

Broad Medical Research Program (BMRP)
The Eli and Edythe Broad Foundation

FINAL REPORTS

1. Final Technical Progress Report

i. A summary of project aims.

Specific microbiological aims were:

1. to evaluate the intestinal dominant microbiota using temporal temperature gradient gel electrophoresis (TTGE);
2. to quantify and characterize mucosa-associated aerobic and facultative anaerobic bacteria, particularly *E. coli*;
3. to define a putative clonal identity of *E. coli* strains;
4. to screen *E. coli* strains for genes associated with pathogenicity
5. to investigate adhesive/invasive properties of isolated *E. coli* strains by analyzing their adhesion to intestinal cell lines

Specific ultrastructural aims were:

1. to evaluate both morphological damages and presence of intracellular bacteria in biopsies;
2. to isolate adherent-invasive lactose-fermenting bacteria by gentamicin protection assay in Crohn's disease (CD) and ulcerative colitis (UC) subjects and to characterize *E. coli* isolates with the aim to identify *adherent-invasive E. coli* (AIEC) strains;
3. to study the characteristic features of bacteria-cell interactions at the intestinal mucosa;
4. to study the interaction between AIEC strains and organ culture by immunofluorescence microscopy.

Specific immunological aims were:

1. to assess that NOD2, an innate immunity gene, is functional and able to interact with RIP2 by showing the presence of the immunocomplex NOD2/RIP2 in the inflamed tissue of patients;
2. to compare CEACAM6 expression in ileal mucosa from controls as well as from uninvolved and involved areas of CD patients;

3. to assess the apical localization of CEACAM6 in the intestinal epithelium of CD patients by immunohistochemistry;
4. to test the ability of new identified adherent-invasive strains to induce in vitro the expression of the receptor CEACAM6 and of pro-inflammatory cytokines, i.e. TNF-alpha and IL-8, in order to demonstrate that they can promote their own colonization in CD patients and activate an inflammatory response;
5. to repeat the same experiments mentioned in the previous step (# 4) in cultured mucosal biopsies from healthy controls.

ii. Accomplishments towards meeting those aims.

The project, performed in pediatric patients with CD through molecular and microbiological techniques, had an overall aim consisting in analyzing the composition of the dominant mucosa-associated microbiota, in quantifying and characterizing specific facultative anaerobic bacteria, particularly *E. coli*, possibly associated to the disease and, finally, in assessing the intestinal innate immune response. Main targets of the project were mostly achieved, as detailed in the next section (significant results).

Specifically, results from the study of the mucosal-associated bacteria underlined the presence of a peculiar microbiota associable to CD in pediatric patients. Indeed, we showed a well-defined separation between the TTGE profiles of the three patients groups (controls, CD and UC), suggesting that the microbiota composition is a sufficient factor to predict the patient category (CD, non-CD). Interestingly, an altered microbial community structure seems to be a common feature of pediatric CD patients and could be a condition existing in susceptible individuals before disease symptoms.

Besides, we assessed that CD patients showed severe morphological alterations, not only in the inflamed, but also in the macroscopically uninfamed intestinal tissue, leading to the loss of gut barrier integrity.

We identified two new AIEC strains, one in a CD and one in a UC patient, suggesting a role of these bacteria strains also in the pediatric disease. A set of experiments showed that they can destroy the integrity of the polarized epithelial cell barrier as well as induce in vitro and ex vivo an up-regulation of the specific receptor, CEACAM6, and an inflammatory response, similarly to the AIEC prototype LF82. Moreover, we demonstrated that these bacteria can survive and replicate inside macrophages.

We also showed that innate immunity genes, NOD2 and RIP2, are functionally active in the intestinal mucosa of CD patients. In addition, we showed a significant increase of CEACAM6 expression not only in the inflamed, but also in the uninfamed ileal tissue of both CD and UC patients as compared to healthy controls, suggesting a possible use of this receptor as a marker predisposing to the disease.

We believe that these findings may contribute to open new directions for future projects aimed at highlighting the relationship between microbiota, epithelial cell barrier and immune response for a better comprehension of the mechanisms underlying the pediatric IBD pathogenesis. In addition, we believe that these results can be of great help in the request for additional funds to continue the study.

iii. A list of significant results (positive or negative).

Microbiological Unit Results:

Aim1

TTGE profiles were analyzed by Doc-It software and XLStat software for bands pattern similarity (Fig.1). The resulting dendrogram showed three well-defined clusters: The first one (1) included almost all control and few CD patient TTGE profiles, while the second and the third (2,3) included mainly CD patients TTGE profiles. These results highlighted the presence of a dominant microbiota related to CD disease, and the presence of different cluster and sub-cluster among CD patients. Moreover, we calculated intra- and inter-individual similarity by Dice index. Intra-individual similarity was around 75% for controls and around 70% for CD patients; inter-individual similarity within each group was 45% for controls and 65% for CD patients.

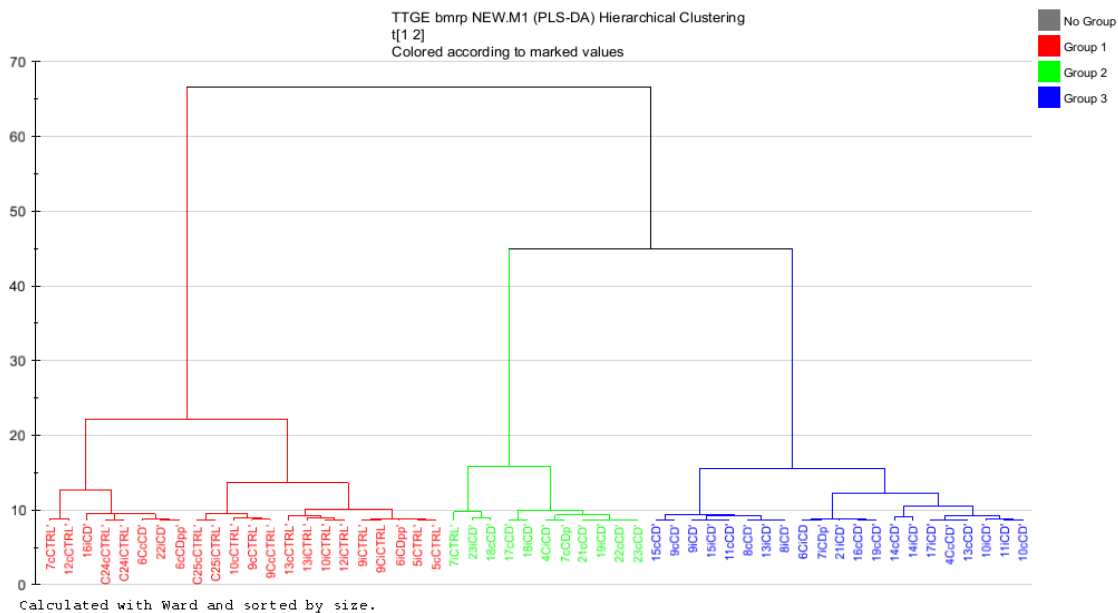


Fig.1

TGGE profile assay with factorial discriminant analysis (FDA) was preceded by the transformation in independent variable (eigenvalue) through a principal component analysis (PCA). Transformed data, used for the FDA analysis, showed a well-defined separation between controls and CD group ($P = 0.0003$) with a total error of 1.85%. (Fig.2). Results of this analysis indicated that the microbiota composition is a sufficient factor to predict the patient category (CD or non-IBD). To improve these results, we performed the partial least square discriminant analysis (PLS-DA) on TTGE profiles. Results obtained showed a predictiveness of 90,74% (Table 1). All these data emphasize the importance of gut microbiota in predicting the disease status.

