Observational Study

Multiplex gene expression profile in inflamed mucosa of patients with Crohn’s disease ileal localization: A pilot study

Francesco Giudici, Letizia Lombardelli, Edda Russo, Tiziana Cavalli, Daniela Zambonin, Federica Logiodice, Ornela Kullolli, Lamberto Giusti, Tatiana Bargellini, Marilena Fazi, Livia Biancone, Stefano Scaringi, Ann Maria Clemente, Eloisa Perissi, Giovanni Delfino, Maria G Torcia, Ferdinando Ficari, Francesco Tonelli, Marie-Pierre Piccinni, Cecilia Malentacchi

ORCID number: Francesco Giudici (0000-0002-6879-5685); Letizia Lombardelli (0000-0001-9986-0956); Edda Russo (0000-0003-3141-1091); Tiziana Cavalli (0000-0001-8673-7190); Daniela Zambonin (0000-0001-8892-2445); Federica Logiodice (0000-0002-3426-304X); Ornela Kullolli (0000-0002-7744-4596); Lamberto Giusti (0000-0002-9972-090X); Tatiana Bargellini (0000-0002-5381-8813); Marilena Fazi (0000-0003-1117-7461); Livia Biancone (0000-0002-9056-1957); Stefano Scaringi (0000-0002-6838-8318); Ann Maria Clemente (0000-0003-0386-590X); Eloisa Perissi (0000-0001-9089-9205); Giovanni Delfino (0000-0002-5434-5923); Maria Gabriella Torcia (0000-0003-4740-4646); Ferdinando Ficari (0000-0003-2248-6243); Francesco Tonelli (0000-0002-0527-9851); Marie-Pierre Piccinni (0000-0003-1810-5209); Cecilia Malentacchi (0000-0002-3315-1396).

Author contributions: Giudici F and Lombardelli L equally contributed to this work. Malentacchi C and Piccinni MP conceived the study. Giudici F, Cavalli T, Zambonin D and Bargellini T collected the samples. Lombardelli L, Logiodice F, Kullolli O, Russo E and Giusti L performed the experiments. Lombardelli L and Clemente AM performed the statistical analysis.

Abstract

BACKGROUND
Crohn’s disease (CD) is a complex disorder resulting from the interaction of genetic, environmental, and microbial factors. The pathogenic process may potentially affect any segment of the gastrointestinal tract, but a selective location in the terminal ileum was reported in 50% of patients.

AIM
To characterize clinical sub-phenotypes (colonic and/or ileal) within the same disease, in order to identify new therapeutic targets.

METHODS
14 consecutive patients undergoing surgery for ileal CD were recruited for this
INTRODUCTION

The pathogenesis of Crohn’s disease (CD), one of the major inflammatory bowel diseases (IBD) together with ulcerative colitis (UC), has been extensively investigated. It is generally accepted that both genetic and environmental factors contribute to the etiology of the disease. In CD patients, strong associations between genes involved in maintaining intestinal barrier function, epithelial antimicrobial defence, and innate immune regulation have been identified.[1] Environmental risk factors involved in the progression of the disease include...
smoking, low-fiber and high-carbohydrate diet, gut microbiota (GM) alteration, and treatments with antibiotics or non-steroidal anti-inflammatory drugs\[3\].

CD is characterized by a transmural inflammation which can potentially affect any segment of the gastrointestinal tract\[4\]. However, recent studies reported a selective location in the terminal ileum in 50% of the patients and location in the colonic district in 20% of the patients. Ileum and colon district were involved in the remaining 30% of the patients. A different clinical course and surgical requirement was reported according to disease’s localization but currently the reasons underlying the differences in the clinical course have not been defined. In addition, the immunological pathways involved in colonic inflammation are different from those involved in ileal inflammation\[5\].

The mutual interplay between GM and the immune system is involved in the pathogenesis and prognosis of intestinal diseases\[6\] as the GM is a key modulator of intestinal inflammation\[7\]. In CD patients, a reduced GM diversity and lower bacterial load in inflamed vs non-inflamed tissues was observed\[8\]. In addition, several evidences report that the small bowel is responsible for the systemic tolerance towards microbes. A recent study revealed that the ileum harbors a distinctive niche of the GM that differs more from the colonic\[9\]. This different GM composition could be attributed to the activation of distinctive immunological pathways.

In the present study, we used the multiplex gene assay\[10,11\] to analyze surgical specimens of CD patients with prevalent ileal localization.

**MATERIALS AND METHODS**

**Patients**

14 consecutive patients undergoing surgery for ileal CD aged 15 to 57 and hospitalized at the Surgery Unit of Azienda Ospedaliero-Universitaria Careggi, University of Florence, were recruited (Table 1). CD was diagnosed based on both histological and clinical/endoscopic criteria. Table 1 reports the clinical characteristics of the patients.

Peripheral blood samples from each patient were collected in EDTA tubes and genomic DNA was extracted using QIA-AMP DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany). The main polymorphisms of the gene Card15/Nod2 (R702W, G908R, and 1007fs) were analysed in each sample\[12\].

**mRNA extraction and multiplex Gene Assay**

Tissue samples were taken both from the tract affected by CD and from the apparently healthy and disease-free margins (internal controls). The surgical specimens were opened longitudinally.

