“FEDERICO II”

UNIVERSITY OF NAPLES

SCHOOL OF MEDICINE AND SURGERY

DEPARTMENT OF TRANSLATIONAL MEDICAL SCIENCES

PhD Program

“Human Reproduction, Development and Growth”

Director

Prof. Claudio Pignata

PhD Thesis

New insights in the pathogenesis and in the management of Pediatric Inflammatory Bowel Disease

Tutor

Prof. Riccardo Troncone

Student

Dr. Caterina Strisciuglio

Academic Year 2015-2016

INDEX

Chapter 1

1. Background

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is characterized by chronic relapsing intestinal inflammation. It is a worldwide health-care problem with increasing incidence. It is thought that IBD results from an aberrant and continuing immune response to the microbes in the gut, catalyzed by the genetic susceptibility of the individual. Although the etiology of IBD remains largely unknown, it involves a complex interaction between the genetic, environmental or microbial factors and the immune responses. Recently, most rapid progress has been made in the genetic study of gut inflammation.

However, the fact that genetic factors account for only a portion of overall disease variance indicates that microbial and environmental factors may interact with genetic elements in the pathogenesis of IBD. Meanwhile, the adaptive immune response has been classically considered to play a major role in the pathogenesis of IBD, although new studies in immunology and genetics have clarified that the innate immune response maintains the same importance in inducing gut inflammation.

Genetics

Over the past decades, there have been huge advances in our understanding of genetic contributions to IBD[1]. This is due to the technological advances in DNA analysis and sequencing and the use of huge multinational databases[2]. Advances in genetic testing and analyzing technologies have allowed for the completion of many genome-wide association studies (GWAS) which identify single nucleotide polymorphisms (SNPs). Recent studies have brought the number of IBD-associated gene loci to 163, of which 110 are associated with both diseases, 30 CD specific and 23 UC specific[3]. Studies of gene loci shared by UC and CD may provide new way to find their common pathogenesis.

The IBD genetic research began in 2001 with the discovery of NOD2 (nucleotide-bindingoligomerization domaincontaining 2), the first susceptibility gene for CD[4]. The NOD2 gene codes for a protein that was originally described as an intracellular receptor recognizing the muramyl dipeptide (MDP), a conserved motif present in peptidoglycan from both Gram-positive and -negative bacteria[5]. MDP stimulation induces autophagy which controls bacterial replication and antigen presentation [6,7], and modulates both innate and adaptive immune responses[8 9].

Genetic analyses have shown an indispensable role for autophagy in immune responses in IBD, and reported two autophagy-related genes named *ATG16L1*and *IRGM*[10,11]. Autophagy is a degradation mechanism originally implicated in the recycling of damaged proteins and provision of amino acids during nutrient starvation.[4] More recent studies have shown that the process is also involved in the degradationof intracellular pathogens and resistance against infection[12]. *ATG16L1* is essential for all forms of autophagy, and the coding mutation T300A is associated with an increased risk of CD. *IRGM* belongs to the p47immunity-related GTPase family. CD-associated polymorphisms in *IRGM* lead to reduced protein expression.

Epithelial cells and dendritic cells containing ATG16L1 and NOD2 variants show defects in antibacterial autophagy[7,13]. GWAS have also pointed out a significant associatiom between IBD and the *IL23R* gene [14]. The *IL23R* gene encodes a subunit of the receptor for the pro-inflammatory cytokine interleukin (IL)-23, a peptide involved in the generation of Th17 cells. The Th17 and IL-23 pathway is well established in the pathogenesis of IBD, with susceptibility gene loci *IL23R*, *IL12B*, *JAK2*, and *STAT3* having been identified in both UC and CD[15,16]. Variants in *IL12B*, which encodes the p40 subunit of IL-12 and IL-23, have been associated with IBD and other immune disorders. Defects in the function of IL-10 have also been associated with CD and UC[17]. Other susceptibility genes that regulate immune function include CARD9,IL1R2, REL, SMAD3 and PRDM1. The expanding numberof susceptibility gene loci described in IBD indicates that genetic influences are critical components of the disease pathogenesis; however the susceptibility loci mentioned above account for only 20%-25% of the heritability. Indeed, more recent theories emphasize the role of epigenetics, which alter cellular features through specific DNA methylation patterns and it has been first of all identified to contribute to asthma[18]. Direct or indirect influence on epigenetic patterns in pertinent cell types may explain the gap in IBD heritability left after GWAS studies. The genetic susceptibility of a human remains constant during lifetime. In contrast, epigenetic changes occur permanently, induced by microbiota components or directly by environmental triggers [18]. These new insights into genetics and heritability of IBD implicate that future explorations of gene-gene interactions, gene-pathway interaction and gene-environment interactions are likely to give us more insights into IBD pathogenesis than finding new rare variants.

Environment

There is no doubt that environmental factors play an important role in the pathogenesis of IBD. A large number of environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography, socialstress, and psychological element[18]. So far in addition to familial aggregation, which is the most important IBD risk factor, only tobacco smoking and appendectomy

have been demonstrated to be strongly associated with IBD incidence[3,5]. The contribution of other potential environmental risk factors, proposed as important in IBD predisposition (including oral contraceptives and diet), has not yet been demonstrated because of discordant results[6,7]. An emerging and interesting theory is the "hygiene hypothesis, which correlates the epidemiological rise in IBD incidence over the 20th century, both in developed and developing countries, with the improvement in general hygienic conditions (i.e. free access to clean water, smaller family size, etc.)[20,21]. This theory asserts that exposure to poor hygiene or infections during childhood can protect from developing IBD or other kind of autoimmune and allergic diseases later in life[23,24]. Moreover, several epidemiological studies suggested a role of perinatal or early life events in the etiology of IBD[10,11,12], such as non-specific (gastroenteritis and other non-specific infections) and specific (vaccines, passive smoking) exposures. A possible explanation is that a decreased prevalence of infections during childhood could lead to a major individual susceptibility in developing IBD later in life. However, there are no definitive data demonstrating a final role of hygiene in IBD development. Understanding the role of environmental factors is important not only for the possible preventive interventions in genetically predisposed individuals, but also to offer a better disease care to those already suffering with IBD.

Microbial factors

The whole human gut microbiome consists of approximately 1150 bacterial species, with each individual host having roughly 160 species[19]. Gut microbiome is established within the first 2 wk of life and then usually remains remarkably stable thereafter. Recent studies have shown disease associations with dysbiosis or abnormal compositions of the gut microbiota [20, 21,22,23], finding a significantly reduced biodiversity in faecal microbiome in IBD patients compared to that in healthy controls[24].

Dysbiosis includes increases in bacterial numbers, a decreased bacterial biodiversity,a increased abundance of the phyla *Proteobacteria* and *Bacteroidetes*, and a decreased abundance of *Firmicutes*[25,26]. Changes to the composition of the microbiome and its interaction with the immune system play a crucial role in the pathogenesis of CD and UC. Genetic susceptibility leads to a dysregulation of the mucosal immune system that result in excessive immunologic responses to normal flora, however in addition also an imbalance exists in the composition of the microbiota elicits a pathologic response from the normal mucosal immune system [27]. In healthy intestine, the Firmicutes and Bacteroidetes phyla predominate, and contribute to the production of epithelial metabolic substrates. In contrast, the microbiota is characterized by a relative lack of Firmicutes and

Bacteroidetes, and an over-representation of enterobacteria in CD; whereas in UC it has been reported a reduction in Clostridium spp. and an increase in *Escherichiacoli* (*E. coli*) [28, 29].
Future research needs to further clarify whether the intestinal dysbiosis in IBD patients is a cause or a result of intestinal inflammation , it is likely that such alterations in bacterial populations has a knock on effect on gut immune system; perhaps disrupting the delicate balance that is maintained in the healthy gut and further contributing to pathology.

Immunological factors

Most studies in the last two decades have focused on the role of abnormal adaptive immune responses in the pathogenesis of IBD.
The focus on the adaptive immune response has ultimately led to the notion that the two main types of IBD represent clearly distinct forms of gut inflammation: CD has long been considered to be driven by a Th1 response and UC has been associated with a non-conventional Th2 response[30,31].
The newly described Th17 cells are also involved in the gut inflammatory response in IBD[32].
However, immunological studies have recently focused on the mucosal innate immune responses, such as epithelial barrier integrity, innate microbial sensing, autophagy and unfolded protein response.

Innate immunity

The innate immune response represents the first line of defense against pathogens and it is initiated by the recognition of microbial antigens by pattern recognition receptors including toll-like receptors (TLRs) on the cell surface and NOD lik ereceptors in the cytoplasm[33]. Recent studies have found that the behavior of the cells mediating innate immunity and the expression and function of both TLRs and NOD proteins are altered signicantly in individuals with IBD. GWAS reveal that the NOD2 muta- tions most commonly associated with CD induce a defective ability of the gut to respond to LPS, and this defect may contribute to disease susceptibility [34]. Although the functional role of NOD2 mutations is still controversial, available evidence suggests that they represent loss-of- function mutations that lead to reduced activation of NF- κB[35].This lacking response might result in reduced antibacterial agent production and pathogenic microbial invasion[36]. Other studies suggest that the loss of function of NOD2 may result in the lack of inhibition of TLR2 stimulation, leading to activation of inflammatory pathways and excessive Th-1 responses[37]. Furthermore, NOD2 also contributes to immune tolerance. These effects are impaired in cells from patients with NOD2 mutation 3020insC[38]. IL-23 is a key cytokine both in innate and adaptive immunity and

possesses a central role in driving early responses against microbes. IL23R polymorphisms have been associated with both CD and UC, suggesting that IL-23 may represent a shared inflammatory molecule in chronic intestinal inflammation. Recent studies have shown that, besides its activity on Th17 cells, IL-23 can also act on cells of the innate immune system. IL-23 has been shown to induce Th17 cytokine production by innate lymphoid cells (ILCs) that share the phenotype of lymphoid tissue-induced cells[39]. CD has also been associated with ATG16L1 and IRGM genes, which are involved in autophagy. Recent studies have shown that autophagy is also involved in the degradationof intracellular pathogens. In patients with mutations in NOD2 or Atg16L1 it has been described decreased bacterial handling. Moreover, also dysregulation of the unfolded protein, which is linked to autophagy and innate immunity response, may contribute to IBD pathogenesis. This response is induced by endoplasmic reticulum stress and induces apoptotic cell death, contribuiting to IBD pathogenesis[40]. In addition, defective epithelial barrier and increased intestinal permeability have been observed in IBD patients[41]. The first physical barrier against intestinal bacteria and food antigens e is represented by the mucous layer that covers the intestinal epithelium and it is important in the prevention of bacterial invasion and intestinal inflammation [42]. The second line of defense is formed by the intestinal epithelium, which form a physical barrier against bacteria, and can secrete different antimirobial peptides. Defective expression of antimicrobial peptides has been observed in patients with CD[43].

Adaptive immunity

As opposed to the innate immune response, the adaptive immunity is highly specific, often takes several days to respond and depends on the type and number of T cells. Th1 cells, induced by IL 12, produce a high amount of IFN-γ, whereas Th2 cells release IL-4, IL-5 and IL-13[44]. An abnormal Th1 immune response is thought to cause intestinal inflammation in CD, and it has been observed that mucosal T cells from CD patients produce higher amounts of IL-2 and IFN-γ than T cells from UC patients or controls[45]. Whereas in UC it has been shown that a typical NK T cells release higher amounts of the Th2 cytokine IL-13 than T cells from controls or CD patients [46,47].Therefore, CD has been thought to be characterized by a Th1 immune response, while UC has been considered as a Th2-mediated disease[48]. Although, there have also been different observations about mucosal Th1 and Th2 cytokines in IBD. Both UC and CD biopsies cultured in vitro release high and comparable amounts of IFN-γ[49]. Lower levels of IL-13 are found in the colonic mucosa of UC patients compared to those in CD patients and subjects of the control group. Collectively, these data should lead us to reconsider the Th1/Th2 paradigm in CD and UC[48].

Th17 cells are a T cell subset characterized by the production of large amounts of IL-17A, IL-17F, IL-21 and IL-22. They are induced by a combination of IL-6 and transforming growth factor (TGF)-β, and their ex- pansion is promoted by IL-23[50]. High transcript levels of IL-17A have been detected both in CD and UC mucosa in comparison to normal gut[51,52]. Moreover, the inflamed IBD mucosa cultured in vitro produces higher levels of IL-17A than the control[45]. Furthermore, Th17 cells are an important source of IL-21, an IL-2-related cytokine which is up-regulated in inflamed IBD mucosa. [53]

1.2 The main aims of this PhD thesis are:

The main aim of this PhD thesis are:

- Deepen the knowledge of IBD pathogenesis:
  - ✓ Investigate clinical and functional implication of impaired autophagy in inflammatory bowel disease
  - ✓ Study the relation between the exposure to environmental factors and the risk to develop UC and CD in a cohort of pediatric patients in Southern Italy.
  - ✓ Correlate serum levels of hepcdin, a protein that controls the amount of iron entering the blood circulation,with disease activity, inflammatory markers, and iron absorption in patients affected by paediatric IBD
  - ✓ Dissection of the cross-talk between the different mucosal compartments to evaluate the cytokine production profile and the activation status of both lamina propria and epithelium cell compartments through an ex vivo analysis of colon biopsies obtained from pediatric patients with IBD.
- Investigate genetic susceptibility in inflammatory bowel disease
  - ✓ Clarify the molecular basis of an early onset ulcerative colitis in an 18-mo-old affected child with consanguineous parents.
  - ✓ Conduct a case-control association analysis between the common CB2-Q63R functional variant of the cannabinoid receptor 2 and IBD in a pediatric cohort of children and evaluate possible correlations between the CB2-Q63R variant and the clinical features .
- Describe peculiar clinical features of pediatric inflammatory bowel disese
  - ✓ Investigate prevalence and disease course of paediatric IBD patients presenting with pancreatitisin a multicenter study.
  - ✓ Evaluate by endoscopy and histology the prevalence of periappendiceal inflammation in children affected by UC.
  - ✓ Study the occurrence and the natural history of C. difficile infection in a multicenter pediatric study.

- Propose new therapeutic strategies for the treatment of IBD.
  - ✓ Analyze the effects of a mixture of 3 Bifidobacterium strains (B. longum, B. breve, B. infantis), on the phenotype and on antigen processing of monocyte-derived DCs in a pediatric cohort with IBD.

- ✓ Evaluate the efficacy of a cow's milk protein elimination diet on induction and maintenance of remission in UC and define the associationwith atopy in children with UC
- ✓ Provide a consensus based recommendations specifically for paediatric gastroenterologists treating children with IBD regarding the use of biosimilars.

1.3 References

1. Gaya DR, Russell RK, Nimmo ER, Satsangi J. New genes in inflammatory bowel disease: lessons for complex diseases? Lancet 2006; 367: 1271-1284.
2. Duerr RH. Genome-wide association studies herald a new era of rapid discoveries in inflammatory bowel disease research. Gastroenterology 2007; 132: 2045-2049 [PMID: 17484895
3. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012; 491: 119-124.
4. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001; 411: 603-606.
5. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nuñez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J BiolChem 2003; 278: 5509-5512.
6. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, Ferguson DJ, Campbell BJ, Jewell D, Simmons A. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med 2010; 16: 90-97 .
7. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhães JG, Yuan L, Soares F, Chea E, Le Bourhis L, Boneca IG, Allaoui A, Jones NL, Nuñez G, Girardin SE, Philpott DJ. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol 2010; 11: 55-62.
8. Shaw MH, Kamada N, Warner N, Kim YG, Nuñez G. The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation. Trends Immunol 2011; 32: 73-79.
9. Franke A, McGovern DP, Barrett JC, Wang K, Radford- Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, et al. Genome-wide metaanalysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010; 42: 1118-1125.
10. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, GoyetteP, Huett A, Green T, Kuballa P et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 2007; 39: 596-604 .
11. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, HuseK, Albrecht M, Mayr G, De La Vega FM, Briggs J, GüntherS, Prescott NJ, Onnie CM, Häsler R, Sipos B, Fölsch

UR,Lengauer T, Platzer M, Mathew CG, Krawczak M, SchreiberS. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease inATG16L1. Nat Genet 2007; 39: 207-211.

12. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature 2011; 474: 307-317.
13. Kuballa P, Huett A, Rioux JD, Daly MJ, Xavier RJ. Impairedautophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One 2008; 3:e3391.
14. 21 Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS,Daly MJ, Steinhart AH, Abraham C, Regueiro M, GriffithsA, Dassopoulos T, Bitton A, Yang H, Targan S, Datta LW, A genome-wide association study identifies IL23R as an inflammatory bowel diseasegene. Science 2006; 314: 1461-1463.
15. Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M,Taylor KD, Lee JC, Goyette P, Imielinski M,Latiano A, LagacéC, Scott R, Amininejad L, Bumpstead S, Baidoo L, BaldassanoMeta-analysis identifies 29 additional ulcerativecolitis risk loci, increasing the number of confirmed associationsto 47. Nat Genet 2011; 43: 246-252.
16. Brand S. Crohn's disease: Th1, Th17 or both? The changeof a paradigm: new immunological and genetic insight simplicate Th17 cells in the pathogenesis of Crohn's disease.Gut 2009; 58: 1152-1167 .
17. Tremelling M, Cummings F, Fisher SA, Mansfield J, GwilliamR, Keniry A, Nimmo ER, Drummond H, Onnie CM,Prescott NJ, Sanderson J, Bredin F, Berzuini C, Forbes A,Lewis CM, Cardon L, Deloukas P, Jewell D, Mathew CG,Parkes M, Satsangi J. IL23R variation determines susceptibility but not disease phenotype in inflammatory bowel disease.Gastroenterology 2007; 132: 1657-1664.
18. Rogler G, Vavricka S. Exposome in IBD: recent insights in environmental factors that influence the onset and course of IBD. Inflamm Bowel Dis. 2015 Feb;21(2):400-8.
19. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464: 59-65.
20. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science 2005; 308: 1635-16384.
21. Frank DN, Amand AL St, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory

bowel diseases. Proceedings of the National Academy of Sciences of the United States of America. Aug 21; 2007104(34):13780–13785.

22. Manichanh C, Rigottier-Gois L, Bonnaud E, et al. Reduced diversity of faecal microbiota in Crohn'sdisease revealed by a metagenomic approach. Gut. Feb; 2006 55(2):205–211.
23. Morgan XC, Tickle TL, Sokol H, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome biology. 2012; 13(9):R79.
24. Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, Vandamme P, Vermeire S. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut 2011; 60: 631-637.
25. SokolH, Lay C, Seksik P, Tannock GW. Analysis of bacterial bowel communities of IBD patients:what has it revealed? Inflammatory bowel diseases. Jun; 2008 14(6):858–867.
26. Ott SJ, Musfeldt M, Wenderoth DF, et al. Reduction in diversity of the colonic mucosa associatedbacterial microflora in patients with active inflammatory bowel disease. Gut. May; 2004 53(5):685–693.
27. Strober W, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest 2007; 117: 514-521.
28. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, SasakiM, Saito Y, Tsujikawa T, Fujiyama Y. Comparison ofthe fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. J Gastroenterol 2011; 46: 479-486.
29. Martinez C, Antolin M, Santos J, Torrejon A, Casellas F, Borruel N, Guarner F, Malagelada JR. Unstable composition of the fecal microbiota in ulcerative colitis during clinical remission. Am J Gastroenterol 2008; 103: 643-648.
30. Cobrin GM, Abreu MT. Defects in mucosal immunity leadingto Crohn's disease. Immunol Rev 2005; 206: 277-295.
31. Targan SR, Karp LC. Defects in mucosal immunity leadingto ulcerative colitis. Immunol Rev 2005; 206: 296-305.
32. Geremia A, Jewell DP. The IL-23/IL-17 pathway in inflammator bowel disease. Expert Rev Gastroenterol Hepatol 2012; 6:223-237.
33. Abreu MT, Fukata M, Arditi M. TLR signaling in the gut in health and disease. J Immunol 2005; 174: 4453-4460.
34. 68 Bonen DK, Ogura Y, Nicolae DL, Inohara N, Saab L, TanabeT, Chen FF, Foster SJ, Duerr RH, Brant SR, Cho JH, Nuñez G.Crohn's disease-associated NOD2 variants share a

signalingdefect in response to lipopolysaccharide and peptidoglycan.Gastroenterology 2003; 124: 140-146.

35. Wehkamp J, Harder J, Weichenthal M, Schwab M, SchäffelerE, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P,Schröder JM, Bevins CL, Fellermann K, Stange EF. NOD2(CARD15) mutations in Crohn's disease are associated mucosal alpha-defensin expression. Gut 2004; 53: 1658-1664.
36. Abraham C, Cho JH. Functional consequences of NOD2 (CARD15) mutations. Inflamm Bowel Dis 2006; 12: 641-650.
37. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helpertype 1 responses. Nat Immunol 2004; 5: 800-808.
38. Noguchi E, Homma Y, Kang X, Netea MG, Ma X. A Crohn'sdisease-associated NOD2 mutation suppresses transcriptionof human IL10 by inhibiting activity of the nuclear ribonucleoproteinhnRNP-A1. Nat Immunol 2009; 10: 471-479.
39. Takatori H, Kanno Y, Watford WT, Tato CM, Weiss G, IvanovII, Littman DR, O'Shea JJ. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. J Exp Med 2009; 206: 35-41.
40. Kaser A, Blumberg RS. Autophagy, microbial sensing, endoplasmicreticulum stress, and epithelial function in inflammatory bowel disease. Gastroenterology 2011; 140: 1738-1747
41. Salim SY, Söderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. Inflamm Bowel Dis2011; 17: 362-381
42. . Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A,Meijerink JP, Van Goudoever JB, Büller HA, Dekker J, VanSeuningen I, Renes IB, Einerhand AW. Muc2-deficient micespontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006; 131: 117-129
43. Wehkamp J, Harder J, Weichenthal M, Mueller O, HerrlingerKR, Fellermann K, Schroeder JM, Stange EF. Inducibleand constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. Inflamm Bowel Dis 2003; 9: 215-223.
44. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17Cells. Annu Rev Immunol 2009; 27: 485-517.
45. Breese E, Braegger CP, Corrigan CJ, Walker-Smith JA, Mac-Donald TT. Interleukin-2- and interferon-gamma-secreting Tcells in normal and diseased human intestinal mucosa. Immunology1993; 78: 127-131.
46. Heller F, Florian P, Bojarski C, Richter J, Christ M, HillenbrandB, Mankertz J, Gitter AH, Bürgel N, Fromm M, ZeitzM, Fuss I, Strober W, Schulzke JD. Interleukin-13 is the key effector

Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. Gastroenterology2005; 129: 550-564.

47. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, Yang Z, Exley M, Kitani A, Blumberg RS, MannonP, Strober W. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. J Clin Invest 2004; 113: 1490-1497.
48. Di Sabatino A, Biancheri P, Rovedatti L, MacDonald TT,Corazza GR. New pathogenic paradigms in inflammatory bowel disease. Inflamm Bowel Dis 2012; 18: 368-371.
49. Rovedatti L, Kudo T, Biancheri P, Sarra M, Knowles CH,Rampton DS, Corazza GR, Monteleone G, Di Sabatino A,Macdonald TT. Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease. Gut 2009; 58: 1629-1636
50. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T,Levy DE, Leonard WJ, Littman DR. IL-6 programs T(H)-17cell differentiation by promoting sequential engagement ofthe IL-21 and IL-23 pathways. Nat Immunol 2007; 8: 967-974.
51. Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, ChinenH, Saito R, Kitazume MT, Nakazawa A, Sugita A, KoganeiK, Isobe K, Hibi T. IL23 differentially regulates theTh1/Th17 balance in ulcerative colitis and Crohn's disease.Gut 2008; 57: 1682-1689.
52. Sugihara T, Kobori A, Imaeda H, Tsujikawa T, AmagaseK, Takeuchi K, Fujiyama Y, Andoh A. The increased mucosal mRNA expressions of complement C3 and interleukin-17 in inflammatory bowel disease. ClinExpImmunol 2010; 160: 386-39.
53. Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F, Stolfi C, Cardolini I, Dottori M, Boirivant M, Pallone F, Macdonald TT, Monteleone G. Regulation of gut inflammation and th17 cell response by interleukin-21. Gastroenterology 2008; 134: 1038-1048.

Chapter 2

2.1 New insights in the pathogenesis of Inflammatory Bowel disease

2.1.1 Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions.

Genome wide association studies have linked SNP in various autophagy related genes to the occurrence of Crohn's disease.[1-3] Autophagy is a degradation mechanism originallyimplicated in the recycling of damaged proteins and provision of amino acids during nutrient starvation.[4] More recent studies have shown that the process is also involved in the degradationof intracellular pathogens such as Salmonella enterica and Mycobacterium tuberculosis.[5-6] The polymorphisms associated with Crohn's disease result in decreased levels of autophagy,leading to decreased bacterial handling. Additionally, ATG16L1 hypomorphic animals display Paneth cell abnormalities, resulting in a deceased release of antimicrobial peptides into the intestinal lumen.[7] The current theory of how autophagy-related SNP contribute to Crohn's disease is therefore based on an indirect effect, where a decrease in bacterial handling results in an altered microbiome.[8-9]

In addition to the function of autophagy in innate immunity, we have recently shown that autophagy also modulates adaptive responses through regulation of DC–T cell interactions.[10] When autophagy levels are insufficient, these interactions are hyperstable, resulting in increased activation of the adaptive immune system. Whether autophagy is also involved in the regulation of other cellular interactions has not been studied thus far.

A particular type cellular interaction which is interesting in the context of Crohn's disease is that between the intestinal epithelium and local DC. It has been shown that DC can sample luminal antigens by extending protrusions in between epithe-lial cells and into the lumen.[11] During this process, epithelial barrier function is maintained because the DC form tight junctions with the surrounding epithelial cells.[12-13] The proper ratio between tight junction formation and release is important for the efficacy of antigen sampling. Additionally, since tight junction protein bonds have been implicated in the activation status of DC, the regulation of tight junction may also influence the induction of tolerance versus immunity by DC.[14] We hypothesized that autophagy is involved in the regulation of DC–epithelial interactions, and thereby influences the ability to sample luminal antigens and the maintenance of a tolerogenic status.