Figure 2

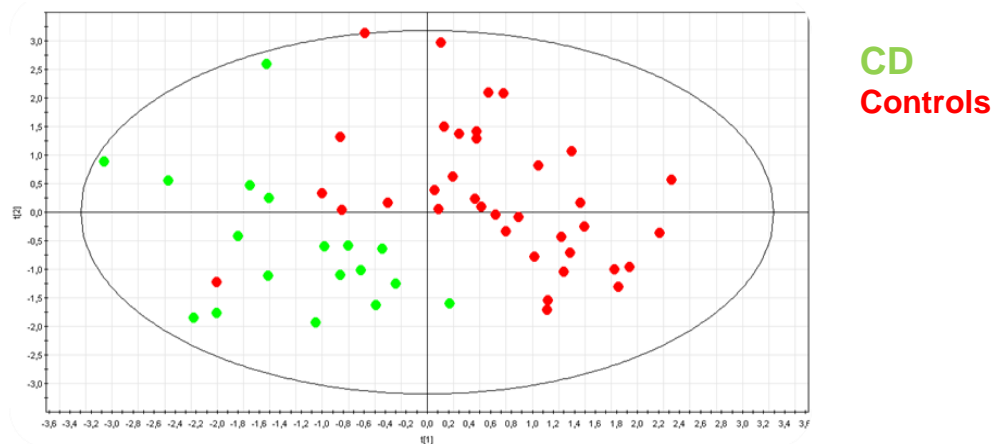


Table 1

Misclassification Table for Model 1					
	1	2	3	4	5
1		Members	Correct	1	2
2	1	36	91,67%	33	3
3	2	18	88,89%	2	16
4	No class	0		0	0
5	Total	54	90,74%	35	19
6	Fishers prob.	6e-009			

1 = CD
2 = CTRL

Results obtained from the mucosal-associated bacteria study underlined the presence of a peculiar microbiota associable to CD in paediatric patients and showed the existence of TTGE CD profile sub-clusters. The presence of the latter could indicate that CD is featured by more sub-types with by different degrees of intestinal inflammation. The altered microbial community structure, which seems to be a common feature of pediatric CD patients, could be a condition typical of susceptible individuals that exists before the disease manifests.

Results obtained by Dice index showed a similarity of 65% between TTGE profiles from CD patients; similarity index values within each cluster were: 78,75% for cluster 1, 85,5% for cluster 2 and 3.

It will be of great interest in the next future to find out the diversity of species or bacterial groups between the different subsets of the disease, addressing a possible role of gut microbiota in establishing different inflammation environments.

Aim2

The concentration of mucosa-associated bacteria from ileal and cecal biptic specimens of CD pediatric patients was compared to that of controls. Results showed that total aerobic, facultative-anaerobe and gram-negative counts were all significantly higher in patients than in controls ($p=0.001$); gram positive bacteria were also increased, but not significantly (Figures 3 and 4).

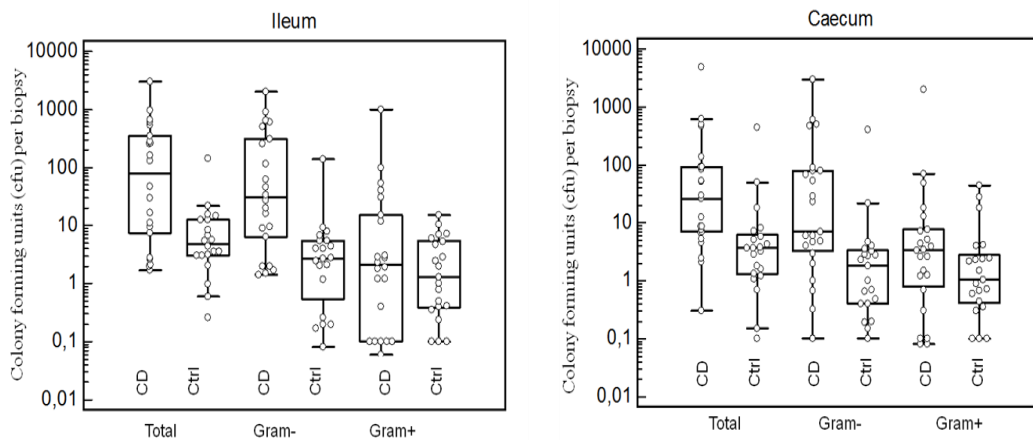


Figure 3. Mucosa-associated bacteria: CD patients and controls. Quantification of aerobic and facultative anaerobic bacteria isolated from the ileal and caecal mucosa after removing of surface mucus by dithiothreitol treatment.

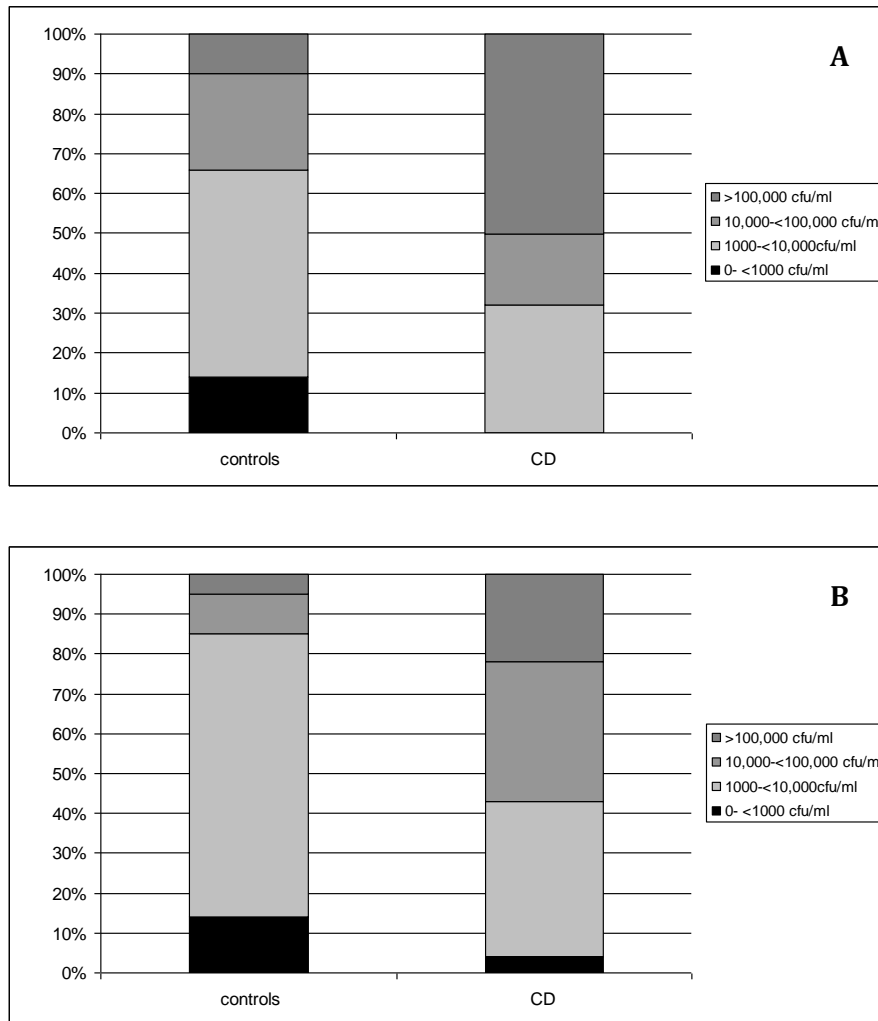


Figure 4. (A) Percentage of patients showing different concentrations of total ileal mucosal bacteria. (B) Percentage of patients showing different concentrations of total caecal mucosal bacteria (undetectable - <1000, 1000 - <10,000, 10,000 - <100,000, and >100,000 cfu/ml).

Bacterial concentration values of CD biopsies were: > 100,000 cfu/ml in 50% of ileal specimens and in 33% of cecal specimens.

Tables 2, 3 show the distribution of aerobic and facultative anaerobic Gram-negative and Gram-positive bacterial species in ileal and cecal mucosa biopsy specimens, reported as number and percentage of isolates found relatively to the total Gram-negative or Gram-positive isolates detected in each patient group. Data analysis showed a generalized similar distribution of bacterial species isolated among all patient groups; most represented species belong to *Escherichia* and *Streptococcus* genera.

Table 2. Distribution of bacterial isolates from aerobic and facultative anaerobic cultures of ileal mucosal samples

	Total Gram negative	Total Gram positive	<i>Escherichia</i>	<i>Klebsielleae</i>	<i>Proteeeae</i>	<i>Streptococcaceae</i>	<i>Staphylococcaceae</i>
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Controls	23 (48)	25(52)	21(91)	2 (9)	0 (0)	21(84)	4 (16)
CD	30(57)	23 (43)	23(77)	5 (17)	2 (6)	18 (78)	5 (22)

Table 3. Distribution of bacterial isolates from aerobic and facultative anaerobic culture of cecal mucosal sample

	Total Gram negative	Total Gram positive	<i>Escherichia</i>	<i>Klebsielleae</i>	<i>Proteeeae</i>	<i>Streptococcaceae</i>	<i>Staphylococcaceae</i>
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Controls	24(52)	22 (48)	21(87)	3(13)	0 (0)	18(82)	4(18)
CD	29 (51)	28 (49)	23(79)	3 (10)	3 (10)	21 (75)	7(25)

Aim 3

Independent groups of investigators have reported an increased number of mucosa-associated *Escherichia coli* in IBD patients. However, it is not known in detail, which specific strains proliferate under disease conditions and which features are responsible for their dominance. *E. coli* genome consists of a conserved part, the so called “core genome”, carrying out the basic cell functions, and a flexible strain-specific part, involved in bacterial fitness and adaptation to different environments. To investigate the presence of *E.coli* strains specifically associated to CD and to assess the intestinal habitat suitability, we performed the analysis of:

- phylogenetic groups;
- *E. coli* intraspecies variability by Random-amplified polymorphic DNA (RAPD);
- adhesive/invasive properties of isolated *E. coli* strains.