All samples were stored in RNA later (Qiagen, Germany) before homogenization. Then each sample was weighed and the appropriate lysis solution was added to a final volume of 150 µL containing 50% Lysis Mixture (Thermo-Fisher, MA, United States) and 1 g/L proteinase. The mixture was agitated for 30 min at 65 °C to lyse the cells. The lysate was stored at -80 °C for later use. We used a microarray panel of 24 genes implicated in CD etiopathogenesis\[10\]. We evaluated the expression of these genes in both non-inflamed and inflamed ileal biopsies. Table 2 indicates the panel of the examined genes, the number of Mendelian Inheritance in Man (MIM) (used as a reference), accession number and their corresponding encoded product and function. To improve the analysis of the results, the selected genes were divided into four groups according to their biological role: (1) Transport across epithelia: ABCB1, SLC40A1, SLC22A4, SLC22A5, HAMP; (2) Immune response: CCR6, IL-17F, IL-17A, MICA, MYD88, STAT3, IL-23R, JAK2, IFNG, NOD2; (3) Antimicrobial activity: HAMP, CAMP, LRRK2, DEFB4; and (4) Physiological activities: STAT3, ESR1, LRRK2, TNFSF15, CARD14, DLG5, BMP2, ATG16L1.

The messenger ribonucleic acid (mRNA) expression for CCR6, IL-17A, IL-17F, BMP2, TNFSF15, ABCB1, IL-23R, DEF4, CARD14, STAT3, SLC40A1, JAK2, SLC22A5, ACTB, ATG16L1, CAMP, DLG5, ESR1, CARD15, MICA, MYD88, SLC22A4, IFN-γ, LRRK2, HAMP, ACTB (high expression housekeeping gene), HPT1 (low expression housekeeping gene) was measured using the QuantiGene® Flex assay (Thermo-Fisher, MA, United States).

A panel of oligonucleotide capture probes was covalently linked to carboxylated fluorescently encoded beads (Luminex, Bio-Rad, MA, United States). Each probe has a unique sequence of 15 bases. Each sample lysate diluted at 1:1 anes was mixed with the pooled capture beads in a round-bottom assay well and hybridized for 16 h at 54 °C (final volume in each well was 100 µL). The assay mixture was moved to a MultiScreen® Filter Plate (Millipore, Billerica, MA, United States) and unbound
Giudici F et al. mRNA levels in CD

Table 1 Clinical characteristics of patients with ileal Crohn’s disease

| Patient | Pt1 | Pt2 | Pt3 | Pt4 | Pt5 | Pt6 | Pt7 | Pt8 | Pt9 | Pt10 | Pt11 | Pt12 | Pt13 | Pt14 |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|------|------|
| Localisation of CDa | L1 | L1 | L1 | L1 | L1 | L1 | L3 | L1 | L3 | L3-L4 | L1 | L3 | L1 | L1 |
| Age of CD onset (yr) | 57 | 15 | 53 | 55 | 25 | 31 | 39 | 42 | 16 | 24 | 19 | 18 | 46 | 30 |
| Surgery / relapse | 1st surgery | 1st surgery | Relapse surgery | 1st relapse | Relapse surgery | Relapse surgery | 1st relapse | Relapse surgery | Relapse surgery | Relapse surgery | 1st surgery |
| Disease behavior | B2 | B2 | B3 | B2 | B2 | B4 | B2 | B3 | B2 | B2 | B2 | B2 | B3 | B2 |
| Therapy | C, F, I, B | C, F, C | C | No | F, C, I | B | B2 | F, C | C, I, F, B | C | F, C, B | C, F, C, I | C, F, B, I | F, F, C |
| Smoking status | No | Cur | No | No | 1010 | No | No | 20/30 | 1010 | Cur | 1020 | No | No | No |
| Genotype | wt | R702 | wt | wt | wt | wt | wt | wt | wt | R702 | wt | wt | wt | wt |

Localization of Crohn Disease: L1: terminal ileum; L2: colon; L3: ileum colon; L4: upper G (gastrointestinal); Disease behavior: B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; B4: perianal disease. Therapy: M: mesalazine; I: immunosuppressant; B: biologics; C: corticosteroids; Ab: antibiotics. Smoking status: No: non-smoker; Ex: ex-smoker; Cur: current smoker, no. cigs per day / no. yr. MIM: Mendelian inheritance in Man. 1: Transport across epithelia; 2: Immune response; 3: Antimicrobial activity; 4: Different physiological activities.

Material was filter-washed from the wells by rinsing 3 times with wash buffer. The plate was hybridized with 100 µL/well of bDNA amplifier in Amplifier Diluent (Panomics, CA, United States) at 54 °C for 1 h. After the plate was filter-washed twice with wash buffer and incubated at 50 °C for 1 h with 100 µL/well of 5’-dT(Biotin)-conjugated label probe (Panomics, CA, United States) diluted in Label Probe Diluent (Panomics, CA, United States). After 2 washes, streptavidin-conjugated R-phycocerythrin diluted in SA-PE diluent (20 mmol/L Tris-HCl, 400 mmol/L lithium chloride, 1 mL/L Tween 20, 1 mL/L bovine serum albumin, and 5 mL/L Micr-O-protect) was added and the plate was shaken and incubated at room temperature for 30 min. We washed the beads to remove unbound SA-PE and then evaluated them with Bio-Plex® 200 system (Bio-Rad, MA, United States). The SA-PE fluorescence measured from each bead was proportional to the number of mRNA transcripts captured by the beads. Expression of target-specific RNA molecules was calculated as the mean values from triplicate cultures and normalized against Actin gene (high expression housekeeping gene).

Polymorphism analysis

A standard non-enzymatic method, using the QIA-AMP® DNA Blood Maxi Kit (Qiagen GmbH, Hilden, Germany) was used to extract Genomic DNA from peripheral blood leukocytes of all CD patients and healthy controls. In addition, DNA samples from 70 healthy Caucasian subjects (140 alleles) were analysed as controls. Three exon of the CARD15/NOD2 gene (Exon 4, Exon 8, Exon 11), were amplified by PCR using pairs of primers derived from the published sequence of the gene (available upon request). Each exon is associated with the three main single-nucleotide polymorphisms (SNPs) (R702W-C2104T; G908R-G2722C; 1007fs-3020insC). These three main variants, associated with susceptibility to CD, represented 32%, 18%, and 31%, respectively, of the total CD mutations.[13-15]

The BigDye® Terminator Cycle Sequencing kit (Applied Biosystems, CA, United States) was used to perform direct sequencing of PCR amplified products (SNPs rs87950, rs127951, and rs137955) of the CARD15/NOD2 gene. The samples were analysed in an ABI Prism® 310 genetic analyzer (Applied Biosystems, CA, United States). The of the sequences were confirmed with the analysis of newly-amplified fragments and the sequencing of both DNA strands.

