visceral pain biomarkers in colon exudates from mice with experimental colitis: an exploratory in vitro study

Elsa Cisneros , Anabel Martínez-Padilla , Casimiro Cardenas , Javier Márquez , Arantxa Ortega de Mues , Carolina Roza

PII: S1526-5900(23)00009-3
DOI: https://doi.org/10.1016/j.jpain.2023.01.001
Reference: YJPAI 4224

To appear in: *Journal of Pain*

Received date: 21 October 2022
Revised date: 22 December 2022
Accepted date: 1 January 2023

Please cite this article as: Elsa Cisneros , Anabel Martínez-Padilla , Casimiro Cardenas , Javier Márquez , Arantxa Ortega de Mues , Carolina Roza , Identification of potential visceral pain biomarkers in colon exudates from mice with experimental colitis: an exploratory in vitro study, *Journal of Pain* (2023), doi: https://doi.org/10.1016/j.jpain.2023.01.001

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© Published by Elsevier Inc. on behalf of United States Association for the Study of Pain, Inc.

Journal Pre-proof

Identification of potential visceral pain biomarkers in colon exudates from mice with experimental colitis: an exploratory *in vitro* study

Elsa Cisneros*[2], Anabel Martínez-Padilla*[3], Casimiro Cardenas[3,4], Javier Márquez[3], Arantxa Ortega de Mues[5], Carolina Roza [#1]

*These authors contributed equally.

[1]Departamento de Biología de Sistemas. Facultad de Medicina. Universidad de Alcalá. Alcalá de Henares, Madrid, Spain.

[2]Facultad de Ciencias de la Salud. Universidad Internacional de la Rioja (UNIR). Logroño, La Rioja, Spain.

[3]Canceromics Lab. Departamento de Biología Molecular y Bioquímica. Facultad de Ciencias e Instituto de Investigación Biomédica de Málaga (IBIMA-Plataforma BIONAND). Universidad de Málaga. Málaga, Spain.

[4]Proteomics Unit. Central Facility of Research Infrastructures (SCAI). Universidad de Málaga, Málaga, Spain.

[5]Fundación Fujitega. Madrid, Spain.

[#]Corresponding author:
Carolina Roza
Dpto. Biología de Sistemas
Edificio de Medicina y Ciencias de la Salud, Campus Universitario
Universidad de Alcalá
Madrid 28871, Spain
Telephone: + 34 91 885 4595
E-mail: carolina.roza@uah.es

Journal Pre-proof

Short runni[1,2]**ng tittle:** Pain biomarkers in experimental colitis model.

Disclosures

This research was supported by grants from MINECO (SAF2016-77585-R), Universidad de Alcalá (CCG19/CCS-013 and GP2020-1) and Junta de Andalucía (UMA18-FEDERJA-082). The authors thank María del Carmen Gomez-García for preliminary analysis in STRING. The authors have no conflicts of interest to declare.

Highlights

- Human biomarkers might be detected in experimental models of pain.
- The proteome can be analyzed at the nociceptor peripheral terminal.
- Hp increases within colon after inflammation.
- Hp is locally released upon noxious distension of the colon.

Journal Pre-proof

Abstract

Chronic visceral pain (CVP) is extremely difficult to diagnose, and available analgesic treatment options are quite limited. Identifying the proteins secreted from the colonic nociceptors, or their neighbor cells within the tube walls, in the context of disorders that course with visceral pain, might be useful to decipher the mechanism involved in the establishment of CVP. Addressing this question in human with gastrointestinal disorders entails multiple difficulties, as there is not a clear classification of disease severity, and colonic secretion is not easy to manage.

We propose using of a murine model of colitis to identify new algesic molecules and pathways that could be explored as pain biomarkers or analgesia targets.

Descending colons from naïve and colitis mice with visceral hyperalgesia were excised and maintained *ex vivo.* The proteins secreted in the perfusion fluid before and during acute noxious distension were evaluated using high-resolution mass spectrometry (MS). Haptoglobin (Hp), PZD and LIM domain protein 3 (Pdlim3), NADP-dependent malic enzyme (Me1), and Apolipoprotein A-I (Apoa1) were increased during visceral insult, whilst Triosephosphate isomerase (Tpi1), Glucose-6-phosphate isomerase (Gpi1), Alpha-enolase (Eno1), and Isoform 2 of Tropomyosin alpha-1 chain (Tpm1) were decreased.

Journal Pre-proof

Most identified proteins have been described in the context of different chronic pain conditions and, according to gene ontology analysis, they are also involved in diverse biological processes of relevance.

Thus, animal models that mimic human conditions in combination with unbiased omics approaches will ultimately help to identify new pathophysiological mechanisms underlying pain that might be useful in diagnosing and treating pain.

Perspective

Our study utilizes an unbiased proteomic approach to determine, first, the clinical relevance of a murine model of colitis and, second, to identify novel molecules/pathways involved in nociception that would be potential biomarkers or targets for chronic visceral pain.

Keywords

Biomarker, Proteomics, Chronic visceral pain, Nociception, Preclinical

Journal Pre-proof

Introduction

Pain arising from the internal organs is the paradigm of chronic pain[27]. Specifically, gastrointestinal disorders, such as inflammatory bowel syndrome (IBS) and inflammatory bowel disorders (IBD), are the major causes of chronic visceral pain (CVP) since they affect 11% and 0.3 % of the global population, respectively[23,44]. Despite its high prevalence and morbidity, the pathophysiological mechanisms by which these conditions produce CVP are far from understood. Therefore, CVP is extremely difficult to diagnose and manage objectively, because commonly prescribed painkillers lack efficacy or are contraindicated due to gut-specific side effects.

During the last decade, enormous progress has been made toward identifying molecules and cellular pathways involved in chronic pain by using quantitative MS-based proteomics analyses . Databases of proteins involved in pain have been recently obtained by evaluating the proteome of primary afferents in several experimental murine models of inflammation, neuropathy, or post-operative pain[5,21,47,53]. Nevertheless, no such approach has been previously made for experimental visceral pain, despite the fact that proteomics is now a common approach to investigate disease-specific markers in plasma and serum . In fact, it is widely used in cardiovascular, cancer, and neurodegenerative research[30]. With regard to visceral pain, potential biomarkers were previously identified in serum and urine samples from patients with CVP[33,63].

Colonic nociceptors are preferentially activated by mechanical distension, and its sensitization (i.e., lowering of their activation threshold) depends on the synthesis and release of endogenous molecules that occurs in response to tissue insults, like inflammation in IBD, but it also takes place in the apparent absence of an underlying cause[28]. Furthermore, active nociceptors release vasoactive neuropeptides from their peripheral terminals that contribute to the inflammatory response, a mechanism called neurogenic inflammation[16,18,42]. Sensitization of peripheral terminals may explain why patients with visceral pain are hypersensitive to physiological contraction and distension of the gut[4,52], a characteristic of CVP[35].

Journal Pre-proof

Journal Pre-proof

Thus, the analysis of the proteins secreted from the colonic nociceptors, or their neighbor cells within the tube walls, in response to IBD and noxious mechanical stimuli might be helpful to identify biomarkers of CVP and hence, to gain insights into its underlying mechanisms.

Collection of the colonic secretion is not easily manageable in humans; however, it is possible in isolated preparations of the mouse colon[24,54]. In this exploratory study, we used unbiased quantitative MS-based proteomics to compare the protein profiles of colon exudates and urine samples of naïve and ulcerative colitis (a representative IBD model) mice before and after acute noxious distension. The protein hits whose levels changed significantly were compared to those identified in experimental models and clinical studies of visceral pain. Furthermore, levels of two protein hits, Hp and Tpm1 were validated by means of Western blotting performed in colon samples or exudates. Raw data are available and can be accessed via ProteomeXchange (PXD033902).

Journal Pre-proof

Methods

Animals

Adult outbred C57BL6 female mice (n = 16, mean body weight 20.22 ± 0.44 g) bred at the Animal Facility of the University of Alcalá were used. Animals were maintained at 22 ºC on a 12-hour light / dark cycle with *ad libitum* access to food and water. European Union and national legislations for the ethical regulation of animal experiments were always followed. All experimental protocols were approved by the University of Alcalá Committee on Animal Research and by the Regional Government (project license: PROEX 129/19).

Induction of experimental colitis and monitoring

Experimental colitis was induced by 2% Dextran Sulphate Sodium (DSS, molecular weight: 36.000–50.000, MP Biomedicals, Illkirch, France), which was added to the drinking water for 6 consecutive days[64]. Monitoring of the course and severity of the colitis was performed by daily body weight measurements, evaluation of stool consistency and/or presence of blood in the stools, and general well-being (see Figure 1 for workflow). To obtain a combined sum score, values were assigned for each category as follows: weight loss: 0, 2, 5, and 10 points (no changes, 5-10%, >10%, and > 20% loss, respectively); stools: 0, 5, 10, and 20 points (for normal, pasty, diarrhea, and presence of blood, respectively); and general well-being: 0, 1, 5, 10, and 20 points (for normal, minor, altered locomotion, lethargic, and apathy, respectively). The severity of the colitis was then graded according to the following scheme: 0-<5 points, no colitis; 5-15, mild colitis; 15-25, moderate colitis; and >25, severe colitis (adapted from[1]/5/2023 3:24:00 PM).