The study was carried out on 5 pediatric patients with active CD (ileum-colonic involvement, moderate-to-severe disease) and 5 age-matched asymptomatic controls.

Results showed that the majority of mucosa-associated *E. coli* from controls belongs to A phylogroup (76%), whereas, those from controls are equally distributed among A, B1 and B2 groups (34%, 24% and 38%) (Fig.6).

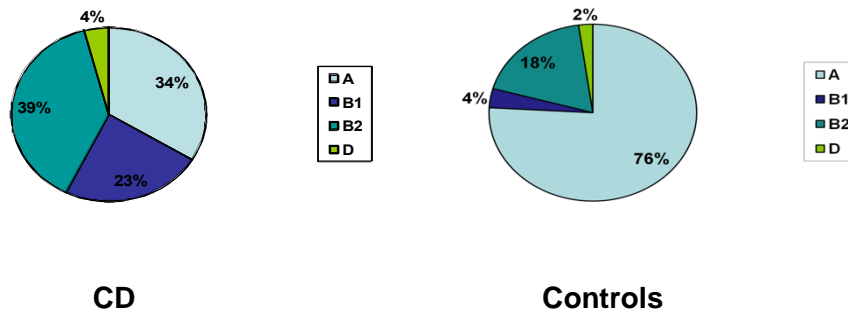


Figure 6

Results from intra-patient mean Dice value were: 0,515 +/- 0,065 in CD patients vs 0,349 +/- 0,046 in controls. A major RAPD profile homogeneity was observed in the CD group as compared to controls, although the difference was not statistically significant: this finding suggests that the inflamed habitat may lead to a biased genomic shaping.

We investigated the association between specific *E. coli* RAPD profiles and phylogenetic groups by a PLS-DA analysis. Results showed a significant separation (Fisher's test $P=1.2 \cdot 10^{-11}$) between three of the four phylogroups (A, B1, B2) in each patient (Fig.7).

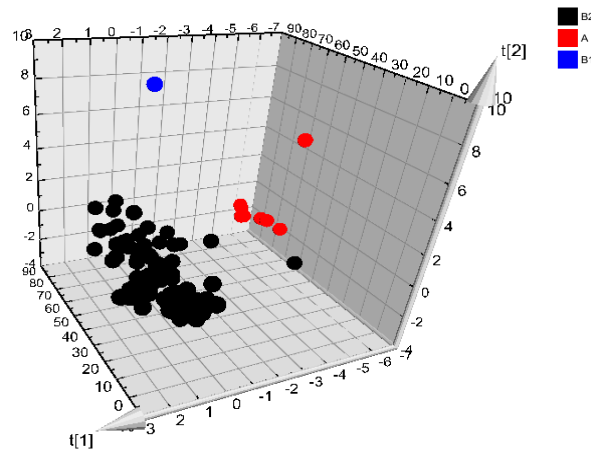


Figure 7. RAPD profiles regressed against phylogenetic groups

It was reported by Giraud *et al.* (2001) that *E.coli* populations, when introduced in germ-free mouse bowel, occupy all available niches, showing a high degree of genomic diversity. Our results showed a reduction of genomic variants of *E.coli* species in CD patients as compared to controls. This could be explained suggesting that the intestinal habitat of diseased individuals is less permissive as compared to that of healthy individuals and, therefore, causes a reduction in the number of genomic variants able to survive. Indeed, in healthy individuals, genomic diversification, within and among species, increases probably to avoid competition phenomena, leading to the colonization of all available ecological niches.

Moreover, an increased presence of more aggressive bacterial phylogenetic groups (B2) in CD and more beneficial groups in controls was displayed. In CD intestinal habitat, phylogenetic groups were randomly distributed and showed a higher heterogeneity of *E.coli* strains. Correlation between phylogenetic groups and RAPD profiles showed peculiar RAPD profiles associable to phylogenetic groups (Fig. 8).

Aim 4

The first part of the study (**A**) was carried out by analyzing only one strain or a maximum of three per patient. A second part of the study (**B**) was carried out by increasing to 77 the number of strains analyzed by subject to better characterize the whole mucosa-associated *E. coli* population.

Results of the study A

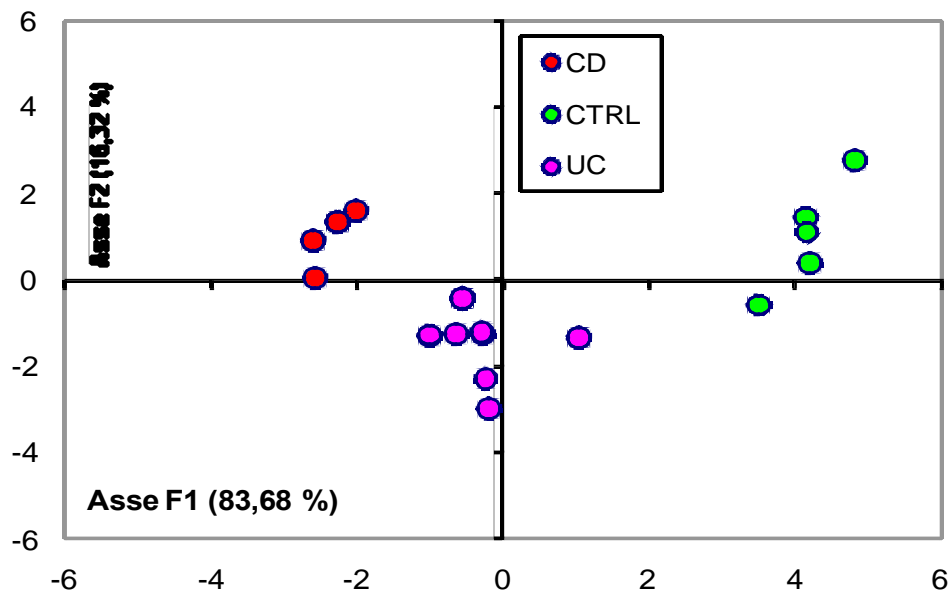
E. coli strains isolated from CD patients and controls were analysed by PCR to assess the frequency of pathogenicity genes associated with virulence as well as the frequency of fimbrial adhesive genes.

No significant difference was found between patients and controls, evaluating one strain for patient. Quite all *E. coli* strains showed a positivity for *fimH* gene, while only a few, randomly distributed, for other adhesivity genes (data not shown).

Sixty-one strains, isolated from biopsies of IBD children and controls (specifically, 28 strains from CD and 20 from UC patients plus 13 from controls), were analysed to find out possible correlations between *fimH* mutational profiles and disease pathogenesis. *FimH* gene was amplified by PCR, sequenced and analyzed for mutation presence.

Results showed the presence of a strong relationship between mutational *fimH* profiles and type of disease (UC or CD, Figure 5), suggesting that *fimH* mutational profiles may predict the specific disease status (CD,UC, control). Moreover, it was evidenced an higher site substitution rate (SSR) in *fimH* gene as well as an higher number of mutations of this gene in IBD. Finally, it was assessed an inverse correlation between mucosal inflammation level and mean number of mutations, in agreement with the higher sequence homogeneity found in CD.

Figure 5



Results of the study B

We analyzed 77 *E. coli* strains from each one of 5 CD patients and 5 controls. Results obtained showed a significant prevalence in the CD group of the following virulence factors: *ibeA* gene (82/154, 53.1%); *traT* gene (142/154, 92.20%); *K1* gene (128/154, 83.1%); *sfaFocDE* gene (28/154, 18,2%); the control group showed a significance prevalence of the following genes: *fyuA* gene (93/154, 60,4%), *papC* gene (135/154, 87,6%), *traT* gene (88/154, 57,1%), *kpsMTII* gene (154/154, 100%).