Statistical analysis

SSPS software vers. 10 (SPSS Inc., IL, United States) was used to perform the statistical analysis. All comparisons of genes mRNA expression in tissues (non-inflamed and inflamed areas) were performed by non-parametric assay (Mann-Whitney test, Wilcoxon test). Data are reported as mean and ranges unless otherwise stated. A P-value < 0.05 was accepted as statistically significant. Furthermore, to better characterize the different clinical CD phenotypes, we compared the results regarding the CARD15, CCR6, interferon gamma, and IL-17A genes to colonic CD patients previously examined for these same genes.
| Symbol  | Complete name                                      | Group                      | Accession number | mim     | Gene product function(s)                                                                 | Ref. |
|---------|---------------------------------------------------|----------------------------|------------------|---------|------------------------------------------------------------------------------------------|------|
| HPRT1   | Hypoxanthine phosphoribosyltransferase 1          | Low expression housekeeping gene | M26434           | 308000  | It plays a central role in the generation of purine nucleotides, chosen as a low expression housekeeping gene | [39] |
| ACTB    | Actin beta provided                               | High expression housekeeping gene | M28424           | 102630  | Is involved in the cell motility, structure, and integrity                                | [40] |
| SLC40A1 | Solute carrier family 40 (iron-regulated transporter), member 1 | 1                         | AF215636         | 604653  | Exports iron from duodenal epithelial cells                                               | [41] |
| ABCB1   | ATP-binding cassette, sub-family B (MDR/TAP), member 1 | 1                         | M14758           | 171050  | Transports various molecules across extra- and intra-cellular membranes. It belongs to a protein sub-family involved in multidrug resistance | [42] |
| SLC22A5 | Solute carrier family 22 (organic cation/carnitine transporter), member 5 | 1                         | AF037164         | 603377  | Transports several small organic cations in the liver, kidney, intestine. It is involved in elimination of drugs and environmental toxins | [43] |
| SLC22A4 | Solute carrier family 22 (organic cation/ergothioneine transporter), member 4 | 1                         | AB007448         | 604190  | Polyspecific transporter of organic cations in the liver, kidney, intestine, and involved in the elimination of these molecules. | [44] |
| CCR6    | Chemokine (C-C motif) receptor6                   | 2                          | U68030           | 601835  | Induces B-lineage maturation and antigen-driven B-cell differentiation                    | [45] |
| IL17A   | Interleukin 17A                                   | 2                          | U32659           | 603149  | Produced by Th17-type CD4+ cells. Regulates the activities of NF-kB and mitogen-activated protein kinases | [46] |
| IL17F   | Interleukin 17F                                   | 2                          | AF384857         | 606496  | Produced by Th17-type CD4+ cells. Stimulates the production of other cytokines, including IL6,IL8. It also inhibits angiogenesis by endothelial cells. | [47] |
| STAT3   | Signal transducer and activator of transcription 3 (acute-phase response factor) | 2-4                        | BC014482         | 102582  | Activates transcription of cell growth and apoptosis' genes as responses to inflammation | [48,49] |
| Gene | Description | Accession | Entrez ID | Function |
|------|-------------|-----------|-----------|----------|
| MICA | MHC class I polypeptide-related sequence A | L14848 | 600169 | Acts as a stress-induced antigen broadly recognized by intestinal intra-epithelial gamma delta T cells. [48,49] |
| MYD88 | Myeloid differentiation primary response gene (88) | U84408 | 602170 | Acts as an essential signal transducer in the interleukin-1 and Toll-like receptor signaling pathways [50] |
| IL23R | Interleukin 23 receptor | AF461422 | 607562 | Expressed on Th17 cells. Involved in the IL23A signaling pathways with the receptor molecule IL12RB1/IL12Rbeta1. [51] |
| JAK2 | Janus kinase 2 | 3717 | 147796 | Is involved in cytokine receptor signaling pathways and is required for responses to gamma interferon [52] |
| IFNG | Interferon, gamma | 3458 | 147570 | It encodes a cytokine with antiviral, immunoregulatory and anti-tumor properties and activates macrophages [53] |
| CAMP | Cathelicidin antimicrobial peptide | BC035089 | 608474 | It is an antimicrobial protein (defensin) [54] |
| CARD15 | Nucleotide-binding oligomerization domain containing 2 | AF1789390 | 605956 | Induces immune response to intracellular bacterial by recognizing the muramyl dipeptide (MDP) [55] |
| DEFB4 | Defensin, beta 4A | AJ314835 | 602215 | Acts as an antibiotic peptide locally regulated by inflammation. [56] |
| HAMP | Hepcidin antimicrobialpeptide | AF309489 | 606464 | It is involved in iron transport, antimicrobial, defence and inflammatory responses [57] |
| LRRK2 | Leucine-rich repeat kinase 2 | AK026076 | 608907 | It is involved in autophagy and implicated in clearance of intracellular bacteria. [58] |
| TNFSF15 | Tumor necrosis facto (ligand) superfamily, member 15 | AF039390 | 604052 | Induces apoptosis in endothelial cells [59] |
| CARD14 | Caspase recruitment domain family, member 14 | AF322642 | 607211 | Regulates the molecular scaffolding process and activates NF-kappa B [60] |
| ATG16L1 | ATG16 autophagy related 16-like 1 | AK000897 | 610767 | Induces autophagy processes involved in degradation of cell organelles [61] |
ESR1  Estrogen receptor 1  4  X03635  133430  Involved in the metabolic pathway of the hormones and in several diseases including osteoporosis [65]