Referred hyperalgesia

Colitis-induced referred hyperalgesia was determined by measuring withdrawal responses to a punctate mechanical stimulation (von Frey hair filaments 0.02 to 2 g, 0.19 to 19.6 mN, Ugo Basile, Gemonio, Italy) of the abdomen, using the simplified up-down paradigm, SUDO[10]. Withdrawal responses were evaluated just before the induction of the colitis (day 1 pre-treatment, n = 10 mice) and upon removal of the DSS (day 7, see Figure 1A). Testing took place during the light phase (from 10:00 to 14:00). Mice were acclimatized, for at least 30 minutes, to test chambers consisting of transparent Plexiglas cages (10x10x10 cm, with a red

frontal wall) on an elevated wire mesh floor. The von Frey filaments were applied to the lower and middle abdomen avoiding perianal and external genitalia areas. A response was considered positive when any of the following behaviors were observed: immediate licking or scratching at the application site, a sharp retraction of the abdomen, or jumping[41].

Sampling of urine

Immediately after behavioral testing, the animals were anesthetized with isoflurane (~2% in pure O_2) and positioned supine. The urinary bladder was manually located and pressed gently to obtain ~100-200 μl of urine, which were collected with an automatic pipette and stored in a plastic test tube at -80ºC. Samples were obtained from 3 mice before induction of colitis (Day 1 pre-treatment) and 1 week after treatment with 2% DSS (Day 7, Figure 1A).

Experimental preparation for colon distension

Experiments were performed as previously described[54]. On day 7, after behavioral testing, the animals were killed in a CO_2 atmosphere, the descending colon was removed in one piece and placed in a dish filled with oxygenated (95% O_2, 5% CO_2) synthetic interstitial fluid (SIF; composition in mM: 108 NaCl, 3.48 KCl, 0.7 $MgSO_4$, 26 $NaHCO_3$, 1.7 NaH_2PO_4, 1.5 $CaCl_2$, 9.6 sodium gluconate, 5.55 glucose, 7.6 sucrose; pH 7.4)[11]. The stools and fecal remains were carefully removed by flushing SIF with a catheter connected to a syringe. Then, a fine plastic tubing (outside diameter 0.8 mm) filled with oxygenated SIF was inserted into one end of the colon segment, and surgical thread was used to tie it to the tubing and to ligate the other end. The mounted preparations were rinsed and equilibrated in SIF at 37 ºC for 30 min. Each preparation was sequentially incubated for 5 min into two test tubes filled with 1 ml of SIF in a shaking bath at 37ºC. Test tube 1 accounts for the pre-stimulus condition. Once transferred to test tube 2, colon preparations were connected to a mobile reservoir filled with oxygenated SIF and elevated above the preparation to exert a 60 mmHg pressure onto the samples (distension condition, Figure 1B). 60 mmHg is a suprathreshold stimulus able to activate colonic nociceptors[54]. The pressure was checked with a pressure transducer (CIBERTEC, Electromedicina, Madrid, Spain).

Journal Pre-proof

As soon as the colon preparation left the test tubes, the incubation fluid was subdivided into two aliquots of 500 μl that were immediately frozen in dry ice. Samples were stored at -80ºC until proteomic analysis.

Exudates were collected from naïve (n = 4) and 2% DSS- treated mice (n = 4). Two sets of experiments were performed; therefore, two controls and two treated colons were driven simultaneously.

Histological assessment of colitis severity

Mice were euthanized by CO_2 inhalation, and colonic fragments of ~0.5 cm were excised and fixed overnight by immersion in 4% PFA in 0.1 M phosphate buffer (PB) at 4ºC. Tissues were cryoprotected with 30% sucrose in PB, embedded in 7.5% gelatin /15 % sucrose in PB, frozen, and sectioned at 15 μm. The sections were stained with hematoxylin and eosin and observed by light microscopy (Olympus BX61, Tokyo, Japan). A blinded observer scored the H&E sections (3-5 for each colon) using the following values: crypt architecture (normal, 0 - severe crypt distortion with loss of entire crypts, 3), degree of inflammatory cell infiltration (normal, 0 - dense inflammatory infiltrate, 3), muscle thickening (base of crypt sits on the muscularis mucosae, 0 - marked muscle thickening present, 3), goblet cell depletion (absent, 0 - present, 1) and crypt abscess (absent, 0 - present, 1). The histological damage score is the sum of each individual score[19,40]. Scores were obtained for naïve mice (n = 5) and for mice treated with 2% DSS for 1 week (n = 5).

Proteomic analysis from colon exudates and urine samples

A quantitative proteomic analysis was performed in exudates collected before and after a 5 min noxious distension of 60 mmHg from naïve (n = 4) and inflamed colons (n = 4). In addition, in 3 of these animals, we were able to obtain urine samples before and 1 week after the induction of the colitis, which were also employed for quantitative proteomic evaluation. The 16 colon samples and the 6 urine samples were analyzed using a Tandem Mass Tag (TMT) isobaric labeling as the method chosen for quantitative proteomics (Figure 1C).

In-solution tryptic digestion of proteins from colon exudates and tandem mass tag (TMT) labeling

Journal Pre-proof

Exudates and urine protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The following steps were carried out with 50 μg of total protein in each sample. Disulfide bonds were reduced with 200 mM Tris-(2-carboxyethyl) phosphine, and cysteines were alkylated with 60 mM iodoacetamide. Proteins were precipitated by incubation with acetone and redissolved in 50 mM triethylammonium bicarbonate buffer (pH 8.5). Subsequently, the proteins were digested with trypsin (Pierce trypsin protease, MS grade; Thermo Fisher Scientific, Waltham, MA) at 1:50 (w/w) trypsin/protein ratio.

For quantitative proteomics, each digested protein sample was labeled with an appropriate TMT Isobaric Label Reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer. Briefly, peptide samples from colon exudates of mice from each experimental condition were labeled with TMT-126, TMT-127, TMT-128, and TMT-129 reagents. In the same way, each urine sample, obtained before and 1 week after the induction of colitis, was labeled with TMT-126 and TMT-127 reagents, respectively. After sample labeling, 5% hydroxylamine was added to quench the TMT reaction. Finally, labeled peptides from colon exudates or urine were combined in equal amounts and dried using a speed vacuum concentrator system (Thermo Fisher Scientific, Waltham, MA).

Liquid chromatography high-resolution mass spectrometry

Solvent A and solvent B were 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively. The peptide samples were dissolved in solvent A and analyzed using a Q-Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) connected to a nano Ultra-Performance Liquid Chromatography system Easy-nLC 1200 UHPLC (Thermo Fisher Scientific, Waltham, MA). Peptide separation was performed using a trap column (Acclaim PepMap 100 C18, 75 µm x 2 cm, 3 µm, 100 A; ThermoFisher Scientific, Waltham, MA) at a flow rate of 20 µL/min; then, peptides were eluted onto a 50 cm analytical column (PepMap RSLC C18, 2 µm, 100 A, 75 µm x 50 cm; ThermoFisher Scientific, Waltham, MA) with a linear gradient of 2% to 20% solvent B for 240 min, followed by a gradient of 20% to 35% solvent B for 30 min, and, finally, to 95% solvent B for 15 min. The peptides were positively ionized using a nanospray ion source, and MS was conducted in top 20 data-dependent mode, with the following settings: ion spray voltage of 2.2 kV; capillary temperature of 320ºC; scan range between 350-1500 for both MS and MS/MS analysis; mass resolution of

Journal Pre-proof

Journal Pre-proof

120,000 for MS and 30,000 for MS/MS; high energy collisional-induced dissociation (HCD) mode (32 eV); isolation window of 0.7 m/z for MS/MS; and dynamic exclusion of 20 s.

Data analysis

The raw data were analyzed using Proteome Discoverer™ 2.4 (Thermo Fisher Scientific, Waltham, MA). MS/MS spectra were searched against *Mus musculus* UniProt database (April 27, 2022) using the Sequest HT search engine.

The search parameters were set as follows: two missed tryptic cleavage sites were allowed; mass tolerance for full MS and MS/MS were 10 ppm and 0.02 Da, respectively; fixed modification of carbamidomethylation on Cys residues, variable modification of oxidation on Met residues, and N-terminal acetylation on proteins were specified. Protein assignments were validated using the Percolator® algorithm[39] by setting the false discovery rate (FDR) to 1%. The results were filtered out so that only proteins with at least two identified peptide sequences were accepted.

Protein abundance ratios were calculated based on precursor intensities. Normalization was performed based on the total peptide amount, and samples were scaled on all averages.

Gene ontology analysis

Relevant proteins (See Statistics section) were uploaded individually to STRING (https://string-db.org/) for examination of protein-protein interaction networks. We established a high confidence level of interaction (0.9) with no more than 5 interactors. Cellular component, biological process, and reactome pathways were essentially considered. When relevant, the Markov Cluster (MCL) algorithm for protein clustering was performed with an inflation parameter of 3.