In the intestine, DC form protrusions in between the epithelial layer, while maintaining barrier function through the continuous formation of tight junction like structures with epithelial cells. We started our investigations by determining whether the level of autophagy regulates the expression of

tight junction proteins. Intestinal epithelial cells and dendritic cells express various tight junction proteins, including ZO-1 and occludin. Upon activation of autophagy using the mTOR inhibitor rapamycin, occludin levels were decreased in both cells tipe, whereas levels of ZO-1 did not change.

Previous studies implicated E-cadherin in formation of intestinal tight junctions. We therefore determined the levels of E-cadherin expression in the presence and absence of autophagy. Rapamycin stimulation resulted in decreased protein levels of E-cadherin, both in epithelial cells and DC. The decreased expression of E-cadherin after activation of autophagy suggests that autophagy functions as a degradation mechanism for this protein. To further confirm this, we performed colocalization analysis with confocal microscope between E-cadherin and the autophagosomal marker LC3 in cultured DC. However, after activation of autophagy, the number of co-localizing spots was significantly enhanced, supporting the hypothesis that autophagy is involved in E-cadherin degradation.Finally to confirm the importance of E-cadherin in DC–epithelial cell interactions, we co-cultured these two cell types and studied the localization of E-cadherin in the resulting cell clusters. Indeed, E-cadherin was found to localize to the site of the interactions. Interestingly, an accumulation was seen at the outer edges of the interaction, suggesting a role in "sealing" the interaction from the environment.

The data described above indicate that autophagy is involved in the degradation of tight junction proteins. As these proteins play an important role in the regulation of cell–cell interaction, we hypothesized that autophagy also regulates DC- epithelial interactions.

To test this,we used a model system for the DC sampling of luminal antigens which has been described previously.[13] Decreased autophagy resulted in a decreased number of protrusions whether autophagy was knocked down in DC, epithelial cells or both. Since formation of these protrusions is important for antigen sampling, decreased protrusions might lead to decreased antigen uptake by DC. To test this, fluorescently labeled E. coli derived particles were applied to the apical side of the transwell culture system. Indeed, when autophagy was knocked down in DC, the amount of antigen taken up by these cells was decreased . When autophagy was decreased in epithelial cells, antigen uptake was decreased in the cultures using Caco-2 cells. To mimic the physiological situation in patients, in whom autophagy defects are present in all cell types, both DC and epithelial cells were treated with siRNA. Under these conditions, the formation of protrusions was impaired and the level of antigen uptake by DC was reduced, indicating that the net outcome of the opposing effects is a decrease in antigen sampling.

In summary, these data show that decreased levels of autophagy result in decreased antigen sampling of intestinal DC, but increased capacity to activate the adaptive immune system. Since

impaired innate immunity but increased adaptive immunity is exactly the phenotype seen in Crohn'sdisease patients, this mechanism may be contributing to the inflammation seen in patients carrying mutations in the autophagy pathway.

Journal of Crohn's and Colitis (2013) 7, 534–541

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Impaired autophagy leads to abnormal dendritic cell–epithelial cell interactions

Caterina Strisciuglio [a,b], Marjolijn Duijvestein [a], Auke P. Verhaar [a], Anne Christine W. Vos [a], Gijs R. van den Brink [a,c], Daniel W. Hommes [a,d], Manon E. Wildenberg [a,c,*]

[a] *Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Paediatrics, University Federico II, Napels, Italy*
[c] *Tytgat Institute for Intestinal and Liver Research and Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands*
[d] *Center for Inflammatory Bowel Diseases, University of California Los Angeles, USA*

Received 12 April 2012; received in revised form 16 August 2012; accepted 16 August 2012

KEYWORDS
Crohn's disease;
Immune regulation;
Dendritic cells;
Cell interactions

Abstract

Background and aims: Dendritic cells (DC) are key players in intestinal immunity, as these cells can direct the immune response to either a tolerogenic or an immunogenic phenotype. In the intestine, DC sample and process luminal antigens by protruding dendrites through the epithelial cell layer. At the same time barrier integrity is maintained through the continuous formation of tight junctions. Aberrations in these interactions may lead to altered antigen sampling and improper immune responses. We have recently shown that autophagy, a process implicated in the pathogenesis of Crohn's disease, regulates cellular interactions in the context of DC and T cells. In this study we aimed to determine whether autophagy also regulates DC–epithelial cell interactions and whether this influences the ensuing immune response.
Methods: DC were generated from peripheral blood monocytes of healthy volunteers. For interaction studies, DC were co-cultured with intestinal epithelial cells on the baso-lateral side of a transwell insert. Modulation of autophagy was achieved using *atg16l1* specific siRNA or pharmacological inhibitors. Intraepithelial protrusion of dendrites was determined by confocal microscopy. Luminal sampling and DC activation status were analyzed by flow cytometry. Protein expression was measured by immunoblotting and cytometric bead assay.
Results: Adhesion molecules E-cadherin and occludin partly localized to autophagosomes and increased autophagy resulted in decreased levels of these proteins. Reduced autophagy in either DC, epithelial cells or both resulted in the decreased formation of transepithelial protrusions by

Abbreviations: DC, dendritic cell; CD, Crohn's disease; SNP, single nucleotide polymorphism.

* Corresponding author at: Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands.
E-mail address: m.e.wildenberg@amc.nl (M.E. Wildenberg).

1873-9946/$ - see front matter © 2012 European Crohn's and Colitis Organisation. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.crohns.2012.08.009

DC as well as a reduction in antigen sampling. Moreover, when autophagy was inhibited in the co-culture model, DC expressed increased levels of HLA-DR and costimulatory molecule CD86. Furthermore, decreased levels of autophagy resulted in lower IL-10 production by DC and these cells induced significantly more T-cell proliferation in an allogeneic mixed lymphocyte reaction. *Conclusions:* In intestinal DC–epithelial cell interactions, autophagy deficiency leads to decreased antigen sampling, increased DC maturation and a more pro-inflammatory type of DC.
© 2012 European Crohn's and Colitis Organisation. Published by Elsevier B.V. All rights reserved.

1. Introduction

Genome wide association studies have linked SNP in various autophagy related genes to the occurrence of Crohn's disease.[1–3] Autophagy is a degradation mechanism originally implicated in the recycling of damaged proteins and provision of amino acids during nutrient starvation.[4] More recent studies have shown that the process is also involved in the degradation of intracellular pathogens such as *Salmonella enterica* and *Mycobacterium tuberculosis*.[5,6] The polymorphisms associated with Crohn's disease result in decreased levels of autophagy, leading to decreased bacterial handling. Additionally, *ATG16L1* hypomorphic animals display Paneth cell abnormalities, resulting in a deceased release of antimicrobial peptides into the intestinal lumen.[7] The current theory of how autophagy-related SNP contribute to Crohn's disease is therefore based on an indirect effect, where a decrease in bacterial handling results in an altered microbiome.[8,9]

In addition to the function of autophagy in innate immunity, we have recently shown that autophagy also modulates adaptive responses through regulation of DC–T cell interactions.[10] When autophagy levels are insufficient, these interactions are hyperstable, resulting in increased activation of the adaptive immune system. Whether autophagy is also involved in the regulation of other cellular interactions has not been studied thus far.

A particular type cellular interaction which is interesting in the context of Crohn's disease is that between the intestinal epithelium and local DC. It has been shown that DC can sample luminal antigens by extending protrusions in between epithelial cells and into the lumen.[12] During this process, epithelial barrier function is maintained because the DC form tight junctions with the surrounding epithelial cells.[13,14] The proper ratio between tight junction formation and release is important for the efficacy of antigen sampling. Additionally, since tight junction protein bonds have been implicated in the activation status of DC, the regulation of tight junction may also influence the induction of tolerance versus immunity by DC.[15] We hypothesized that autophagy is involved in the regulation of DC–epithelial interactions, and thereby influences the ability to sample luminal antigens and the maintenance of a tolerogenic state.

2. Materials and methods

2.1. Cell culture (incl. transfection and T cell activation)

Monocytes and lymphocytes were isolated from buffy coats using Ficoll and Percoll density gradients according to a previously described protocol.[16] For generation of DC, monocytes were then cultured for 6–8 days in AIM-V culture medium (Invitrogen, Carlsbad, CA) in the presence of recombinant human GM-CSF and IL-4 (both 100 ng/ml, RnD Systems, Inc. Minneapolis, MN). When applicable, DC were labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (Sigma Aldrich, Deisenhofen, Germany). HT29, Caco-2 and HCT116 colorectal carcinoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. For transwell experiments, Caco-2 cells were seeded onto transwell insert filters and allowed to differentiate for a minimum of two weeks.

ON-TargetPlus ATG16L1 specific siRNA pools were obtained from Dharmacon (Epsom, United Kingdom) and transfected using Dharmafect reagent (Dharmacon) according to the manufacturer's protocol. Off target effects were controlled by using non-specific control siRNA. Knockdown efficacy was determined by RT-PCR in every experiment and typically ranged 35–70%. Plasmid encoding eGFP-LC3 fusion protein was described previously (Addgene plasmid 11546[17]) and was transfected using Lipofectamine 2000 reagent (Invitrogen). Where applicable, cells were treated using rapamycin (50 nM, Sigma Aldrich), 3-methyladenine (3-MA, 10 μM, Sigma Aldrich) or vehicle control.

For mixed lymphocyte reactions, DC were co-cultured with allogeneic lymphocytes for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation assay. Cytokine levels were determined using cytometric bead array (BD Biosciences, San Jose, CA).

2.2. Transwell system

Transwell experiments were performed as described previously.[14] Briefly, HT29 cells were grown to confluency in the upper chamber of a 3 μm pore transwell insert (Falcon, Franklin Lakes, NJ). Transwell inserts were inverted and monocyte derived DC were applied to the basolateral side of the filter for two hours. Inserts were then reverted and stimuli were applied to the apical surface for 2 hours. Stimuli were applied where indicated and consisted of FITC labeled-*Escherichia coli* Bioparticles (20 μg/ml, Invitrogen) or fMLP (100 nM, Sigma Aldrich). Subsequently, stimuli were removed and cells were further incubated for 20 hours and processed for further analysis.

2.3. Confocal analysis

For confocal analysis of transwell cultures, inserts were incubated in 4% paraformaldehyde for a minimum of 60 minutes and then mounted using SlowFade Gold (Invitrogen). For confocal analysis of cell cultures, cells were adhered to

poly-L-lysine (Sigma Aldrich) coated coverslips, fixed in 4% paraformaldehyde, stained in permeabilisation buffer (PBS containing 0.05% Triton X-100) and embedded in SlowFade Gold (Invitrogen). Images were obtained on a Leica TCS SP2 confocal system equipped with 405 nm UV, 488 nm argon and 543 HeNe lasers (Leica, Mannheim, Germany) and processed using ImageJ software. Antibodies used included anti-E-cadherin (BD), anti-GFP (Invitrogen), anti-pancytokeratin (Santa Cruz).

2.4. Flow cytometry

Anti-CD86, anti-HLA-DR-FITC and isotype controls were obtained from BD Biosciences. For flow cytometric analysis of transwell experiments, DC were harvested from the basolateral side of the transwell insert. Cells were then washed and stained in PBS/0.5% BSA when applicable, washed again and fixed in 2% PFA. Samples were analyzed using a FACSCalibur (Becton Dickenson) and FlowJo software (Tree Star Inc., Ashland, OR).

2.5. Immunoblotting

Anti-E-cadherin was obtained from BD Biosciences and anti-ZO-1, anti-occludin and anti-claudin-1 were obtained from Cell Signaling Technologies (Beverly, MA) and all were used in a 1:500 dilution. Secondary antibodies were obtained from DAKO (Glostrup, Denmark) and used in a 1:1000 dilution. For Western Blot analysis, cells were lysed in lysis buffer (Cell Signaling Technologies) containing Protease Inhibitor Cocktail (MP Biomedicals Inc., Solon, OH), and homogenized by ultrasound sonication. Samples were run on SDS–PAGE gels under reducing conditions and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked by incubation in 5% blocking powder (Bio-Rad, Hercules, CA) and incubated with primary and secondary antibodies in 1% blocking powder (Bio-Rad). Expression was detected by Lumilight Plus (Roche, Woerden, The Netherlands).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad (GraphPad software Inc., La Jolla, CA). For statistical comparison, Mann–Whitney *U*-test was used. Data were considered significant if $P<0.05$. Due to normal donor-to-donor variability, for graphs containing data from multiple experiments data were normalized per individual donor. The siRNA sample was set to 1 (relative units, relU = 1), and the other values were calculated accordingly.

3. Results

3.1. Activation of autophagy reduces protein levels of several tight junction proteins

In the intestine, DC form protrusions in between the epithelial layer, while maintaining barrier function through the continuous formation of tight junction like structures with epithelial cells. We started our investigations by determining whether the level of autophagy regulates the expression of tight junction proteins. Intestinal epithelial cells express various tight junction proteins, including ZO-1 and occludin. Upon activation of autophagy using the mTOR inhibitor rapamycin, occludin levels were decreased, whereas levels of ZO-1 did not change (Fig. 1A/B). This is in line with the previously described differences in regulation between these proteins.[18] Treatment of DC or epithelial cells using rapamycin in the presence of the autophagy inhibitor 3-MA rescued the expression level of occludin, emphasizing the autophagy dependence of the degradation (Fig. 1A).

As previously described, DC expressed occludin, but not ZO-1 (Fig. 1A/B). Also in these cells, activation of autophagy resulted in the degradation of occludin levels, which could be rescued using 3-MA (Fig. 1B).

Previous studies implicated E-cadherin in formation of intestinal tight junctions. We therefore determined the levels of E-cadherin expression in the presence and absence of autophagy. Rapamycin stimulation resulted in decreased protein levels of E-cadherin, both in epithelial cells and DC (Fig. 1C). In addition, we tested the involvement of autophagy using DC treated with siRNA against ATG16L1. These cells display impaired autophagy, and in line with our hypothesis, rapamycin stimulation did not result in decreased E-cadherin levels after ATG16L1 siRNA treatment (Fig. 1D).

3.2. E-cadherin co-localizes with autophagosomes

The decreased expression of E-cadherin after activation of autophagy suggests that autophagy functions as a degradation mechanism for this protein. To further confirm this, we performed co-localization analysis between E-cadherin and the autophagosomal marker LC3 in cultured DC. In resting cells, very little E-cadherin and LC3 co-localization was observed (Fig. 2A, top). However, after activation of autophagy, the number of co-localizing spots was significantly enhanced (Fig. 2A, bottom), supporting the hypothesis that autophagy is involved in E-cadherin degradation.

To confirm the importance of E-cadherin in DC–epithelial cell interactions, we co-cultured these two cell types and studied the localization of E-cadherin in the resulting cell clusters. Indeed, E-cadherin was found to localize to the site of the interactions (Fig. 2B). Interestingly, an accumulation was seen at the outer edges of the interaction, suggesting a role in "sealing" the interaction from the environment.

3.3. Effect of decreased autophagy on DC–epithelial interactions: protrusion and sampling

The data described above indicate that autophagy is involved in the degradation of tight junction proteins. As these proteins play an important role in the regulation of cell–cell interaction, we hypothesized that autophagy also regulates DC–epithelial interactions. To test this, we used a model system for the DC sampling of luminal antigens which has been described previously.[14] In this system, DC are cultured on the basolateral side of a transwell containing colonic epithelial cells. Stimuli can then be added to the apical side of the epithelial layer, leading to the formation of dendritic protrusions and sampling of antigen (Fig. 3A). In this system, we treated either DC, epithelial cells with siRNA against ATG16L1, thus decreasing the level of autophagy in the respective cell types. Decreased autophagy resulted in a decreased number of protrusions,

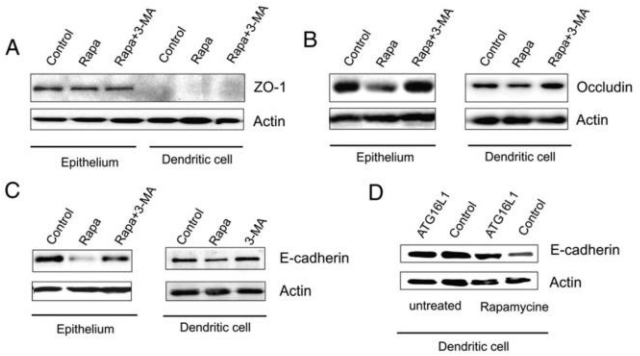


Figure 1 (A–C) Autophagy regulation of tight junction protein expression. HCT116 colonic epithelial cells or monocyte derived DC were cultured in the presence of rapamycin (50 nM) and/or 3-MA (10 μM) or vehicle control for 24 hours. Protein expression levels were determined by Western blotting. (D) Monocyte derived DC were treated with siRNA for ATG16L1 or control for 48 hours and then stimulated using rapamycin for 24 hours. Representative image of 3–4 experiments is shown.

whether autophagy was knocked down in DC, epithelial cells or both (Fig. 3B). Similar data were obtained both for the HT29 cell line and the more differentiated Caco-2 cells, although data for the latter did not reach statistical significance. Since formation of these protrusions is important for antigen sampling, decreased protrusions might lead to decreased antigen uptake by DC. To test this, fluorescently labeled *E. coli* derived particles were applied to the apical side of the transwell culture system. Indeed, when autophagy was knocked down in DC, the amount of antigen taken up by these cells was decreased (Fig. 3C). When autophagy was decreased in epithelial cells, antigen uptake was decreased in the cultures using Caco-2 cells, but remained unaltered in the HT29 system, possibly due to the less differentiated phenotype of the latter cell type. To mimic the physiological situation in patients, in whom

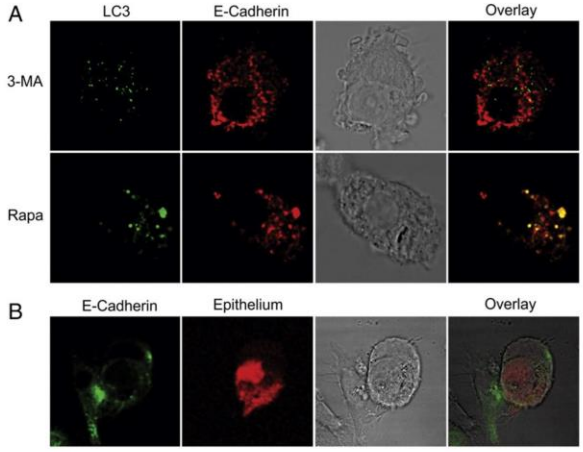


Figure 2 E-cadherin co-localizes with autophagosomes in cultured DC. (A) Monocyte-derived DC were transfected with GFP-LC3 and cultured in the presence of either rapamycin (50 nM) or 3-MA (10 μM) for 24 hours. DC were then stained for E-cadherin (red) and LC3 (green) and analyzed by confocal microscopy. Representative image of 3 experiments shown. (B) Monocyte derived DC were co-cultured with RFP expressing HCT116 for 24 hours. Cells were stained for E-cadherin (green) and analyzed by confocal microscopy. Magnification 400×.

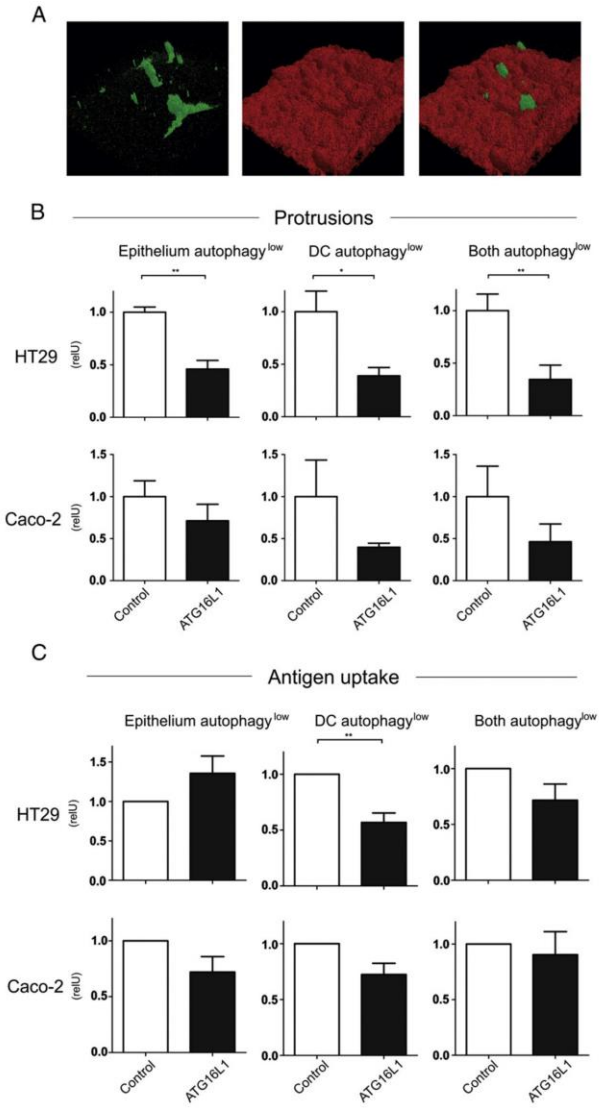


Figure 3 Impaired autophagy results in decreased transepithelial antigen sampling by DC. HT29 or differentiated Caco-2 epithelial cells were cultured on a transwell insert. Monocyte-derived DC were treated with siRNA and adhered to the basolateral side of the filter. Stimuli (B: fMLP, C: *E. coli* particles) were added to the apical side for 2 hours. (A,B) Filters were fixed and analyzed by confocal microscopy. (A) 3D reconstruction of DC protruding through the epithelial cell layer. (B) Image analysis of the amount of protrusions detectable on the apical side of the filter. (C) DC were harvested from the transwell insert and analyzed by flow cytometry. * indicates $P<0.05$, ** indicates $P<0.01$. Error bars represent s.e.m.

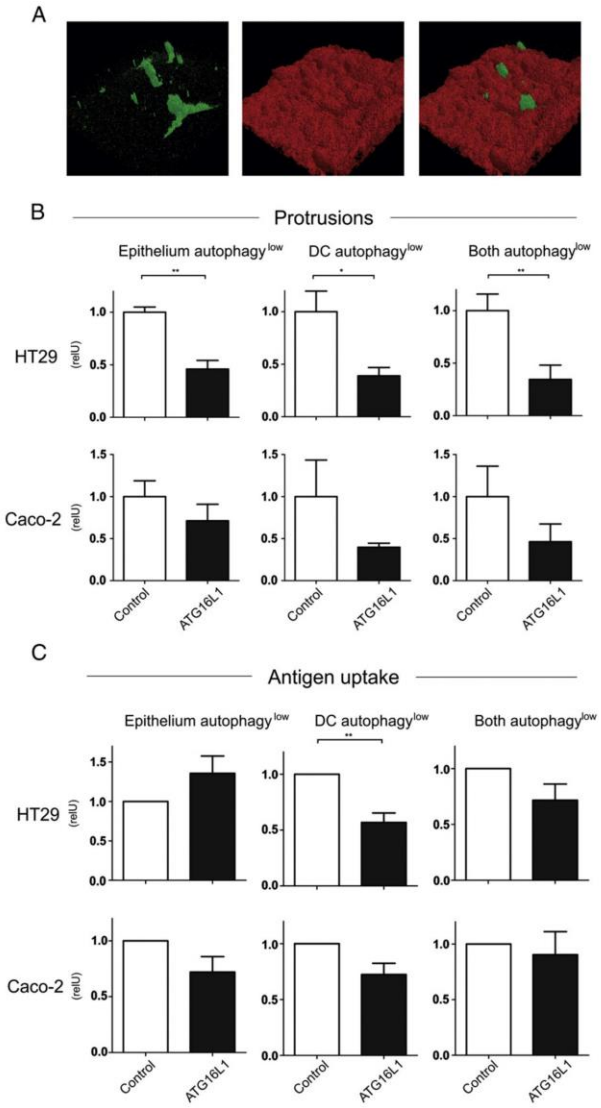


Figure 3 Impaired autophagy results in decreased transepithelial antigen sampling by DC. HT29 or differentiated Caco-2 epithelial cells were cultured on a transwell insert. Monocyte-derived DC were treated with siRNA and adhered to the basolateral side of the filter. Stimuli (B: fMLP, C: *E. coli* particles) were added to the apical side for 2 hours. (A,B) Filters were fixed and analyzed by confocal microscopy. (A) 3D reconstruction of DC protruding through the epithelial cell layer. (B) Image analysis of the amount of protrusions detectable on the apical side of the filter. (C) DC were harvested from the transwell insert and analyzed by flow cytometry. * indicates $P<0.05$, ** indicates $P<0.01$. Error bars represent s.e.m.

autophagy defects are present in all cell types, both DC and epithelial cells were treated with siRNA. Under these conditions, the formation of protrusions was impaired and the level of antigen uptake by DC was reduced (Fig. 3B/C), indicating that the net outcome of the opposing effects is a decrease in antigen sampling.

3.4. Effect of decreased autophagy on DC activation and T cell proliferation

DC sampling of luminal antigens has been shown to occur under non-inflammatory, steady-state conditions, and is therefore thought to be involved in the maintenance of intestinal immune tolerance. Therefore, we determined the activation status of DC in our model system. Under conditions of decreased autophagy in epithelial cells, DC displayed a moderately more activated phenotype with higher expression levels of CD86 and HLA-DR (Fig. 4A). Decreasing autophagy in DC alone did not alter their activation status, which is in agreement with our previous data.[10] In addition, production of IL-10 was also decreased in DC, especially after inhibition of autophagy in DC themselves (Fig. 4B). As expected, the net outcome of decreasing autophagy in both epithelial cells and DC was a moderate increase in expression of HLA-DR and CD86 combined with a clearly decreased production of IL-10 (Fig. 4C). Together this suggests a less regulatory phenotype of DC.