BMP2  Bone morphogenetic protein 2  4  650  112261  Induces bone and cartilage formation [66]

DLG5  Discs, large homolog 5  4  U61843  604090  It encodes for scaffolding molecules involved in cell-cell contact and in the maintenance of epithelial cell integrity. Its products are also involved in the transmission of extracellular signals [67]

Histological analysis
Once removed, tissue samples were rinsed in 0.1 M, pH 7.0 cacodylate buffer, the same used in prefixation and further steps of histological preparation. Samples were then placed in Karnovsky (1965)[16], aldehyde solution, and after 3 h prefixation (4°C), underwent prolonged washing in the buffer. Surgery specimens were reduced into approximately 20 mm³ fragments that were post-fixed (1 h 30 min, 4°C) with 1% OsO₄ in cacodylate. These specimens were washed in the buffer, dehydrated in graded ethanol series, soaked in propylene oxide, and embedded in Epon 812. Flat blocks were obtained after polymerization, which were reduced into semi-thin sections (1.5 µm thick), using an 8800 ULTROTOME III LKB equipped with glass knives. Semi-thin sections were stained with borax buffered 1% toluidine blue, and observed with a LEITZ DMRB, in order to collect LM digital images (JPG) for structural analysis. Subsequent ultrastructural observations were carried out on ultrathin sections, obtained with an ULTROTOME NOVA LKB, using a DIATOME diamond knife. Ultrathin sections with gold yellow to silver gray interference colour were selected and collected on uncoated 200-300 mesh copper grids to be electron-dense stained with a hydroalcoholic saturated solution (25 mg/mL) of uranyl acetate, followed by alkaline lead citrate (2 mg/mL). These sections where finally observed (80 KV) with a PHILIPS 201 TEM (BIO, UNIFI), and analogic images were collected, which were later acquired and stored as digital TIFF files using a DIMAGE SCAN DUAL (MINOLTA).

RESULTS

Expression of CD susceptibility genes in the inflamed ileum tissue
The simultaneous expression of 24 genes involved in the pathogenesis of CD was studied in surgical specimens from 14 CD patients with ileal localization of disease. The expression of genes in inflamed ileal mucosa was compared to that of non-inflamed ileal sites collected from the same patient. We observed a significant increase in mRNA levels of twelve genes compared to internal control (Figure 1).

Figure 1 shows that genes related to innate immune response (NOD2, ATG16L1, DEFB4), and to adaptive immune response (CCR6, IL17A, IL17F, IL23R, IFN-γ) were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites. Moreover, the levels of mRNA for genes involved in physiological functions of epithelial cells, such as JAK2, TNFSF15, and SLC22A4 were higher in inflamed mucosa compared to non-inflamed mucosa and the differences in expression reached statistical significance.

Detection of CARD15 polymorphism
DNA samples obtained from peripheral blood were sequenced to investigate the presence of polymorphisms of CARD15/NOD2 gene. The results of this analysis showed that four patients (28.5%) included in this study are carriers of at least one of the polymorphisms investigated, suggesting that genetic factors might contribute to the dysregulated expression of CARD15/NOD2 gene[17-19].
Figure 1  Quantitative evaluation of gene expression using multiplex gene assay in surgical ileum specimens of CD patients. The abscissa shows the genes evaluated in inflamed and not inflamed tissue; the axis of the ordinates shows the value of expression of the gene normalized to the housekeeping actin (gene/β actin ratio). The first seven genes are more closely related to immunity. *P value is reported only when statistically significant (*P < 0.05).

Morphological analysis

In order to observe the morphology of inflamed tissue samples, light microscopy (LM) and TEM micrographs were obtained. Both gut wall and Lieberkühn crypts retain usual features in both lining epithelium and lamina propria. Epithelial cells consist of constitutive enterocytes along with goblet mucocytes, whereas the underlying connective tissue contains large amounts of cells with wide morphological variety (Figure 2). Goblet cells are involved in impressive secretory processes, releasing a moderately opaque product into cryptal and gut lumen through gaps between enterocytes apices (Figure 3). As a consistent pattern, the lamina propria contains granulocytes and plasma cells.

DISCUSSION

Among the numerous genes that have been studied so far with respect to CD, strong and replicated associations have been identified with \textit{NOD2}, \textit{IL23R}, and \textit{ATG16L1} genes\cite{20}. Environmental factors like smoking, low-fiber and, high-carbohydrate diet, altered GM, and medications such as non-steroidal anti-inflammatory drugs interact with genetic background and induce abnormal inflammation and dysregulation of the immune response. Clinical symptomatology relates to such dysregulation.

The clinical course of CD is conditioned by several parameters such as disease location, extra-intestinal manifestation, and age at onset\cite{21}. Strictures and fistulas are more frequent in patients with ileal disease, whereas Crohn’s colitis remains uncomplicated for many years. On the whole, almost 80% of patients with CD require intestinal surgery, with a permanent stoma required by almost 10%. The presence of selected mutations in the \textit{NOD2} gene (see, e.g., 605956.0001-605956.0003) (IBD1; 266600) has been associated with susceptibility to ileum-localized CD\cite{20}; patients homozygous for the 1007fs mutation had an early disease onset with long-segment ileal stenoses and entero-enteral fistulas; they frequently needed surgical intervention and had a high risk of recurrence\cite{22, 23}. Beside \textit{NOD2} gene, huge genome-wide linkage-analyses and meta-analyses have described several CD susceptibility regions including IBD5 locus, DLG5, and autophagy-related 16-like 1 (ATG16L1) gene, JAK2, STAT3 interleukin-23 receptor (IL23R), SLC22A4 and SLC22A5 TNFSF15\cite{14}.