Validation of hits by Western blotting

Colon tissue was individually homogenized with a pestle in RIPA buffer (50 mM Tris pH 8, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with cOmplete™ Mini Protease Inhibitor cocktail (Roche, Basel, Switzerland) and centrifuged at 12000 x g for 20 min. The same colon exudates that were used for proteome analysis were

freeze-dried to concentrate proteins for Western blot validation. Protein concentrations were measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) for normalization purposes. After heating at 95°C for 5 min, total proteins (20 μg) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in TBST buffer (20 mM Tris, 50 mM NaCl, 0.1% w/v Tween 20) at RT for 1 h and then incubated with the appropriate primary antibodies. The antibodies were: Hp (1:20000 dilution, Abcam, ab256454, Cambridge, MA); Tpm1 (1:1000 dilution, Abcam, ab109505, Cambridge, MA); and β-Actin (1:1000, Merck, Darmstadt, Germany), used as a loading control. The membranes were washed and incubated with goat anti-rabbit IgG peroxidase antibody (1:80000 dilution A0545, Merck, Darmstadt, Germany) in 5% BSA in TBST, followed by enhanced chemiluminescence (ECL) detection system (SuperSignal™ West Pico, Thermo Fisher Scientific, Waltham, MA). Immune-reactive bands were visualized in a ChemiDoc™ Gel Imaging System (Bio-Rad, Hercules, CA) and quantified using Image Lab software (Bio-Rad Hercules, CA). In brief, each band was detected and selected, and background signal was discarded. After that, net values corresponding to the signal of each band was normalized to β-Actin signal.

Statistics

Statistical analyses were performed with Graph Pad Prism, and Microsoft Excel 365, including the assessment of colitis and validation of proteins hits. All data are represented as mean ± standard error of the mean (SEM) for each group. Differences between mean values were analyzed with parametric (unpaired Student t-test) or nonparametric tests (Wilcoxon signed-rank test) as adequate. Correlation between colitis severity and SUDO values decrease after colitis were assessed by simple linear regression. A significant difference was accepted for $p < 0.05$.

For proteomics experiments, statistical analyses were performed with Proteome Discoverer™ 2.4 (Thermo Fisher Scientific, Waltham, MA). The normalized and scaled relative abundance of every protein was expressed as the mean (grouped protein abundance) of the biological replicates. The variability was calculated as the coefficient of variation (see database via ProteomeXchange PXD033902). Protein ratios were directly calculated from the grouped protein abundances. Abundance ratio p-values and Benjamini-Hochberg (BH) adjusted p-values were calculated by ANOVA, based on the abundance of individual proteins. To strengthen our

Journal Pre-proof

Journal Pre-proof

confidence in the changes in protein levels, we considered that a particular protein abundance changed significantly when BH p-value < 0.05. Proteins fulfilling this criterion were considered relevant hits and further researched (see Gene Ontology Analysis and Systematic literature search sections). Additionally, the comparisons that yielded an unadjusted p-value < 0.05 have been listed in Supplementary Tables.

Systematic literature search

We performed a systematic literature search in Medline to assess whether relevant proteins have already been investigated in the context of visceral pain in clinical and preclinical studies. We search for each protein in combination with the following terms: visceral pain, pain, mice or rat, human, proteomics, and biomarker (see Supplementary Table 1). The identified publications were reviewed and considered relevant when fulfilling the inclusion criteria: clinical conditions present with pain, experimental models of pain, quantitative identification of the protein, or use of proteomics. We excluded publications as obviously irrelevant by reading the titles and abstracts and those that were focused on “cancer” or “inflammation”. A comprehensive reading of the selected publications and the literature used in the introduction yielded further publications of interest.

Journal Pre-proof

Results

Assessment of colitis and abdominal pain

All the animals treated with 2% DSS for 1 week developed colitis, as indicated by macroscopic, histological, and behavioral scores.

The severity of colitis was macroscopically scored based on weight loss, stool consistency, signs of rectal bleeding, and general well-being. Four mice developed severe colitis (mean score 31.5 ± 3.7), 1 had moderate colitis (20), and the remaining 5 mice had mild colitis (mean score 9.6 ± 0.6, Figure 2A). Thus, under our experimental conditions, mice more frequently develop mild colitis.

All the animals developed referred hyperalgesia (5.9 ± 0.2 to 2 ± 0.3 SUDO values; n = 10, p-value < 0.01, Wilcoxon signed-rank test; Figure 2B), although abdominal pain was not correlated with the severity of the colitis (lineal correlation test, p = 0.06). Abdominal pain is a characteristic symptom in IBD patients; therefore, this DSS-induced colitis model is helpful in identifying visceral pain biomarkers.

Finally, histological scores significantly increased in DSS-treated colons (6.7 ± 1.0, n = 6) when compared with water-treated controls (0.7 ± 0.3, n = 5; independent samples t-test, p-value < 0.01; Figure 2C).

Proteome signature during colitis: comparison of proteomes from naïve and colitis colon exudates and urine samples

During IBD, like ulcerative colitis, structural and functional changes within the gut wall might contribute to pain. Although numerous inflammatory mediators and ion channels have been identified as fundamental to nociception, the lack of effectivity of current treatments indicates that unidentified molecules must be involved.

It is expected that some analytes might be continuously released from the colon during visceral inflammation contributing to nociceptor sensitization and pain signaling. Therefore, to identify a proteome signature of acute colitis, we compared the proteome of exudates from naïve and inflamed colon samples in a latent condition without any additional insult.

Journal Pre-proof

In this line, we also hypothesized that proteins released from the colon to the extracellular media would eventually end in systemic circulation. Hence, we decided to evaluate the proteomics of urine samples obtained from mice before and one week after the DSS-inducted colitis.

All analyses were performed in mice with mild colitis, as it was the more frequent output of DSS treatment under our specific experimental conditions (see Figure 2A).

Among the 265 proteins detected in the colon exudates, only **Haptoglobin** (Hp) significantly changed in colitis exudates (n = 4 for each naïve and colitis group; ANOVA, BH p-value < 0.05; see Table 1). Hp was the only protein detected in all biological and technical replicates in exudates from inflamed colons and was undetected in control colons.

Furthermore, when evaluating urine samples from the same mice before and 1 week after DSS-induced colitis (n = 3), we could quantify 203 proteins, including Hp. However, none changed their relative abundance levels significantly.

Supplementary Tables 2 and 3 list the proteins that changed in colon exudates and urine samples, respectively, using a less stringent statistical cut-off (ANOVA, unadjusted p-value < 0.05). **Proteome signature during noxious distension: comparison of proteomes from naïve and colitis colons before and after acute distension**

It is generally accepted that abdominal pain experienced by patients with CVP arises from muscle spasms that activate sensitized nociceptors[14]. Therefore, we hypothesized that acute distension of the colon would evoke the release of analytes that could be used as biomarkers indicative of noxious stretch.

In naïve colons, 266 proteins were detected in the exudates upon acute distension of ~60 mmHg. However, only **Haptoglobin** (Hp), **PZD and LIM domain protein 3** (Pdlim3), and **NADP-dependent malic enzyme** (Me1) significantly changed when compared to pre-stimulus condition (n = 4 naïve mice for each pre-stimulus and stimulus condition; ANOVA, BH p-value < 0.05; see Table 1). None of these proteins was detected before distension (i.e., in pre-stimulus condition). Under distension, Hp was detected in all biological replicates, Pdlim3 in 75%, and Me1 in 50%. Interestingly, only one of these proteins, Pdlim3, significantly increased in exudates of the colitis colons after distension and was detected in all biological replicates (n = 4

Journal Pre-proof

colitis mice for each pre-stimulus and stimulus condition; ANOVA, BH p-value < 0.05; see Table 1).

A direct comparison of distension-evoked exudates from naïve and colitis colons revealed that 2 proteins were significantly increased in animals suffering colitis: **Hp** (~13-fold increased) and **Apolipoprotein A-I** (Apoa1, ~4-fold increased). Another 4 proteins were significantly downregulated by ~80%: **Triosephosphate isomerase** (Tpi1), **Glucose-6-phosphate isomerase** (Gpi1), **Alpha-enolase** (Eno1), and **Isoform 2 of Tropomyosin alpha-1 chain** (Tpm1) (n = 4 for each naïve and colitis, ANOVA BH p-value < 0.05). These data are presented in Table 2.

Table 1 summarizes the significant protein abundance changes in our experimental models. The results indicate that Hp significantly increase in response to both inflammation and noxious distension, whereas Pdlim3 and Me1 protein levels increase only in response to noxious distension. No protein was found that changed exclusively after inflammation. The abundance of Apoa1, Tpi1, Gpi1, Eno1, and Tpm1 only changed in colons where inflammation and distension occurred simultaneously. Finally, the combination of inflammation and distension abolished the increase of Me1 in response to distension.

Supplementary Tables 4 and 5 list the proteins that changed using a less stringent statistical cut-off (ANOVA, unadjusted p-value < 0.05).

Gene ontology analysis and literature mining

Lastly, we used STRING to identify the networks that may be related to those proteins that changed significantly.

Gene ontology (GO) analysis covers three categories: biological process, molecular function, and cellular component. Biological process refers to a biological objective to which the gene or gene product contributes. A process is accomplished via one or more ordered assemblies of molecular functions. Molecular function is the biochemical activity of a gene product (enzyme, transporter, ligand). It basically describes what is done without specifying where or when the event occurs. Cellular component refers to the place where the gene product is active[2]. Here we used STRING for GO analysis. Only biological process/reactome pathways and cellular component of each of the proteins evaluated were annotated.