Interestingly, the *ibeA* gene, encoding for an endothelial invasine, was present only in the CD group and was always associated to B2 and D phylogenetic bacterial groups; we suggest that this gene may be related to a higher virulence of strains.

In conclusion, we believe that the inflamed intestinal mucosa environment, which characterizes the active phase of the disease and is associated with a consistent presence of pro-inflammatory cytokines, aggressive metabolites and inflammatory mediators, may contribute to reduce the habitat suitability. This condition may shape the intestinal microbiota by selecting positively only few genomic variants.

Aim 5

The first part of the study (**A**) was carried out by analyzing only one strain or a maximum of three per patient. A second part of the study (**B**) was carried out by increasing to 77 the number of strains analyzed by subject to better characterize the whole mucosa-associated *E. coli* population.

Results of the study A

Adhesivity of *E. coli* isolated strains were tested *in vitro* by using CaCo-2 and Hep-2 cell lines. Results showed that all isolated strains, from both CD patient and control groups, were strongly adhesive. *E.coli* invasive strains were no detected.

Results of the study B

Almost all strains isolated resulted to be strongly adhesive by using CaCo-2 and Hep-2 cell lines, with no significant difference between the two patients group studied *E.coli* invasive strains were isolated with at low percentage (1-5%) in both CD and control. The screening is still in progress.

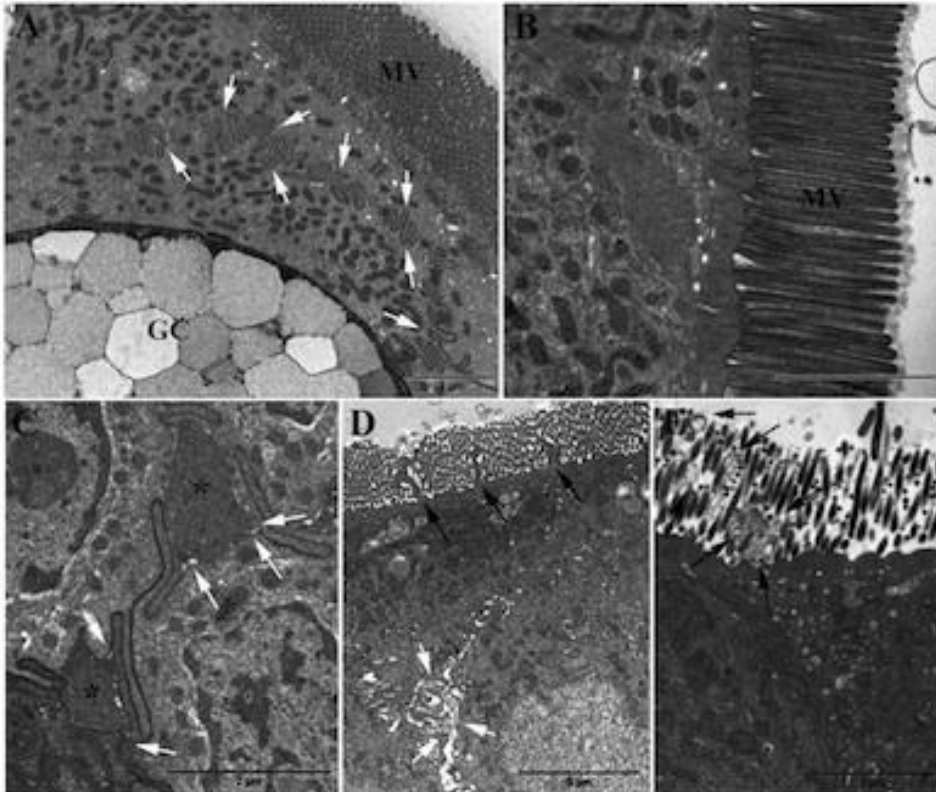
Ultrastructural Unit Results:

Aim 1

Ultrastructural analysis of intestinal biopsies from CD and UC pediatric patients was performed and compared to that of healthy controls. For this purpose, pathological specimens were taken close to macroscopically inflamed ileal and colonic areas of patients. As expected, intestinal epithelium of healthy controls (Figure 1 A, B) showed microvilli (MV) which were extremely regular in shape, dimension, and organization

together with well connected tight junctions (white arrows) maintaining their structural integrity (Figure 1 A). Differently, TEM analysis of biopsies from CD patients (Figure 1 C-E) showed microvilli altered in size and shape, frequently fused together (Figure 1 D, arrows), with an architectural organization broken up by the presence of numerous vesicular bodies (Figure 1 E, arrows). Enterocyte alterations were characterized by cytoplasm derangement with deep vacuolation and dilatation of the endoplasmic *reticulum cisternae* (Figure 1 E). In some areas, the mucosal epithelium had lost its typical organization, above all, tight junctions were altered at different degrees with an increased distance between enterocyte membranes (Figure 1 C-D, white arrows) and the intercellular spaces, often filled with edema (Figure 1 C, asterisks).

Figure 1

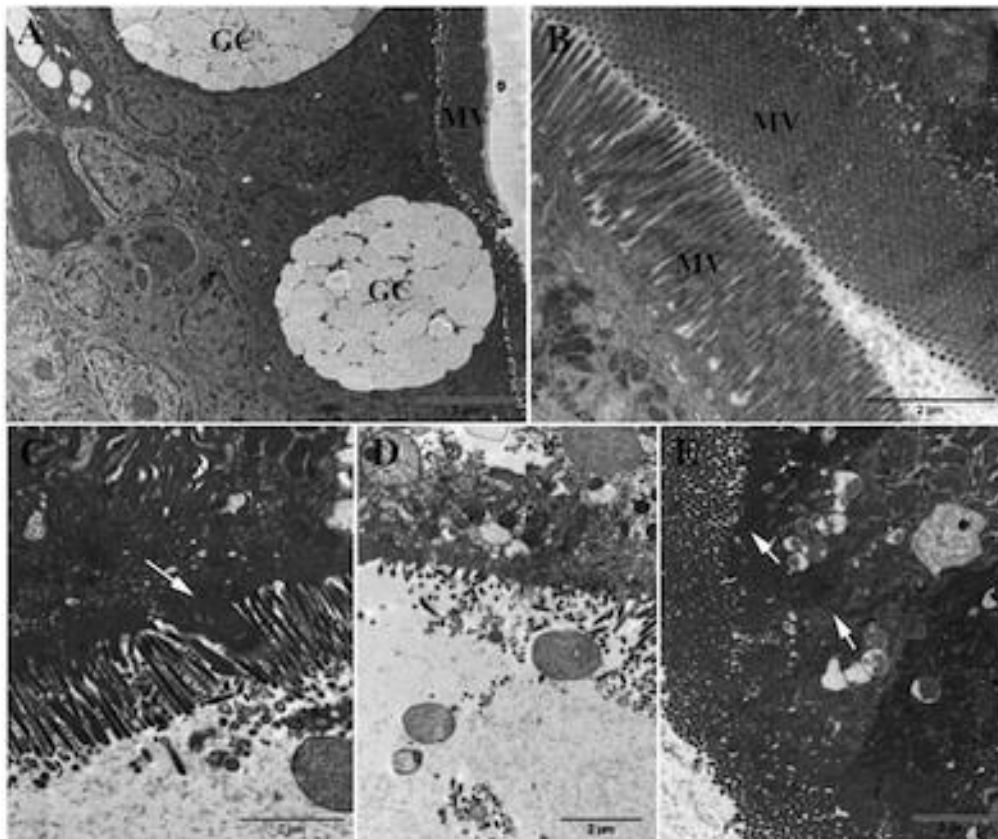


Colonic biopsies from children with UC (Figure 2 C-E) showed microvillar alterations comparable to those described in CD patients (Figure 2 C, D). Microvilli were frequently fused together (Figure 2 C, white arrow) and, in some areas, nearly absent (Figure 2 D); a number and size reduction of goblet cell was found (Figure 2 E). Colonic mucosa of these patients was less regular as compared to controls and showed a reduction of TJ strands, with a loss of the classical membrane finger-like structure (Figure 2 E, white arrows). Enterocyte alterations were characterized by cytoplasm derangement with higher electron-dense zones and deep vacuolation (Figure 2 C, D).

Electron microscopy analysis showed severe morphological alterations, not only in the inflamed, but also in the macroscopically uninfamed intestinal tissue, leading to the loss of gut barrier integrity.