In this paper, we evaluated the expression of 24 genes that were associated to CD susceptibility\cite{20}. mRNA was extracted from gut specimens obtained from patients with CD ileal localization of CD, undergoing surgery. We used a multiplex gene assay which directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the inflammation process for each patient\cite{11}. The same technique was used to quantify gene expression in colonic mucosa from surgical specimens or endoscopic bioptic fragments obtained by CD patients with predominant colonic (L2) location\cite{10}.

The analysis revealed a clear activation of immune-adaptive Th17 response in association with a Th1 response in inflamed mucosa of patients undergoing surgery.
Here gene expression analysis of inflamed ileal mucosa revealed an increased expression of genes involved in adaptive immune response compared to non-inflamed tissue. In particular, we found a significant increase of IL17A and IL17F, IL23R and CCR6 gene expression suggesting an activation of a Th17 adaptive response similar to that found in gut mucosa of patients with colonic localization. According with this hypothesis, three additional genes involved in Th17 differentiation as JAK2, STAT3 and TNFSF15 were found to be overexpressed in inflamed ileal mucosa of CD patients compared to non-inflamed sites. Furthermore, as we expected, the expression of the antimicrobial peptides as defensin (DEFB4)[29] and Hepcidin 6 (HAMP)[30] were significantly increased in inflamed mucosa of CD patients compared with non-inflamed sites, suggesting the overwhelming stimulation of epithelial cells by commensal GM. Indeed, while the human β-defensin (HBD) 1 is constitutively expressed, other genes, like HBD2 (gene name DEFB4), show pathogen and/or inflammation dependent upregulation[31] while also being inducible by probiotic bacteria[32]. Conversely, HAMP transcription mediates the effects of host defence and inflammation. Shanmugam et al. provided persuasive evidence in support of an important role for the GM composition in influencing hepcidin expression during intestinal inflammation in mouse models of colitis[33].

As the position of the pathogenic tissue may condition not only the clinical course of the disease but also the probability to require surgery, we also compared with the same methodology (Quantigene 2.0) the expression of selected genes (IFN-γ, CCR6, IL17A, NOD2) involved in immune responses in inflamed mucosa with predominant ileal location with the one previously studied[10] in inflamed mucosa with colonic location. mRNA expression for IFN-γ appeared significantly higher in ileal site compared to colonic site (ileal CD = 2.7 ± 1.5; colonic CD = 0.2 ± 0.06; P = 0.01). The mRNA for IL-17 and NOD2 appeared to be expressed at higher levels in ileal site compared to colonic site, even if the difference is not statistically significant (P ≥ 0.05). The significant differences in the expression levels of IFN-γ gene (higher expression in specimens from patients with ileal localization compared to patients with colonic localization) may suggest an increased damage of the ileal mucosa due to the simultaneous presence of Th1 and Th17 effector cells and/or the shift of Th17 cells to Th1 effectors functionally more aggressive than Th17 unshifted cells[34,35].

Furthermore, according to a worse clinical course of patient with ileal localization of CD compared with patient with colonic localization[36], the increased expression of IL17 and NOD2 in mucosal fragments from patients with ileal CD compared to patients with colonic CD is in agreement with the NOD2-dependent regulation of immunity in mouse intestinal tract[37]. We suppose that the above differences between the two gut tracts (ileal and colonic) may be due to the Paneth cells at the bottom of the crypts of Lieberkühn in the small intestine, which produce antimicrobial peptides and hinder commensal GM and pathogenic bacteria to penetrate gut mucosa. Initially described as innate immune cells producing antimicrobial products, Paneth cells have recently been suggested to constitute a cardinal component of the intestinal stem cell niche. In fact, Paneth cells contribute to controlling the luminal flora as well as repairing the intestinal barrier following an insult. Genomic alterations that impede the Paneth cell compartment functionality can potentially increase the propensity to
As a consistent trait, cryptal goblet cells produce large amounts of mucus that performs the double role of barrier and holder of antimicrobial products. The microscopic anatomy analysis aims to provide some details that illustrate phenotypic features: the large cell variety in the lamina propria includes immune lines that represent a further defense tool. Although these morphological traits are not directly related to specific gene outputs, they illustrate the tissue responses to key gene deregulation.

As a pilot study, our study presents a low number of subjects investigated which may have influenced the statistical power of the results. To confirm these results, studies with a larger number of patients are needed. In addition, gene expression was evaluated with Multiplex Gene Assay only. This method directly quantifies the mRNA amounts without need of reverse transcription and gives a detailed picture of the metabolic processes for each patient but it should be validated by comparisons with additional techniques to evaluate gene expression.

One of the main purposes of our research is therefore to identify new molecules involved in metabolic pathways that could potentially represent new biological drugs to identify the appropriate therapy in relation to the clinical phenotype of the CD patient.

**ARTICLE HIGHLIGHTS**

**Research background**
The interplay of environmental, genetic and microbial elements influences the etiopathogenesis of Crohn’s disease (CD). Differences in the clinical course of CD have recently been reported in patients with ileal or colonic localization of the inflammatory process.

**Research motivation**
Aim of this study was to define biochemical and histological differences in intestinal biopsies from patients with ileal or colonic localization of Crohn disease in order to identify new assays which can be useful for planning individual therapeutic strategies.

**Research objectives**
Main objective of the current research was to investigate the expression of genes involved in immune-inflammatory pathways in gut mucosa from patients with ileal or colonic localization of CD and to correlate the results of gene expression with those obtained through a classical morphological analysis of surgical biopsies.

**Research methods**
A Multiplex Gene Assay was used to assess the simultaneous expression of 24 genes related to immune-inflammatory process and to CD pathogenesis. Structural and ultrastructural features of gut samples were also evaluated through Light microscopy (LM) and Transmission Electron Microscopy (TEM) techniques.