Putting together all the data (see Table 1), **Hp** appears as the central/main protein of interest, as it was increased in the colon after the induction of colitis and is undetected in colons extracted from naïve mice. Of note, upon noxious distension, Hp was also significantly released even from naïve colons. Hp is an abundant plasmatic protein mainly synthesized by the liver that, under physiological conditions, plays an antioxidant role by binding free hemoglobin (Hb) and forming a complex that is cleared by the macrophage receptor CD163. Hp belongs to the family of acute-phase proteins and, hence, its levels increase after inflammatory or traumatic insults in a wide variety of diseases, to exert an immunomodulatory effect[22]. Analysis of the main interacting networks related to this hit using the bioinformatic software STRING showed that **Hp** is highly related to **Apoa1**, which, under distension conditions, is increased within colitis colons when compared with naïve colons. Apoa1 is a member of Apolipoprotein family predominantly produced by the liver (80%) and the gut (20%). It is involved in the biosynthesis of high-density lipoproteins (HDL) and has anti-inflammatory properties in the endothelium[9]. Among the other interactors, the macrophage receptor CD163 was not detected in colon exudates; while Alb, Hpx and Hba1-a1 were detected but did not change between the different conditions (Figure 3A).

Regarding Apoa1 interactors (Figure 3B), it is worth mentioning **Ampb,** which increased 5-fold in urine after colitis, although with a less restrictive statistical cut-off (Supplementary Table 3, Figure 3B). **Ampb** is synthesized in the liver and transported in the blood to the extravascular space of all organs, where cells can internalize it. Under physiological conditions, it reduce oxidation products, scavenge radicals, and bind free heme molecules. Due to its small size, it is almost freely filtrated, and some quantity is excreted in the urine, where its high concentration is clinically used as an indicator of tubular renal damage[8].

Me1 was released upon distension, but only in naïve animals. Me1 is involved in regulation of redox balance, cellular energy, and biosynthesis of molecules. It catalyzes the oxidative conversion of malate to pyruvate and mediates lipid and fatty acid biosynthesis through the generation of NADPH. Its interactors are metabolic proteins (data not shown).

Pdlim3 was released only after noxious distension in both naïve and colitis colons. Pdlim3 is an actin-associated LIM protein expressed in cardiac, smooth and skeletal muscle[48]. It has a role in cellular proliferation and differentiation, but also participates in the stabilization of the

Journal Pre-proof

cytoskeleton. In fact, it is upregulated during the differentiation of myoblast cellular lines[49]. The only protein belonging to its network is Pdlim7 (data not shown), and both might be related to the organization of actin filament arrays within muscle cells. These proteins have only been detected intracellularly.

The protein hits Tpi1, Gpi1, and Eno1 were identified in colon exudates and were significantly downregulated upon distension in colitis mice. The three proteins are included in metabolic pathways mostly related to glycolysis/gluconeogenesis and biosynthesis of amino acids (Figure 3C-D). None of these proteins exerts their function in the extracellular space, even though they were detected in the exudates under our experimental conditions.

Finally, Tmp1 also decreased in colitis subjected to distension. It is a widely expressed actin-binding protein first identified in striated muscle, where its role in contraction is well characterized. Nevertheless, its role in regulating actin cytoskeleton in other cell types is unclear. In the nervous system, Tpm1 has been found in synapses and has been linked with neurodegenerative diseases and trauma[12]. The network for Tpm1 is exclusively involved in biological processes related to muscle contraction (Figure 3E).

Table 3 relates the proteins that changed significantly their abundance in our study and were also altered in previous clinical and preclinical pain studies. Their role in pain and colitis is discussed below.

Validation of hits

In order to validate some of the most relevant hits characterized in our proteomic study, we evaluated the expression of Hp and Tpm1 by using an orthogonal method: Western blotting protein. Protein levels of these two protein hits were examined in the exudates obtained during the 5 minutes of acute distension in naïve and colitic colons. We also determined changes in the expression of Hp and Tpm1 in protein extracts from colonic tissue obtained from naïve and inflamed mice, thus, according to our working hypothesis, proteins in the exudates must be released from the colonic nociceptors, or their neighbor cells within the tube walls.

Western blot analysis showed that Hp protein was undetected in healthy tissue, but in sharp contrast, its levels were significantly increased in colons 6 days after induction of the colitis (from 0.00 ± 0.00 in naïve to 0.70 ± 0.15 in colitis, values of protein signal intensity normalized

Journal Pre-proof

Journal Pre-proof

vs β-actin; n = 3 for each condition). On the other hand, Tpm1 expression was similar in naïve and inflamed colons (0.12 ± 0.01 and 0.10 ± 0.01, respectively, values of protein signal intensity normalized vs β-actin; n = 3 for each condition) (see Figure 4A).

After distension, Hp accumulated in exudates from inflamed colons, but it was barely detected in exudates from naïve colons (3.04 ± 0.71 and 0.00 ± 0.00, respectively, values of protein signal intensity normalized vs β-actin; n = 3 and 2 for each condition). In clear contrast, Tpm1 decreased by ~16-fold in the exudates of inflamed colons when compared to exudates from naïve tissue (0.54 ± 0.20 vs 0.03 ± 0.01, values of protein signal intensity normalized vs β-actin; n = 3 and 2 for colitis and naïve, respectively) (Figure 4B).

Journal Pre-proof

Discussion

Overview

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders characterized by acute relapsing-remitting manifestations such as diarrhea, blood loss, and abdominal pain. Although targeting inflammation of the gut mucosa is the first line of treatment, one-third of patients with healed mucosa still suffer abdominal pain indicating that, besides inflammatory mediators, other signals arising from the gut wall, as yet unidentified, might contribute to pain[28]. Therefore, these signals could be useful for diagnosing and treatment of visceral pain. Nonetheless, in depth analysis of human colon exudates entails multiple technical limitations.

In this context, we used a murine model of ulcerative colitis for inducing visceral hypersensitivity[64] and, thus, allowing to perform an exploratory study to detect molecules released from the colon during active colitis but also upon acute mechanical distension. Extrinsic innervation of the colon includes low threshold mucosal, muscular and muscular/mucosal afferents, which signal physiological distension caused by fecal matter and are involved in defecation. The largest proportion of mechanosensitive afferents (indistinctly named “serosal”, “nociceptors”, “high-threshold” or “vascular” afferents) responds to noxious intensity of distension and to a wide variety of inflammatory and immune mediators. Finally, mechanically insensitive “silent” afferents become mechano-sensitive following exposure to inflammatory mediators, with properties that resemble vascular/serosal afferents[13,34,58]. During colitis, sensitization of these afferents, particularly high-threshold/distension-sensitive, together with the recruitment of mechanically insensitive afferents, will lead to an increase in sensory signaling from the periphery to the spinal cord. Consequently, this enhanced peripheral activity will trigger sensitization of viscero-somatic and viscero-visceral second-order neurons, explaining the increased pain sensitivity in somatic and visceral areas of referral[15]. Central and peripheral sensitization, strictures, and bowel dysmotility are among the underlying mechanisms of pain during remitting (post-inflammatory) IBD[59]. Therefore, identification of molecules with potential sensitizing effects is crucial.

Comparison between these four scenarios that we analyzed here (naïve vs acute colitis, latent vs noxious mechanical distension/stretch) allows for the identification of a protein signature

Journal Pre-proof

indicative of visceral inflammation and a protein signature indicative of distension-induced nociceptor activity, likely altered during inflammation.

We focus on proteins secreted from the serosal side of *ex vivo* colons preparations as it was shown that the content of neuropeptides (CGRP and SP) and PGE2 secreted upon distension was significantly larger when the serosal side was exposed to the superfusion fluid than when the mucosal side was out. Our approach yielded the identification of a discrete number of proteins released to the extracellular space after chemical and/or mechanical noxious insults to the colon, and, therefore, probably involved in nociception in several ways. These proteins might be synthesized and secreted by different cellular types within the colonic wall, including nociceptors as a part of the neurogenic response, and thereafter reach nociceptor terminals or even the bloodstream. Both scenarios clearly point to the gut-brain axis, since diverse brain functions, such as nociception, are influenced by the gut system. In fact, IBD is often referred to as a disorder of the gut-brain axis and has been linked to other chronic pain conditions such as migraine[1,20].

Journal Pre-proof

Proteins increased in colon exudates under noxious conditions

Among the proteins that significantly changed their abundance, **Hp** attracted first our attention. Hp is not present in the exudates of naïve colons, but it was upregulated in all biological replicates in response to DSS-induced inflammation and to noxious distension in both colitis and healthy colons. Thus, these two types of harmful conditions can trigger the expression of Hp. Additionally, we have confirmed that Hp protein levels are increased within colitis colons and exudates by Western blotting. This validation by an orthogonal method to our proteomic analysis supports the findings of our exploratory study.

In the context of pain, Hp isoforms were significantly upregulated in serum obtained from mice and rats with experimental neuropathy compared to control animals[7,60]. Clinical studies have described the upregulation of Hp within the serum of patients suffering different types of chronic pain, such as widespread pain[61], musculoskeletal pain[31], and fibromyalgia[51]. Moreover, Hp is also increased in muscle dialysates in patients with myalgia[46], in cerebrospinal fluid of patients with neuropathic pain[3], and within the stools from a pediatric IBD cohort[57].

Our results are in line with previous literature that links inflammatory conditions, including IBD, with an increment in Hp levels. Besides, we show that a noxious mechanical stimulus is enough to trigger Hp. Interestingly, Hp levels inversely correlate with pressure pain thresholds in patients with chronic widespread pain[29]. Together, these results might indicate a relation between Hp and nociceptor sensitization, maybe through the neurogenic inflammation process. More investigation is needed to clarify the role of Hp in visceral pain, but convergent evidence strongly indicates that Hp is a potential biomarker.