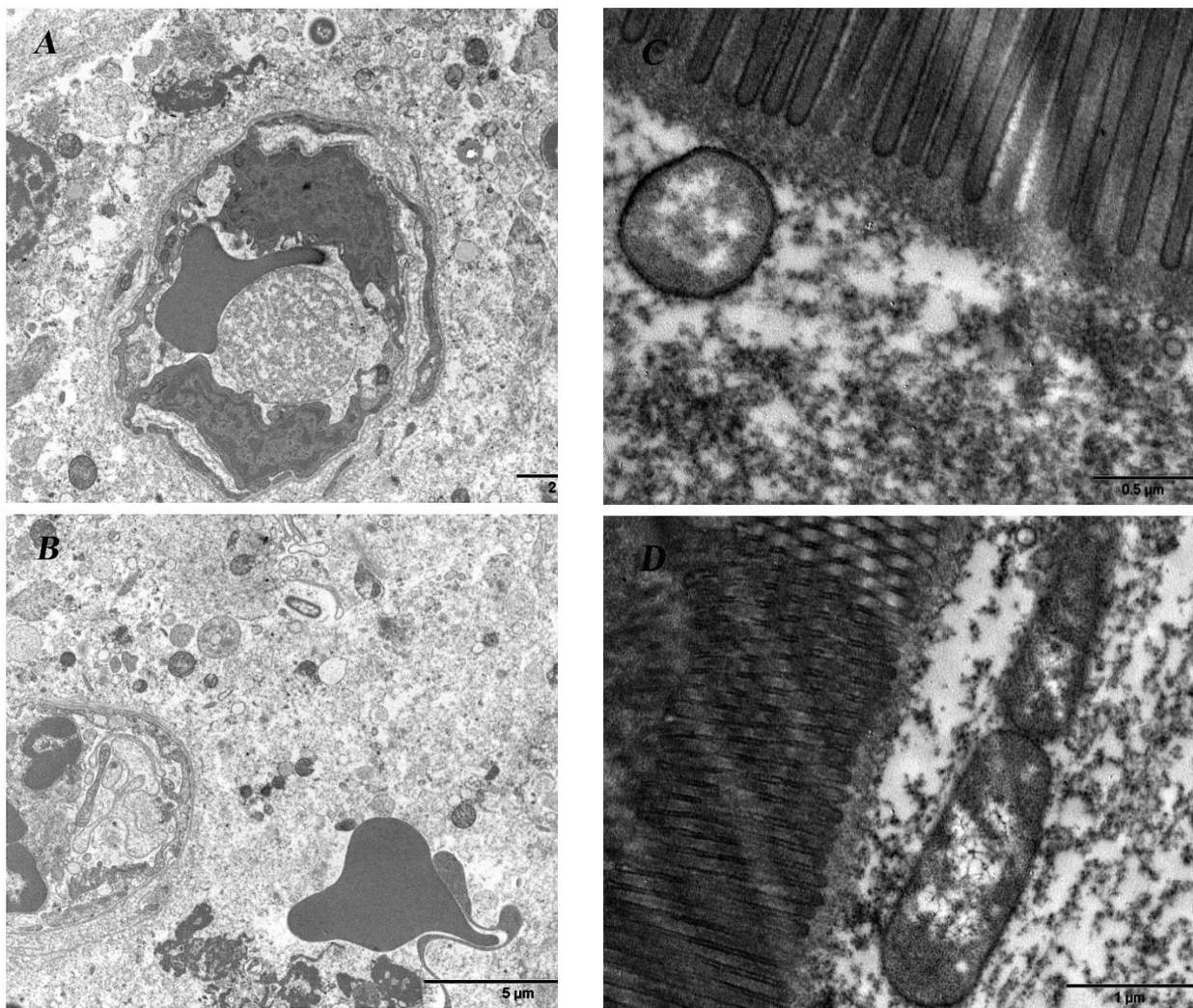
Gaps along the intestinal barrier were in agreement with the increased microbial antigen entry in the mucosa and the worsening of inflammation.

Figure 2



Bacteria were frequently observed in proximity of microvilli (Figure 3 C, D), but rarely inside the biopsy specimens (Figure 3 A, B).

Figure 3



Aim 2

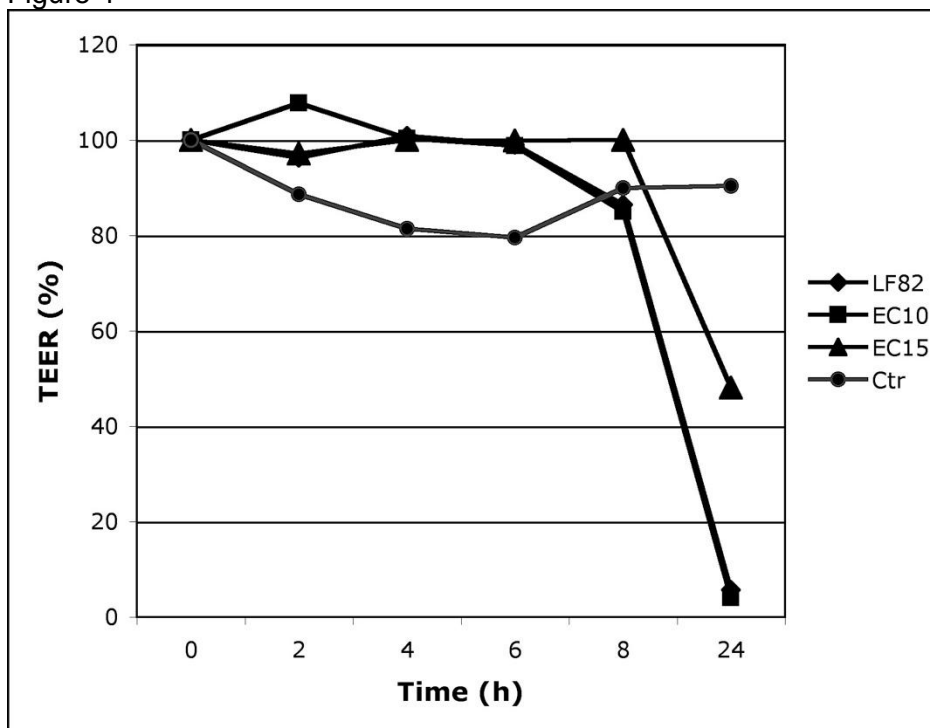
Gentamicin protection assay was performed in bioptic specimens from 24 CD and 10 UC patients and from 18 controls to identify the presence of intracellular bacteria. Invasion *in vitro* assay on HEp-2 cells allowed to identify and isolate two invasive lactose-fermenting bacterial strains, one in a CD and one in a UC patient, which were named EC10 and EC15, respectively. Invasivity indexes were 1.4% and 0.7% for EC10 and EC15, respectively (invasivity index of LF82, used as a positive control: 1.29%). Both these strains were determined to be *E. coli* and to belong to A (EC10) and D (EC15) phylogenetic groups. They were negative for the principal virulence genes typical of pathogenic *E. coli* strains: intimin, verotoxin (shiga-like-toxin) and enteroaggregative factor gene, while positive for the adhesive fimbrial factor H gene (FimH).

Aim 3

a) Regulation of epithelial barrier functions in response to invasive *E. coli* infection

As it is known that the increased intestinal permeability is a feature of the disease, we assessed *in vitro* the transepithelial electrical resistance (TEER) values of EC10 and EC15. CaCo2 cells, as a model of human intestinal epithelium, were cultured in presence or absence of bacterial strains and TEER was then measured. Results showed that both strains induced comparable and significant decrease in TEER, although EC15 didn't suppress it completely as compared to mock-infected cells (Figure 4).