**Research results**
We observed a strong activation of genes involved in TH-1- and TH-17 immune response in patients with ileal localization of CD compared to patients with colonic localization. In addition, the expression of genes for antimicrobial peptides as DEFB4 and HAMP was found highly stimulated in ileal mucosa from CD patients suggesting a possible interference with microbial
commensals at this site.

**Research conclusions**

Our results indicate that patients with ileal localization of CD have a stronger activation of TH-1 and TH-17 immune-inflammatory responses compared with patients with colonic localization of the disease thus defining a clear subclinical phenotype of CD.

**Research perspectives**

These results may suggest that therapeutic strategies with biological drugs in CD patients can be differentiated depending on the location of the disease.

**ACKNOWLEDGEMENTS**

We would like to thank Dr. Michele Tanturli for the statistical support, Dr. Giulia Ricciardi for additional manuscript revision, and all the patients who participated to this study.

**REFERENCES**

1. **Boyapati R**, Satsangi J, Ho GT. Pathogenesis of Crohn's disease. *F1000Prime Rep* 2015; 7: 44 [PMID: 26907717 DOI: 10.12703/p.97-44]
2. **Ramos GP**, Papadakis KA. Mechanisms of Disease: Inflammatory Bowel Diseases. *Mayo Clin Proc* 2019; 94: 155-165 [PMID: 30611442 DOI: 10.1016/j.mayocp.2019.09.013]
3. **Abegunde AT**, Muhammad BH, Ibbati O, Ali T. Environmental risk factors for inflammatory bowel diseases: Evidence based literature review. *World J Gastroenterol* 2016; 22: 6296-6317 [PMID: 27468219 DOI: 10.3748/wjg.v22.i27.6296]
4. **Li N**, Shi RH. Updated review on immune factors in pathogenesis of Crohn's disease. *World J Gastroenterol* 2018; 24: 15-22 [PMID: 29358878 DOI: 10.3748/wjg.v24.i1.15]
5. **Weiser M**, Simon JM, Kochar B, Tovar A, Israel JW, Robinson A, Gipson GR, Schaner MS, Herfarth HH, Sartor RB, McGovern DPH, Rahbar R, Sadiq TS, Korudai MJ, Furey TS, Sheikh Z. Molecular classification of Crohn's disease reveals two clinically relevant subtypes. *Gut* 2018; 67: 36-42 [PMID: 27734276 DOI: 10.1136/gutjnl-2016-312518]
6. **Russo E**, Taddei A, Ringressi MN, Ricci F, Amedei A. The interplay between the microbiome and the adaptive immune response in cancer development. *Therap Adv Gastroenterol* 2016; 9: 594-605 [PMID: 27366226 DOI: 10.1177/1756283X16635082]
7. **Sartor RB**. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; 134: 577-594 [PMID: 18242222 DOI: 10.1053/j.gastro.2007.11.059]
8. **Sepehr S**, Kotlowski R, Bernstein CN, Krause DO. Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; 13: 675-683 [PMID: 17262808 DOI: 10.1002/ibd.20101]
9. **Villanueva IC**, Haag ES, Ulvestad E, Grode N, Stenstad T, Halland A, Kommiedal O. Species Level Description of the Human Ileal Bacterial Microbiota. *Sci Rep* 2018; 8: 4736 [PMID: 29549283 DOI: 10.1038/s41598-018-23198-5]
10. **Russo E**, Lombardelli L, Giudici F, Cavalli T, Faci F, Fazi M, Scarinzi S, Biancone L, Logiodice F, Nesi M, Latiano A, Annese V, Torcia MG, Bechi P, Tonelli F, Piccinini MP, Malentaacchi C. Crohn's Colitis: Development of a multiplex gene expression assay comparing mRNA levels of susceptibility genes. *Clin Res Hepatol Gastroenterol* 2017; 41: 435-444 [PMID: 28365139 DOI: 10.1016/j.clinre.2017.02.004]
11. **Piccinini MP**, Lombardelli L, Logiodice F, Tisi D, Kullolli O, Biagiotti R, Giudici M, Romagnani S, Maggi E, Faciara G. Potential pathogenic role of Th17, Th6, and Th2 cells in erosive and reticular oral lichen planus. *Oral Dis* 2014; 20: 212-218 [PMID: 23556506 DOI: 10.1111/odi.12094]
12. **Palmieri O**, Bossa F, Valvano MR, Corriore G, Latiano T, Martin G, D’Inci R, Caciarella S, Pastore M, D’Altilia M, Scimeca D, Biscaglia G, Andruiti A, Latiano L. Crohn's Disease Localization Displays Different Predisposing Genetic Variants. *PLoS One* 2017; 12: e0168821 [PMID: 28052082 DOI: 10.1371/journal.pone.0168821]
13. **Barreiro-de Acosta M**, Peña AS. Clinical applications of NOD2/CARD15 mutations in Crohn's disease. *Acta Gastroenterol Latinoam* 2007; 37: 49-54 [PMID: 17486745]
14. **Huang H**, Fang M, Jostins L, Umićević Mirkov M, Boucher G, Anderson CA, Andersen V, Cleynen I, Cortes A, Seiderer J, Schnitzler F, Brand S, Staudinger T, Pfennig S, Herrmann K, Hofbauer K, Dambacher J, Palmieri O, Sepehr S, Kotlowski R, Bernstein CN, Krause DO. Microbial diversity of inflamed and noninflamed gut biopsy tissues in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; 13: 675-683 [PMID: 17262808 DOI: 10.1002/ibd.20101]
15. **Keh C**, Shatari T, Yamamoto T, Menon A, Clark MA, Keighley MR. Jejunal Crohn's disease is associated with a higher postoperative recurrence rate than ileocelecal Crohn's disease. *Colorectal Dis* 2005; 7: 366-368 [PMID: 15932560 DOI: 10.1111/j.1463-1318.2005.00766.x]
16. **Karnovsky MJ**. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* 1965; 27: 137A
17. **Seiderer J**, Schmitzler F, Brand S, Staudinger T, Pfennig S, Herrmann K, Hofbauer K, Dambacher J, Tillack C, Sackmann M, Gieke B, Lobhes P, Ochsenekühn T. Homozygosity for the CARD15 frameshift mutation 1007fs is predictive of early onset of Crohn's disease with ileal stenosis, entero-enteral fistulas, and frequent need for surgical intervention with high risk of re-stenosis. *Scand J Gastroenterol* 2006; 41: 1421-1432 [PMID: 17101573 DOI: 10.1080/00365520600705900]
18. **Siddiq T**, Yoshitama S, Downs I, Kobayashi KS. Nov2: A Critical Regulator of Ileal Microbiota and
Giudici F et al. mRNA levels in CD