On the other hand, Hp might exert a protective effect. Activating CD163 macrophage receptor by Hb-Hp complexes might contribute to the release of anti-inflammatory compounds and inflammation resolution[25]. DSS-induced colitis was more severe in Hp-deficient mice compared to control littermates, and this is accompanied by an increase of Th1 and Th17 cytokines[43].

Thus, we consider that Hp is a potential biomarker of visceral pain elicited by inflammation or noxious distension. Nonetheless, more studies are needed to determine whether Hp is directly involved in nociception or, alternatively, it is only reflecting an attempt to restore homeostasis after a noxious stimulus. The protein **Pdlim3** was released upon colon distension in both naïve and colitis mice, but did not change in response to inflammation. Therefore, Pdlim3 could be indicative of noxious distension. Its expression has been previously associated with dilated cardiomyopathy[62]. Most recently, after comparing genetic databases obtained from normal and ectopic endometrial human tissue samples, it has been proposed as a candidate biomarker for endometriosis, a disorder featured by pelvic pain[26]. Pdlim3 is associated with cytoskeleton stability in muscular cells. In our context, the increase of Pdlim3 might reflect the reorganization of the cytoskeleton of smooth muscle cells after excessive distension or stretch. With regard to the increase of **Me1**, this hit was released upon distension, but only in naïve animals,then, it could be postulated as possible marker of distension. To the best of our knowledge, there is no data about the involvement of Me1 in pain or colitis. This protein maintains redox homeostasis, but it is challenging to address the link of this process with this distension.

Finally, Apoa1 protein levels rose significantly only when naïve and colitis colons under distension were compared. Apoa1, a protein with anti-inflammatory properties[9], is overexpressed in the serum of a rat model of neuropathic pain, and its transcripts levels are increased in the lumbar spinal cord[7]. Additionally, Apoa1 was upregulated in the serum from

Crohn's patients that do not respond, or respond partially, to IFX (an anti-TNF agent accepted for IBD treatment), when compared to those patients that achieve clinical and serological remission[27]. Apoa1 has been related to nervous tissue healing after damage and it is speculated that exerts its effect activating the ERK pathway[55]. On this matter, we detected Apoa1 release in the condition in which we could expect greater tissue damage, which might activate nociception but also regenerative responses.

Proteins decreased in colon exudates under noxious conditions

The protein hits Tpi1, Gpi1, and Eno1 were identified in colon exudates and their concentratios were greatly reduced upon distension in colitis mice. These proteins, related to energy metabolism pathways, have already been shown susceptible of regulation in clinical studies. In colon biopsies from patients with ulcerative colitis, Tpi1 was one of the identified proteins downregulated as compared with control colons[36]. Eno1 levels were decreased in colon biopsies from inflamed tissue when compared with noninflamed tissue from the same UC patient[50,56]. Nevertheless, in burning mouth syndrome, Eno1 was upregulated in the saliva of the patients when compared to healthy volunteers[37].

Regarding the remaining deregulated protein, Tpm1, literature mining retrieved no results involving Tpm1 with clinical studies. However, it was the only hit in our study already identified in DRG extracts obtained from mice under experimental conditions of incisional and neuropathic pain[5,47]. According to GO analysis, Tpm1 is exclusively involved in biological processes related to muscle contraction, but our unbiased exploratory study indicates that Tpm1 can be released, and its regulation might be relevant during neurogenic inflammation.

Potential biomarkers in urine

Besides being an excellent source for biomarker identification, analyzing urine samples optimizes the follow-up for diagnostic purposes. Several advantages can be outlined for urine samples: less complex than plasma samples, collected in a noninvasive and unrestricted way, and stable composition in comparison with other biological fluids[38]. We hypothesized that molecules secreted in the extracellular space would eventually reach the bloodstream and, consequently, accumulate in urine, which was not the case. However, data mining of proteomic results by GO analysis revealed an interconnection between **Hp**, **Apoa1**, and **Ampb**, the latter

Journal Pre-proof

being detected in urine. Interestingly, the three proteins were localized at the extracellular space. Of note, Ambp was upregulated in urine samples from UC patients[63] and from women with post-menopause migraine[6]. Our exploratory study supports the relevance of urine as a potential niche for biomarkers.

Relevance for patients with IBD

Patients who exhibit healing and reduced inflammation continue suffering pain, suggesting that other factors, besides the renowned inflammatory mediators, might contribute to pain signaling. Identification of these factors is relevant for objectively diagnosing visceral pain in IBD patients, and, mostly, prescribing effective treatment.

Our exploratory study has pointed to protein targets with a variety of molecular functions beyond inflammation, and most of them have been related to different classes of pain in previous preclinical and clinical studies. The protein hits Hp, Pdlim3, and Apoa1 can be postulated as potential biomarkers, as they are released by the colon after noxious chemical and/or mechanical stimuli. On the other hand, it is noteworthy that Tpi1, Gpi1, Eno1, and Tpm1 were downregulated when both inflammation and distension occurred. Interestingly, these proteins have not yet been described in the extracellular space, but they might be also involved in signaling. Together, these results open the possibility of defining a pain footprint and targeting new molecules for the treatment of CVP.

Limitations and future lines

It is becoming increasingly clear that robust differences exist in the genetic, molecular, cellular, and systems-level mechanisms of acute and chronic pain processing in male and female rodents and humans; actually, most patients with chronic pain are women [44]. On the other hand, DSS-induced colitis is less aggressive in rodent females than in males[17]. For all these reasons, in this exploratory study we have focused only in one sex, females. Thus, the results might not apply to male individuals.

We want also to stress that experiments were performed 1 week after the colitis induction, which is an early time point. Thus, it would be interesting to have a time course of the levels of the biomarkers and analyze other fluids, like serum.

Journal Pre-proof

Journal Pre-proof

Finally, to establish Hp and other hits identified in this work as visceral pain biomarkers, a future study needs to identify their cellular sources and targets, mechanism of regulation, and specific role in nociception.

Conclusions

- This is the first time that proteomic analysis has been achieved in exudates of an *ex vivo* preparation from an animal model which might be useful to basic research. It allows for studying physiopathological mechanisms and testing of experimental compounds.
- The fact that some of the hits identified here were already proposed as pain biomarkers in different experimental models and clinical studies reinforces the significance of our approach.
- We have validated two proteomic hits: Hp and Tmp1, by using Western blotting as an orthogonal method. This fact stresses the usefulness of our unbiased proteomic exploratory study.
- At least one hit in the exudate was indirectly linked with one of the biomarkers that was detected in urine samples.

Journal Pre-proof

References

1. Arzani M, Jahromi SR, Ghorbani Z, Vahabizad F, Martelletti P, Ghaemi A, Sacco S, Togha M, School of Advanced Studies of the European Headache Federation (EHF-SAS): Gut-brain Axis and migraine headache: a comprehensive review. J Headache Pain 21:15, 2020.

2. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25:25–9, 2000.

3. Bäckryd E, Ghafouri B, Carlsson AK, Olausson P, Gerdle B: Multivariate proteomic analysis of the cerebrospinal fluid of patients with peripheral neuropathic pain and healthy controls - a hypothesis-generating pilot study. J Pain Res 8:321–33, 2015.

4. Barbara G, Cremon C, De Giorgio R, Dothel G, Zecchi L, Bellacosa L, Carini G, Stanghellini V, Corinaldesi R: Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. Curr Gastroenterol Rep 13:308–15, 2011.

5. Barry AM, Sondermann JR, Sondermann J-H, Gomez-Varela D, Schmidt M: Region-Resolved Quantitative Proteome Profiling Reveals Molecular Dynamics Associated With Chronic Pain in the PNS and Spinal Cord. Front Mol Neurosci 11:259, 2018.

6. Bellei E, Bergamini S, Rustichelli C, Monari E, Dal Porto M, Fiorini A, Tomasi A, Ferrari A: Urinary Proteomics Reveals Promising Biomarkers in Menstrually Related and Post-Menopause Migraine. J Clin Med 10:1854, 2021.

7. Bellei E, Vilella A, Monari E, Bergamini S, Tomasi A, Cuoghi A, Guerzoni S, Manca L, Zoli M, Pini LA: Serum protein changes in a rat model of chronic pain show a correlation between animal and humans. Sci Rep 7:41723, 2017.

8. Bergwik J, Kristiansson A, Allhorn M, Gram M, Åkerström B: Structure, Functions, and Physiological Roles of the Lipocalin α1-Microglobulin (A1M). Frontiers in Physiology [Internet] 12:, 2021 [cited 2022 Nov 28]. Available from: https://www.frontiersin.org/articles/10.3389/fphys.2021.645650

9. Bhale AS, Venkataraman K: Leveraging knowledge of HDLs major protein ApoA1: Structure, function, mutations, and potential therapeutics. Biomedicine & Pharmacotherapy [Internet] 154:113634, 2022 [cited 2022 Nov 27]. Available from: https://www.sciencedirect.com/science/article/pii/S075333222201023X

10. Bonin RP, Bories C, De Koninck Y: A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Mol Pain 10:26, 2014.

11. Bretag AH: Synthetic interstitial fluid for isolated mammalian tissue. Life Sci 8:319–29, 1969.

12. Brettle M, Patel S, Fath T: Tropomyosins in the healthy and diseased nervous system. Brain Research Bulletin [Internet] 126:311–23, 2016 [cited 2022 Nov 28]. Available from: https://www.sciencedirect.com/science/article/pii/S0361923016301289

13. Brierley SM, Hibberd TJ, Spencer NJ: Spinal Afferent Innervation of the Colon and Rectum. Frontiers in Cellular Neuroscience [Internet] 12:, 2018 [cited 2022 Nov 15]. Available from: https://www.frontiersin.org/articles/10.3389/fncel.2018.00467

14. Brierley SM, Linden DR: Neuroplasticity and dysfunction after gastrointestinal inflammation. Nat Rev Gastroenterol Hepatol [Internet] 11:611–27, 2014 [cited 2022 Nov 15]. Available from: http://www.nature.com/articles/nrgastro.2014.103

15. Bulmer DC, Roza C: Visceral Pain. In: Wood JN, editor. The Oxford Handbook of the

Journal Pre-proof

Neurobiology of Pain [Internet] Oxford University Press; page 02020 [cited 2022 Nov 28]. Available from: https://doi.org/10.1093/oxfordhb/9780190860509.013.12

16. Carlton SM: Nociceptive primary afferents: they have a mind of their own. J Physiol 592:3403–11, 2014.

17. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M: Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol 104:15.25.1-15.25.14, 2014.