Figure 4

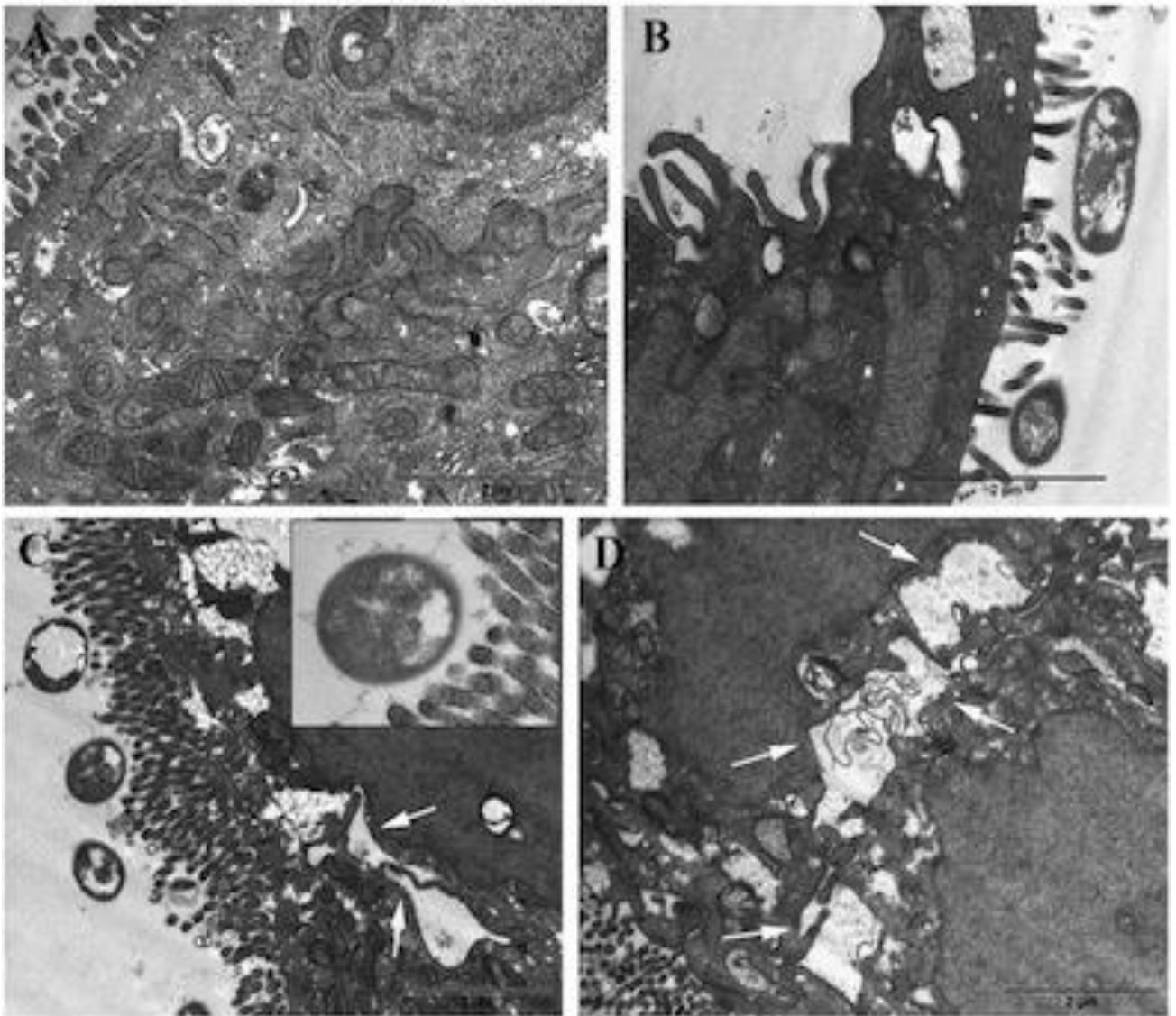


b) Transmission electron microscopic examination of bacterial-infected CaCo-2 cells.

After 24 h from infection, EC10 adhered close up to CaCo2 cells (Figure 5 B, C) and induced microvilli elongation. These bacteria were able to attach microvilli by fimbriae (Figure 5 C inset); this was a very interesting observation as *fimbriae* have been recognized as a key virulence factor for adherent-invasive strains. Moreover, this strain disrupted epithelial tight junctions of infected cells as compared to controls (Figure 5 C, D, white arrows).

These findings, which confirm previous TEER results, suggested that EC10 can affect the integrity of the polarized epithelial cell barrier in a way that is comparable to that of the positive control, LF82.

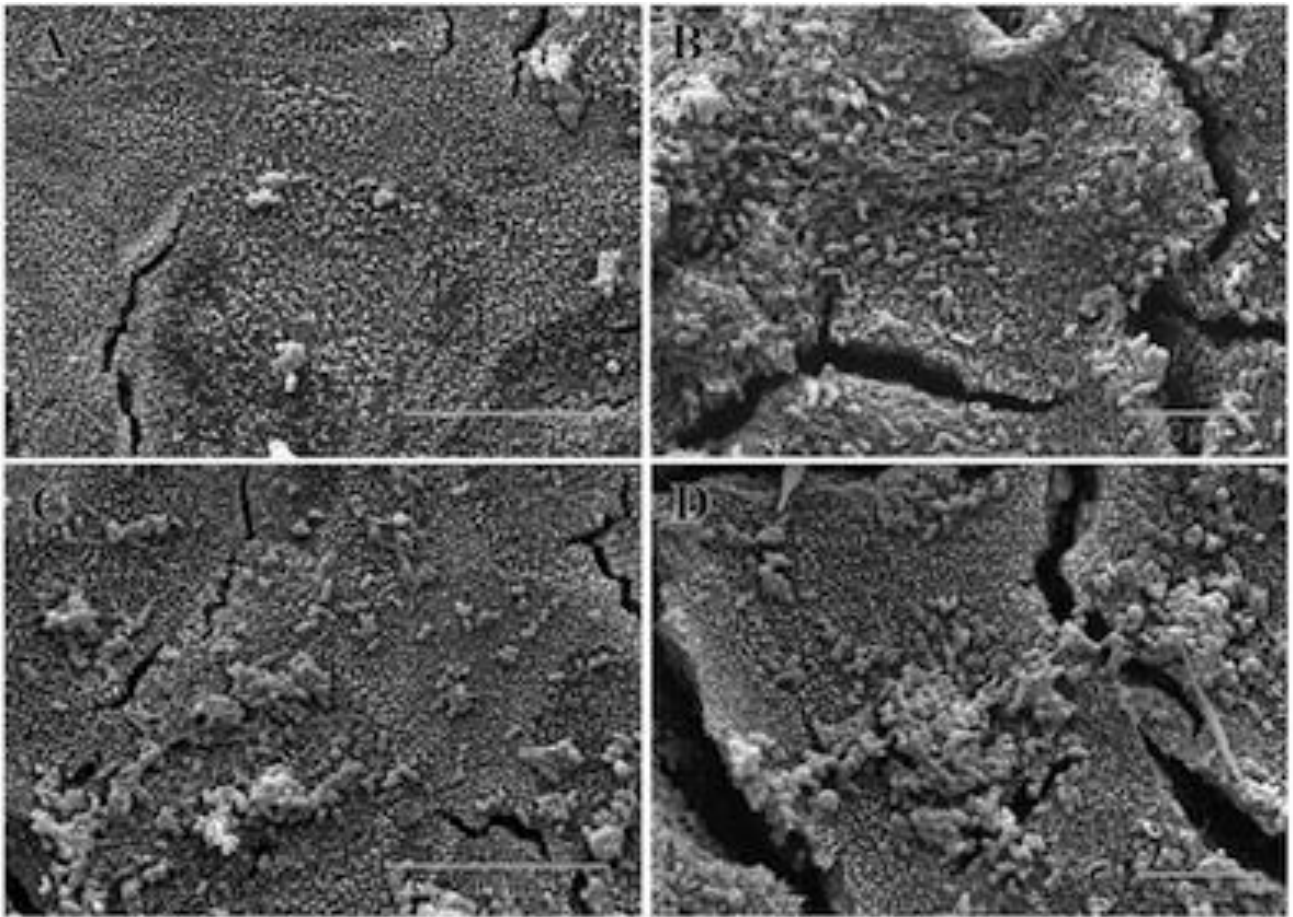
Figure 5



c) Scanning electron microscopic examination of bacterial-infected CaCo-2 cells.

The interaction between isolated pediatric *E. coli* strains and target cells was examined by scanning electron microscopy (SEM) and compared to that of AIEC prototype, LF82 (Figure 6). SEM analysis showed that mock-infected CaCo-2 cells appeared fully differentiated, displaying a developed brush border with well-defined apical microvilli (Figure 6 A). Besides, the analysis showed that bacteria-cell interaction was partially damaged due to the microvilli elongation on the brush border (Figure 6 B-D). Concerning the adherence pattern, EC15 strain (Figure 6 C) showed a pattern similar to that of LF82 (Figure 6 D); indeed, bacteria appeared more aggregate as compared to EC10 strain (Figure 6 B). These findings seem to be strictly related to the amount of biofilm formation.

Figure 6



Aim 4

We believe that, in our experimental conditions, the immunofluorescence assay is not suitable to investigate the interaction between Isolated AIEC bacteria and cells/cultured tissues, mainly due to the low sensitivity of specific antibodies used. Thus, we abandoned this method and decided to perform only ultrastructural assays.