To test the functional consequences of this altered phenotype, DC were isolated from the transwell culture and placed in a secondary co-culture with allogeneic T cells. In accordance with our previous data, DC in which autophagy was knocked down induced more T cell proliferation, probably at least in part due to increased T cell interactions (data not shown). However, DC obtained from cultures where autophagy was knocked down in epithelial cells only also induced stronger T cell proliferation, supporting a more pro-inflammatory phenotype of these cells (Fig. 4C).

4. Discussion

In mice, various intestinal DC subsets have been described, each with their own function and phenotype.[11] Commonly, a distinction is made between CD103+ DC, which are mainly present in the mesenchyme, and CX3CR1+ DC which are located directly under the epithelial layer.[19] CX3CR1+ DC form the majority of intestinal DC, and are considered the more resident population.[19] They sample luminal antigens, but are rather refractory to TLR stimulation.[12] Instead, this subset is involved in a two step bacterial clearance. After ingestion of luminal pathogens such as *Salmonella*, CX3CR1+ DC migrate into the lumen, while at the same time digesting the pathogen.[20] In our model system, DC are placed directly under the epithelial layer, mimicking the positioning of CX3CR1+ DC. Indeed, these cells form protrusions through the epithelial layer, and sample luminal antigen, all reminiscent of the DC performing *in situ* functions. When autophagy was decreased in this system, both formation of transepithelial protrusion and antigen sampling was decreased. Others have shown previously that autophagy deficient DC are also less capable of degrading pathogens once ingested.[6,21] In combination, the decreased sampling and decreased bacterial degradation may result in bacterial overgrowth in the intestine.

In addition, DC display a more activated phenotype after antigen uptake when autophagy was decreased in the culture system. These cells expressed more CD86 and HLA-DR, and produced less IL-10 resulting in increased T cell stimulatory capacity. This indicates that while innate immune functions such as bacterial uptake are decreased, stimulation of adaptive immunity is increased by the same cell type. Interestingly, this combination is precisely what has been implicated in the pathogenesis of Crohn's disease.[8,9] We have previously shown that decreasing autophagy in DC in itself does not alter the activation status of these cells.[10] Therefore, the altered DC phenotype may be a secondary effect, resulting from altered cell–cell interactions. Similar to the data describing the maturation of DC after disruption of homotypic E-cadherin bonds,[15] DC show increased expression of activation markers, but no increased expression of pro-inflammatory cytokines. These data suggest that the increased activation seen in this model may also be the result of mechanical disruption of E-cadherin bonds. During DC–T cell interactions, autophagy is induced and molecules associated with the interaction are taken up by the autophagosome.[10] When autophagy can not be induced properly, for example as a consequence of SNPs in the ATG16L1 alleles, the interactions are hyperstable and result in increased T cell activation. Along these lines, it can be envisaged that in the current DC–epithelial model, E-cadherin would be partially degraded by autophagy, leaving less molecules available for bond formation. Indeed, activation of autophagy results in increased co-localization of E-cadherin with autophagosomes. Under autophagy^{low} conditions, this does not occur, and the level of homotypic bonds may increase. Since DC are highly motile cells, the bonds will still be disrupted, but with more mechanical force, resulting in the increased maturation phenotype observed.

Our data suggest that in DC, decreased autophagy results in decreased antigen processing, but increased T cell activation. This is in contrast to earlier reports, which show that both antigen handling and presentation are decreased. The difference between these results and ours is most likely due to differences in the experimental set-up. For example, Cooney et al. show that expression of the ATG16L1 T300A allele in DC results in decreased T cell responses to specific pathogens.[22] Indeed, this paper also shows that DC carrying the risk allele display less maturation upon activation with MDP, but not upon activation by a TLR2 ligand. This suggests that responses to certain pathogens may be hampered, whereas others are not. In addition, Lee et al. have shown that in mice lacking Atg5 specifically in their DC, autophagosome-to-lysosome fusion is impaired, also resulting in decreased antigen presentation and T cell activation.[23] In this system, autophagy is completely blocked in DC, whereas our model is a partial deficiency. The complete knockout is a good system to determine whether a gene has any role in a given biological system, but our model may be more reflective of the situation in human Crohn's disease patients, who have hypomorphic alleles.

Our data implicate autophagy in the regulation of tight junction proteins, as activation of autophagy results in lower protein levels. Previously, it has been shown that the various tight junction molecules do not all show similar patterns of regulation. For example, upon stimulation by TNF-alpha, occludin is internalized by intestinal epithelial cells, while ZO-1 is not.[18] In this model, occludin internalization is mediated by caveolin-1 and co-localization of caveolin-1 and the autophagic

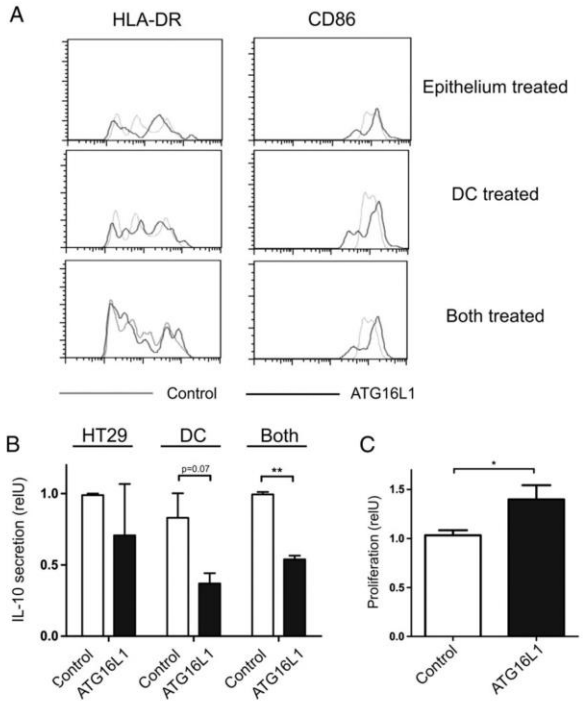


Figure 4 Impaired autophagy results in DC with a more pro-inflammatory phenotype. Colonic epithelial cells were cultured on a transwell insert (A: Caco-2, B/C:HT29). Monocyte-derived DC were adhered to the basolateral side of the filter. fMLP (100 nM) was added to the apical side for 2 hours. DC were harvested from the inserts. (A) DC were analyzed by flow cytometry, (B) DC were placed in culture for 24 hours, supernatant was removed for analysis of cytokine production, (C) DC were co-cultured with allogeneic T cells for 96 hours and proliferation was determined by 3H-thymidine incorporation assay. * indicates $P<0.05$, ** indicates $P<0.01$. Bars represent means of 3–4 separate experiments in individual donors, and error bars represent s.e.m.

marker LC3 has been described.[24] Interestingly, a recent study showed that autophagy is involved in degradation of gap junctions, another type of cellular interaction.[25] Whether autophagy is involved in the trafficking of tight junction molecules to autophagosomes in the context of the intestine is a topic of current investigation.

In summary, these data show that decreased levels of autophagy result in decreased antigen sampling of intestinal DC, but increased capacity to activate the adaptive immune system. Since impaired innate immunity but increased adaptive immunity is exactly the phenotype seen in Crohn's disease patients, this mechanism may be contributing to the inflammation seen in patients carrying mutations in the autophagy pathway.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

This study was financially supported by a fellowship of the European Crohn's and Colitis Organization. Author contributions: C.S. carried out experiments, analyzed data and participated in drafting the article, M.D. and A.C.W.V. carried out experiments and analyzed data, A.P.V. provided critical technical advice, G.R.B. and D.W.H. designed the study and provided conceptual advice, M.E.W. designed the study and drafted the article.

References

1. Barrett JC, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008;**40**:955–62.
2. Franke A, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010;**42**:1118–25.
3. Latiano A, et al. Replication of interleukin 23 receptor and autophagy-related 16-like 1 association in adult- and pediatric-

onset inflammatory bowel disease in Italy. *World J Gastroenterol* 2008;**14**:4643–51.
4. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 2010;**22**: 124–31.
5. Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J Biol Chem* 2006;**281**:11374–83.
6. Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 2006;**313**:1438–41.
7. Cadwell K, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 2008;**456**:259–63.
8. Hayee B, Rahman FZ, Sewell G, Smith AM, Segal AW. Crohn's disease as an immunodeficiency. *Expert Rev Clin Immunol* 2010;**6**:585–96.
9. Marks DJ, Rahman FZ, Sewell GW, Segal AW. Crohn's disease: an immune deficiency state. *Clin Rev Allergy Immunol* 2010;**38**:20–31.
10. Wildenberg ME, et al. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. *Gastroenterology* 2012;**142**:1493–503.
11. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol* 2008;**8**:435–46.
12. Niess JH, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 2005;**307**: 254–8.
13. Chieppa M, Rescigno M, Huang AY, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med* 2006;**203**:2841–52.
14. Rescigno M, Rotta G, Valzasina B, Ricciardi-Castagnoli P. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* 2001;**204**:572–81.
15. Jiang A, et al. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* 2007;**27**:610–24.
16. Repnik U, Knezevic M, Jeras M. Simple and cost-effective isolation of monocytes from buffy coats. *J Immunol Methods* 2003;**278**:283–92.
17. Jackson WT, et al. Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol* 2005;**3**:e156.
18. Marchiando AM, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* 2010;**189**:111–26.
19. Schulz O, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* 2009;**206**:3101–14.
20. Arques JL, et al. *Salmonella* induces flagellin- and MyD88-dependent migration of bacteria-capturing dendritic cells into the gut lumen. *Gastroenterology* 2009;**137**:579–87 587.
21. Travassos LH, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 2010;**11**:55–62.
22. Cooney R, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* 2010;**16**:90–7.
23. Lee HK, et al. In vivo requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* 2010;**32**:227–39.
24. Chen ZH, et al. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. *Proc Natl Acad Sci U S A* 2010;**107**:18880–5.
25. Fong JT, Kells RM, Gumpert AM, Marzillier JY, Davidson MW, Falk MM. Internalized gap junctions are degraded by autophagy. *Autophagy* 2012;**8**.

2.1.2 T300A variant of autophagy ATG16L1 gene is associated with decreased antigen sampling and processing by dendritic cells in paediatric Crohn's disease.

We have previously shown that if autophagy is impaired, DC form hyperstable interactions with T cells[15], and their antigen sampling through epithelial cell monolayers is greatly decreased[16]. In these studies, autophagy was inhibited by the use of siRNA or by pharmacological inhibitors, and though very informative, it remains an indirect reflection of the physiological condition in subjects carrying the risk allele of the *ATG16L1*.

In the present study we aimed to investigate the physiological relevance of an impaired autophagy machinery for human disease by analysing phenotype and function of DC obtained from CD patients carrying the risk allele of *ATG16L1* gene. In particular, since very little is known on the role of autophagy in paediatric inflammatory bowel disease (IBD), we have investigated the phenotype and antigen sampling capacity of DC in children carrying the *ATG16L1* T300A variant.

In this study we demonstrate that the monocyte-derived DC from paediatric patients with Crohn's disease carrying the *ATG16L1* polymorphism T300A, associated with a defective autophagy, are impaired in proper antigen sampling and processing. We found a marked reduction of bacteria particle localization in DC from CD children with the *ATG16L1*risk variant compared to DC of children with *ATG16L1* wild type variant, indicating an impairment of particulate antigen sampling. DC from CD patients with *ATG16L1*risk variant were also impaired in processing of soluble antigen, as shown by reduced intracellular degradation of ovalbumin. Furthermore, we found that DC from the *risk* group almost completely failed in up-regulating the HLA-DR and CD86, the early activation markers, after a brief incubation with particulate or soluble antigens.

Interestingly, the DC from children that were heterozygous for *ATG16L1*T300A polymorphism were hampered in bacteria particles, but not ovalbumin, phagocytosis. Moreover, DC from the *ATG16L1* heterozygous group did not upregulate HLA-DR on the surface membrane after exposure to bacteria particles or to ovalbumin. The impairment of *het* DC functionality suggested a dose dependent effect of the T300A allele that has never been described, if excepted only one study that reported a dose effect of this allele with CD risk.[17] One possible explanation of this difference in antigen handling by DC from risk variant and heterozygous groups is the structure of the processed antigens, requiring a more complex degradation machinery for bacteria compared to the a soluble protein of dietary origin, such as ovalbumin.

DC sampling of luminal antigens has been shown to occur under non inflammatory, steady state conditions, and is therefore thought to be involved in the maintenance of intestinal immune tolerance. In line with this, and with the finding that autophagy is involved in the degradation of

tight junction proteins, we showed that autophagy also regulates DC–epithelial interactions in the model system.[16] Others have shown previously that DC form protrusions through the epithelial layer, and sample luminal antigen.[18] We found that DC of patients carrying the T300A allele of *ATG16L1* form less transepithelial protrusion through the Caco2 monolayer compared to *wild type* and heterozygous patients. In addition, it is known that autophagy deficient DC are also less capable of degrading pathogens sampled through the intestinal mucosa. [19-20] In line with these observations, we found that DC from *risk* children are less efficient in sampling bacteria particulate fragments when added to the apical side of the transwell filter in a co-colture system with Caco2 epithelial cells. Furthermore, upon bacteria mucosal load, these DC were highly impaired in upregulating DR and CD86, two key molecules for activating T cell-mediated immune response.

In conclusion, our data indicates defects in DC antigen sampling and activation in pediatric CD patients carrying the T300A *ATG16L1* allele. The fact that there are a number of autophagy stimulating compounds already in clinical practice, makes us more confident that this may be a new therapeutic option, and a viable pathway, for intervention in CD, in particular for those patients carrying one, or more, SNPs associated with the autophagy machinery (approximately 40% of the total CD population) .[21]

T300A Variant of Autophagy *ATG16L1* Gene is Associated with Decreased Antigen Sampling and Processing by Dendritic Cells in Pediatric Crohn's Disease

Caterina Strisciuglio, MD, Erasmo Miele, MD, PhD,* Manon E. Wildenberg, Dr Biol Sc, PhD,† Francesca P. Giugliano, MD,* Marialuisa Andreozzi, MD,* Alessandra Vitale, Dr Biol Sc,* Francesca Capasso, PhD,* Alessandra Camarca, PhD,*‡ Maria V. Barone, MD, PhD,* Annamaria Staiano, MD,* Riccardo Troncone, MD,* and Carmela Gianfrani, Dr Biol Sc*‡*

Background: The single-nucleotide polymorphism T300A of *ATG16L1*, a Crohn's disease (CD)–associated gene, is responsible for decreased autophagy. This study aimed to investigate the effects of this single-nucleotide polymorphism on the uptake and processing of antigens by dendritic cells (DCs) and the interaction between DC and intestinal epithelium in pediatric patients with CD.

Methods: Pediatric patients who homozygously carry either the protective (wild type, n = 7) or risk allele (risk, n = 13) of *ATG16L1*, as well as heterozygous patients (het, n = 13) were enrolled. The monocyte-derived DC were analyzed for phenotype, antigen sampling, and processing by flow cytometry, whereas the capability of DC to form transepithelial protrusions was determined by confocal microscopy.

Results: DC generated from wild type patients showed higher bacteria sampling and antigen processing compared with risk patients. Additionally, after exposure to either bacteria particles or the antigen DQ-ovalbumin, wild type DC showed a significant increase in the expression of the HLA-DR and CD86 when compared with risk DC. Interestingly, also het patients showed an impairment in bacteria uptake and expression of activation marker when compared with the wild type. In the Caco2/DC coculture, the formation of transepithelial protrusions were less numerous in risk DC compared with wild type and the antigen uptake decreased.

Conclusions: DC of pediatric patients with CD carrying the T300A allele showed a marked impairment of antigen uptake and processing and defective interactions between DC and intestinal epithelium. Collectively, our results suggest that an autophagy defect is associated with an impairment of intestinal innate immunity in pediatric CD.

(Inflamm Bowel Dis 2013;19:2339–2348)

Key Words: autophagy, Crohn's disease, immunity

Significance of this study

What is already known about this subject?

1. Genetic variation in the autophagy gene *ATG16L1* (T300A) has been associated to Crohn's disease (CD) susceptibility.
2. Functional studies have confirmed a dramatic alteration of autophagy capability in adult inflammatory bowel disease (IBD) patients, whereas little is known on this cellular degradation machinery in pediatric IBD.
3. The current view, that children with IBD are a patient population with specificities different from adult IBD, strongly encourages the analysis of intestinal immunity in pediatric IBD.
4. The *ATG16L1*-mediated autophagy has been shown to be required for both bacteria handling and antigen presentation in dendritic cells (DCs) from adult patients with CD.

What are the new findings?

5. DC of pediatric patients with CD carrying the T300A allele show a marked impairment of antigen uptake and processing, and a decreased expression of activation markers, suggesting a defect in immune response.
6. The T300A polymorphism also regulates DC—epithelial interactions resulting in a reduced formation of transepithelial protrusions and decreased antigen uptake and processing through the enterocyte monolayer.
7. The presence of the T300 allele in the DC of pediatric patients with CD is responsible simultaneously for decreased innate immune function and for impaired stimulation of adaptive immunity.

Received for publication June 22, 2013; Accepted July 24, 2013.

From the *Department of Pediatrics with Department of Translational Medical Science, Section of Pediatrics and European Laboratory for the Investigation of Food-Induced Diseases (E.L.F.I.D), University of Naples Federico II, Naples, Italy; †Departments of Gastroenterology and Hepatology and Tytgat Institute, Academic Medical Center, Amsterdam, the Netherlands; and ‡Institute of Food Sciences-CNR, Avellino, Italy.

The authors have no conflicts of interest to disclose.

Reprints: Riccardo Troncone, MD, Department of Pediatrics with Department of Translational Medical Science, Section of Pediatrics and European Laboratory for the Investigation of Food-Induced Diseases, University of Naples Federico II, Via Sergio Pansini 5, I-Naples 80131, Italy (e-mail: troncone@unina.it).

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1097/MIB.0b013e3182a6a11c

Published online 9 September 2013.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

How might it impact on clinical practice in the foreseeable future?

8. The DC of pediatric patients with CD carrying the risk allele of the *ATG16L1* T300A polymorphism showed an impaired functionality, as well as already reported in adults. This finding, together with the fact that there are a number of autophagy stimulating compounds already in clinical practice, makes us more confident that this may be a viable pathway for intervention in CD, especially for children who may have a worse disease phenotype.

An altered immune response to intestinal antigens, particularly from microflora, is hypothesized to play a key role in Crohn's disease (CD), a chronic inflammatory bowel disorder.[1] Recently, genome-wide association studies indicated that *ATG16L1* is one of the main susceptibility genes of CD and suggested a role for autophagy in its pathogenesis.[2] Autophagy is a lysosome-dependent degradation pathway through which the cells metabolize proteins of either external or internal sources.[3] *ATG16L1* when bound to phagosomal vesicle is responsible for the proper function of the autophagy machinery.[4] Firstly described as a cell survival mechanism under nutrient starvation,[5] autophagy in the recent time has been implicated in immune defense against microorganisms.[3] *ATG16L1* hypomorphic mice display Paneth cell abnormalities, resulting in a deceased release of antimicrobial peptides into the intestinal lumen,[6] and an increased susceptibility to dextran sulfate sodium–induced colitis.[7,8] Additionally, even if the *ATG16L1* T300A variant is not clearly associated with a classical autophagy defect,[9] a decreased level of autophagy was associated with a marked decrease of bacteria handling in patients with CD homozygously carrying the risk allele of the *ATG16L1* single-nucleotide polymorphism (SNP) rs2241880.[10]

The intestinal mucosa is continuously exposed to a large amount of foreign (non-self) substances, such as bacteria, viruses, and food antigens. The balance between immune activation and tolerance is maintained by a variety of cells, including enterocytes and immune competent cells, such as dendritic cells (DCs) and T lymphocytes.[1] Changes in the specific interactions between these various cell types may affect the outcome of the response to antigens, and therefore lead to a lack of immunity or an exaggerated response to nonharmful substances, as it occurs in CD.[11]

DCs resident in the intestinal mucosa have an important role in maintaining immune balance in the gut. Lamina propria DC sample luminal antigens by protruding their dendrites in between the enterocytes while the epithelial barrier function is maintained.[12–16] We have previously shown that if autophagy is impaired, DCs form hyperstable interactions with T cells,[17] and their antigen sampling through epithelial cell monolayers is greatly decreased.[18] In these studies, autophagy was inhibited by the use of siRNA or by pharmacological inhibitors, and although very informative, it remains an indirect reflection of the physiological condition in subjects carrying the risk allele of the *ATG16L1*.

In this study, we aimed to investigate the physiological relevance of an impaired autophagy machinery for human disease by analyzing phenotype and function of DC obtained from patients with CD carrying the risk allele of *ATG16L1* gene. In particular, because very little is known on the role of autophagy in pediatric inflammatory bowel disease (IBD), we have investigated the phenotype and antigen sampling capacity of DC in children carrying the *ATG16L1* T300A variant.

MATERIALS AND METHODS

Study Population and Genotyping

The study population included a total of 34 children (mean age 14 yr; range 1–18 yr; female/male: 8/25). Demographic and clinical characteristics of enrolled patients are described in Table 1. For each patient, clinical activity of the disease was evaluated at the time of diagnosis using the Pediatric Crohn Disease Activity Index.[19] Children were genotyped for the SNPs *ATG16L1* rs2241880, *NOD2* rs2066844, *NOD2* rs2066845, and *NOD2* rs2066847 variants using the TaqMan system (7900HT sequence detection system; Applied Biosystems, Foster City, CA), as previously described.[18] Patients were stratified into 3 groups on the basis of *ATG16L1* polymorphism: (1) the AA homozygous wild type (wild type) group, (2) the GG homozygous risk (variant risk) group, and (3) the AG heterozygous (het) group. Genotype analysis is summarized in Table 2. Written informed consent was obtained from participants' parents or from patients themselves if older than 10 years of age. The study was approved by the Institutional Ethical Committee of the University "Federico II" of Naples (Protocol 67/12).

Cell Culture

DCs were generated from peripheral blood mononuclear cells according to a well-established procedure.[20] Briefly, adhered monocytes were cultured for 6 to 8 days in complete culture medium (RPMI supplied with penicillin/streptomycin, nonessential amino acids, and 10% fetal calf serum, thereafter indicated as complete medium) in the presence of recombinant human GM-CSF and IL-4 (both 100 ng/mL; RnD Systems, Inc., Minneapolis, MN). In experiments set-up to evaluate protrusions, DC were labeled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (Sigma-Aldrich, St. Louis, MO). Colorectal carcinoma cells Caco2 were maintained in complete medium supplemented with penicillin/streptomycin and 10% fetal calf serum.

Antigen Uptake

Monocyte-derived DC were suspended at 3 to 5×10^5/mL in complete medium and incubated in the presence or absence of 20 μg/mL Alexa-488–labeled *E. coli* bioparticles (hereafter indicated as bacteria particles; Invitrogen, Carlsbad, CA). After 2 hours of incubation, cells were extensively washed and analyzed by flow cytometry or at the confocal microscope, as previously described.[17] In the experiments evaluating antigen processing, DCs were incubated with DQ-ovalbumin (DQ-OVA; Molecular Probes, Eugene, OR) 90 μg/mL for 4 hours. After antigen pulsing, cells were washed and analyzed at flow cytometry.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

TABLE 1. Demographic and Clinical Characteristics in 33 Children with CD

Characteristics	AA Wild Type	GG Risk	AG Het
Patients (n)	7	13	14
Sex			
Male/Female	4/3	9/4	12/2
Age, mean (range), yr	13.85 (1–22)	16.7 (8–21)	14.6 (8–24)
Duration of disease (mean ± SD, yr)	3.6 ± 3.2	4.9 ± 3.7	3.3 ± 2.5
Therapy (n)			
Aminosalicylates	8	5	8
Immunosuppressants	2	1	3
Aminosalicylates and immunosuppressants	3	1	3
Pediatric Crohn Disease Activity Index (mean ± SD)	27.8 ± 9.57	24.7 ± 9.27	26.4 ± 9.54

Caco2-DC Transwell Experiments

Transwell experiments were performed as described previously.[15] Briefly, Caco2 cells were grown for 10 days in the upper chamber of a 3-μm pore transwell insert (Falcon, Bedford, MA) until the cells reached the confluence and transepithelial electric resistance of approximately 300 Ohm was achieved. Thereafter, transwell inserts were inverted, and DCs were applied to the basolateral side of the filter for 2 hours. Inserts were then reverted and stimuli, either bacteria particles or 100 nm formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich), were applied to the apical surface for an additional 2 hours. Subsequently, stimuli were removed by washing the insert, and cells were further incubated for 20 hours before the flow cytometry and confocal analysis were done.

TABLE 2. Genotype Analysis of 34 Children with CD

NOD2 Genotypes	Wild type *ATG16L1*	Risk *ATG16L1*	Het *ATG16L1*
NOD2 rs2066844, n (%)			
Wild type	7 (100%)	13 (100%)	13 (93%)
Risk	0 (0%)	0 (0%)	0 (0%)
Het	0 (0%)	0 (0%)	1 (8%)
NOD2 rs2066845, n (%)			
Wild type	5 (71%)	12 (92%)	14 (100%)
Risk	0 (0%)	0 (0%)	0 (0%)
Het	2 (28%)	1 (8%)	0 (0%)
NOD2 rs2066847, n (%)			
Wild type	6 (86%)	13 (100%)	10 (71%)
Risk	0 (0%)	0 (0%)	0 (0%)
Het	1 (14%)	0 (0%)	4 (28%)

Flow Cytometry

Fluorochrome-conjugated anti-CD86, anti-CD11c, anti-HLA-DR, and isotype control antibodies were obtained from BD Biosciences (San Jose, CA) or Miltenyi Biotec (Bologna. Italy). DCs used in transwell experiments were harvested from the basolateral side of the insert. Cells were washed and stained in phosphate buffered saline/0.5% bovine serum albumin and analyzed at FACSCalibur (Becton Dickenson, Franklin Lakes, NJ) using the CellQuest software.