18. Chiu IM, von Hehn CA, Woolf CJ: Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nat Neurosci 15:1063–7, 2012.

19. Cooper HS, Murthy SN, Shah RS, Sedergran DJ: Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 69:238–49, 1993.

20. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, Codagnone MG, Cussotto S, Fulling C, Golubeva AV, Guzzetta KE, Jaggar M, Long-Smith CM, Lyte JM, Martin JA, Molinero-Perez A, Moloney G, Morelli E, Morillas E, O'Connor R, Cruz-Pereira JS, Peterson VL, Rea K, Ritz NL, Sherwin E, Spichak S, Teichman EM, van de Wouw M, Ventura-Silva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G, Dinan TG: The Microbiota-Gut-Brain Axis. Physiol Rev 99:1877–2013, 2019.

21. de Clauser L, Kappert C, Sondermann JR, Gomez-Varela D, Flatters SJL, Schmidt M: Proteome and Network Analysis Provides Novel Insights Into Developing and Established Chemotherapy-Induced Peripheral Neuropathy. Front Pharmacol 13:818690, 2022.

22. di Masi A, De Simone G, Ciaccio C, D'Orso S, Coletta M, Ascenzi P: Haptoglobin: From hemoglobin scavenging to human health. Mol Aspects Med 73:100851, 2020.

23. Enck P, Aziz Q, Barbara G, Farmer AD, Fukudo S, Mayer EA, Niesler B, Quigley EMM, Rajilić-Stojanović M, Schemann M, Schwille-Kiuntke J, Simren M, Zipfel S, Spiller RC: Irritable bowel syndrome. Nat Rev Dis Primers 2:16014, 2016.

24. Engel MA, Leffler A, Niedermirtl F, Babes A, Zimmermann K, Filipović MR, Izydorczyk I, Eberhardt M, Kichko TI, Mueller-Tribbensee SM, Khalil M, Siklosi N, Nau C, Ivanović-Burmazović I, Neuhuber WL, Becker C, Neurath MF, Reeh PW: TRPA1 and substance P mediate colitis in mice. Gastroenterology 141:1346–58, 2011.

25. Etzerodt A, Moestrup SK: CD163 and Inflammation: Biological, Diagnostic, and Therapeutic Aspects. Antioxid Redox Signal [Internet] 18:2352–63, 2013 [cited 2022 Nov 29]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3638564/

26. Gan L, Sun J, Sun J: Bioinformatical analysis identifies PDLIM3 as a potential biomarker associated with immune infiltration in patients with endometriosis. PeerJ 10:e13218, 2022.

27. Gazouli M, Anagnostopoulos AK, Papadopoulou A, Vaiopoulou A, Papamichael K, Mantzaris G, Theodoropoulos GE, Anagnou NP, Tsangaris GT: Serum protein profile of Crohn's disease treated with infliximab. J Crohns Colitis 7:e461-470, 2013.

28. Gebhart GF, Bielefeldt K: Physiology of Visceral Pain. Compr Physiol 6:1609–33, 2016.

29. Gerdle B, Wåhlén K, Ghafouri B: Plasma protein patterns are strongly correlated with pressure pain thresholds in women with chronic widespread pain and in healthy controls-an exploratory case-control study. Medicine (Baltimore) 99:e20497, 2020.

30. Geyer PE, Holdt LM, Teupser D, Mann M: Revisiting biomarker discovery by plasma proteomics. Mol Syst Biol 13:942, 2017.

31. Ghafouri B, Carlsson A, Holmberg S, Thelin A, Tagesson C: Biomarkers of systemic inflammation in farmers with musculoskeletal disorders; a plasma proteomic study. BMC Musculoskelet Disord

Journal Pre-proof

Journal Pre-proof

17:206, 2016.

32. Gomez-Varela D, Barry AM, Schmidt M: Proteome-based systems biology in chronic pain. J Proteomics 190:1–11, 2019.

33. Goo YA, Cain K, Jarrett M, Smith L, Voss J, Tolentino E, Tsuji J, Tsai YS, Panchaud A, Goodlett DR, Shulman RJ, Heitkemper M: Urinary proteome analysis of irritable bowel syndrome (IBS) symptom subgroups. J Proteome Res 11:5650–62, 2012.

34. Grundy L, Erickson A, Brierley SM: Visceral Pain. Annual Review of Physiology [Internet] 81:261–84, 2019 [cited 2022 Nov 28]. Available from: https://doi.org/10.1146/annurev-physiol-020518-114525

35. Houghton LA, Whorwell PJ: Towards a better understanding of abdominal bloating and distension in functional gastrointestinal disorders. Neurogastroenterol Motil 17:500–11, 2005.

36. Hsieh S-Y, Shih T-C, Yeh C-Y, Lin C-J, Chou Y-Y, Lee Y-S: Comparative proteomic studies on the pathogenesis of human ulcerative colitis. Proteomics 6:5322–31, 2006.

37. Ji EH, Diep C, Liu T, Li H, Merrill R, Messadi D, Hu S: Potential protein biomarkers for burning mouth syndrome discovered by quantitative proteomics. Mol Pain 13:1744806916686796, 2017.

38. Kalantari S, Jafari A, Moradpoor R, Ghasemi E, Khalkhal E: Human Urine Proteomics: Analytical Techniques and Clinical Applications in Renal Diseases. Int J Proteomics 2015:782798, 2015.

39. Käll L, Canterbury JD, Weston J, Noble WS, MacCoss MJ: Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat Methods 4:923–5, 2007.

40. Kim JJ, Shajib MS, Manocha MM, Khan WI: Investigating intestinal inflammation in DSS-induced model of IBD. J Vis Exp :3678, 2012.

41. Laird JMA, Martinez-Caro L, Garcia-Nicas E, Cervero F: A new model of visceral pain and referred hyperalgesia in the mouse. Pain 92:335–42, 2001.

42. Maggi CA: The mammalian tachykinin receptors. Gen Pharmacol 26:911–44, 1995.

43. Márquez L, Shen C, Cleynen I, Hertogh GD, Steen KV, Machiels K, Perrier C, Ballet V, Organe S, Ferrante M, Henckaerts L, Galicia G, Rutgeerts P, Ceuppens JL, Vermeire S: Effects of haptoglobin polymorphisms and deficiency on susceptibility to inflammatory bowel disease and on severity of murine colitis. Gut [Internet] BMJ Publishing Group; 61:528–34, 2012 [cited 2022 Nov 28]. Available from: https://gut.bmj.com/content/61/4/528

44. Mogil JS: Qualitative sex differences in pain processing: emerging evidence of a biased literature. Nat Rev Neurosci 21:353–65, 2020.

45. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, Panaccione R, Ghosh S, Wu JCY, Chan FKL, Sung JJY, Kaplan GG: Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 390:2769–78, 2017.

46. Olausson P, Gerdle B, Ghafouri N, Larsson B, Ghafouri B: Identification of proteins from interstitium of trapezius muscle in women with chronic myalgia using microdialysis in combination with proteomics. PLoS One 7:e52560, 2012.

47. Pogatzki-Zahn EM, Gomez-Varela D, Erdmann G, Kaschube K, Segelcke D, Schmidt M: A proteome signature for acute incisional pain in dorsal root ganglia of mice. Pain 162:2070–86, 2021.

48. Pomiès P, Macalma T, Beckerle MC: Purification and Characterization of an α-Actinin-binding PDZ-LIM Protein That Is Up-regulated during Muscle Differentiation*. Journal of Biological

Journal Pre-proof

Chemistry [Internet] 274:29242–50, 1999 [cited 2022 Nov 28]. Available from: https://www.sciencedirect.com/science/article/pii/S0021925819519676

49. Pomiès P, Pashmforoush M, Vegezzi C, Chien KR, Auffray C, Beckerle MC: The Cytoskeleton-associated PDZ-LIM Protein, ALP, Acts on Serum Response Factor Activity to Regulate Muscle Differentiation. MBoC [Internet] American Society for Cell Biology (mboc); 18:1723–33, 2007 [cited 2022 Nov 28]. Available from: https://www.molbiolcell.org/doi/10.1091/mbc.e06-09-0815

50. Poulsen NA, Andersen V, Møller JC, Møller HS, Jessen F, Purup S, Larsen LB: Comparative analysis of inflamed and non-inflamed colon biopsies reveals strong proteomic inflammation profile in patients with ulcerative colitis. BMC Gastroenterol 12:76, 2012.