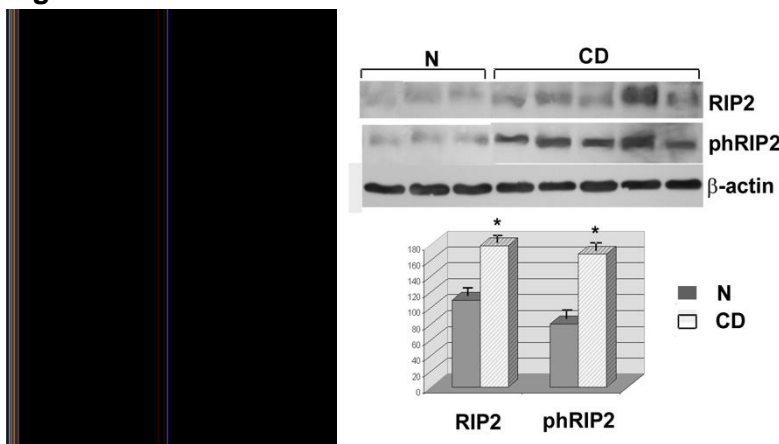
Immunological Unit Results:

Aim1

In order to describe the downstream events triggered specifically by Nod2 induction and demonstrating that the protein, other than overexpressed, is active, we analysed ex vivo the ability of Nod2 to form the immunocomplex with the kinase Rip2. Indeed, previous studies had shown that Nod2 is able to stimulate NF- κ B activity dependently on the co-expression of the adapter protein Rip2; thus, the latter is an essential and specific mediator of Nod1 and Nod2 signalling. Moreover, as it has been reported that Rip2 is activated through an autophosphorylation process, we also analysed the phosphorylated Rip2 expression. Experiments were carried out in a group of 15 children with CD and 10 age-matched healthy controls.

Results showed that immunocomplex Nod2/Rip2 was detectable in both CD and healthy specimens, but in the former it was significantly ($p < 0,05$) more expressed; furthermore, Rip2 protein is over expressed and present principally as phosphorylated active form in the inflamed mucosa of patients, as compared to controls (Figure1). In conclusion, we provide for the first time ex vivo evidence of physiologically relevant protein interactions between Nod2 and Rip2, that are able to trigger the innate immune response in intestinal mucosal specimens of children with CD. It was also suggested that both these molecules can represent useful targets for future biological therapies in IBD.

Figure 1



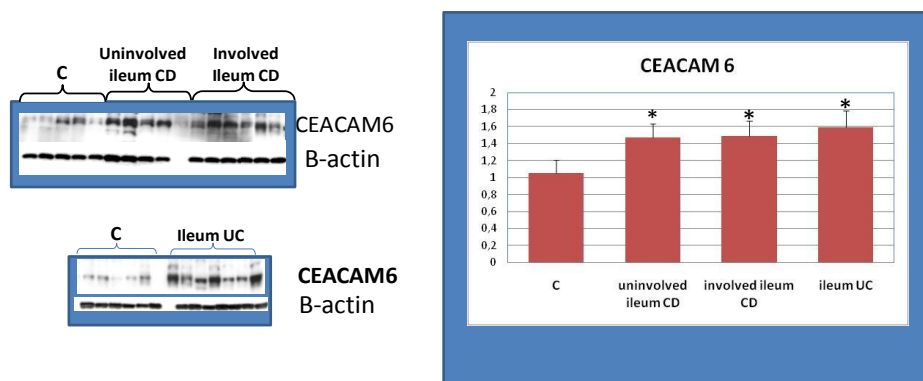
Aim2

The ileal mucosa of CD patients is supposed to be abnormally colonized by bacterial strains. In particular, an association between particular adherent-invasive *E. coli* strains

(AIEC) and IBD has been recently hypothesized. These bacteria specifically recognize the CEACAM6 surface receptor. Therefore, we analysed by western blot CEACAM6 protein expression in ileal bioptic specimens of CD and UC pediatric patients and compared results to those of healthy controls. A significant ($p < 0.05$) increase of CEACAM6 expression was detected in the inflamed ileum of CD patients as compared to controls. Moreover a significant increase of CEACAM6 expression was also detected in uninvolved areas of ileum of CD and UC patients ($p < 0.05$). (Figure2). The increased CEACAM6 expression in the uninvolved ileal mucosa of some CD and UC patients, compared to that in controls, suggests that some patients are genetically predisposed to express that molecule. In patients expressing high basal level of CEACAM6, the presence of AIEC bacteria and the secretion of $IFN\gamma$ and $TNF\alpha$ could lead to amplification of the inflammation.

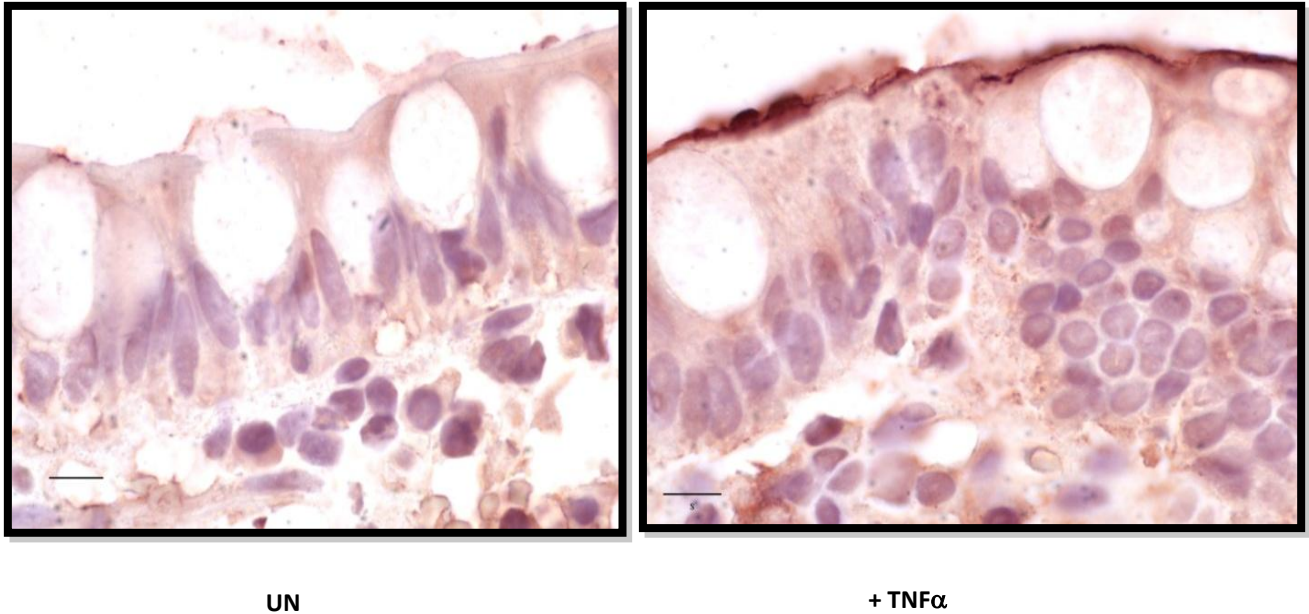
Even if invasive bacteria are known to be typically associated to the ileal mucosa, as reported by literature data, we investigated CEACAM6 expression level also in the colon of both CD and UC patients. Result didn't show any difference between inflamed colonic mucosa of CD and UC patients as compared to controls.

Figure 2



Aim 3

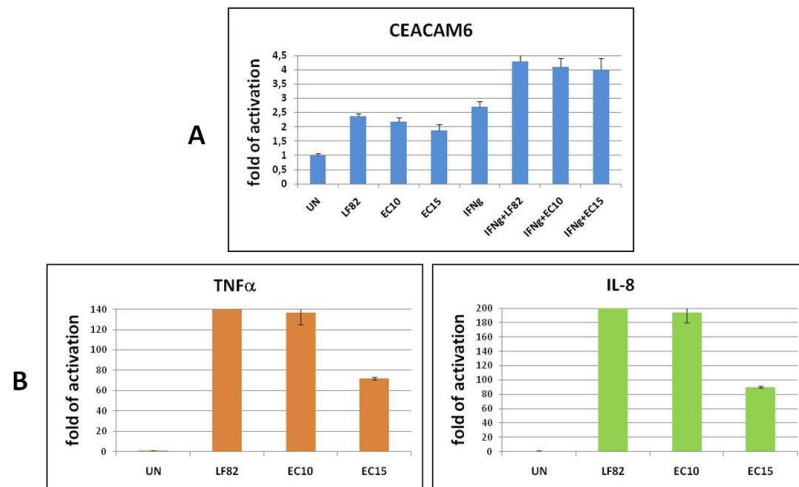
In order to assess whether the increased protein evidenced in the ileum of CD patients was localized on the surface of inflamed cells rather than dispersed throughout, we set up intestinal mucosal sections from inflamed tissues taken from fresh as well as cultured biopsies (healthy tissue incubated with pro-inflammatory cytokines). Results showed that CEACAM6 protein is always strongly up-regulated on the inflamed epithelial surface in agreement with the hypothesis that it works as a surface bacterial receptor (Fig.3).