Confocal Microscope Analysis

For confocal analysis of transwell cultures, inserts were incubated in 4% paraformaldehyde for a minimum of 60 minutes and then mounted using SlowFade Gold (Invitrogen). For confocal analysis, cells were adhered to poly-L-lysine–coated coverslips (Sigma-Aldrich), fixed in 4% paraformaldehyde, stained in permeabilization buffer (phosphate buffered saline containing 0.05% Triton X-100) and embedded in SlowFade Gold (Invitrogen). Images were obtained on a LSM Zeiss 510 confocal system equipped with 405 nm UV, 488 nm argon, and 543 HeNe lasers (ZEISS Germany) and processed using AIS Zeiss software. Anti-pancytokeratin (Dako, Carpinteria, CA) allowed detection of Caco2 cells.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software package for Windows (version 13.0; SPSS, Chicago, IL). The χ^2 and Fisher's exact tests for categorical variables, the Student's t test, and the Mann–Whitney U test for normally distributed variables were used where appropriate. Statistical significance was predetermined because $P < 0.05$ was considered significant.

RESULTS

Effect of T300A Allele on Direct DC Bacteria Particle Uptake

Since it has been shown that in adult CD the presence of specific SNP in autophagy related genes is associated with defective bacteria handling by DC, we aimed to investigate if this also occurs in DCs of pediatric patients with CD. DC were cultured from peripheral blood and defined as the HLA-DR and CD11c double positive population. Bacteria particle uptake was measured in the $HLA\text{-}DR^{+}CD11c^{+}$ cells obtained from each subject (Fig. 1A). Although we found that the majority of DCs from all 3 groups were able to sample particulate antigens, as indicated by the high percentage of stained cells (Fig. 1A), the amount of particles taken up, evaluated as the mean fluorescence intensity was much lower in risk (mean ± SD: 1558 ± 1747) and in het patients (mean ± SD: 1728 ± 1760) compared with wild type (mean ± SD: 3892 ± 2581) ($P = 0.01$ and 0.03, respectively; Fig. 1B, C). These flow cytometry findings were next confirmed by confocal microscopy in which the images showed a reduced uptake of bacteria particles by DC from risk children with CD (Fig. 1D).

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

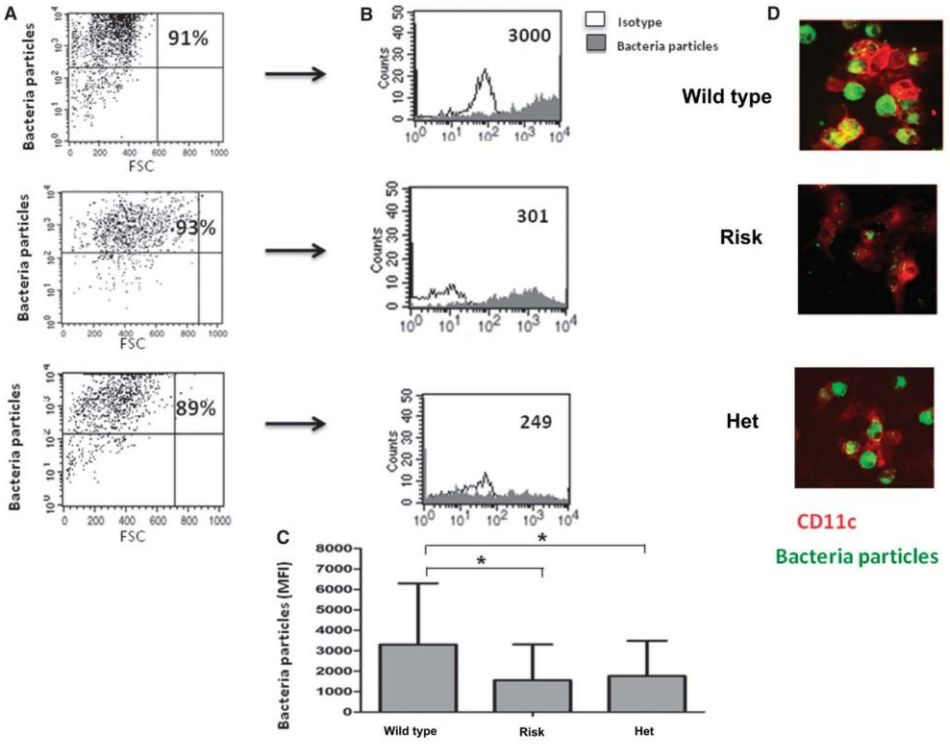


FIGURE 1. Uptake of bacteria particles is reduced in DC from pediatric patients with CD with *ATG16L1* risk allele. Monocyte-derived DCs were obtained from pediatric patients with CD carrying the AA homozygous allele (wild type), the GG homozygous allele (risk), or the AG heterozygous allele (het) of the autophagy-associated gene *ATG16L1*. The DCs were incubated for 2 hours with Alexa-488–labeled *E. coli* bioparticles and soon after stained for surface phenotype, fixed, and analyzed by flow cytometry and confocal microscope. DC population are analyzed in gate of HLA-DR$^+$CD11c$^+$ cells. A, Staining of DC positive for bacteria particles uptake, the numbers indicate the percentage of positive cells. B, Intensity of bacteria particles uptake in DC of a representative patient from each group (numbers indicate MFI). C, Overall bacteria particles staining in 7 wild type, 13 risk, and 13 het patients. Data are shown as MFI (mean ± SD). D, Confocal image of DC from a representative patient from each group after 2 hours of incubation with Alexa fluor 488-coniugated bacteria particles (green). DCs were stained for CD11c (in red). Dot plots (A), histograms (B), and confocal images (D) are representative of DC from 1 patient of each group. *$P < 0.05$. MFI, mean fluorescence intensity.

Effect of T300A Allele on DC Maturation

To further examine whether the presence of the *ATG16L1* T300A variant might affect the maturation of DC occurring upon particulate antigen exposure, we determined the expression of activation markers HLA-DR and CD86 after 2 hours of bacteria particle incubation. The expression of HLA-DR in DC from wild type patients markedly increased after exposure to the bacteria particles compared with unstimulated cells ($P = 0.02$); by contrast, the membrane levels of HLA-DR remained almost unchanged in DC from either risk or het patients. Furthermore, the HLA-DR expression induced on exposure to bacteria particles in wild type DC was significantly higher compared with the levels induced in either risk or het DC ($P = 0.008$ and 0.006, respectively), (Fig. 2A, B).

We next looked at the expression of CD86, another marker of early immune activation. In contrast to HLA-DR, the basal levels of CD86 in DC surface membrane did not substantially differ among the 3 groups. Notwithstanding, the exposure to bacteria particles induced a marked increase of CD86 expression in the DC from wild type children for *ATG16L1* ($P = 0.04$), whereas the CD86 level observed in the DC from the risk group

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

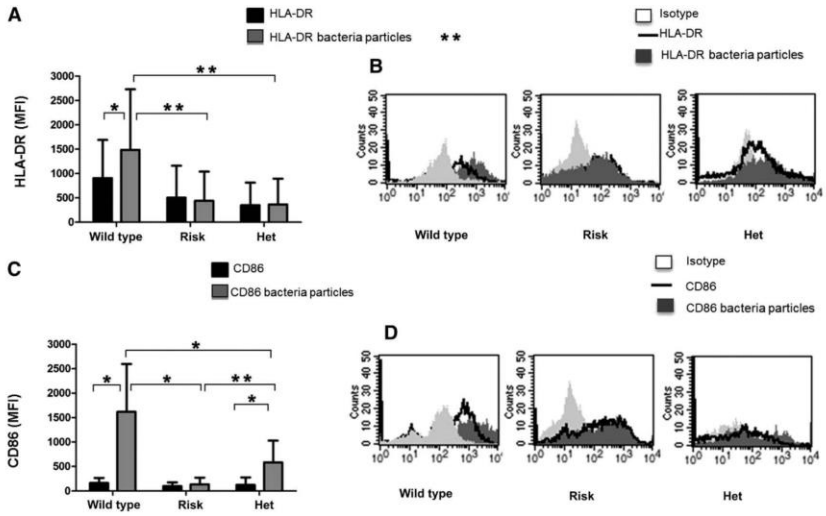


FIGURE 2. Both HLA-DR and CD86 expression is reduced in DC from *ATG16L1* risk children with CD after uptake of bacteria particles. DCs stimulated with bacteria particles as described in Figure 1 were analyzed by flow cytometry for the expression of HLA-DR (A and B) and CD86 (C and D) on surface membrane. A, HLA-DR was evaluated before and after bacteria particles exposure among the HLA-DR$^+$CD11c$^+$ cells. Data are shown as mean fluorescence intensity (MFI) (mean ± SD) of DC obtained from 7 wild type, 13 risk, and 14 het patients. B, Representative histograms of HLA-DR expression on DC from an individual patient. C, CD86 expression in DC from 5 wild type, 7 risk, and 11 het pediatric CD patients and (D) representative CD86 MFI in DC for each analyzed group. $^*P < 0.05$, $^{**}P < 0.01$.

remained almost unchanged ($P = 0.6$), (Fig. 2C, D). In contrast to the HLA-DR findings, in heterozygous patients, the expression of CD86 significantly increased after bacteria handling compared with the levels of the unexposed cells ($P = 0.02$). To strengthen this observation, the expression of CD86 in both wild type and het DC detected after the brief bacteria exposure resulted in significantly higher levels compared with the CD86 level in risk DC ($P = 0.03$ and 0.008, respectively; Fig. 2C, D). Collectively, these findings show that the T300A SNP is associated with a dysregulated membrane mobilization of the immune activation markers HLA-DR and CD86 in DCs from pediatric patients with CD.

Effect of T300A Allele on DC Antigen Processing

Uptake of an antigen is the first step for its presentation to immune competent cells and is followed by a proteolytic processing. To study this second process and to further investigate whether the T300A polymorphism affects the processing of the antigens, as well as the uptake, we tested the ability of DC to phagocytize and process DQ-OVA, a dietary protein labeled with a quenched fluorochrome that becomes detectable only on proteolytic degradation in antigen-presenting cells. As expected, DC from wild type patients showed a statistically significant increase of uptake and processing compared with the risk group ($P = 0.04$). Interestingly the het group, displayed a more efficient DQ-OVA uptake when compared with the risk group, although the difference did not reach a statistical significance ($P = 0.09$; Fig. 3A, B).

When we looked at the expression of the HLA-DR and CD86 after 4 hours of DQ-OVA sampling, significant upregulation of both the activation markers occurred only in the wild type DCs when compared with unstimulated cells, consistent to the findings obtained after bacteria particle exposure ($P = 0.02$ and 0.04 for DR and CD86, respectively; Fig. 3D, F).

Effect of T300A Allele on DC–Epithelial Interactions: Transepithelial Protrusions

We have reported that autophagy regulates the interaction of DC with enterocytes in a model system for intestinal antigen sampling.[18] In this model, DCs are cultured on the basolateral side of a transwell containing differentiated Caco2 cells. Stimuli in the form of chemotactic molecules or antigens are then applied to the apical surface to induce the DC antigen sampling through the epithelial layer.[18] By using this system, we tested the ability of DC from patients with CD to form protrusions through an epithelial cell layer and to take up antigen. DC from wild type, het, and risk CD groups were stained with carboxyfluorescein succinimidyl ester and were adhered to the basolateral side of the filter with differentiated CaCo2 cells. Formyl-methionyl-leucyl-phenylalanine

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

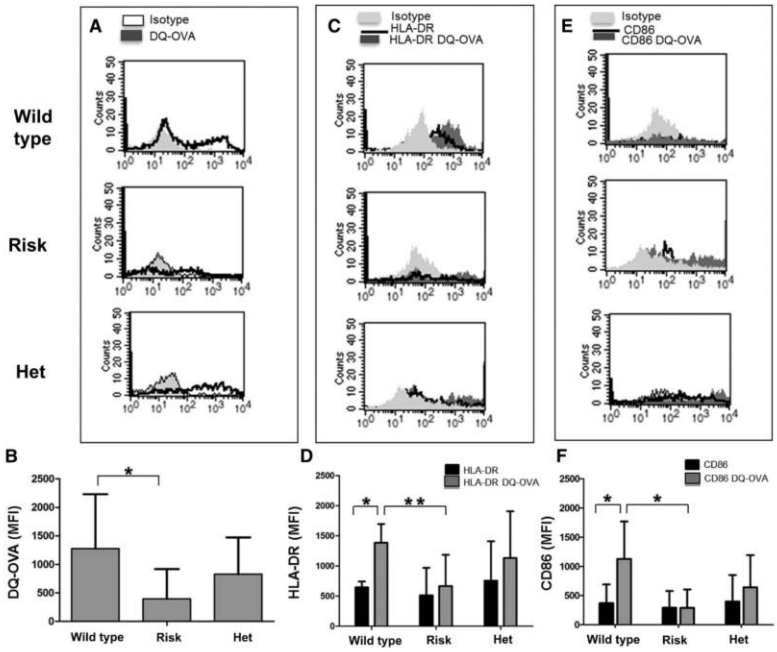


FIGURE 3. Processing of soluble protein (DQ-OVA) is unpaired in DC from *ATG16L1* risk children with CD. DCs were incubated for 4 hours with a fluorochrome-labeled ovalbumin (DQ-OVA) and thereafter stained, fixed, and analyzed by flow cytometry. Mean fluorescence intensity (MFI) of DQ-OVA (A and B), HLA-DR (C and D), and CD86 (E and F) stainings in DC was assessed either at basal level (unstimulated) or after DQ-OVA incubation. Histograms are representative of 1 patient of each group. Graphs indicated the overall MFI (mean ± SD) of DQ-OVA, HLA-DR, and CD86 expression in DC obtained from 6 wild type, 8 risk, and 9 het pediatric CD patients. $*P < 0.05$, $**P < 0.01$. DQ-OVA, DQ-ovalbumin.

was added as a stimulus to allow formation of protrusions. Confocal analysis showed that risk DC displayed a decreased formation of transepithelial protrusions compared with those from the wild type and the het patients ($P = 0.03$; Fig. 4A, B).

Effect of T300A Allele on DC–Epithelial Interactions: Sampling

To test the functional consequences of the decreased formation of transepithelial protrusions by risk DC, we repeated the experiments adding fluorescent bacteria particles to the apical side of the Caco2 monolayer. After 24 hours, uptake of particles by DC was measured by flow cytometry. The results indicated that in patients from the risk group, sampling was severely compromised ($P = 0.01$). The het DC appeared to have an intermediate phenotype, with less sampling than wild type but more than risk, although the difference did not reach statistical significance (Fig. 5A, B).

Similarly to the experiments done with DC alone, phenotypic analysis did not show differences in HLA-DR expression between the various genotypes of DC after coculture with epithelial cells alone. However, addition of bacteria particles to the system resulted in an increase in the expression of HLA-DR in wild type DC, but not in risk or het DC (Fig. 5C, D). As a result, staining of membrane HLA-DR after bacteria load was markedly higher in wild type DC than that in risk DC ($P = 0.01$) or in het DC ($P = 0.01$).

Interestingly, after coculture with epithelial cells, expression of CD86 was significantly higher in wild type DC compared with risk DC ($P = 0.04$), even in the absence of an antigenic stimulus (Fig. 5E, F). Although the exposure to the bacteria particles resulted in an increased expression of CD86 in all genotypes, the levels detected in wild type and het DC were significantly higher than in risk DC ($P = 0.004$ and 0.009, respectively; Fig. 5E, F).

DISCUSSION

In this study, we demonstrate that the monocyte-derived DC from pediatric patients with CD carrying the *ATG16L1*

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

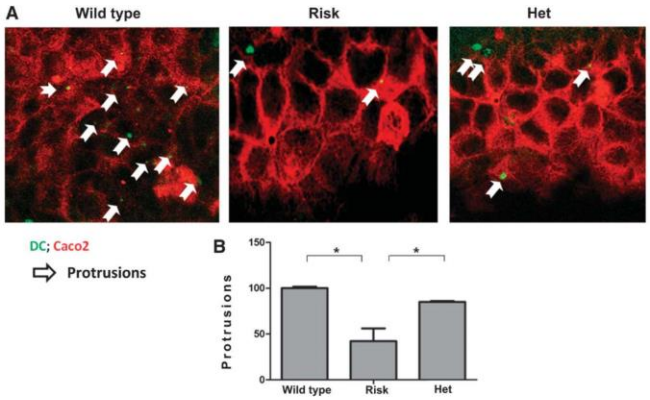


FIGURE 4. DCs from *ATG16L1* risk children with CD form less transepithelial protrusions through CaCo2 layer. CaCo2 epithelial cells (red) were cultured on transwell inserts, and monocyte-derived DCs (green) were adhered to the basolateral side of the filter. Stimuli (formyl-methionyl-leucyl-phenylalanine) were added to the apical side for 2 hours. Filters were fixed and analyzed by confocal microscopy. A, Confocal images of DC protrusions trough the epithelial cell layer. White arrows indicate the DC protrusions through the CaCo2 epithelial layer. B, Image analysis of the amount of protrusions detectable on the apical side of the filter. Data are shown as the mean ± SD of the protrusions of DC obtained from 5 wild type, 4 risk, and 3 het pediatric patients with CD. *$P < 0.05$.

polymorphism T300A, associated with a defective autophagy, are impaired in proper antigen sampling and processing. We found a marked reduction of bacteria particle localization in DC from children with CD with the *ATG16L1* risk variant compared with DC of children with *ATG16L1* wild type variant, indicating an impairment of particulate antigen sampling. DCs from patients with CD *ATG16L1* risk variant were also impaired in processing of soluble antigen, as shown by reduced intracellular degradation of ovalbumin. Furthermore, we found that DC from the risk group almost completely failed in upregulating the HLA-DR and CD86, the early activation markers, after a brief incubation with particulate or soluble antigens.

Genome-wide association studies have shown a strong association of CD with genes encoding for *ATG16L1*, *NOD2* family proteins, *IRGM1*, and *ULK1*, involved in autophagy machinery, either in adult or, more recently, in pediatric patients.[21–23] *ATG16L1* protein has been implicated in the clearance of intracellular pathogens, such as *Mycobacterium tubercolosis*, Salmonella spp, and more recently *Helicobacter pylori*.[21,24,25] Because intestinal microorganisms have been suggested to be one of the causes responsible of the bowel inflammation,[1] it is likely that an alteration of autophagy process might lead to an uncontrolled microorganism growth in the intestine of patients with IBD.

Some, but not all, functional studies have next confirmed a dramatic alteration of autophagy capability in adult patients with IBD carrying the T300A polymorphism, whereas little is known on this cellular degradation machinery in pediatric IBD.[9] However, the distinction between adult and pediatric IBD is a current argument due to the rising in the incidence of pediatric IBD.[26] Children with IBD seem to be a distinctive population with specificities requiring highly skilled and specialized approach for diagnosis and treatment.[27]

Because only a subset of patients affected by CD are mutant for the *ATGL16L1* gene, we have analyzed the autophagy in DC from either children with CD not carrying the *ATG16L1* risk allele (wild type) and in those carrying the T300A SNP allele in homozygous (risk) or heterozygous (het) combinations.

To our knowledge, very little is known about the phenotype and function of DC from pediatric CD, and our study aimed to investigate the ability these key cells to phagocytize, process, and present particulate antigens of microbial origin. We found that DCs are markedly impaired in antigen sampling and processing in children homozygous for CD-associated T300A *ATGL16L1* polymorphism. Besides *ATG16L1* T300A, additional variants, independent of rs2241880, could be the causative allele implicated in CD pathogenesis.[28] Also, because it has been reported that *NOD2* interacts with *ATG16L1* in the activation of autophagy process,[29] we have genotyped all the 34 patients recruited in the study for the 3 most frequent *NOD2* SNPs *(NOD2* rs2066844, *NOD2* rs2066845, and *NOD2* rs2066847*)* previously found associated to genetic risk of CD, other than for *ATG16L1* SNP rs2241880, as shown in Table 2. We found that the majority of our study children (>86%) were wild type for the 3 main *NOD2* SNPs, and none of them carried in homozygosity any of the 3risk alleles analyzed. In particular, in the children with CD carrying the *ATG16L1* risk polymorphism T300A (rs2241880), only 1 child was heterozygous

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

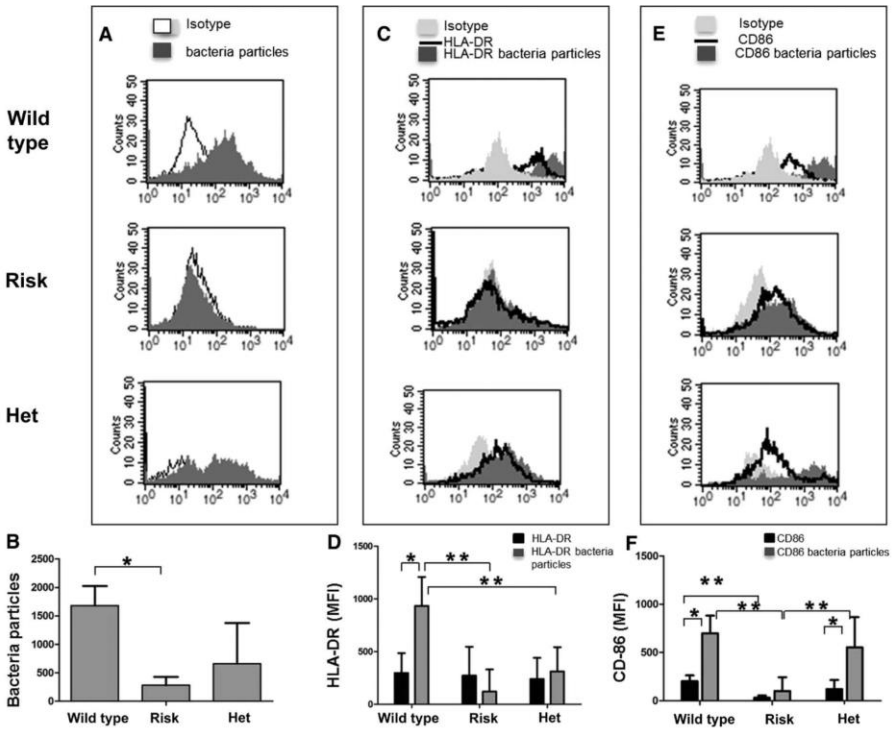


FIGURE 5. Transepithelial antigen sampling by DC from *ATG16L1* risk patients with CD is unpaired. CaCo2 epithelial cells and DCs were cultured on transwell inserts as described in Figure 3. Alexa 488–labeled *E. coli* bioparticles were added to the apical side for 2 hours and then DC were removed from the filter, stained, and analyzed by flow cytometry. A, Intensity of bacteria particles uptake in representative DC ($HLA\text{-}DR^{+}CD11c^{+}$ gated cells), and (B) from 4 wild type, 3 risk, and 5 het CD pediatric patients. C and D, HLA-DR expression at basal level and after bacteria particles exposure in representative DC and from 4 wild type, 5 risk, and 6 het are shown. E and F, Basal CD86 in DC expression and after bacteria incubation from 5 wild type, 7 risk, and 11 het pediatric patients with CD are shown. Data in panels (B), (D), and (F) are mean ± SD of mean fluorescence intensity (MFI). $^{*}P < 0.05$, $^{**}P < 0.01$.

for the *NOD2* rs2066845 SNP; therefore, we assume that the defect in antigen sampling and processing, seen on the DC, is most likely due to the *ATG16L1* T300A allele.

Interestingly, the DC from children that were heterozygous for *ATG16L1* T300A polymorphism were hampered in bacteria particles, but not ovalbumin, phagocytosis. Moreover, DC from the *ATG16L1* heterozygous group did not upregulate HLA-DR on the surface membrane after exposure to bacteria particles or to ovalbumin. The impairment of het DC functionality suggested a dose-dependent effect of the T300A allele that has never been described, except only 1 study that reported a dose effect of this allele with CD risk.[30] One possible explanation of this difference in antigen handling by DC from risk variant and heterozygous groups is the structure of the processed antigens, requiring a more complex degradation machinery for bacteria compared with the a soluble protein of dietary origin, such as ovalbumin.

Cooney et al,[10] in their work on adult patients with IBD, showed that both *NOD2*-mediated and *ATG16L1*-mediated autophagy is required for both bacteria handling and generation of antigen-specific $CD4^{+}$ T-cell responses, because homozygous risk patients had an impaired DC activity. In fact, in this elegant study, monocyte-derived DC from T300A CD patients showed aberrant bacteria handling and were defective in the upregulation of surface DR after 24 h of bacteria exposure. Our results are in complete agreement with these findings, although we have analyzed a pediatric CD population.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

DC sampling of luminal antigens has been shown to occur under noninflammatory steady-state conditions and is therefore thought to be involved in the maintenance of intestinal immune tolerance. In line with this, and with the finding that autophagy is involved in the degradation of tight junction proteins, we showed that autophagy also regulates DC–epithelial interactions in the model system.[18] Others have shown previously that DCs form protrusions through the epithelial layer and sample luminal antigen.[31] We found that DCs of patients carrying the T300A allele of *ATG16L1* form less transepithelial protrusion through the Caco2 monolayer compared with wild type and heterozygous patients. In addition, it is known that autophagy-deficient DC are also less capable of degrading pathogens sampled through the intestinal mucosa.[21,29] In line with these observations, we found that DC from risk children are less efficient in sampling bacteria particulate fragments when added to the apical side of the transwell filter in a coculture system with Caco2 epithelial cells. Furthermore, on bacteria mucosal load, these DC were highly impaired in upregulating DR and CD86, the 2 key molecules for activating T-cell–mediated immune response. Indeed, the physiological environment in the gut includes, besides enterocyte, a large variety of cells, such as goblet cells, Paneth cells, and the connective tissue of the lamina propria. The ideal model, of course, would be the use of entire highly viable human intestinal specimens in culture. Therefore, it would be highly interesting to expand such an analysis to intestinal DC derived from biopsies of childhood CD.