51. Ramírez-Tejero JA, Martínez-Lara E, Rus A, Camacho MV, Del Moral ML, Siles E: Insight into the biological pathways underlying fibromyalgia by a proteomic approach. J Proteomics 186:47–55, 2018.

52. Ritchie J: Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. Gut 14:125–32, 1973.

53. Rouwette T, Sondermann J, Avenali L, Gomez-Varela D, Schmidt M: Standardized Profiling of The Membrane-Enriched Proteome of Mouse Dorsal Root Ganglia (DRG) Provides Novel Insights Into Chronic Pain. Mol Cell Proteomics 15:2152–68, 2016.

54. Roza C, Reeh PW: Substance P, calcitonin gene related peptide and PGE2 co-released from the mouse colon: a new model to study nociceptive and inflammatory responses in viscera, in vitro. Pain 93:213–9, 2001.

55. Sengupta MB, Saha S, Mohanty PK, Mukhopadhyay KK, Mukhopadhyay D: Increased expression of ApoA1 after neuronal injury may be beneficial for healing. Mol Cell Biochem [Internet] 424:45–55, 2017 [cited 2022 Nov 29]. Available from: https://doi.org/10.1007/s11010-016-2841-8

56. Shkoda A, Werner T, Daniel H, Gunckel M, Rogler G, Haller D: Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease. J Proteome Res 6:1114–25, 2007.

57. Soomro S, Venkateswaran S, Vanarsa K, Kharboutli M, Nidhi M, Susarla R, Zhang T, Sasidharan P, Lee KH, Rosh J, Markowitz J, Pedroza C, Denson LA, Hyams J, Kugathasan S, Mohan C: Predicting disease course in ulcerative colitis using stool proteins identified through an aptamer-based screen. Nat Commun 12:3989, 2021.

58. Spencer NJ, Kyloh M, Duffield M: Identification of different types of spinal afferent nerve endings that encode noxious and innocuous stimuli in the large intestine using a novel anterograde tracing technique. PLoS One 9:e112466, 2014.

59. Takahashi K, Khwaja IG, Schreyer JR, Bulmer D, Peiris M, Terai S, Aziz Q: Post-inflammatory Abdominal Pain in Patients with Inflammatory Bowel Disease During Remission: A Comprehensive Review. Crohn's & Colitis 360 [Internet] 3:otab073, 2021 [cited 2022 Nov 29]. Available from: https://doi.org/10.1093/crocol/otab073

60. Vincenzetti S, Pucciarelli S, Huang Y, Ricciutelli M, Lambertucci C, Volpini R, Scuppa G, Soverchia L, Ubaldi M, Polzonetti V: Biomarkers mapping of neuropathic pain in a nerve chronic constriction injury mice model. Biochimie 158:172–9, 2019.

61. Wåhlén K, Olausson P, Carlsson A, Ghafouri N, Gerdle B, Ghafouri B: Systemic alterations in plasma proteins from women with chronic widespread pain compared to healthy controls: a proteomic study. J Pain Res 10:797–809, 2017.

62. Wang D, Fang J, Lv J, Pan Z, Yin X, Cheng H, Guo X: Novel polymorphisms in PDLIM3 and PDLIM5 gene encoding Z-line proteins increase risk of idiopathic dilated cardiomyopathy. J Cell Mol Med 23:7054–62, 2019.

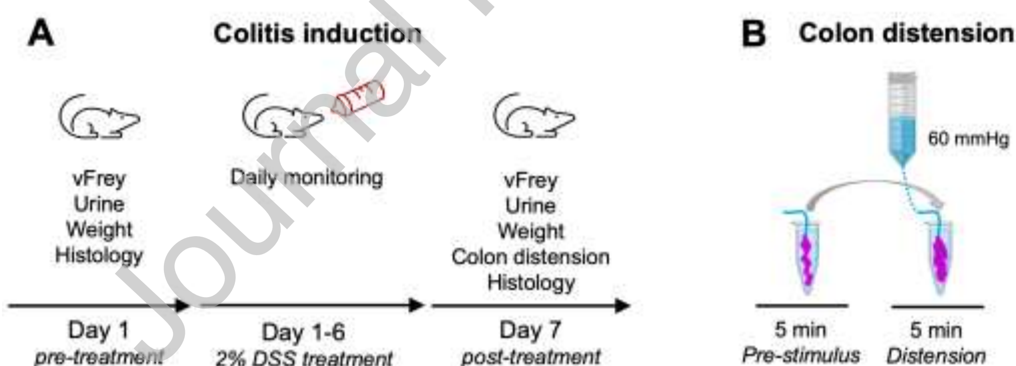
Journal Pre-proof

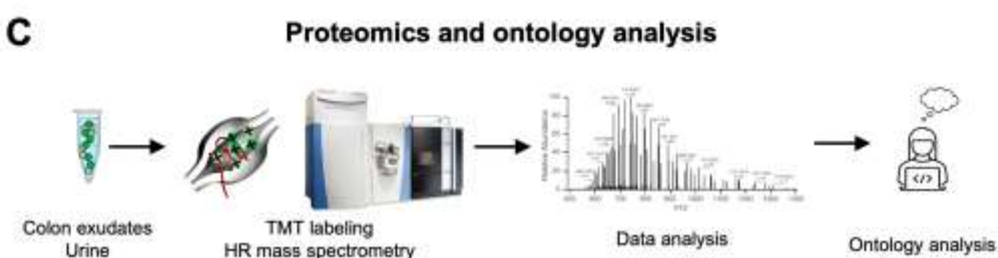
63. Wasinger VC, Yau Y, Duo X, Zeng M, Campbell B, Shin S, Luber R, Redmond D, Leong RWL: Low Mass Blood Peptides Discriminative of Inflammatory Bowel Disease (IBD) Severity: A Quantitative Proteomic Perspective. Mol Cell Proteomics 15:256–65, 2016.

64. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, Neurath MF: Chemically induced mouse models of acute and chronic intestinal inflammation. Nat Protoc 12:1295–309, 2017.

Figure legends

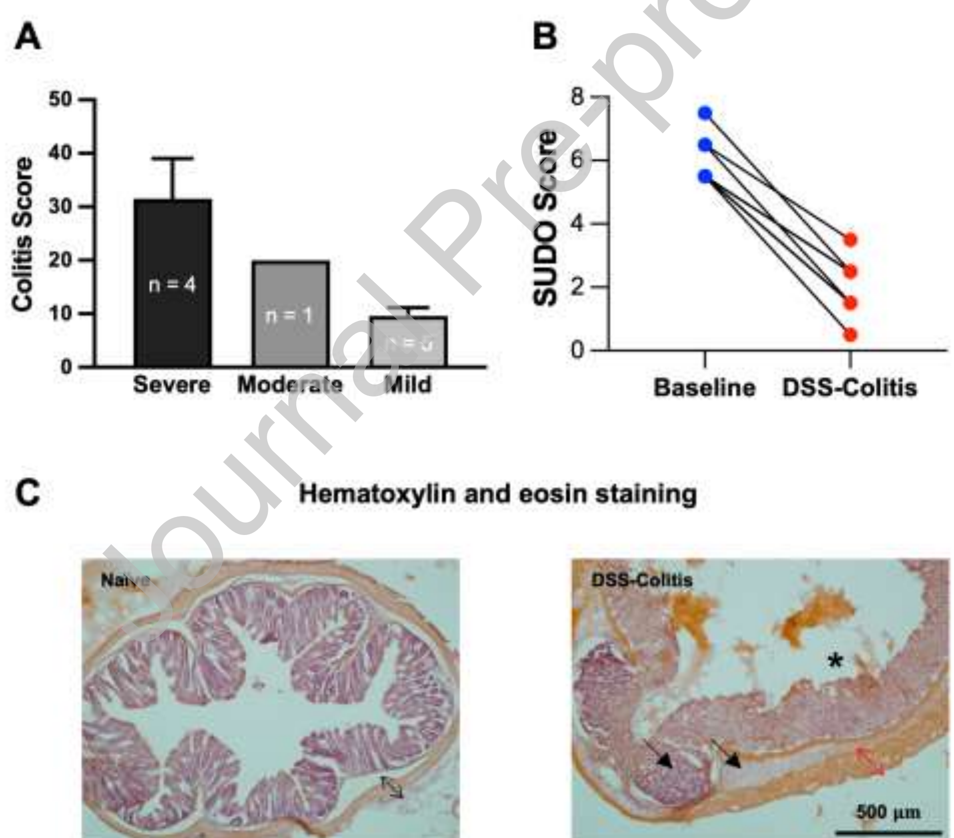
Figure 1: Experimental workflow. A. Day 1. Representation of procedures for colitis induction and assessment, as well as time points for sample collection. **B.** *Ex vivo* colon preparations from naïve and colitis mice were incubated in SIF media and exudates were collected following 5 min of incubation without stimulus (pre-stimulus), and then after 5 min of incubation during the application of 60 mmHg pressure (distension). **C.** Peptides samples from colon exudates and urine samples were labeled with TMT and subjected to high-resolution spectrometry. The obtained data were analyzed using Proteome Discoverer software. Interaction networks for each of the significantly regulated proteins were examined using STRING.