Crohn's Disease. Front Immunol 2016; 7: 367 [PMID: 27703457 DOI: 10.3389/fimmu.2016.00367]

19

Cucchiara S, Latiano A, Palmieri O, Stanino AM, D'Inca R, Guariso G, Vinci G, Rutigliano V, Borrelli O, Valvano MR, Ammese V. Role of CARD15, DLG5 and OCTN genes polymorphisms in children with inflammatory bowel diseases. World J Gastroenterol 2007; 13: 1211-1229 [PMID: 17471203]

20

Naser SA, Arce M, Khaja A, Fernandez M, Naser E, Elwassia S, Thaniqchalam S. Role of ATG16L1, NOD2 and IL23R in Crohn's disease pathogenesis. World J Gastroenterol 2012; 18: 412-424 [PMID: 22346247 DOI: 10.3748/wjg.v18.i4.412]

21

Lazarev M, Huang C, Bilton A, Cho JH, Duerr RH, McGovern DP, Proctor DD, Regueiro M, Rioux JD, Schumm PP, Taylor KD, Silverberg MS, Steinhart AH, Haffless S, Brant SR. Relationship between proximal Crohn's disease location and disease behavior and surgery: a cross-sectional study of the IBDD Genetics Consortium. Am J Gastroenterol 2013; 108: 106-112 [PMID: 23229423 DOI: 10.1038/ajg.2012.389]

22

Noble CL, Abbas AR, Lees CW, Cornelius J, Toy K, Modrusan Z, Clark HF, Arndt ID, Penman ID, Satsangi J, Diehl L. Characterization of intestinal gene expression profiles in Crohn's disease by genome-wide microarray analysis. Inflamm Bowel Dis 2010; 16: 1717-1728 [PMID: 20848455 DOI: 10.1002/ibd.21263]

23

Quezada SM, Steinberger EK, Cross RK. Association of age at diagnosis and Crohn's disease phenotype. Age Ageing 2013; 42: 102-106 [PMID: 22918090 DOI: 10.1093/ageing/asu107]

24

Sehgal R, Berg A, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA. NOD2/CARD15 mutations correlate with severe pouchitis after ileal pouch-anal anastomosis. Dis Colon Rectum 2010; 53: 1487-1494 [PMID: 20940596 DOI: 10.1007/DCR.0b013e31122635]
associated with inflammatory bowel disease. Am J Hum Genet 2003; 73: 1282-1292 [PMID: 14610718 DOI: 10.1086/379927]

43. Peltohkova VD, Winstead RF, Rubin LA, Amos CI, Huang Q, Gu X, Newman B, Van Oene M, Cescon D, Greenberg G, Griffiths AM, St George-Hyslop PH, Siminovich KA. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nat Genet 2004; 36: 471-475 [PMID: 15107849 DOI: 10.1038/ng1339]

44. Girardin M, Dionne S, Guyette P, Rioux J, Bitton A, Elinnani I, Charlebois P, Qureshi I, Levy E, Seidman EG. Expression and functional analysis of intestinal organic cation/l-amino transporter (OCTN) in Crohn's disease. J Crohns Colitis 2012; 6: 189-197 [PMID: 23235173 DOI: 10.1016/j.crohns.2011.08.003]

45. Fransen K, van Sommeren S, Westra HJ, Veenstra M, Lamberts LE, Modderman R, Djikstra G, Fu J, Wijmenga C, Franke L, Weersma RK, van Diemen CC. Correlation of genetic risk and messenger RNA expression in a Th17/Th23 pathway analysis in inflammatory bowel disease. Inflamm Bowel Dis 2014; 20: 777-782 [PMID: 24660257 DOI: 10.1097/MIB.0000000000000312]

46. Ueno A, Ghosh A, Hung D, Li J, Jijon H. Th17 plasticity and its changes associated with inflammatory bowel disease. World J Gastroenterol 2015; 21: 12283-12295 [PMID: 26604637 DOI: 10.3748/wjg.v21.i43.12283]

47. Nguyen PM, Potoczek TI, Ernst M. STAT3-Activating Cytokines: A Therapeutic Opportunity for Inflammatory Bowel Disease? J Interferon Cytokine Res 2015; 35: 340-350 [PMID: 25760898 DOI: 10.1089/jir.2014.0225]

48. Allee M, Tieng V, Nakazawa A, Treton X, Pacault V, Dalphy N, Caillat-Zuconn S, Paul P, Gornet JM, Douay C, Ravel S, Tamouza R, Charron D, Léemann M, Mayer L, Toubert A. CD4+NGK2D+ T cells in Crohn's disease mediate inflammatory and cytotoxic responses through MICA interactions. Gut 2007; 52: 2346-2358 [PMID: 17570210 DOI: 10.1053/j.gut.2007.03.025]