Little information exists at present about the degree to which combinations of CD susceptibility variants contribute to specific cellular pathways or clinical phenotypes in CD. Cleynen et al[32] by a large multicenter study found that NOD2 and early immunomodulator use are the clinically most meaningful predictors for CD clinical course. However, preliminary data in our pediatric cohort of patients affected by CD suggested that the T300A SNP is associated with a more aggressive disease course, including changes in clinical behavior, a higher number of relapses, and an earlier use of immunosuppressant (Strisciuglio et al, submitted). Additional multicenter studies will be required to confirm this information in the future and to help in identifying molecular pathways that could be used as therapeutic targets in CD.

In conclusion, our data indicate defects in DC antigen sampling and activation in pediatric patients with CD carrying the T300A *ATG16L1* allele. The fact that there are a number of autophagy stimulating compounds already in clinical practice makes us more confident that this may be a new therapeutic option and a viable pathway for intervention in CD, in particular for those patients carrying 1, or more, SNPs associated with the autophagy machinery (~40% of the total CD population).[33]

Author Contributions: C.S. performed the flow cytometry experiments, data analysis, and manuscript drafting; E.M. did patients' recruitment and gastrointestinal endoscopy; M.E.W. contributed in study design and critical revision and manuscript drafting; F.P.G., M.A., A.V., and F.C. did all the DC generation and transwell experiments; A.C. provided assistance in flow cytometry and data analysis; M.V.B. performed the confocal analysis; A.S. contributed to the study design and critical revision of manuscript; R.T. and C.G. conceived the study design, the critical revision of experiments, and the manuscript drafting.

REFERENCES

1. Blumberg R, Cho J, Lewis J, et al. Inflammatory bowel disease: an update on the fundamental biology and clinical management. *Gastroenterology*. 2011;140:1701–1703.
2. Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy*. 2007;3:649–651.
3. Deretic V. Autophagy in infection. *Curr Opin Cell Biol.* 2010;22:252–262.
4. Kuma A, Mizushima N, Ishihara N, et al. Formation of the approximately 350-kDa Apg12-Apg5.Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. *J Biol Chem.* 2002; 277:18619–18625.
5. Nakatogawa H, Suzuki K, Kamada Y, et al. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol.* 2009; 10:458–467.
6. Cadwell K, Patel KK, Maloney NS, et al. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. *Cell*. 2010;141:1135–1145.
7. Biswas D, Qureshi OS, Lee WY, et al. ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages. *BMC Immunol.* 2008;9:35.
8. Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature*. 2008;456:264–268.
9. Fujita N, Saitoh T, Kageyama S, et al. Differential involvement of Atg16L1 in Crohn disease and canonical autophagy: analysis of the organization of the Atg16L1 complex in fibroblasts. *J Biol Chem.* 2009;284: 32602–32609.
10. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacteria handling and antigen presentation. *Nat Med.* 2010;16:90–97.
11. McCarroll SA, Huett A, Kuballa P, et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat Genet.* 2008;40:1107–1112.
12. Brain O, Cooney R, Simmons A, et al. Functional consequences of mutations in the autophagy genes in the pathogenesis of Crohn's disease. *Inflamm Bowel Dis.* 2012;18:778–781.
13. Siddiqui KR, Laffont S, Powrie F. E-cadherin marks a subset of inflammatory dendritic cells that promote T cell-mediated colitis. *Immunity*. 2010;32:557–567.
14. Iliev ID, Matteoli G, Rescigno M. The yin and yang of intestinal epithelial cells in controlling dendritic cell function. *J Exp Med.* 2007;204:2253–2257.
15. Rimoldi M, Chieppa M, Salucci V, et al. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nat Immunol.* 2005;6:507–514.
16. Chieppa M, Rescigno M, Huang AY, et al. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med.* 2006;203:2841–2852.
17. Wildenberg ME, Vos AC, Wolfkamp SC, et al. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. *Gastroenterology*. 2012;142:1493–1503.e6.
18. Strisciuglio C, Duijvestein M, Verhaar AP, et al. Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions. *J Crohns Colitis*. 2013;7:534–541.
19. Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr.* 1991;12:439–447.
20. Russell RK, Drummond HE, Nimmo EE, et al. Genotype-phenotype analysis in childhood-onset Crohn's disease: NOD2/CARD15 variants consistently predict phenotypic characteristics of severe disease. *Inflamm Bowel Dis.* 2005;11:955–964.
21. Singh SB, Davis AS, Taylor GA, et al. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science*. 2006;313:1438–1441.
22. Rioux JD, Xavier RJ, Taylor KD, et al. Genome wide association study identifies new susceptibility loci for Crohn's disease and implicates autophagy in disease phatogenesis. *Nat Genet.* 2007;39:596–604.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

23. Gammoh N, Florey O, Overholtzer M, et al. Interaction between FIP 200 and ATG16L1 distinguishes ULK1 complex dependent and independent autophagy. *Nat Struct Mol Biol.* 2013;20:144–149.
24. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 1994;179:1109–1118.
25. Birmingham CL, Smith AC, Bakowski MA, et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem.* 2006;281:11374–11383.
26. Raju D, Hussey S, Jones NL. Crohn disease ATG16L1 polymorphism increases susceptibility to infection with Helicobacter pylori in humans. *Autophagy.* 2012;8:1387–1388.
27. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology.* 2008;135:1114–1122.
28. Van Limbergen J, Kabakchiev B, Stempak JM, et al. Hypothesis-free analysis of ATG16L1 demonstrates gene-wide extent of association with Crohn's disease susceptibility. *Gut.* 2013;62:331–333.
29. Benchimol EI, Fortinsky KJ, Gozdyra P, et al. Epidemiology of paediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis.* 2011;17:423–439.
30. Okazaky T, Wang MH, Rawsthorne P, et al. Contributions of *IBD5, IL23R, ATG16L1*, and *NOD2* to Crohn's Disease Risk in a Population-Based Case-Control Study: Evidence of Gene–Gene Interactions. *Inflamm Bowel Dis.* 2008;14:1528–1541.
31. Rescigno M, Rotta G, Valzasina B, et al. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology.* 2001;204:572–581.
32. Cleynen I, González JR, Figueroa C, et al. Genetic factors conferring an increased susceptibility to develop Crohn's disease also influence disease phenotype: results from the IBDchip European Project. *Gut.* [published online ahead of print December 21, 2012]. doi: 10.1136/gutjnl-2011-300777.
33. Palomino-Morales RJ, Oliver J, Gomez-Garcia M, et al. Association of ATG16L1 and IRGM genes polymorphisms with inflammatory bowel disease: a meta-analysis approach. *Genes Immun.* 2009;10:356–364.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

2.1.3 Authophagy Genes Variants and Pediatric Crohn's disease phenotype: a single-center experience.

The molecular basis of the pathogenesis of CD is not completely understood. Strong epidemiological evidence for a genetic predisposition has stimulated recent efforts to identify the susceptibility genes.[22-23] In 1996 the discovery of NOD2/CARD15, the first gene identified to be associated to CD [24], represented one of the first success story in the genetics of a complex polygenic disease. Genome wide association studies (GWAS) have provided evidence for several determinants, including genes encoding ATG16L1 (autophagy-related 16-like 1) and IRGM1 (immunity-related GTPase family M), providing further insights into disease pathogenesis.[25] These genes are involved in a biological process known as autophagy, which plays a role in protein degradation, antigen processing, regulation of cell signaling, and many other pathways essential to the regulation of inflammation.[26] Different studies found in pediatric patients a strong association between NOD/CARD15 SNPs and an early-onset disease as well as a major ileal or ileo-colonic localization of CD.[27-28] However, regarding ATG16L1, a correlation between ATG16L1 risk allele and a more frequent ileal involvement of CD has been described in adults [29], but there are no evidences in children. In addition, only one pediatric study [30] analysed the recurrence of IRGM1 SPNs and CD risk, but no associations were found between these SPNs and the clinical course of the disease. The purpose of our study is to evaluate the relationship between the main risk alleles of NOD2/CARD15 (rs2066844; rs2066845; rs2066847), ATG16L1 (rs2241880) and IRGM1 (rs13361189; rs4958847), and the clinical features in our cohort of children affected by CD.

To our knowledge, this is the first pediatric study reporting an association between the presence of rs2241880 risk polymorphism of ATG16L1 in children with CD and a more severe phenotype of disease. We clearly demonstrated that patients carrying the homozygous risk allele show a significant trend to change from an inflammatory to a structuring behaviour. Hypothetically, the more severe phenotype may be somehow linked with the early development of fibrosis. In line with this phenotype, we demonstrated an association of rs2241880 risk polymorphism and the lack of perianal disease, suggesting that the ATG16L1 risk allele may exert a protective effect on the development of this manifestation in CD children. To support the hypothesis of worst phenotype in homozygous patients, we found that rs2241880 risk polymorphism was related to a major incidence of clinical relapses and with the introduction of immunosuppressants. In addition, children carrying rs2241880 risk allele show higher values of fecal calprotectin and CRP at diagnosis compared with patients carrying ATG16L1 heterozygous or wild type variants. Although, the role of fecal and serologic markers in predicting IBD disease course is still controversial, the hypothesis that higher

values at diagnosis could predict a more severe disease course has been claimed. Our data were strengthened by either a discriminant function, either a multivariate logistic regression analysis. Indeed, the multivariate logistic regression analysis demonstrated that development of structuring behaviour, followed by relapse during the first year of disease and absence of perianal disease, were respectively the most significant variables associated with ATG16L1 risk allele. In accordance with, Our data from functional studies are in accordance with these genotype-phenotype results, indeed we show that the monocyte-derived dendritic cells (DC) from pediatric patients with CD carrying the ATG16L1 risk polymorphism are impaired in the proper antigen sampling and processing. Furthermore, we found that DC from the risk group almost completely failed in upregulating the HLA-DR and CD86, the 2 key molecules for activating T-cells mediated immune response. Since intestinal microorganisms have been suggested to be one of the causes responsible of the bowel inflammation, it is likely that an alteration of autophagy process might lead to an uncontrolled microorganism growth in the intestine of IBD patients.[31] The IRGM1 gene has been recently identified to play a role in the development of CD and also shown to be involved in autophagy. In contrast, however, the extensive amount of work that has been accomplished in identifying and characterizing the complex network of ATG molecular mechanisms, there is much less known about the role of the IRGM1 related autophagic pathways.[32] We found that the presence of IRGM1 rs13361189 variant allele was associated with a lower use of immunosuppressant therapy, highlighting a possible role on the development of a milder phenotype. However, none of the associations were confirmed at the multivariate logistic regression analysis and further studies are needed to provide definitive conclusions about IRGM1 rs13361189. Several studies supported the concept of more frequent small bowel disease in children and adolescents [33,34] and a significant association between ileal disease and the carriage of one or more NOD2/CARD15 variant alleles. According to the current evidences, our analyses of genotype-phenotype correlation showed that patients carrying the NOD2/CARD15 rs2066847 (1007fs) heterozygous allele had a more frequent ileal involvement than children showing the wild type variant for the same SNP. Our study has some limitations, including the small cohort of patients. However, the associations were confirmed with powerful statistical tests in order to avoid potential bias.

In conclusion, we assume that genetic susceptibility may have a more important role in the etiology of pediatric-onset IBD. [35-36] IBD has certainly a multifactorial origin, and the importance of environmental is widely recognized. However, if genetic susceptibility is greater, than pediatric IBD patients can be expected to have a more severe clinical course of the disease. Within pediatric-onset CD, specific genotype-phenotype associations can be found. In our pediatric cohort, ATG16L homozygous risk allele resulted to be associated with a more aggressive disease course, including

development of structuring behaviour, early relapse and premature use of immunosuppressants. As previously demonstrated, the presence of heterozygous allele of NOD2/CARD15, significantly correlated with major ileal disease. These data stress the importance of genetic susceptibility research in larger pediatric onset IBD cohorts in order to find new genes and to allow an early treatment stratification of these patients.

Digestive and Liver Disease 46 (2014) 512–517

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary Tract

Autophagy genes variants and paediatric Crohn's disease phenotype: A single-centre experience

Caterina Strisciuglio[a,1], Renata Auricchio[a,1], Massimo Martinelli[a], Annamaria Staiano[a,*], Francesca Paola Giugliano[a], Marialuisa Andreozzi[a], Marina De Rosa[b], Eleonora Giannetti[a], Carmela Gianfrani[a,c], Paola Izzo[b], Riccardo Troncone[a], Erasmo Miele[a]

[a] Department of Translational Medical Sciences, Section of Pediatrics, University of Naples "Federico II", Italy
[b] Department of Molecular Medicine and Medical Biotechnology and CEINGE, University of Naples "Federico II", Italy
[c] Institute of Protein Biochemistry-CNR, Naples, Italy

ARTICLE INFO

Article history:
Received 9 October 2013
Accepted 18 February 2014
Available online 19 March 2014

Keywords:
Autophagy
Crohn's disease
Genome wide association studies
Inflammatory bowel diseases

ABSTRACT

Background and aims: Little evidence demonstrating the correlation between several single nucleotide polymorphisms and a specific phenotype of Crohn's disease has been reported in children. We investigated the relationship between autophagy genes variants and clinical features in our children with Crohn's disease.

Methods: Genotyping for *ATG16L1*, *NOD2/CARD15*, and *IRGM1* was performed in 80 consecutive patients with Crohn's disease (median age: 11 years; range: 0.7–17.9 years). Crohn's disease location and behaviour were classified using the Paris classification. Additional data were collected from clinical records on patients' demographics, age at symptom onset and diagnosis, extraintestinal manifestations, therapy, clinical relapses, and need of surgical intervention.

Results: Patients homozygous for the risk allele *ATG16L1* (T300A) showed a trend towards switching to a stricturing phenotype during the course of disease compared to children either homozygous for the wild-type allele or heterozygous for the *ATG16L1* single nucleotide polymorphism ($p = 0.01$). Homozygosity for the *ATG16L1* risk allele was associated with a major recurrence of clinical relapses and earlier introduction of immunosuppressants ($p = 0.006$ and $p = 0.04$, respectively). Heterozygosity for the *NOD2* rs2066847 allele was associated with major ileal involvement ($p = 0.01$).

Conclusion: In patients carrying the T300A variant, Crohn's disease follows a more aggressive clinical course.

© 2014 Editrice Gastroenterologica Italiana S.r.l. Published by Elsevier Ltd. All rights reserved.

1. Introduction

The molecular basis of the pathogenesis of Crohn's disease (CD) is not completely understood. Strong epidemiological evidence for a genetic predisposition has stimulated recent efforts to identify the susceptibility genes [1,2]. In 1996, the discovery of *NOD2/CARD15*, the first gene identified as being associated with CD [3], represented one of the first success stories in the genetics of a complex polygenic disease. Subsequently, in 2001, the characterization of the gene at the IBD1 locus as *NOD2/CARD15* was a landmark observation [4]; since then, a large number of replication studies confirmed the association of the *NOD2/CARD15* variant alleles with an increased susceptibility to CD in adults [5]. Genome-wide association studies (GWAS) provided evidence for several determinants, including genes encoding *ATG16L1* (autophagy-related 16-like 1) and *IRGM1* (immunity-related GTPase family M), providing further insights into disease pathogenesis [6]. These genes are involved in a biological process known as autophagy, which plays a role in protein degradation, antigen processing, regulation of cell signalling, and many other pathways essential to the regulation of inflammation [7]. The group of Hampe et al. was the first to implicate the autophagy pathway in CD pathogenesis [8]. An association of *NOD2/CARD15*, *ATG16L1*, and *IRGM1* risk alleles with CD susceptibility has already been confirmed both in adults and children; however, there is little evidence describing a possible correlation between these single nucleotide polymorphisms (SNPs) and a spe-

* Corresponding author at: Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5 80131 Naples, Italy. Tel.: +39 081 746 2679; fax: +39 081 546 9811.
E-mail address: staiano@unina.it (A. Staiano).
[1] These authors participated equally in this study.

http://dx.doi.org/10.1016/j.dld.2014.02.016
1590-8658/© 2014 Editrice Gastroenterologica Italiana S.r.l. Published by Elsevier Ltd. All rights reserved.

Table 1
Clinical features of 80 paediatric patients with Crohn's disease.

Clinical features	Total *n* (%)
Median age at diagnosis (years; range)	11 (0.7–17.9)
Gender	
Male	53 (66.2)
Median disease duration (months; range)	36.4 (12–154)
Positive family history	2 (2.5)
Location of disease	
Ileum only (L1)	17 (21.2)
Colon only (L2)	27 (33.8)
Ileum and colon (L3)	35 (43.8)
Upper gastrointestinal tract (L4a)	1 (1.2)
Perianal disease	20(25)
Extraintestinal manifestation	15 (18.7)
Disease behaviour	
Inflammatory (B1)	68 (85)
Stricturing (B2)	8 (10)
Fistulizing (B3)	4 (5)
Change in clinical behaviour	12 (15)
Need for surgery	10 (12.5)

cific CD phenotype in children. Various studies found a strong association, in paediatric patients, between the *NOD/CARD15* SNPs and early-onset disease, as well as a major ileal or ileo-colonic localization of CD [9,10]. However, as regards ATG16L1, a correlation between the *ATG16L1* risk allele and a more frequent ileal involvement of CD has been described in adults [11], but not in children. In addition, only one paediatric study [12] analyzed the recurrence of *IRGM1* SPNs and CD risk, and no associations were found between these SPNs and the clinical course of the disease. The purpose of our study is to evaluate the relationship between the main risk alleles of *NOD2/CARD15* (rs2066844; rs2066845; rs2066847), *ATG16L1* (rs2241880), and *IRGM1* (rs13361189; rs4958847) and the clinical features in our cohort of children affected by CD.

2. Materials and methods

2.1. Study population

The study population included 80 children diagnosed with CD between January 2001 and August 2013. Demographic and clinical data of each patient were retrospectively collected from medical records (Table 1). Only children with at least 1 year of follow-up were included in this study. Diagnosis of CD was based on clinical, endoscopic, radiological, and histopathological criteria [13]. For the diagnosis, all children underwent ileo-colonoscopy, upper GI endoscopy, and imaging analyses, including abdominal ultrasound and entero-MRI or small bowel follow-through.

2.2. Phenotype analysis

CD disease location was categorized using the Paris classification: L1 for cecal and distal ileum involvement, L2 for colonic disease, L3 for ileocolonic disease, L4a for upper disease proximal to the ligament of Treitz, and L4b for upper disease distal to the ligament of Treitz. Perianal disease was defined by the presence of fissures, perianal ulcers, abscesses or fistulae, and skin (tags). Disease behaviour based on clinical history, was categorized as fistulising, stricturing, or inflammatory using the Paris classification guidelines criteria [14]. Stricturing disease referred to the presence of a constant luminal narrowing diagnosed radiologically, endoscopically, or surgically. Penetrating disease referred to radiographic, endoscopic, surgical, or clinical evidence of an abscess or fistula in any location. Patients who had neither stricturing nor fistulising disease at diagnosis and throughout follow-up were classified as having inflammatory disease behaviour. Moreover, information regarding changes from the inflammatory to the fistulising or stricturing patterns, as well as any clinical relapses, was collected.

For each patient, clinical activity of the disease was evaluated at the time of diagnosis using the paediatric Crohn's disease activity index (PCDAI) [15]. Clinical remission was defined as a PCDAI score of <10, while clinical response to the induction treatment was identified by a change in the PCDAI score of at least 15 points from baseline. Clinical relapse was defined as the occurrence or worsening of symptoms accompanied by a PCDAI score of >10 points, sufficient to require rescue treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery [16]. Family history was defined as positive if at least one first or second-degree relative was diagnosed with Inflammatory Bowel Disease (IBD). We also considered clinical therapy at disease onset and during follow-up. Two expert paediatric gastroenterologists (AS and EM) made all decisions regarding therapeutic interventions, in line with the validated international guidelines [17]. Exclusive enteral nutrition for 6–8 weeks or oral steroid treatment (oral methylprednisolone: 1 mg/kg/day, max 40 mg/day per 4 weeks) was used as induction therapy in all patients. Aminosalicylates (mesalazine 50 mg/kg/day) were used as maintenance therapy in patients with mild disease. Patients in whom standard induction therapy had failed or patients with early relapse (<6 months) were treated with azathioprine (2–2.5 mg/kg/day). Methotrexate was used as second-line immunosuppressant in those patients intolerant or refractory to azathioprine. infliximab (5 mg/kg/dose at weeks 0, 2 and 6, and then 8-weekly) was given as a first biological agent in patients refractory or intolerant to steroids and immunomodulators. Patients refractory or intolerant to infliximab therapy were treated with adalimumab (loading dose: 160/80 mg or 80/40 mg at weeks 0 and 2, respectively in patients weighing ≥40 kg or <40 kg; maintenance dose: 80 mg and 40 mg every 2 weeks, respectively in patients weighing ≥40 kg or <40 kg). Extraintestinal manifestations included eye, joint, skin, or liver involvement and persistent fever, defined as temperature ≥38 °C for 3 days during the week before the treatment. The laboratory tests used for inflammation parameters included: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), haemoglobin concentration, and faecal calprotectin. Written, informed consent was obtained from the participants' parents, and assent was obtained for all patients older than 10 years of age. The study was approved by the Institutional Review Board of the University of Naples "Federico II".

2.3. Genotype analysis

Genomic DNA was isolated from 10 ml of peripheral venous blood anticoagulated with EDTA and extracted using the modified salting out technique [18]. DNA samples of the patients were analyzed for the following gene variants: *NOD2/CARD15* rs2066844 and rs2066845 (gene accession number: NC_000016.9, region: 50721584.50772911), *IRGM1* rs13361189 and rs4958847 (gene accession number: NC_000005, region: 150225762.150228552), and *ATG16L1* rs2241880 (gene accession number: NC_000002.11, region: 234153601.234210934). The PCR allelic discrimination assay was performed using a predesigned TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) performed in 20-μL reactions consisting of 5 ng of genomic DNA and including primer, probes, and Universal PCR Master Mix. Cycling conditions were: 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. The implemented software (Eppendorf) automatically called and plotted genotypes based on a two-parameter plot using fluorescence intensities of carboxyfluorescein (FAM) and 2-chloro-7-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Indeterminate calls were excluded from the analysis. A combination of PCR, SSCP, and direct sequencing of amplified fragments was used to analyze NOD2/CARD15 rs2066847. A fragment of

Table 2
Genotype frequencies based on Hardy–Weinberg equilibrium.

Polymorphism	Wild-type	Heterozygous	Risk	χ^2 test	*p*
	AA	AG	GG		
ATG16L1rs2241880	8 (10%)	46 (57.5%)	26 (32.5%)	3.5	0.06
	TT	TC	CC		
IRGM rs13361189	45 (56.3%)	22 (27.5%)	13 (16.3%)	9.5	0.002
	GG	GC	CC		
IRGM rs4958847	50 (62.5%)	27 (33.8%)	3 (3.8%)	0.07	0.7
	CC	CG			
NOD2/CARD15 rs2066844	70 (87.5%)	10 (12.5%)		0.5	0.35
	GG	GC	CC		
NOD2/CARD15 rs2066845	67 (32.5%)	10 (47.5%)	3 (10%)	7.4	0.006
	CC	CG			
NOD2/CARD15 rs2066847	58 (78.4%)	16 (21.6%)		0.08	0.2

202 bp in molecular weight was amplified using the following primer pairs: NOD2-rs2066847-FPGGACAGGTGGGCTTCAGTAG; NOD2-rs2066847RPGCCTTACCAGACTTCCAGGAT. The oligonucleotides used were designed with the primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR-SSCP analysis was performed as previously described [19]. Sequencing analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of the NOD mRNA sequence (GenBank accession number: NM_022162.1).

2.4. Statistical analysis

Statistical analysis was performed using the SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). The patients' allele frequencies were tested for Hardy-Weinberg equilibrium (HWE) by comparing expected and observed genotypes with the Fisher's exact test. All markers showed no statistically significant deviation from the HWE, except for *IRGM1* rs13361189 ($p = 0.002$) and *NOD2/CARD15* rs2066845 ($p = 0.006$) (Table 2). The Student's *t*-test and the Mann–Whitney test were used for comparison of continuous variables, and the χ^2 and Fisher's exact tests were used for categorical variables, as appropriate. In order to explore the weight of clinical and laboratory factors, discriminating homozygous patients from wild-type and heterozygous patients, a stepwise discriminant analysis was performed. Multivariate conditional logistic regression analysis was used to explore the odds associated with each polymorphism status. *ATG16L1*, *IRGM1*, and *NOD2/CARD15* were used as dependent variables, while the effect of all the above mentioned parameters for phenotype/expression was analyzed by a stepwise procedure.

3. Results

3.1. Clinical features

A total of 80 consecutive CD patients were included in the study [males: 53 (66.2%); median age: 11 years, range 0.7–17.9 years]. Clinical features of the study population are summarized in Table 1. Eighteen out of 80 patients (22.5%) underwent induction treatment with steroids at diagnosis, while 58 (72.5%) received nutritional therapy, and 4 (5%) had both steroid and nutritional therapy. Thirty-one out of 80 patients (38.7%) relapsed during the first year of disease. The median time of relapse was 6 months (range, 15 days to 12 months). At diagnosis, 68 out of 80 patients (85%) showed inflammatory disease behaviour, while 8 presented with a stricturing phenotype, and 4 with a fistulising pattern. During the course of disease, 12 out of 68 patients (17.6%) with an initial inflammatory disease changed their clinical phenotype: 8 (66.6%) switched to a stricturing CD, while 4 (33.4%) developed a penetrating phenotype. The median period in which patients switched their phenotype was 29 months (range 9–132 months). Twenty-nine patients (36.2%) needed to start immunosuppressant therapy for relapse of disease during the first year of follow-up. Sixteen patients (57%) were treated with azathioprine (AZT), 4 (14%) with methotrexate (MTX), and 8 (28%) firstly with AZT and then, due to lack of response, with MTX. Ten out of 80 patients (12.5%) needed to start biological therapy with infliximab for refractoriness to conventional therapy, and in 3 of them (30%) infliximab therapy was later replaced with adalimumab due to a lack of efficacy. In 10 out of 80 patients (12.5%) surgical intervention was needed for complications of disease. Fifteen out of 80 patients (18.7%) presented extraintestinal manifestations, 8 (53.3%) were affected by arthropathy, 3 (20%) by erythema nodosum, and 2 (13.3%) by persistent fever. Moreover, 1 of these 15 patients (6.6%) suffered from pancreatitis, while another presented with vaginal ulcers.