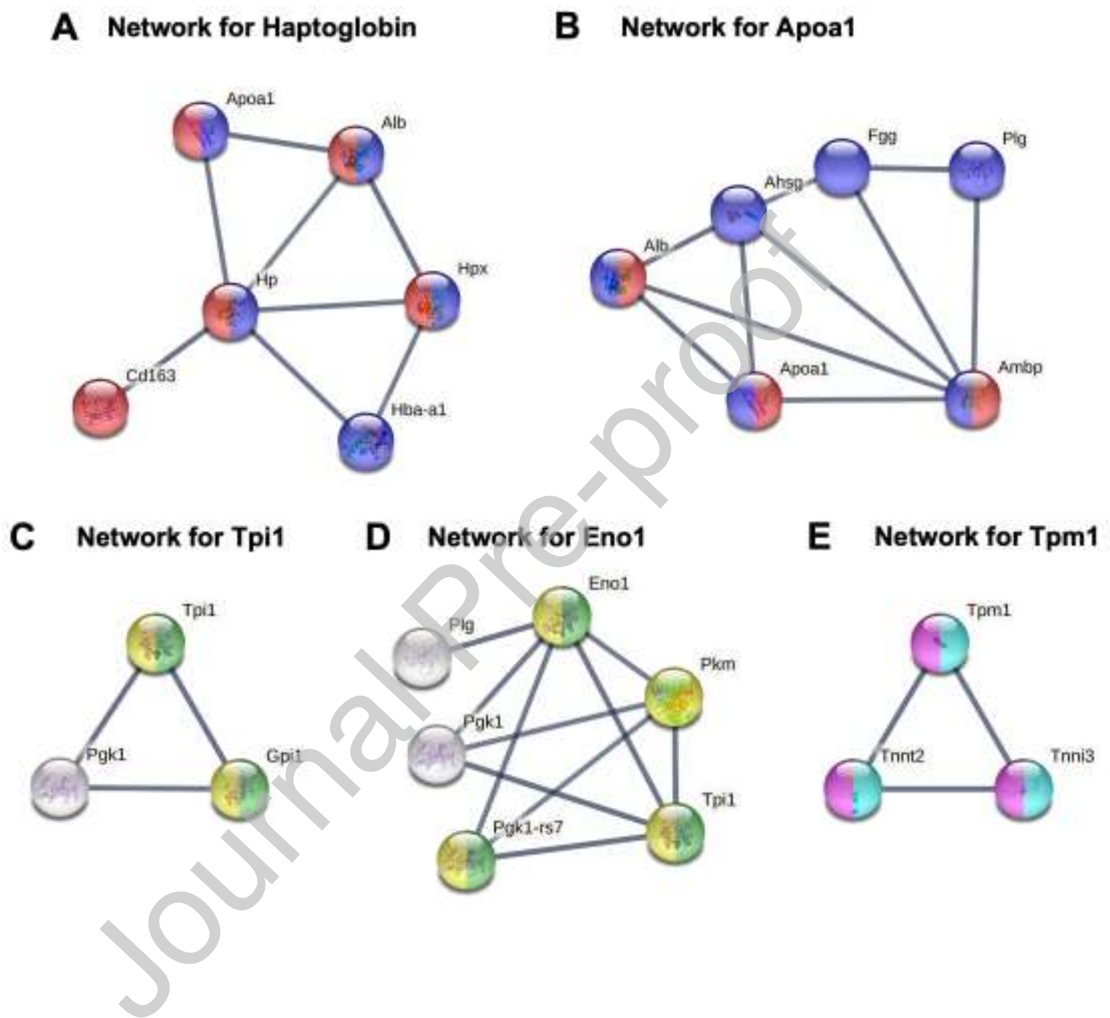
Journal Pre-proof

Figure 2: Macroscopic, behavioral, and histological scores for colitis assessment. A. Colitis scores for mice treated with 2% DSS for 1 week (n = 29). The severity of colitis was severe (>25 points) in 4 mice, moderate (15-25 points) in 20, and mild (5-15 points) in 5. **B.** Withdrawal responses to mechanical stimulations were measured previously to colitis induction (Baseline) and after treatment with 2% DSS (DSS-Colitis) during 6 d. All animals developed referred hyperalgesia after colitis induction, as indicated by the drop of SUDO in all animals (n = 10). **C.** Colon sections from naïve (left) and DSS-treated animals (right). Note that DSS-treated colons show distortion of crypt architecture (*), inflammatory cell infiltration (arrows), and muscle thickening (red arrow).



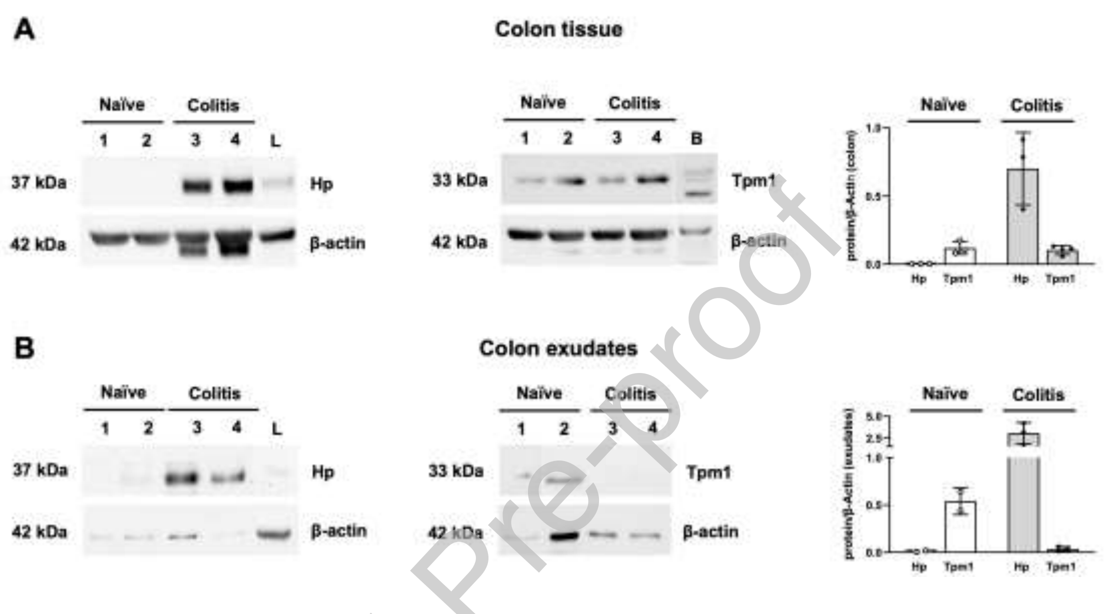
Journal Pre-proof

Figure 3: Protein networks obtained with STRING for significantly deregulated proteins in colon exudates. Code color denotes the cellular compartment (extracellular space: dark blue; striated muscle thin filament: light blue) and the reactome pathways (scavenging of heme from plasma: red; gluconeogenesis: green; glycolysis: yellow).



Journal Pre-proof

Figure 4: Hp validation. A. Representative Westerns blots for Hp and Tmp1 in colon extracts from naïve and DSS-treated mice. **B.** Representative Westerns blots for Hp and Tmp1 in colon exudates obtained after noxious distension in naïve and DSS-treated mice. Liver lysate (L) and brain lysate (B) were used as a positive control for Hp and Tmp1, respectively. β-actin was used as the loading control. Plotted data indicate the mean ± SEM for β-actin normalized signal values.



Journal Pre-proof

Tables

Table 1: Summary of proteins that changed significantly (BH p-value < 0.05) in colon exudates after colitis and/or noxious distension. The ratios were calculated as Colitis/Naïve or Distension/Pre-stimulus.

	Pre-stimulus	Distension	Ratios
Naïve			↑ Hp, Pdlim3, Me1
Colitis			↑ Pdlim3
Ratios	↑ Hp	↑ Hp, Apoa1	

		↓ Tpi1, Gpi1, Eno1, Tpm1	

Table 2: Proteins that changed significantly (BH p-value < 0.05) when compared naïve and colitis colons (distension condition).

Uniprot ID	Gene	Protein	Colitis/Naïve ratio	BH p-value	Identified peptides
Q61646	Hp	Haptoglobin	13.10	0.001	2
Q00623	Apoa1	Apolipoprotein A-I	4.15	0.011	8
P17751	Tpi1	Triosephosphate isomerase	0.33	0.01	8
P06745	Gpi1	Glucose-6-phosphate isomerase	0.32	0.027	6
P17182	Eno1	Alpha-enolase	0.26	0.046	14
P58771-2	Tpm1	Isoform 2 of Tropomyosin alpha-1 chain	0.23	0.045	7

Journal Pre-proof

Table 3: Relevant hits in our study that have been reported in clinical and preclinical pain studies.

Colitis pre-stim. Naïve pre-stim. Naïve distension. Colitis distension.

Protein	vs	vs	vs	vs	Studies
Hp	↑	↑		↑	*Preclinical* Neuropathic pain[7,60] *Clinical* Widespread pain[61] Musculoskeletal pain[31] Fibromyalgia[51] Myalgia[46] Neuropathic pain[3] Ulcerative Colitis[57]
Pdlim3		↑	↑		*Clinical* Endometriosis[26]
Me1		↑			
Apoa1				↑	*Preclinical* Neuropathic pain[7] *Clinical* Chron's Diseases[27]
Ambp*	↑ **Urine**				*Preclinical* IBD[63] Post-menopause migraine[6]
Tpi1				↓	*Clinical* Ulcerative Colitis[36]
Gpi1				↓	
Eno1				↓	*Clinical* Ulcerative Colitis[50,56] Burning moth[37]
Tpm1				↓	*Preclinical* Neuropathic pain[5,47]

Journal Pre-proof