49. Muro M, López-Hernández R, Mrowiec I. Immunogenetic biomarkers in inflammatory bowel diseases: role of the IBD3 region. World J Gastroenterol 2014; 20: 15037-15048 [PMID: 25386502 DOI: 10.3748/wjg.v20.i41.15037]

50. Santarlasci V, Conmi L, Maggi L, Liotta F, Ammuniato F, IL-1 and T Helper Immune Responses. Front Immunol 2013; 4: 182 [PMID: 23874332 DOI: 10.3389/fimmu.2013.00182]

51. Danese S, Grisham M, Hodge J, Telliez JB. JAK inhibition using tofacitinib for inflammatory bowel disease treatment: a hub for multiple inflammatory cytokines. Am J Physiol Gastrointest Liver Physiol 2016; 310: G155-G162 [PMID: 26608188 DOI: 11.1152/ajpgi.00311.2015]

52. Tailler T, Atar S, Ruppin E, Gurevich M, Achiron A. Common and specific signatures of gene expression and protein-protein interactions in autoimmune diseases. Genes Immun 2013; 14: 67-82 [PMID: 23190644 DOI: 10.1038/gene.2012.55]

53. Koczulla AR, Bals R. Antimicrobial peptides: current status and therapeutic potential. Drugs 2003; 63: 389-406 [PMID: 12555461 DOI: 10.2165/00003495-200363040-00005]

54. Hugot JP. CARD15/NOD2 mutations in Crohn's disease. Nat Rev Genet 2003; 4: 1571-1586 [PMID: 12106649 DOI: 10.1038/nrm1108]

55. Wehkamp J, Stange EF, Fellermann K. Defensin-immunology in inflammatory bowel disease. Gastroenterol Clin Biol 2009; 33 Suppl 3: S157-S164 [PMID: 17173337 DOI: 10.1016/S0399-8320(09)73149-5]

56. Gersemann M, Becker S, Kübler I, Kosloski M, Wang G, Herrlinger KR, Griger J, Fritz P, Fellermann K, Schwab M, Wehkamp J, Stange EF. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. Differentiation 2009; 77: 84-94 [PMID: 19281767 DOI: 10.1016/j.diff.2008.09.008]

57. Verga Falzacappa MV, Muckenthaler MU. Heparin: anti-hormone and anti-microbial peptide. Gene 2005; 364: 37-44 [PMID: 16203112 DOI: 10.1016/j.gene.2005.07.023]

58. Mleczko-Sanecka K, Casanovas G, Ragab A, Breitkopf K, Müller A, Boutros M, Dooley S, Hentze MW, Muckenthaler MU. SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expression. Blood 2010; 115: 2657-2665 [PMID: 20407671 DOI: 10.1182/blood-2009-09-238105]

59. Gardet A, Bentia T, Li C, Sands BE, Ballester I, Stevens C, Korzenik JR, Rioux JD, Daly MJ, Xavier RJ, Podolsky DK. LRRK2 is involved in the IFN-gamma response and host response to pathogens. J Immunol 2010; 185: 5577-5585 [PMID: 20921534 DOI: 10.4049/jimmunol.1000548]

60. Schapansky J, Nardozzi JD, Felizia F, LaVoie MJ. Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy. Hum Mol Genet 2014; 23: 4201-4214 [PMID: 24862598 DOI: 10.1093/hmg/ddu135]

61. Bamias G, Martin C, Marini M, Hoang S, Mishina M, Ross WG, Sacheneda MA, Friel CM, Mize J, Buckston SJ, Pizarro TT, Wei P, Cominelli F. Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. J Immunol 2003; 171: 4688-4678 [PMID: 14568967 DOI: 10.4049/jimmunol.171.9.4686]

62. Tsou LC, Spain SL, Knight J, Ellinghaus E, Stuart PE, Capon F, Ding J, Li Y, Tejiasvi S, Gudjonsson JE, Kang HM, Allen MH, McManus R, Novelli G, Samanenlou S, Schalkwijk J, Stähle M, Burden AD, Smith CH, Cork MJ, Estivill X, Bowcock AM, Knuever GG, Weger W, Worthington J, Tazi-Ahnini R, Nestle FO, Hayday A, Hoffmann P, Winkler M, Wijmenga C, Langford C, Edkins S, Andrews R, Blackburn H, Strange A, Band G, Pearson RD, Vulcovic D, Spencer CC, Deloukas P, Mowriez U, Schreiber S, Weidinger S, Koks S, Kingo K, Metspalu A, Lim HW, Voorhees JJ, Wijmenga C, Wang G, Herrlinger KR, Griger J, Fritz P, Fellermann M, Steinberg ME, Cardon LR, Schapansky J, Nardozzi JD, Felizia F, LaVoie MJ. Membrane recruitment of endogenous LRRK2 precedes its potent regulation of autophagy. Hum Mol Genet 2014; 23: 4201-4214 [PMID: 24862598 DOI: 10.1093/hmg/ddu135]
Giudici F et al. mRNA levels in CD

66 Cejalvo T, Sacedón R, Hernández-López C, Diez B, Gutierrez-Frias C, Valencia J, Zapata AG, Varas A, Vicente A. Bone morphogenetic protein-2/4 signalling pathway components are expressed in the human thymus and inhibit early T-cell development. *Immunology* 2007; 121: 94-104 [PMID: 17425602 DOI: 10.1111/j.1365-2567.2007.02541.x]

67 Stoll M, Cornelissen B, Costello CM, Waetzig GH, Mellgard B, Koch WA, Rosenstiel P, Albrecht M, Croucher PJ, Steegert D, Nikolaus S, Hampe J, Lengauer T, Pierreou S, Foelsch UR, Mathew CG, Lagerstrom-Fermer M, Schreiber S. Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat Genet* 2004; 36: 476-480 [PMID: 15107852 DOI: 10.1038/ng1345]