3.2. Genotype/phenotype correlation

3.2.1. ATG16L1

The evaluation of the *ATG16L1* polymorphism, rs2241880, was performed in 80 CD patients. The overall frequency of the G allele was 61%. Homozygosity for the risk allele G was found in 26 out of 80 patients (32.5%), while the number of subjects carrying the wild-type and the heterozygous alleles was 8 (10%) and 46 (57.5%), respectively. The *ATG16L1* genotype/phenotype correlations are summarized in Table 3. The presence of the rs2241880 risk polymorphism was significantly associated with the development of stricturing behaviour. Six out of the 8 patients (75%) who developed stricturing behaviour were homozygous for the *ATG16L1* risk allele ($p = 0.01$). This mutation was more greatly associated with the occurrence of relapses during the first year of disease and with the use of immunosuppressant therapies than were the wild-type and heterozygous variants ($p = 0.006$ and $p = 0.04$, respectively). Moreover, homozygosity for the risk allele was significantly associated with a higher value of CRP and faecal calprotectin at diagnosis ($p = 0.05$, $p = 0.007$, respectively). Conversely, homozygous risk allele carriers showed a trend towards a lower risk of developing perianal disease than heterozygous and wild-type patients ($p = 0.06$ for both). Homozygosity for the risk allele occurred more frequently in males than in females, with a trend towards statistical significance ($p = 0.07$). Furthermore, the rs2241880 risk polymorphism of *ATGL16L1* was associated with earlier age at diagnosis, specific clinical behaviour, change in fistulising phenotype, family history of IBD, disease localization, PCDAI and ESR scores at diagnosis, surgery, and the start of biological therapy (Table 3).

3.2.2. IRGM1

All enrolled patients were genotyped for the *IRGM1* risk alleles rs13361189 and rs4958847. Thirteen out of 80 patients (16.3%) were homozygous for the rs13361189 risk allele of *IRGM1*, while 22 (15%) were heterozygous, and 45 (68.7%) carried only the wild-type

Table 3
Characteristics of patients with Crohn's disease according to ATG16L1 s2241880 gene status.

Variables (n, %)	Risk	Het + WT	p
Sex			0.07[c]
Male	21/26 (80.8)	32/54 (59.3)	
Median age at diagnosis (years, range)	11 (2.7–17.9)	11 (0.7–16.8)	0.4[a]
Family history	1/26 (4)	1/54 (2)	1[b]
Disease behaviour at diagnosis			0.7[b]
Inflammatory (B1)	23/26 (88.4)	45/54 (83.3)	
Stricturing (B2)	3/26 (11.5)	5/54 (9.3)	
Fistulizing (B3)	0/26 (0)	4/54 (7.4)	
Development of stricturing phenotype	6/26 (23.1)	2/54 (3.7)	0.01[b]
Development of fistulising phenotype	2/26 (7.7)	2/54 (3.7)	0.5[b]
Localization of disease at diagnosis			0.6[b]
Ileum only (L1)	7/26 (26.9)	10/54 (18.5)	
Colon only (L2)	7/26 (26.9)	20/54 (37)	
Ileum and colon (L3)	12/26 (46.2)	23/54 (42.6)	
Upper gastrointestinal tract (L4a)	0 (0)	1/54 (1.9)	
Perianal disease	3/26 (11.5)	17/54 (31.5)	0.06[b]
Extraintestinal manifestations	5/26 (19.2)	10/54 (18.5)	1[b]
Relapse during the first year of disease	16/26 (61.5)	15/54 (27.8)	0.006[b]
Immunosuppressants	13/26 (50)	13/54 (24.5)	0.04[b]
Biologic therapy	4/26 (15.4)	5/54 (9.3)	0.4[b]
Surgery	3/26 (11.5)	7/54 (13)	1[b]
PCDAI score (median, range)	28.7 (10–48)	27.7 (5–60)	0.6[a]
CRP (median, range)	9.7 (0–174)	4.8 (0–165)	0.05[a]
ESR (median, range)	33 (2–90)	25 (2–102)	0.1[a]
Calprotectin (median, range)	500 (150–1470)	375 (15–790)	0.007[a]

Het: heterozygous; WT: wild type; PCDAI: paediatric Crohn disease activity Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.
[a] Mann–Whitney test.
[b] Fisher's test.
[c] χ^2 test.

Table 4
Characteristics of Crohn's disease patients according to NOD2/CARD15 1007 fs gene status.

Variables (n, %)	Risk	WT	p
Sex			0.7[c]
Male	12/16 (75)	39/58 (67.2)	
Median age at diagnosis (years, range)	10.3 (6.4–15.7)	11.2 (2–17.9)	0.2[a]
Family history	0/16 (0)	2/58 (3.4)	1[b]
Disease behaviour at diagnosis			0.4[b]
Inflammatory (B1)	15/16 (93.8)	47/58 (81)	
Stricturing (B2)	1/16 (6.2)	7/58 (12.5)	
Fistulizing (B3)	0/16 (0)	4/58 (6.9)	
Development of stricturing phenotype	5/16 (8.6)	3/58 (18.8)	0.1[b]
Development of fistulising phenotype	4/16 (6.9)	0/58 (0)	0.5[b]
Localization of disease at diagnosis			0.008 [b]
Ileum only (L1)	8/16 (50)	8/58 (13.8)	
Colon only (L2)	1/16 (6.2)	22/58 (37.9)	
Ileum and colon (L3)	7/16 (43.8)	27/58 (46.6)	
Upper gastrointestinal tract (L4a)	0/16 (0)	1/58 (1.7)	
Perianal disease	4/16 (25)	16/58 (27.6)	1[a]
Extraintestinal manifestations	1/16 (6.2)	13/58 (22.4)	0.2[a]
Relapse during the first year of disease	5/16 (31.2)	25/58 (43.1)	0.5[b]
Immunosuppressants	5/16(31.2)	21/58 (36.2)	0.7[b]
Biologic therapy	2/16 (12.5)	7/58 (12.1)	1[b]
Surgery	2/16 (12.5)	8/58 (13.8)	1[b]
PCDAI score (median, range)	25 (18–50)	27.5 (5–60)	0.6[a]
CRP (median, range)	1.8 (0–61)	5.8 (0–174)	0.2[a]
ESR (median, range)	30 (2–84)	25 (3–102)	0.9[a]
Calprotectin (median, range)	418 (15–500)	450 (60–1470)	0.5[a]

WT: wild type; PCDAI: paediatric Crohn disease activity index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.
[a] Mann–Whitney test.
[b] Fisher's test.
[c] χ^2 test.

allele. The overall frequency of the risk allele was 30%. Homozygosity for the risk allele was significantly associated with less frequent relapse during the first year of the disease and with a lower use of immunosuppressant therapy ($p = 0.001$ and $p = 0.05$, respectively). Among the 80 patients, 3 (3.8%) were homozygous for the *IRGM1* rs4958847 risk polymorphism, 27 (33.8%) were heterozygous, and 50 (62.5%) carried only the wild-type allele. *IRGM1* rs4958847 was not associated with any of the clinical features analyzed.

3.2.3. NOD2/CARD15

The evaluation of the *NOD2/CARD15* polymorphism, rs2066847, was possible in 74 of the CD patients. The overall frequency of the G allele was 10.8%. Fifty-eight (78.4%) out of 74 patients carried only the wild-type allele, while 16 (21.6%) were found to be heterozygous for the rs2066847 polymorphism. The heterozygous variant was significantly associated with an ileal localization of the disease ($p = 0.01$, Table 4). Allelic variants of rs2066844 and rs2066845 were not significantly associated with any of the clinical features analyzed.

3.3. Discriminant analysis

In order to explore the weight of the clinical and laboratory parameters discriminating homozygous patients from wild-type and heterozygous patients, we attempted a discriminant analysis to progressively select the factors that can best distinguish patients, having considered all the other factors in a multivariate fashion. Wilks-Lambda is an estimate of the discriminant capacity of each factor, while the variance rate, *F*, assigns a weight to each discriminant factor. Once the discriminant function is developed, a discriminant score is assigned to each subject, regardless of his phenotype. The classification analysis shows the efficiency of the discrimination based on the selected variables. Development of stricturing behaviour, relapse during the first year of life, faecal calprotectin, and male gender correctly classified 77.5% of the ATG16L1 rs2241880 allele-carrying patients. In order to validate the analysis, a jack-knifing procedure was applied (Table 5). Considering the few variables involved in discriminating *NOD2/CARD15* rs2066847 and *IRGM1* rs13361189 and rs4958847 it was not feasible to outline a discriminant function.

3.4. Multivariate logistic regression analysis

In a multivariate logistic regression model, the *ATG16L1* rs2241880, *NOD2/CARD15* rs2066847, and *IRGM1* rs13361189 polymorphisms were used as dependent variables. The variables

Table 5
Discriminant analysis – Wilk's Lambda for ATG16L1 polymorphism.

Variables	WL	F	p
ATG16L1			
Development of stricturing phenotype	0.87	12.5	0.001
Faecal calprotectin	0.76	11.6	0.0001
Relapse during the first year of disease	0.71	10.3	0.0001
Gender	0.6	9.4	0.0001

WL: Wilk's-Lambda; *F*: variance rate *F*.

Table 6
Multivariate logistic regression analysis.

	OR	CI 95%	*p*
ATG16L1			
Development of stricturing behaviour	18.4	2.5–135	0.001
Relapse during the 1st year of disease	1.2	1–1.4	0.002
Perianal disease	0.2	0.05–1	0.05
NOD2/CARD15 1007fs			
Ileal disease	5.4	1.6–18	0.01

OR: odds ratio; CI: confidence interval.

that were still significantly associated with the presence of the *ATG16L1* risk allele in homozygosity were the development of stricturing behaviour (OR = 18.4; $p = 0.004$) and the relapse during the first year of disease (OR = 1.2; $p = 0.002$) (Table 6). The presence of perianal disease was independently associated with homozygosity of the *ATG16L1* risk allele as a protective factor (OR 0.2; $p = 0.05$) (Table 6). The sole variable found to be associated with heterozygosity of the *NOD2/CARD15* allele was the presence of ileal disease (OR = 5.4; $p = 0.001$) (Table 6). None of the variables were independently correlated with the presence of the *IRGM1* rs13361189 and rs4958847 risk alleles in homozygosity.

4. Discussion

To our knowledge, this is the first paediatric study reporting an association between the presence of the rs2241880 risk polymorphism of *ATG16L1* in children with CD and a more severe phenotype of the disease. It was not the aim of our paper to reassess the role of ATG16L1 in susceptibility to paediatric Crohn's disease, since it has already been widely described in paediatric literature [20–23]. Nonetheless, in our cohort of paediatric CD patients, the frequency of the G allele (61%) was comparable with that reported by the single Italian multicentre study published by Latiano and colleagues (59%) [20]. Instead, we here tried to look for possible phenotypic associations, and we clearly demonstrated that homozygous risk allele carriers show a significant trend towards changing from inflammatory to stricturing behaviour, suggesting that the ATG16L1 risk allele may be somehow linked with the early development of fibrosis. This finding is in agreement with those of Fowler and colleagues [24]. These authors genotyped a large population of Australian adults with CD and found that the GG variant was independently associated with some more complicated disease courses, such as the development of stricturing disease and intra-abdominal penetrating disease. Consistent with Fowler's data [24], we here demonstrated an association between the rs2241880 risk polymorphism and the absence of perianal disease, suggesting that the *ATG16L1* risk allele may exert a protective effect at least on this manifestation in CD children. To support the hypothesis of a more severe phenotype in homozygous patients, we found that the rs2241880 risk allele was related to a higher incidence of clinical relapses and also to the introduction of immunosuppressants. In addition, children carrying the rs2241880 risk allele showed higher values of faecal calprotectin and CRP at diagnosis compared to heterozygous and wild-type *ATG16L1* patients. However, the role of faecal and serologic markers in predicting IBD disease course is still controversial, and the hypothesis that higher values of these markers at diagnosis could predict a more severe course of disease has been claimed [25]. Our data were strengthened by both a discriminant function and a multivariate logistic regression analysis. Indeed, our multivariate logistic regression analysis demonstrated that development of stricturing behaviour, followed by relapse during the first year of disease and absence of perianal disease, were the most significant variables associated with the ATG16L1 risk allele. In accordance with these genotype-/phenotype correlations, our data from functional analyses show that the monocyte-derived dendritic cells (DC) from paediatric patients with CD carrying the *ATG16L1* risk polymorphism are antigen-sampling and processing impaired. We found a marked reduction of bacteria particle localization in DC from CD children with the rs2241880 risk variant of ATG16L1 compared to DC of children with the wild-type variant. Furthermore, we found that DC from the risk group almost completely failed to upregulate HLA-DR and CD86, the 2 key molecules for the activation of the T-cell-mediated immune response [26]. Since intestinal microorganisms have been suggested to be one of the causes responsible for bowel inflammation [27], it is likely that an alteration of the autophagy process might lead to uncontrolled microorganism growth in the intestine of IBD patients [26].

In agreement with Lauriola et al., we found that the *ATG16L1* risk allele is not associated with a positive family history or with the presence of extraintestinal manifestations [28]. The association between the rs2241880 risk polymorphism of *ATG16L1* and CD localization is controversial. The rs2241880 variant was described as being associated with an ileal CD phenotype in children [11,22,29], while, according to Lakatos et al., ATG16L1 homozygosity for rs2241880 was associated with colon disease only [30]. In our study population we did not find any specific disease localization according to the stratification of CD with the Paris classification.

The *IRGM1* gene has been recently identified as playing a role in the development of CD and has also been shown to be involved in autophagy. In contrast to the extensive amount of work that has been accomplished in identifying and characterizing the complex network of *ATG* molecular mechanisms, little is known about the role of *IRGM1*-related autophagic pathways [31]. No Italian study has ever assessed the IRGM1 frequency in a CD population. We found that the presence of the *IRGM1* rs13361189 variant allele was associated with a lower use of immunosuppressant therapy, highlighting a possible role in the development of a milder phenotype. However, none of the associations were confirmed by the multivariate logistic regression analysis; thus, further studies are needed to provide definitive conclusions regarding *IRGM1* rs13361189.

Several studies supported a significant association between ileal disease and the carriage of one or more *NOD2/CARD15* variant alleles [32–34]. In our cohort of paediatric CD patients, the frequency of the G allele for NOD2/CARD15 rs2066847 (10.6%) was comparable to that reported by an Italian multicentre study by Annese and colleagues (9.3%) [35]. According to the current evidence, our analyses of genotype/phenotype correlation showed that patients heterozygous for the *NOD2/CARD15* rs2066847 (1007 fs) allele had more frequent ileal involvement than children showing the wild-type variant for the same SNP. In contrast with previous studies [36,37], we did not find any significant relationship between the three *NOD2/CARD15* risk genes studied and parameters such as a specific pattern of CD, positive family history, extraintestinal manifestations, perianal disease, or surgery. Our study has some limitations, including the small cohort of patients; despite this, the associations were confirmed by powerful statistical tests as to avoid potential bias.

In conclusion, we believe that genetic susceptibility may have a very important role in the aetiology of paediatric-onset IBD. Certainly, IBD has a multifactorial origin, and environmental factors are crucial in the development of the disease. Nevertheless, if genetic susceptibility is greater, paediatric IBD patients can be expected to have a more severe clinical course of the disease. Within paediatric-onset CD, specific genotype/phenotype associations can be found. In our paediatric cohort, homozygosity for the ATG16L risk allele was associated with a more aggressive disease course, including development of stricturing behaviour, early relapse, and premature use of immunosuppressants. As previously demonstrated, heterozygosity for the NOD2/CARD15 was significantly correlated with major ileal disease. These data highlight the

importance of genetic susceptibility research in larger paediatric-onset IBD cohorts in order to find new genes and allow an early stratification for the treatment of these patients.

Conflict of interest
None declared.

References

[1] Trinh TT, Rioux JD. Understanding association and causality in the genetic studies of inflammatory bowel disease. Gastroenterology 2005;129:2106–10.
[2] Mathew CG. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. Nature Reviews Genetics 2008;9:9–14.
[3] Hugot JP, Laurent-Puig P, Gower-Rousseau C, et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 1996;379:821–3.
[4] Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603–6.
[5] Economou M, Trikalinos TA, Loizou KT, et al. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a meta-analysis. American Journal of Gastroenterology 2004;99:2393–404.
[6] Van Limbergen J, Russell RK, Nimmo ER, et al. The genetics inflammatory bowel disease. American Journal of Gastroenterology 2007;102:2820–31.
[7] Klionsky DJ. Crohn's disease, autophagy, and the Paneth cell. New England Journal of Medicine 2009;360:1785–6.
[8] Hampe J, Franke A, Rosenstiel P, et al. A genome wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nature Genetics 2007;39:207–11.
[9] Weiss B, Shamir R, Bujanover Y, et al. NOD2/CARD15 mutation analysis and genotype–phenotype correlation in Jewish pediatric patients compared with adults with Crohn's disease. Journal of Pediatrics 2004;145:208–12.
[10] De Ridder L, Weersma RK, Dijkstra G, et al. Genetic susceptibility has a more important role in pediatric-onset Crohn's disease than in adult-onset Crohn's disease. Inflammatory Bowel Diseases 2007;13:1083–92.
[11] Prescott NJ, Fisher SA, Franke A, et al. A nonsynonymus SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5. Gastroenterology 2007;132:1665–71.
[12] Wagner J, Sim WH, Ellis JA, et al. Interaction of Crohn's disease susceptibility genes in an Australian paediatric cohort. PLoS ONE 2010;5:e15376, http://dx.doi.org/10.1371/journal.pone.0015376.
[13] IBD Working Group of the European Society for Paediatric Gastroenterology. Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis – the Porto criteria. Journal of Pediatric Gastroenterology and Nutrition 2005;41:1–7.
[14] Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. Inflammatory Bowel Diseases 2011;17:1314–21.
[15] Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a pediatric Crohn's disease activity index. Journal of Pediatric Gastroenterology and Nutrition 1991;12:439–47.
[16] Hyams J, Crandall W, Kugathasan S, et al. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. Gastroenterology 2007;132:863–73.
[17] Biancone L, Michetti P, Travis S, et al. European evidence-based consensus on the management of ulcerative colitis: special situations. Journal of Crohn's and Colitis 2008;2:63–92.
[18] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 1988;16:1215.
[19] De Rosa M, Scarano MI, Panarello L, et al. The mutation spectrum of the APC gene in FAP patients from southern Italy: detection of known and four novel mutation. Human Mutation 2003;21:655–6.
[20] Latiano A, Palmieri O, Valvano MR, et al. Replication of interleukin 23 receptor and autophagy-related 16-like 1 association in adult- and pediatric-onset inflammatory bowel disease in Italy. World Journal of Gastroenterology 2008;14:4643–51.
[21] Van Limbergen J, Russell RK, Nimmo ER, et al. Autophagy gene ATG16L1 influences susceptibility and disease location but not childhood-onset in Crohn's disease in Northern Europe. Inflammatory Bowel Diseases 2008;14:338–46.
[22] Gazouli M, Pachoula I, Panayotou I, et al. NOD2/CARD15, ATG16L1 and IL23R gene polymorphisms and childhood-onset of Crohn's disease. World Journal of Gastroenterology 2010;16:1753–8.
[23] Lacher M, Schroepf S, Ballauf A, et al. Autophagy ATG16L1 rs2241880 G allele is associated with Crohn's disease in German children. Acta Paediatrica 2009;98:1835–40.
[24] Fowler EV, Doecke J, Simms LA, et al. ATG16L1 T300A shows strong associations with disease subgroups in a large Australian IBD population: further support for significant disease heterogeneity Elizabeth V. American Journal of Gastroenterology 2008;103:2519–26.
[25] Lewis JD. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. Gastroenterology 2011;140:1817–26.
[26] Strisciuglio C, Miele E, Wildenberg ME, et al. T300A variant of autophagy ATG16L1 gene is associated with decreased antigen sampling and processing by dendritic cells in pediatric Crohn's disease. Inflammatory Bowel Diseases 2013;19:2339–48.
[27] Blumberg R, Cho J, Lewis J, et al. Inflammatory bowel disease: an update on the fundamental biology and clinical management. Gastroenterology 2011;140:1701–3.
[28] Areola M, Ugolini G, Rivetti S, et al. IL13, NOD2/CARD15, ATG16L1 and PHOX2B polymorphisms in a group of patients with Crohn's disease and correlation with sub-phenotypes. International Journal of Molecular Medicine 2011;27:469–77.
[29] Amre DK, Mack DR, Morgan K, et al. Autophagy gene ATG16L1 but no IRGM is associated with Crohn's disease in Canadian children. Inflammatory Bowel Diseases 2009;15:501–7.
[30] Lakatos PL, Szamosi T, Szilvasi A, et al. ATG16L1 and IL23 receptor (IL23R) genes are associated with disease susceptibility in Hungarian CD patients. Digestive and Liver Disease 2008;40:867–73.
[31] Xavier RJ, Huett A, Rioux JD. Autophagy as an important process in gut homeostasis and Crohn's disease pathogenesis. Gut 2008;57:717–20.
[32] Bonen DK, Cho JH. The genetics of inflammatory bowel disease. Gastroenterology 2003;124:521–36.
[33] Lesage S, Zouhali H, Cezard JP, et al. CARD15/NOD2 mutational analysis and genotype–phenotype correlation in 612 patients with inflammatory bowel disease. American Journal of Human Genetics 2002;70:845–57.
[34] Ahmad T, Armuzzi A, Bunce M, et al. The molecular classification of the clinical manifestations of Crohn's disease. Gastroenterology 2002;122:854–66.
[35] Annese V, Lombardi G, Perri F, et al. Variants of CARD15 are associated with an aggressive clinical course of Crohn's disease – an IG-IBD study. American Journal of Gastroenterology 2005;100:84–92.
[36] Russell RK, Drummond HE, Nimmo EE, et al. Genotype–phenotype analysis in childhood-onset Crohn's disease: NOD2/CARD15 variants consistently predict phenotypic characteristics of severe disease. Inflammatory Bowel Diseases 2005;11:955–64.
[37] Helio T, Halme L, Lappalainen M, et al. CARD15/NOD2 gene variants are associated with familially occurring and complicated forms of Crohn's disease. Gut 2003;52:558–62.

2.2 Serum Hepcidin and Iron Absorption in Paediatric Inflammatory Bowel Disease

Anaemia is the most frequent extra-intestinal manifestation of in ammatory bowel disease [IBD], with a great impact on the patient's quality of life.[37-38]

Hepcidin, a 25 amino-acid peptide mainly secreted by hepatocytes, controls the amount of iron entering the blood circulation by binding and downregulating ferroportin, a plasma membrane transporter that pumps iron out of phagocytes and duodenal enterocytes. The hepcidin expression is regulated transcriptionally in response to changing serum iron levels. Elevated serum iron promotes hepcidin expression, leading to downregulation of ferroportin and decreased entry of iron into the circulation.

The role of hepcidin in the mechanisms of anaemia in paediatric IBD is limited and shows con icting results. Increased urine[39]or serum [40] hepcidin has been reported in two studies, correlating with the rise of IL-6 levels, ferritin, and disease activity. Conflicting results may suggest that hepcidin in human IBD is likely to be in uenced by various factors such as age, type of disease, and disease activity. The primary aim of this study was to correlate hepcidin serum levels in patients affected by paediatric IBD with disease activity, in ammatory markers, and iron absorption. The secondary aims were to compare serum hepcidin levels of IBD patients with a group of coeliac and healthy patients, and to establish which iron parameter better correlates with hepcidin.

To the best of our knowledge, this is the paediatric study evaluating iron absorption and serum hepcidin levels in IBD paediatric patients. Our data show that IBD children with active disease tend to have impaired iron absorption, driven through the hepcidin pathway. Indeed, serum hepcidin levels were signicantly higher in IBD patients with moderate to severe activity as compared with all other groups, including patients with mild activity or in remission, coeliac patients, and healthy controls. In addition, a signicant inverse correlation was found between hepcidin levels and iron absorption.

Despite its relative high prevalence, costs, and impact on patient quality of life, it is rarely considered and adequately treated.[38,41,42] In our study population, the prevalence of anaemia among IBD patients was 34%. The development of ACD is caused by numerous factors, among which hepcidin is now considered the leading actor. Indeed, the decrease in ferroportin expression, which results from elevated hepcidin levels, would block entry of iron into the circulation, with consequent erythropoiesis impairment. Furthermore, since intestinal absorption of iron is inhibited by downregulation of ferroportin on enterocytes, the anaemia would be resistant to oral iron supplementation.[43-44] Therefore, serum hepcidin may represent an useful surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as rst-line option. Indeed, serum hepcidin may serve as a useful, sensitive, surrogate marker to distinguish

patients with impaired iron absorption in whom intravenous iron could be used as first line option, avoiding the waste of time with a futile and possibly harmful cycle of oral iron. Further studies are needed to better elucidate the role of hepcidin in both iron metabolism and in ammation in IBD, in order to design new therapeutic strategies for ACD in paediatric IBD.

Though the etiopathogenesis of IBD is still unknown, it has been suggested that the interaction of environmental factors and host immune response with genetic individual susceptibility play an important role.[37] Both human and animal studies have demonstrated the central role of intestinal microbiota in flaring up and perpetuating inflammation in IBD patients.[37]

IBD are associated with significant shifts in the composition of the normal enteric microbiota compared to control subjects, with an almost depletion of Lactobacillus and Bifidobacterium [45], normally present in healthy intestinal conditions, and increase of harmful species, such as Proteobacteria and Actinobacteria.[46] This kind of dysbiosis might determine in the host organism the loss of tolerance towards microbes and induce an inappropriate inflammation.