Figure 3**Aim 4**

To assess whether AIEC strains, EC10 and EC15, previously isolated by our group, were able to alter CEACAM6 mRNA expression, monolayers of Caco2 were incubated for 3 hrs with EC10, EC15 and LF82 as a positive control; untreated cells were used as a negative control. Moreover, to evaluate the effect of a combined treatment, aliquots of cells were firstly treated for 48 hrs with INF-gamma and then, for other 3 hrs, with each bacterial strains. Control cells were treated with INF-gamma alone for 48 hrs.

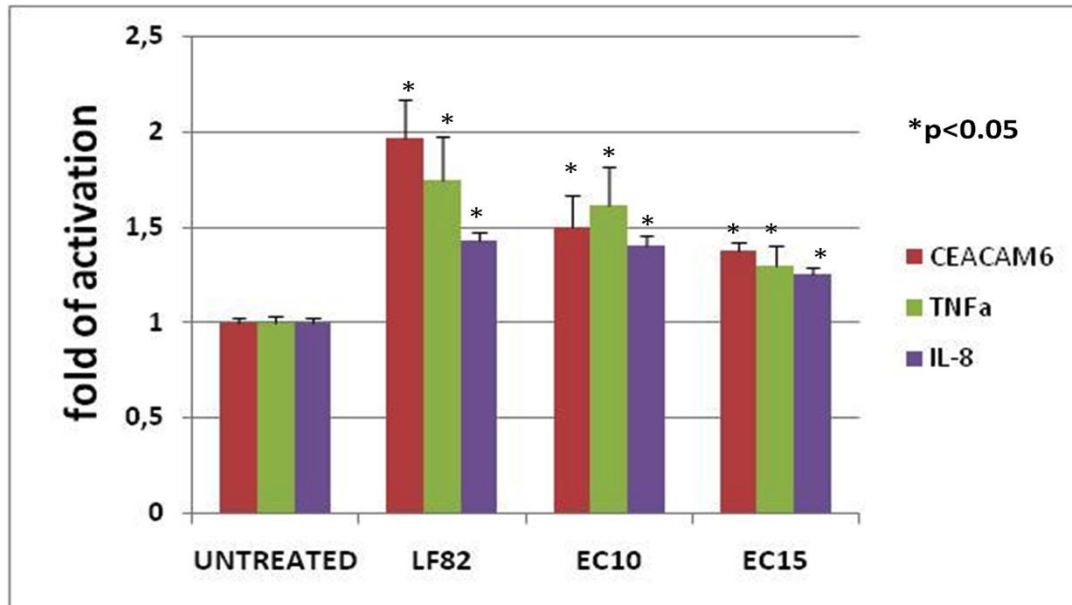
Results showed that both EC10 and EC15 were able to induce a significant ($p < 0.01$) increase of CEACAM6 gene expression, similarly to that produced by LF82 and INF-gamma. Results from the combined treatment showed a significant increase of CEACAM6 gene expression ($p < 0.01$), as compared to the effect of bacteria or IFN-gamma alone (Figure 4a). Furthermore, the ability of EC10 and EC15 to trigger an inflammatory response was assessed by measuring the gene up-regulation of two pro-inflammatory cytokines, TNF-alpha and IL-8. Results showed that EC10 could significantly increase pro-inflammatory cytokines expression as much as LF82 ($p < 0.01$), while EC15 a bit less ($p < 0.05$) (Figure 4b).

Finally, the gentamicin protection assay demonstrated that EC10 and EC15 strains were able to survive and replicate within the macrophage cell line, RAW 264.7.

Figure 4**Aim 5**

Above experiments were replicated using ileal mucosa explants from two healthy controls to confirm previous results with Caco2 cells. Biopsic specimens were cultured for 24 hrs in presence of EC10, EC15 or LF82 as a positive control; untreated tissues were used as a negative control. Results were fully confirmed: both strains could induce a significant up-regulation ($p < 0.05$) of CEACAM6, TNF α and IL-8, comparable to that obtained with LF82 (Fig.5).

Figure 5



LAY SUMMARY

Inflammatory bowel diseases (IBD) that include two classical entities such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory disorders causing severe morbidity and poor quality of life. The incidence of these diseases varies widely between different countries, but overall has increased greatly in recent years, and IBD is now a major public health problem. Almost 25% of IBD are diagnosed in pediatric age and in the adolescence.

Mechanisms underlying IBD come from loss of immunological tolerance and homeostasis towards commensal intestinal bacteria in subjects genetically predisposed. Bacterial colonization of the intestine is a key step for several physiologically processes such as formation of intestinal epithelial barrier, development of mucosal immunity and resistance to pathogen colonization. Due to the complexity of microbial community in the gut it is a great challenge to establish which components of the microbial flora specifically contribute to the development of intestinal inflammation in IBD. Previous reports on the characterization of the microbial communities in IBD indicate that individuals with IBD have reduced bacterial diversity, while compositional comparisons have generally identified reductions in components of the *Firmicutes* phylum often with concurrent increases in *Bacteroidetes* and facultative anaerobes such as *Enterobacteriaceae* (mainly *E. Coli*), a phenomenon known as "dysbiosis". An exciting development was the discovery of a group of microorganisms, called adherent and invasive *E. coli* (AIEC), associated with biopsy tissues in a subset of CD patients: these strains adhere to and invade epithelial cells via carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6), and replicate in macrophages.

This study was performed in pediatric IBD and has investigated different aspects of the innate intestinal immune response in relation to some gut microbiota components. In particular, the authors aimed at evaluating the intestinal dominant microbiota in a pediatric population with CD, by characterizing mucosa-associated aerobic and facultative anaerobic bacteria, screening *E. coli* strains for genes associated with pathogenicity and evaluating their adhesive/invasive properties. The authors also aimed at assessing the mucosal expression of genes involved in the innate immune response in relation to components of the gut microbiota; furthermore, they evaluated the expression of the receptor CEACAM6 for adhesive-invasive *E. coli* strains and the ability of novel identified adherent-invasive strains to induce the expression of the receptor CEACAM6 and of other pro-inflammatory cytokines.

The results of the study can be summarized as follows.

Results from the study of the mucosal-associated bacteria underlined the presence of a peculiar microbiota associable to CD in pediatric patients. Indeed, it was shown a well-defined separation between the profiles of the three subject groups (controls, CD and UC), suggesting that the microbiota composition is a sufficient factor to predict the patient category (CD versus non-CD). Interestingly, an altered microbial community structure seems to be a common feature of pediatric CD patients and could be a condition existing in susceptible individuals before disease symptoms.

It was also assessed that CD patients showed severe morphological alterations, not only in the inflamed, but also in the macroscopically uninfamed intestinal tissue, leading to the loss of gut barrier integrity.

Two new AIEC strains, one in a CD and one in a UC patient, were identified, suggesting a role of these strains also in the pediatric disease. A set of experiments showed that they can destroy the integrity of the polarized epithelial cell barrier as well as induce *in vitro* and *ex vivo* an up-regulation of the specific receptor, CEACAM6, and an inflammatory response, similarly to the classical AIEC prototype, known as LF82. It was also shown that these bacteria can survive and replicate inside macrophages.

The study showed that innate immunity genes, NOD2 and RIP2, are functionally active in the intestinal mucosa of CD patients and that a significant increase of CEACAM6 expression occur in the inflamed and uninfamed ileal tissue of both CD and UC patients as compared to healthy controls, suggesting a possible use of this receptor as a marker predisposing to the disease.

These findings highlight the relationship between microbiota, epithelial cell barrier and immune response for a better comprehension of the mechanisms underlying the pediatric IBD pathogenesis. It is strongly expected that novel therapeutic targeted strategies can originate from detailing the pathways leading to inflammation of the gut in IBD.