Journal of Crohn's and Colitis Advance Access published January 18, 2016

Journal of Crohn's and Colitis, 2016, 1–9
doi:10.1093/ecco-jcc/jjv242
Original Article

Original Article

Serum Hepcidin and Iron Absorption in Paediatric Inflammatory Bowel Disease

Massimo Martinelli,[a,†] Caterina Strisciuglio,[b,†] Annalisa Alessandrella,[a] Francesca Rossi,[b] Renata Auricchio,[a] Natascia Campostrini,[c] Domenico Girelli,[c] Bruno Nobili,[b] Annamaria Staiano,[a] Silverio Perrotta,[b] Erasmo Miele[a]

[a]Department of Translational Medical Science, Section of Paediatrics, University of Naples Federico II, Naples, Italy [b]Department of Woman, Child and General and Specialized Surgery, 2nd University of Naples, Naples, Italy; [c]Department of Medicine, Section of Internal Medicine, University of Verona, Verona, Italy
[†]Drs Martinelli and Strisciuglio participated equally in this study.

Corresponding author: Annamaria Staiano, MD, Department of Translational Medical Science, Section of Paediatrics, University of Naples Federico II, Via S. Pansini, 5, 80131 Naples, Italy. Tel.: +39.081.7462679; fax: +39.081.5469811; email: staiano@unina.it

Abstract

Background and Aims: We sought to correlate hepcidin levels in inflammatory bowel disease [IBD] children with disease activity, inflammatory markers, and iron load test [ILT] and to compare IBD patients with coeliac and healthy patients.
Methods: Between December 2012 and June 2013, 145 subjects [50 IBD patients, 45 coeliac patients and 50 healthy controls] were included in the study. All patients underwent the following examinations: blood count, iron status, erythropoiesis parameters, serum hepcidin, C-reactive protein [CRP], and erythrocyte sedimentation rate [ESR]. In order to evaluate the efficacy of iron absorption, ILT was performed in IBD patients. Disease activity indexes and IBD duration, localisation, and therapy were also evaluated, and a faecal sample for calprotectin collected.
Results: Serum hepcidin was significantly higher in IBD patients with active disease compared with both coeliac and healthy patients [$p = 0.005$, $p = 0.003$ respectively]. In a multivariate logistic regression model, having a Paediatric Crohn's Disease Activity Index [PCDAI] / Paediatric Ulcerative Colitis Activity Index [PUCAI] ≥ 30 resulted in the only variable independently associated with a positive serum hepcidin (odds ratio [OR] = 6.87; 95% confidence interval [CI] 1.4–33, $p = 0.01$]]. Patients with iron malabsorption [IM] showed higher values of ESR, CRP, and hepcidin [$p = 0.02$, $p = 0.001$, and $p = 0.06$, respectively]. Eight out of 12 [66.7%] children with IM showed an active disease compared with 6/31 [19.3%] children with normal ILT [$p = 0.01$]. Hepcidin levels correlated negatively with ILT [$r = -0.451$, $p = 0.002$], and positively with ferritin and CRP [$r = 0.442$, $p = 0.0001$; $r = 0.243$, $p = 0.009$, respectively]
Conclusions: Our study demonstrates that serum hepcidin is increased in IBD children with active disease and it is responsible for IM.

Keywords: Hepcidin; IBD; iron absorption

Copyright © 2016 European Crohn's and Colitis Organisation (ECCO). Published by Oxford University Press. All rights reserved.
For permissions, please email: journals.permissions@oup.com

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

1. Introduction

Anaemia is the most frequent extra-intestinal manifestation of inflammatory bowel disease [IBD], with a great impact on the patient's quality of life.[1,2] The prevalence of anaemia in IBD varies between 15% and 75%, depending on the definition and the subgroup of examined patients.[3,4] The main types of anaemia in IBD are iron deficiency anaemia [IDA], anaemia of inflammatory aetiology, anaemia of chronic disease [ACD], and combined IDA + ACD.[4,5] IDA is mainly the result of the chronic blood loss from the gastrointestinal [GI] tract, due to prolonged inflammation of the small and large intestine epithelium. On the other hand inflammation, through an inflammatory cytokines-mediated mechanism, leads to a decreased iron level in the circulation and thus to a limited availability of iron for erythroid cells.[6] Hepcidin, a 25 amino-acid peptide mainly secreted by hepatocytes, controls the amount of iron entering the blood circulation by binding and downregulating ferroportin, a plasma membrane transporter that pumps iron out of phagocytes and duodenal enterocytes. The hepcidin expression is regulated transcriptionally in response to changing serum iron levels. Elevated serum iron promotes hepcidin expression, leading to downregulation of ferroportin and decreased entry of iron into the circulation. Conversely, low serum iron leads to reduced hepcidin expression, elevated ferroportin, and increased movement of iron into the circulation.[7,8] In addition to iron status, inflammatory cytokines can also influence transcription of the hepcidin gene. Interleukin-6 [IL-6] has been shown to increase hepcidin expression in vitro and in vivo, and IL-6 induced hepcidin upregulation has been proposed to play an important role in the pathogenesis of ACD.[9,10] The role of hepcidin in the mechanisms of anaemia in paediatric IBD is limited and shows conflicting results. Increased urine[11] or serum[12] hepcidin has been reported in two studies, correlating with the rise of IL-6 levels, ferritin, and disease activity. Conversely, Arnold *et al.* found significantly decreased hepcidin levels in IBD patients compared with healthy controls.[13] Hepcidin precursor, pro-hepcidin, has also been evaluated in three different studies in IBD patients, with variable results.[12,14,13,15] Conflicting results may suggest that hepcidin in human IBD is likely to be influenced by various factors such as age, type of disease, and disease activity.

The primary aim of this study was to correlate hepcidin serum levels in patients affected by paediatric IBD with disease activity, inflammatory markers, and iron absorption. The secondary aims were to compare serum hepcidin levels of IBD patients with a group of coeliac and healthy patients, and to establish which iron parameter better correlates with hepcidin.

2. Materials and Methods

2.1. Study population

We conducted a comparative, cross-sectional, single-centre study in paediatric patients with a diagnosis of IBD. Children and adolescents aged from 2 to 18 years with a diagnosis of IBD were prospectively enrolled between December 2012 and June 2013 at the Department of Translational Medical Science, Section of Paediatrics, University of Naples 'Federico II', Italy. The diagnosis of IBD was established on the basis of clinical, endoscopic, radiological, and histological criteria according to the Porto criteria.[16] During the same study period, we also recruited a group of children who were referred to our centre with suspected coeliac disease due to pathological serum levels of anti-tissue transglutaminase [tTG] [> 7 U/ml] and/or positive anti-endomysium [EMA] antibodies. Only patients with a confirmed diagnosis of coeliac disease or potential coeliac disease [positive serology and normal duodenal architecture] were finally included in the study. In addition, we enrolled a group of healthy children referred to our primary care centre for routine well-child visits. Exclusion criteria from the study were: age ≤ 2 years or > 18 years; patients with suspected coeliac disease without confirmation of diagnosis; the presence of other comorbidities; patients having iron supplementation during the month preceding the enrolment; and inability or unwillingness to give informed consent. At the time of the enrolment, all patients underwent the following examinations: full blood count, reticulocytes, serum iron, ferritin, transferrin, soluble transferrin receptor [STfR], total iron-binding capacity [TIBC], transferrin saturation [Tsat], and inflammatory indexes (C-reactive protein [CRP], erythrocyte sedimentation rate [ESR]). In addition, blood samples from all enrolled patients were also obtained for hepcidin 25 isoform analysis. After being centrifuged, the serum of all patients was stored at -80°C in aliquots in order to avoid multiple-frozen thaw. Once collected, all samples were sent to the Section of Internal Medicine, Department of Medicine, University of Verona for the analysis. Hepcidin 25 isoform was measured through a validated mass spectrometry-based assay, as previously described.[17] Synthetic hepcidin 25 [Peptides International, Louisville, KY] was used for external calibration and a synthetic hepcidin analogue [Hepcidin 24, Peptides International] as an internal standard. The lower sensitivity limit of the assay was 0.55 nM. All samples were measured in duplicate.[18] For the IBD group, type of IBD, anatomical distribution of disease, symptoms, disease activity, and treatments, including surgery, were recorded. For the purpose of this manuscript, disease location was described according to the Paris classification.[19] Disease activity was scored by the Paediatric Crohn's Disease Activity Index [PCDAI] or the Paediatric Ulcerative Colitis Activity Index [PUCAI] for CD and UC, respectively.[20, 21] In addition, a stool sample for faecal calprotectin determination was also obtained. For the coeliac group, tTG, EMA, and duodenal histology [according to Marsh grading] were also evaluated.

2.2. Differential diagnosis of anaemia

Anaemia was defined on the basis of World Health Organization Criteria [22]: in boys aged > 15 years, as Hb < 13 g/dl; in non-pregnant girls aged > 15 years, as Hb < 12 g/dl; in children aged 5–11 years, as Hb < 11.5 g/dl; and in children aged < 5 years, as Hb < 11 g/dl. Iron deficiency was defined as a ferritin < 12 ng/ml in children aged < 5 years, or ferritin < 15 ng/ml in children aged > 5 years, when the corresponding CRP was < 1 mg/dl and Tsat < 20%.[22] In the presence of biochemical evidence of inflammation, the diagnostic criteria for ACD were a serum ferritin > 100 ng/ml and TfS < 20%, whereas if the serum ferritin level was ≤ 100 ng /ml, a combination of true iron deficiency and ACD was diagnosed.[23]

2.3. Iron absorption in IBD patients

In order to evaluate the efficacy of iron absorption, an iron load test [ILT] was performed in children affected by IBD. We used a previously described protocol.[24] After an overnight fast and baseline serum iron determination, ferrous sulphate [dosed as 1 mg/kg elemental iron with a 60-mg maximum] was administered orally as a liquid preparation, followed by determination of serum iron after 2 h. The change in iron levels between the baseline and the 2-h period (Δ[Fe]2hr) was calculated. Iron malabsorption [IM] was defined using the normative data, when the increase of serum iron after 2 h from the ILT was lower than the fifth percentile.[24]

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

2.4. Ethical approval

All parents or guardians signed a consent form indicating their awareness of the investigative nature of the study and possible risks. When appropriate, we also obtained children's assent. The study was approved by the Institutional Review Board of University of Naples Federico II.

2.5. Statistical analysis

Variables were screened for their distribution, and appropriate parametric or non-parametric tests were adopted as necessary. Student's t-test, the ANOVA test, and the Mann-Whitney test for continuous variables, and the chi-square and Fisher's exact tests for categorical variables, were used where appropriate. Multivariate conditional logistic regression analysis was used to explore the odds associated with a positive serum hepcidin and a pathological ILT. Serum hepcidin and ILT were used as dependent variables, and the effect of all the parameters was analysed by a stepwise procedure. Serum hepcidin was considered positive for values higher than the measurable cut-off [0.55 nM]. Correlations between serum hepcidin and Δ[Fe]2hr with continuous variables were evaluated through linear regression and expressed by Pearson's correlation coefficient. Variables not normally distributed [serum hepcidin and ferritin] were log-transformed before performing the correlations. Statistical significance was predetermined as $p < 0.05$. Percentages were rounded to the nearest whole numbers. SPSS version 15 was used for all statistical analyses. The sample size of 50 children in each group was estimated with a 90% power to detect a difference of at least 20% between the three groups, with an alpha of 0.05.

3. Results

Initially, 150 subjects were enrolled between December 2012 and June 2013, comprising 50 IBD patients (UC: 28; CD: 20; IBD-unclassified

Table 1. Demographic and clinical characteristics of enrolled patients.

Characteristics [n, %]	IBD patients	Coeliac patients	Healthy controls	*p*
	N = 50	*N* = 45	*N* = 50	
Age, years [range]	12.6 ± 3.5 [4–18]	8 ± 3.2 [2–14]	11.1 ± 4.2[3.2–18]	0.01
Gender [*n*, %]				
Male	27 [54]	20 [44.4]	25 [50]	0.6
Coeliac disease diagnosis [*n*, %]				NA
Coeliac disease	-	38 [84.4]	-	
Potential coeliac disease	-	7 [15.6]	-	
tTG	-	110.8 ± 87.6 [3.4–200]	-	
EMA positive	-	41 [91.1]	-	
Marsh grading [*n*, %]	-		-	
T1	-	3 [6.6]	-	
T2	-	0		
T3a	-	0		
T3b	-	12 [26.6]	-	
T3c	-	21 [46.6]	-	
IBD type [*n*, %]				NA
UC	28 [56]	-	-	
CD	20 [40]	-	-	
IBD-U	2 [4]	-	-	
IBD disease location [*n*, %]		-	-	NA
UC				
Proctosigmoiditis [E1]	9 [32]	-	-	
Left-sided colitis [E2]	2 [8]	-	-	
Extensive colitis [E3]	6 [21]	-	-	
Pancolitis [E4]	11 [39]	-	-	
CD		-	-	
Ileum only [L1]	1 [5]	-	-	
Colon only [L2]	5 [25]	-	-	
Ileum and colon [L3]	14 [70]	-	-	
Upper gastrointestinal tract [L4a]	2 [10]	-	-	
-	-			
IBD disease activity indexes				NA
PUCAI	13.8 ± 16.4 [0–47.5]	-	-	
PCDAI	11.8 ± 14.4 [0–60]	-	-	
IBD therapy [*n*, %]				NA
Steroids	10 [20]	-	-	
Enteral nutrition	3 [6]	-	-	
Azathioprine	11 [22]	-	-	
Methotrexate	1 [2]	-	-	
Biologicals	2 [4]	-	-	
Mesalazine	31 [62]	-	-	

All continuous variables values are expressed as means ± standard deviation [range].

CD, Crohn's disease; EMA, anti-endomysium; tTg, anti-tissue transglutaminase; IBD, inflammatory bowel disease; UC, ulcerative colitis; IBD-U, inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; NA, not applicable.

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

Table 2. Laboratory parameters of enrolled patients.

Parameters	IBD patients [*n* = 50]	Coeliac patients [*n* = 45]	Healthy controls [*n* = 50]	*p**
Hb, g/dl	12.2 ± 1.7 [8.3–14.9]	12.1 ± 1 [9.1–14.6]	12.8 ± 1 [11–16]	0.04
MCV, fl	78.6 ± 8.1 [60–94]	78.5 ± 5.3 [60.3–91]	82.5 ± 4.1 [74–92]	0.002
Serum iron, μg/dl	56.6 ± 32.5 [13–148]	60.4 ± 33.4 [19–176]	80.7 ± 26.9 [28–138]	0.001
Ferritin, ng/ml	45.8 ± 36.8 [6–217]	33.8 ± 30.5 [6–157]	56.8 ± 31 [10–179]	0.0 001
Transferrin, g/l	2.5 ± 0.6 [1.3–4.3]	2.7 ± 0.3 [2.1–3.4]	1.4 ± 0.3 8 [0.7–2.6]	0.02
STfR, mg/dl	1.9 ± 1.2 [0.8–8.5]	1.7 ± 0.4 [1.1–2.8]	1.4 ± 0.3 [0.4–2.6]	0.02
Tsat, %	16.1 ± 9.8 [3–41]	15 ± 8.6 [5–42]	21.4 ± 8.3 [6–44]	0.01
TIBC, μg/dl	315.5 ± 77.7 [139–460]	365.1 ± 72.8 [236–576]	350.8 ± 43.8 [268–450]	0.3
Reticulocytes, %	3.8 ± 3.6 [0.6–9]	4.0 ± 3.3 [1–4]	3.2 ± 3.4 [0.6–9]	0.01
Serum hepcidin, nM	4.3 ± 8.3 [0.55–49.2]	2.1 ± 3.1 [0.55–15.5]	2 ± 2.6 [0.55–11.3]	0.06
ESR, mm	12.2 ± 11.8 [1–46]	9.5 ± 6.1 [2–23]	6.2 ± 3.9 [1–17]	0.03
CRP, mg/dl	1.4 ± 3.1 [0.33–18.6]	0.35 ± 0 .04 [0.33–0.4]	0.36 ± 0.2 [0.33–2]	0.0 001

All values are expressed as means ± standard deviation [range].

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, haemoglobin; MCV. mean corpuscolate volume; STfR, soluble transferrin receptor; TIBC, total iron binding capacity; TSat, transferrin saturation.

* ANOVA test.

Table 3. Serum hepcidin levels in different groups of patients.

Groups [*n*]	Serum hepcidin*	*p*
IBD with PCDAI/PUCAI ≥ 30 [11]	9.4 ± 15.8 [0.55–49.2]	0.02
IBD with PCDAI/PUCAI < 30 [39]	2.8 ± 3.7 [0.55–19.2]	
IBD type		0.1
UC [28]	3.3 ± 6.2 [0.55–28.5]	
CD [20]	5.7 ± 10.9 [0.55–49.2]	
IBD-U [2]	1.8 ± 1.7 [0.55–3]	
IBD with PCDAI/PUCAI ≥ 30 [11]	9.4 ± 15.8 [0.55–49.2]	0.005
Coeliac patients [45]	2.1 ± 3.1 [0.55–15.5]	
IBD with PCDAI/PUCAI ≥ 30 [11]	9.4 ± 15.8 [0.55–49.2]	0.003
Healthy controls [50]	2.1 ± 2.6 [0.55–11.3]	
Coeliac patients [45]	2.1 ± 3.1 [0.55–15.5]	0.9
Healthy controls [50]	2.1 ± 2.6 [0.55–11.3]	
Coeliac disease group		0.3
Coeliac disease [38]	1.9 ± 3.0 [0.55–15.5]	
Potential coeliac disease [7]	3.1 ± 3.3 [0.55–8.3]	
Patients with anaemia		
IDA [8]	0.55 [0.55]	0.001
ACD [2]	25.1 ± 34 [1–49.2]	
ACD [2]	25.1 ± 34 [1–49.2]	0.001
IDA + ACD [12]	1.9 ± 4.3 [0.55–15]	
IDA [8]	0.55 [0.55]	0.06
IDA + ACD [12]	1.9 ± 4.3 [0.55–15]	

*Values are expressed as means ± standard deviation [range].

ACD, anaemia of chronic disease; CD, Crohn's disease; IDA, iron deficiency anaemia; IBD, inflammatory bowel disease; IBD-U: inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index; UC, ulcerative colitis.

[IBD-U]: 2; mean age ± standard deviation [SD:] 12.6 ± 3.5; range 4 to 18 years; M/F: 27/23), 50 children with a suspicion of coeliac disease, and 50 healthy controls [mean age ± SD: 11.1 ± 4.2 years; range 3.2 to 18 years; M/F: 25/25]. Among the coeliac disease group, 5 patients [10%] were subsequently excluded from the study for non-confirmed positive coeliac serology and therefore only 45 coeliac patients were included in the study [mean age ± SD: 8 ± 3.2 years; range 2 to 14 years; M/F: 20/25]. Of these 45 children, 38 [84.4%] were affected by coeliac disease and 7 [15.6%] by potential coeliac disease. The baseline and laboratory characteristics of all subjects included in the study are described in Table 1 and Table 2.

3.1. Prevalence of anaemia among different study groups

The prevalence of anaemia in IBD patients was significantly higher compared with both the coeliac group and the healthy controls (17/50 [34%] versus 5/45 [11.1%]; OR = 4.1; 95% CI 1.3 to 12.3, $p = 0.01$; 17/50 [34%] versus 0/50 [0%]; $p = 0.001$; OR 2.5; 95% CI 1.9 to 2.3, respectively]. In detail, 3 out of 17 IBD patients [17.6%] were affected by IDA, 2/17 [11.7%] by ACD, and in 12 out of 17 [70.5%] IBD children a combination of IDA and ACD was identified. All five anaemic coeliac patients were affected by IDA. In addition, an iron deficiency status without anaemia was found in 1/50 [2%] IBD patients, 7/45 [14%] coeliac patients, and 2/50 [4%] healthy controls [$p = 0.03$].

3.2. Serum hepcidin among different groups

Serum hepcidin was significantly higher in IBD patients with a PCDAI/PUCAI ≥ 30 compared with patients with PCDAI/PUCAI < 30, coeliac patients, and healthy controls [$p = 0.02$, $p = 0.005$, $p = 0.003$, respectively] [Table 3]. In IBD patients with PCDAI/PUCAI < 30, serum hepcidin values were higher than in coeliac or in healthy children, but statistical significance was not reached [$p = 0.3$, $p = 0.2$, respectively]. No difference was observed when comparing coeliac patients with healthy controls [$p = 0.9$] [Table 3]. In addition, serum hepcidin was significantly higher in patients with ACD compared with children with IDA and ACD + IDA [$p = 0.001$, $p = 0.001$, respectively]. Patients with ACD + IDA showed higher values of serum hepcidin compared with patients with IDA, with a trend toward statistical significance [$p = 0.06$] [Table 3]. In detail, none of the patients with pure IDA showed hepcidin values higher than the lower limit of the assay [0.55 nM], whereas 7 out of 12 [58.3%] patients with combined IDA and ACD showed values > 0.55 nM. Both of the two patients [100%] with pure ACD showed values higher than the cut-off.

3.2.1. Multivariate analysis

In a multivariate logistic regression model, serum hepcidin was considered positive for values > 0.55 nM. Having a PCDAI/PUCAI ≥ 30 resulted in the only variable independently associated with a positive serum hepcidin (9/11 [81.8%] patients with a PCDAI/PUCAI ≥ 30 versus 53/134 [39.5%] of the remaining patients; OR = 6.87; 95% CI 1.4–33, $p = 0.01$). None of the other variables was associated with positive serum hepcidin.

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

3.3. Iron absorption in IBD patients

Off 50 IBD patients, 43 [86%] performed the ILT. Twelve of the 43 [27.9%] patients showed a pathological ILT. Iron absorption was not associated with patient age or gender [p = 0.6 and p = 0.3, respectively] [Table 4]. Specific type of IBD and duration and extension of disease did not associate with the IM [p = 0.1, p = 0.1, and p = 0.7, respectively]. Patients with IM showed significant higher values of ESR and CRP compared with patients with normal iron absorption [p = 0.02 and p = 0.001, respectively]. Active disease was more frequent in children with a pathological ILT when compared with children with normal iron absorption (8/12 [66.7%] versus 6/31 [19.3%] [p = 0.01]. In particular, a PUCAI/PCDAI ≥ 30 was more common in children with pathological ILT when compared with children with normal ILT (5/12 [41.6%] versus 1/31 [1.3%]; p = 0.004). Mean PUCAI and PCDAI are shown in Table 4. In addition, IBD children with abnormal ILT were significantly more often taking immunosuppressive therapy (9/12 [75%] versus 11/31 [35.5%]; p = 0.03). Baseline serum iron, haemoglobin, transferrin, and Tsat values were significantly lower in patients with IM [p = 0.001, p = 0.005, p = 0.04, and p = 0.003, respectively] [Table 4]. Although baseline ferritin and faecal calprotectin were found to be higher in patients with IM, these differences were not statistically significant [p = 0.2 and p = 0.6, respectively] [Table 4]. Serum hepcidin was higher in patients with pathological ILT, with a trend toward statistical significance [p = 0.06] [Table 4]. In a multivariate logistic regression model, being affected by active IBD resulted in the only variable independently associated with a pathological ILT [OR = 15.4; 95% CI 1.4–160.2, p = 0.007].

3.4. Correlations of serum hepcidin and Δ[Fe]2hr

In order to determine correlations, we included all the patients [n = 145] in the analysis, except for Δ[Fe]2hr, PUCAI, PCDAI, calprotectin, and tTg, which were determined only in IBD patients or in coeliac patients. Log-tranformed hepcidin levels correlated negatively with Δ[Fe]2hr [r = -0.451, p = 0.002] [Figure 1A], and positively with log-transformed ferritin and CRP [r = 0.442, p = 0.0 001; r = 0.243, p = 0.009, respectively] [Figure 1B and C]. An inverse relationship was found with serum transferrin [r = -0.249, p = 0.005, respectively] [Figure 1D]. A direct correlation with a trend toward statistical significance was found between log-transformed hepcidin and ESR [r = 0.158, p = 0.09]. No specific correlation was found comparing log-transformed hepcidin with age, duration of IBD, serum iron, STfR, TIBC, or reticulocytes. Among coeliac patients, no significant correlation was identified between log-transformed hepcidin levels and tTG titres [r = -0.201, p = 0.2]. Δ[Fe]2hr was found to be inversely correlated with log-transformed ferritin and CRP [r = -0.588, p = 0.0001; r = -0.585, p = 0.0001, respectively] [Figure 2A and B]. A direct correlation was found between Δ[Fe]2hr and transferrin [r = 0.613, p = 0.0001] [Figure 2C]. An inverse correlation with a trend toward statistical significance was found with ESR, PUCAI, and faecal calprotectin [r = -0.296, p = 0.07; r = -0.292, p = 0.06; r = -0.325, p = 0.08, respectively]. None of the other variables correlated with Δ[Fe]2hr.

Table 4. Characteristics associated with iron malabsorption in IBD children.

Characteristics	Pathological ILT [n = 12]	Normal ILT [n = 31]	p
Mean age [years, range]	12.9 ± 3.6 [2–18]	12.3 ± 3.5 [2–18]	0.6
Gender [*n*, %]			0.3
Male	4 [33.3]	17 [54.8]	
Duration of disease [months]	25.5 ± 29.6 [0–82]	43.8 ± 36.3 [1–113]	0.1
IBD type [*n*, %]			0.1
CD	6 [50]	10 [32.3]	
UC	5 [41.7]	20 [64.5]	
IBD-U	1 [8.3]	1 [3.2]	
Active disease [*n*,%]	8 [66.7]	6 [19.4]	0.01
PCDAI/PUCAI ≥ 30 [*n*, %]	5 [41.7]	1 [3.2]	0.004
Disease activity indexes			
PCDAI	17.1 ± 17.4 [0–47.5]	3.7 ± 4.6 [0–10]	0.03
PUCAI	25.8 ± 15.3 [0–40]	7.5 ± 5.1 [0–35]	0.002
Immunosuppressants [*n*,%]	9 [75]	11 [35.5]	0.03
Laboratory parameters			
Haemoglobin, g/dl	11.3 ± 1.5 [9.1–14.5]	12.8 ± 1.5 [8.3–14.9]	0.005
Pre-load sideraemia, μg/dl	29.7 ± 14.4 [13–52]	71.6 ± 31 [18–148]	0.001
Ferritin, ng/ml	46.8 ± 28 [6–99]	36.7 ± 23.9 [10–115]	0.2
Serum hepcidin, nM	4.8 ± 7.8 [0.55–28.5]	2.7 ± 3.9 [0.55–19.2]	0.06
Transferrin, g/l	2.3 ± 0.8 [1.3–4.3]	2.7 ± 0.4 [1.6–3.6]	0.04
Tsat, %	9.9 ± 5.7 [4–21]	19.8 ± 9.5 [3–41]	0.003
STfR, mg/dl	1.8 ± 0.5 [0.8–2.4]	1.9 ± 1.5 [0.9–8.5]	0.8
TIBC, μg/dl	321 ± 51 [220–370]	327.6 ± 78.2 [139–460]	0.8
Reticulocytes,%	2.6 ± 3 [0.8–9]	4.6 ± 3.9 [0.6–9]	0.1
CRP, mg/dl	1.7 [0.2–5.1]	0.4 [0–2.6]	0.001
ESR, mm	16.7 ± 11.4 [5–35]	8.4 ± 9.6 [1–46]	0.02
Calprotectin, μg/g	316 [25–485]	234.1 [30–493]	0.6

All continuous variables values are expressed as means ± standard deviation [range].

CD, Crohn's disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; IBD-U, inflammatory bowel disease unclassified; PCDAI, Paediatric Crohn's Disease Activity Index; PUCAI, Paediatric Ulcerative Colitis Activity Index; STfR, soluble transferrin receptor; TIBC, total iron binding capacity; Tsat, transferrin saturation; UC, ulcerative colitis.

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

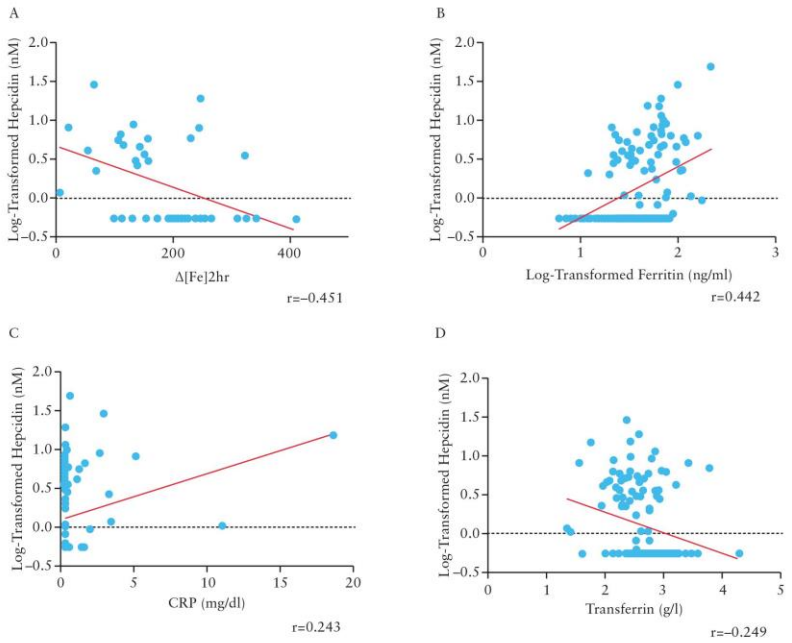


Figure 1. Correlations between log-transformed serum hepcidin and Δ[Fe]2hr [r = -0.451, $p = 0.002$] in inflammatory bowel disease [IBD patients]. [A] Correlations between log-transformed serum hepcidin and log-transformed ferritin [r = 0.442, $p = 0.0\ 001$]. [B] C-reactive protein [CRP] [r = 0.243, $p = 0.009$] and [C] transferrin [r = -0.249, $p = 0.005$], [D] in all the enrolled patients.

4. Discussion

To the best of our knowledge, this is the first paediatric study evaluating iron absorption and serum hepcidin levels in IBD paediatric patients. Our data show that IBD children with active disease tend to have impaired iron absorption, driven through the hepcidin pathway. Indeed, serum hepcidin levels were significantly higher in IBD patients with moderate to severe activity as compared with all other groups, including patients with mild activity or in remission, coeliac patients, and healthy controls. In addition, a significant inverse correlation was found between hepcidin levels and iron absorption. Our results are in agreement with the paper from Semrin and colleagues, the only paediatric study investigating the relationship between iron absorption and hepcidin.[11] The authors, using urinary hepcidin as a proxy for serum hepcidin, enrolled 19 paediatric patients with CD and found that hepcidin was increased in those with active disease and inversely correlated with iron absorption.[11]

Anaemia is a relevant problem frequently occurring during IBD management. Despite its relative high prevalence, costs, and impact on patient quality of life, it is rarely considered and adequately treated.[2,25,26] In our study population, the prevalence of anaemia among IBD patients was 34%. It is well known that the origin of IBD-related anaemia is usually multifactorial.[23] Not surprisingly, the majority of the anaemic patients in our study population showed a combined IDA and ACD [70.5%]. This finding once more highlights the difficulties in the therapeutic management of IBD-related anaemia. Indeed, in those cases with a mixed pathogenesis, the clinician usually faces the dilemma whether or not to use oral iron. As previously reported, the treatment with oral iron has significant limitations in IBD, being less efficacious than the intravenous route.[27] In addition, absorption of iron from the GI tract is limited, and unabsorbed iron is exposed to the intestinal surface. Iron mucosal harm has been described in IBD.[28] Studies in animal models of colitis indicate that luminal iron may exacerbate disease activity.[29,30] In a more recent study, iron supplementation affected microbiota and increased faecal calprotectin.[31] Taking into account this possible warning, the recently published ECCO guidelines state that the intravenous route should be preferred in those patients with suspected ACD.[23] It is therefore important to detect ACD in order to avoid an unnecessary and possibly harmful oral iron therapy.

The development of ACD is influenced by numerous factors, among which hepcidin is now considered the leading actor. Indeed, the decrease in ferroportin expression, which results from elevated hepcidin levels, would block entry of iron into the circulation, with consequent erythropoiesis impairment. Furthermore, since intestinal absorption of iron is inhibited by downregulation of ferroportin on enterocytes, the anaemia would be resistant to oral iron supplementation.[7,8] Therefore, serum hepcidin may represent an useful

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

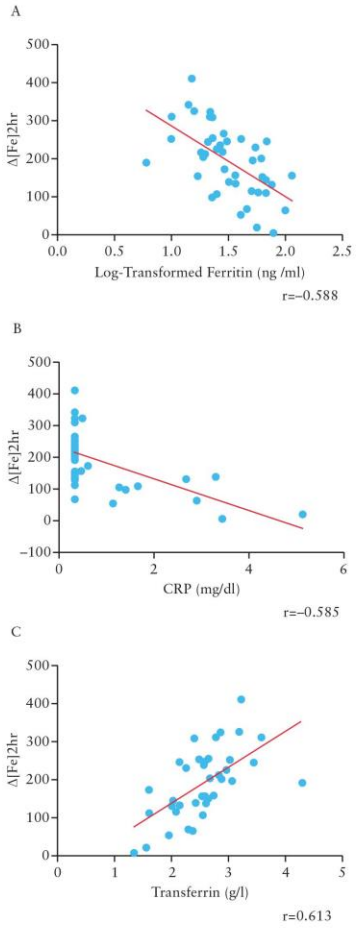


Figure 2. Correlations between Δ[Fe]2hr and log-transformed ferritin [r = -0.588, $p = 0.0\ 001$]. [A] C-reactive protein [CRP] [r = -0.585, $p = 0.0\ 001$] and [B] transferrin [r = 0.613, $p = 0.0\ 001$], [C] in inflammatory bowel disease [IBD] patients.

surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as first-line option. To date, no paediatric study has evaluated serum hepcidin sensitivity and specificity in IBD-related anaemia. As previously reported in adult populations, the low specificity together with the lack of standardisation should be considered the main limitations of this marker.[17,32] Although strongly limited by the small sample of anaemic patients, our data suggest that patients with pure IDA have undetectable hepcidin levels. This finding, if confirmed by larger series, may at least allow hepcidin use in the differential diagnosis of pure IDA from ACD. On the other hand, hepcidin's role in the diagnosis of combined IDA and ACD is still questionable and needs to be addressed by further studies.

Based on a recent paper published by Wang and colleagues, hepcidin may also act beyond the simple role of iron regulator.[33] The authors, inhibiting hepcidin expression in a mouse model of colitis, not only corrected IBD-related anaemia but also reduced colonic inflammatory cytokine expression.[33] This finding suggests that hepcidin may be some way involved in perpetuating inflammation, representing a new potential IBD therapeutic target. Our study does not provide information about hepcidin's role in the inflammatory process. Nevertheless, IBD children with pathological ILT showed higher values of disease activity indexes and acute phase reactants and were more often under immunosuppressive therapy, indicating the more severe phenotype of disease. In addition, the positive correlations of hepcidin with ESR and CRP, confirming that inflammation plays a major role in hepcidin induction, may also indicate that hepcidin could be directly implicated in the IBD inflammatory cascade. Anyhow, targeted studies are still necessary to confirm hepcidin's role in the pathogenesis of IBD's inflammatory course.

Our study was also meant to find out which iron parameters better correlate with hepcidin levels, and serum ferritin was found to be the most strictly correlated. This finding is in agreement with previous literature,[17,34] and once more demonstrates that ferritin is the primary biochemical marker correlated to hepcidin concentration. However, 58.3% of patients, with a diagnosis of combined IDA + ACD and normal ferritin values, showed an increased hepcidin, demonstrating that the ferritin value is not sufficient in the differential diagnosis of IBD-related anaemia.

This study has some limitations. First of all, intestinal iron absorption was simply determined using the ILT based on the increment of iron level 2 h after administrating an iron load, and this constitutes a methodological limitation. However, studies using dual stable iron isotope techniques, which are considered the gold standard for iron absorption determination, have shown a similar inverse correlation between serum hepcidin and iron absorption in healthy controls.[35,36] In addition, ILT has already been correlated with urinary hepcidin in IBD.[11] Finally, due to the earlier diagnosis, coeliac children were not age-matched with IBD and healthy controls and this may have influenced differences in hepcidin levels.

4.1. Conclusion

In conclusion, this comparative, cross-sectional study demonstrates that serum hepcidin is increased in IBD children with active disease and plays an important role in the process of iron malabsorption. ACD is significantly prevalent in paediatric IBD and should be taken in consideration before starting oral iron therapy. If confirmed by further studies, the negative correlation between hepcidin levels and iron malabsorption during the ILT may have important practical implications for a tailored management of anaemia in children with IBD-associated IDA. Indeed, serum hepcidin may serve as a useful, sensitive, surrogate marker to distinguish patients with impaired iron absorption in whom intravenous iron could be used as first-line option, avoiding the waste of time with a futile and possibly harmful cycle of oral iron. Further studies are needed to better elucidate the role of hepcidin in both iron metabolism and inflammation in IBD, in order to design new therapeutic strategies for ACD in paediatric IBD.

Funding

This work was partially supported by the Italian Ministry of Health Grant RF-2010-2312048 to DG and by the Department of Women, Children and of

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

General and Specialist Surgery at the Second University of Naples [Grant on Normal and Pathological Hematopoiesis] to SP.

Author Contributions

MM: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. CS: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. AA: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. FR: substantial contributions to conception and design, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published. RA: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. NC:substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. DG: substantial contributions to conception and design, analysis and interpretation of data, drafting the article, and final approval of the version to be published. BN: revising the article critically for important intellectual content and final approval of the version to be published. AS: revising the article critically for important intellectual content and final approval of the version to be published. SP: revising the article critically for important intellectual content and final approval of the version to be published. EM: substantial contributions to conception and design, interpretation of data, revising the article critically for important intellectual content, and final approval of the version to be published.

Potential Competing Interests

The authors declare no conflict of interest to disclose regarding this paper; AS is a speaker for Valeas Angelini, Milte Italia, and Danone, and consultant of D.M.G. Italy and Sucampo.

References

1. Gasche C, Lomer MCE, Cavill I, Weiss G. Iron, anemia, and inflammatory bowel diseases. *Gut* 2004;**53**:1190–7.
2. Wells CW, Lewis S, Barton JR, Corbett S. Effects of changes in hemoglobin level on quality of life and cognitive function in inflammatory bowel disease patients. *Inflamm Bowel Dis* 2006;**12**:123–30.
3. Goodhand JR, Kamperidis N, Rao A, *et al.* Prevalence and management of anemia in children, adolescents, and adults with inflammatory bowel disease. *Inflamm Bowel Dis* 2012;**18**:513–9.
4. Oustamanolakis P, Koutroubakis IE, Kouroumalis EA. Diagnosing anemia in inflammatory bowel disease: beyond the established markers. *J Crohns Colitis* 2011;**5**:381–91.
5. Zhu A, Kaneshiro M, Kaunitz JD. Evaluation and treatment of iron deficiency anemia: a gastroenterological perspective. *Dig Dis Sci* 2010;**55**:548–59.
6. Weiss G, Gasche C. Pathogenesis and treatment of anemia in inflammatory bowel disease. *Haematologica* 2010;**95**:175–8.
7. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001;**276**:7806–10.
8. Ganz T. Hepcidin. a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* 2003;**102**:783–8.
9. Nemeth E, Rivera S, Gabayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;**113**:1271–6.
10. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc Natl Acad Sci U S A* 2005;**102**:1906–10.
11. Semrin G, Fishman DS, Bousvaros A, *et al.*. Impaired intestinal iron absorption in Crohn's disease correlates with disease activity and markers of inflammation. *Inflamm Bowel Dis* 2006;**12**:1101–6.
12. Oustamanolakis P, Koutroubakis IE, Messaritakis I, Malliaraki N, Sfiridaki A, Kouroumalis EA. Serum hepcidin and prohepcidin concentrations in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2011;**23**:262–8.
13. Arnold J, Sangwaiya A, Bhatkal B, Geoghegan F, Busbridge M. Hepcidin and inflammatory bowel disease: dual role in host defence and iron homoeostasis. *Eur J Gastroenterol Hepatol* 2009;**21**:425–9.
14. Kaya Z, Yildiz E, Gursel T, *et al.* Serum prohepcidin levels in children with solid tumors, inflammatory bowel disease and iron deficiency anemia. *J Trop Pediatr* 2011;**57**:120–5.
15. Nagy J, Lakner L, Poór VS, *et al.* Serum prohepcidin levels in chronic inflammatory bowel diseases. *J Crohns Colitis* 2010;**4**:649–53.
16. IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis the Porto criteria. *J Pediatr Gastroenterol Nutr* 2005;**41**:1–7.
17. Bergamaschi G, Di Sabatino A, Albertini R, *et al.* Serum hepcidin in inflammatory bowel diseases: biological and clinical significance. *Inflamm Bowel Dis* 2013;**19**:2166–72.
18. Campostrini N, Castagna A, Zaninotto F, *et al.* Evaluation of hepcidin isoforms in hemodialysis patients by a proteomic approach based on SELDI-TOF MS. *J Biomed Biotechnol* 2010;**2010**:329646.
19. Levine A, Griffiths A, Markowitz J, *et al.* Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis* 2011;**17**:1314–21.
20. Hyams JS, Ferry GD, Mandel FS, *et al.* Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr* 1991;**12**:439–47.
21. Turner D, Otley AR, Mack D, *et al.* Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology* 2007;**133**:423–32.
22. WHO, UNICEF, UNU. *Iron Deficiency Anemia: Assessment, Prevention and Control. Report of a joint WHO/UNICEF/UNU consultation.* Geneva: World Health Organization, 1998.
23. Dignass AU, Gasche C, Bettenworth D, *et al.* European consensus on the diagnosis and management of iron deficiency and anaemia in inflammatory bowel diseases. *J Crohns Colitis* 2015;**9**:211–22.
24. De Vizia B, Poggi V, Conenna R, Fiorillo A, Scippa L. Iron absorption and iron deficiency in infants and children with gastrointestinal diseases. *J Pediatr Gastroenterol Nutr* 1992;**14**:21–6.
25. Ott C, Liebold A, Takses A, Strauch UG, Obermeier F. High prevalence but insufficient treatment of iron-deficiency anemia in patients with inflammatory bowel disease: results of a population-based cohort. *Gastroenterol Res Pract* 2012;**2012**:595970.
26. Lugg S, Beal F, Nightingale P, Bhala N, Iqbal T. Iron treatment and inflammatory bowel disease: What happens in real practice? *J Crohn's Colitis* 2014;**8**:876–80.
27. Lee TW, Kolber MR, Fedorak RN, van Zanten SV. Iron replacement therapy in inflammatory bowel disease patients with iron deficiency anemia: a systematic review and meta-analysis. *J Crohns Colitis* 2012;**6**:267–75.
28. De Silva AD, Tsironi E, Feakins RM, Rampton DS. Efficacy and tolerability of oral iron therapy in inflammatory bowel disease: a prospective, comparative trial. *Aliment Pharmacol Ther* 2005;**22**:1097–105.
29. Seril DN, Liao J, West AB, Yang GY. High-iron diet: foe or feat in ulcerative colitis and ulcerative colitis-associated carcinogenesis. *J Clin Gastroenterol* 2006;**40**:391–7.
30. Oldenburg B, Berge Henegouwen GP, Rennick D, Van Asbeck BS, Konigsberger JC. Iron supplementation affects the production of pro-inflammatory cytokines in IL-10 deficient mice. *Eur J Clin Invest* 2000;**30**:505–10.
31. Zimmermann MB, Chassard C, Rohner F, *et al.* The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. *Am J Clin Nutr* 2010;**92**:1406–15.
32. Oustamanolakis P, Koutroubakis IE. Soluble transferrin receptor-ferritin index is the most efficient marker for the diagnosis of iron deficiency anemia in patients with IBD. *Inflamm Bowel Dis* 2011;**17**:E158–9.

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

33. Wang L, Trebicka E, Fu Y, *et al.* The bone morphogenetic protein-hepcidin axis as a therapeutic target in inflammatory bowel disease. *Inflamm Bowel Dis* 2012;**18**:112–9.
34. Mecklenburg I, Reznik D, Fasler-Kan E, Drewe J, Beglinger C, Hruz P. On behalf of the Swiss IBD Cohort Study Group. Serum hepcidin concentrations correlate with ferritin in patients with inflammatory bowel disease. *J Crohns Colitis* 2014;**8**:1392–7.
35. Young MF, Glahn RP, Ariza-Nieto M, *et al.* Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. *Am J Clin Nutr* 2009;**89**:533–8.
36. Roe MA, Collings R, Dainty JR, Swinkels DW, Fairweather-Tait SJ. Plasma hepcidin concentrations significantly predict interindividual variation in iron absorption in healthy men. *Am J Clin Nutr* 2009;**89**:1088–91.

Downloaded from http://ecco-jcc.oxfordjournals.org/ by guest on January 23, 2016

2.3 *Impact of Environmental and Familial Factors in a cohort of pediatric patients with Inflammatory Bowel Disease*

In the last years, epidemiological and molecular studies stressed the importance of genetic susceptibility in causing IBD onset.[47,48] However, important features such as the significant geographical variation in disease incidence, the incomplete penetrance among monozygotic twins, and the striking rise of IBD over the course of the past century, can't be explained with genetic predisposition alone and reinforce the environmental hypothesis for IBD etiology. [49-51] So far, in addition to familial aggregation, which is the most important IBD risk factor, only tobacco smoking and appendectomy have been demonstrated to be strongly associated with IBD incidence.[52-54]

An emerging and interesting theory supporting the environmental influence on IBD onset, is the "hygiene hypothesis", which correlates the epidemiological rise in IBD incidence over the 20th century, both in developed and developing countries, with the improvement in general hygienic conditions (i.e. free access to clean water, smaller family size, etc.). [55-57]

Understanding the role of environmental factors is important not only for the possible preventive interventions in genetically predisposed individuals, but also to offer a better disease care to those already suffering with IBD. Therefore, the purpose of our work was to investigate the relation between the exposure to some environmental factors and the risk to develop UC and CD in a cohort of pediatric patients in Southern Italy.

Understanding the role of environmental factors is important not only for the possible preventive interventions in genetically predisposed individuals, but also to offer a better disease care to those already suffering with IBD. Therefore, the purpose of our work was to investigate the relation between the exposure to some environmental factors and the risk to develop UC and CD in a cohort of pediatric patients in Southern Italy. Our study suggests numerous evidences supporting the influence of environmental factors as possible explanation for the significant increase of IBD incidence in the last decades.[58] To the best of our knowledge this is the first pediatric study addressing the hypothesis that the development of IBD may be related to a lower adherence to Mediterranean diet, assessed with a validated questionnaire.

As previously reported, having a first degree relative with IBD represents the single most important factor determining an individual's risk for developing the disease[59], confirming the importance of genetic background. However the fact that environmental factors play a major role in IBD development is corroborated by many of our results. We confirmed that antibiotics areassociated with increased risk of CD.[60,61] The use of antibiotics may alter the composition of the gut microbiome with the loss of potentially beneficial bacteria and the emergence of potentially

pathogenic bacteria. Alternatively, the loss of immune interaction with potentially pathogenic bacteria at an early age fails to prime the immune system to harmful organisms that it may encounter later in life.[60,61] Moreover, our study confirms an association between crowded housing and protection against the development of both IBD forms: owning pets, reduced number of toilets at home and higher number of siblings were associated with a lower risk for both CD and UC. Family size can be used to indicate the level of overcrowding in a home, which has been associated with potential exposure to infection. A small family size and thus a less propensity for exposure to infections have been associated with a higher risk for IBD.[62] Siblings may influence the development of IBD altering exposure patterns to microorganisms in early life, affecting acute manifestation of infections, or influencing age of transmission and severity.[63]

Above all, the striking difference in Mediterranean diet adherence, between cases and controls, has to be considered the most innovative finding of our study. Indeed, the Mediterranean diet is perhaps considered one of the healthiest dietary models currently existing. Numerous epidemiological and experimental nutrition studies have demonstrated how Mediterranean countries benefit from lower rates of chronic disease morbidity and higher life expectancy.[64] There are several proposed mechanisms of action to explain the association between IBD and dietary choices. These proposed mechanisms involve a direct effect of dietary antigens, alteration of gut permeability, and the auto inflammatory response of the mucosa due to changes in the microbiota

Impact of Environmental Factors in a Pediatric IBD cohort: a Case-Control Study

Caterina Strisciuglio[1,2], MD, Francesca Paola Giugliano[1], Massimo Martinelli[1], MD, MD, Sabrina Cenni[1], MD, Luigi Greco[1], MD, Annamaria Staiano[1], MD, and Erasmo Miele[1], MD, PhD.

[1]Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Italy
[2]Department of Woman, Child and General and Specialized Surgery, Second University of Naples, Italy

#Strisciuglio C, Giugliano FP and Martinelli M participated equally in this study.

Address all correspondence to:

Erasmo Miele, M.D.
Department of Translational Medical Science
Section of Pediatrics
University of Naples "Federico II"
Via S. Pansini, n. 5, 80131, Naples, Italy
Tel: +39.081.746.45765
Fax: +39.081.746.3116
E-mail: erasmo.miele@unina.it

Short title: Risk Factors in Pediatric IBD

Abbreviations: CD: Crohn's disease; H. Pylori: Helicobacter Pylori; IBD: Inflammatory Bowel Disease, versus: vs, UC: Ulcerative Colitis.

Funding Source: No funding was secured for this study.

Financial Disclosure: The authors have no financial relationships relevant to this article to disclose.

Conflict of Interest: Annamaria Staiano A. served as investigator and member of advisory board for the following companies: D.M.G, Valeas, Angelini, Miltè, Danone, Nestlé, Sucampo, Menarini. The remaining authors have no conflict of interest to declare.

What's known in this subject

The increased incidence of IBD over the last decades and the rise of diagnosis in pediatric age underline the importance of environmental contribution on IBD onset. Smoke and appendectomy have been demonstrated to be strongly associated to IBD incidence.

What This Study Adds

This is the first pediatric study describing the protective role of adherence to Mediterranean diet on IBD development. Our study confirms that environmental factors are closely linked to IBD onset and can explain the rise of IBD in developed countries.

Contributors' Statements:

Caterina Strisciuglio- substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article and final approval of the version to be published
Massimo Martinelli- substantial contributions to conception and design, analysis and interpretation of data, drafting the article and final approval of the version to be published
Francesca Paola Giugliano- substantial contributions to conception and design, acquisition of data drafting the article and final approval of the version to be published
Luigi Greco- substantial contributions to conception and design, revising the article critically for important intellectual content and final approval of the version to be published
Sabrina Cenni- substantial contributions to conception and design, acquisition of data, and final approval of the version to be published
Annamaria Staiano- substantial contributions to conception and design, drafting the article and final approval of the version to be published
Erasmo Miele- substantial contributions to conception and design, interpretation of data, revising the article critically for important intellectual content and final approval of the version to be published.
All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

ABSTRACT

Objectives: The primary role of environment on Inflammatory Bowel Disease (IBD) onset has been recently stressed. We aimed to investigate the impact of environmental factors in an IBD pediatric cohort. **Methods:** A total of 467 subjects [(264 IBD and 203 controls] were enrolled. All patients underwent a questionnaire including 5 different groups of environmental risk factors: family history of IBD and autoimmune diseases, perinatal period, home amenities and domestic hygiene, childhood diseases and vaccinations, diet. **Results:** In a multivariate model, IBD family history resulted the most significant factor associated with the risk of being affected by Crohn's disease (CD) and Ulcerative Colitis (UC) (Odds Ratio (OR): 79; 95% Confidence Interval 6.6-946.3; OR: 22.7; 6.8-76, respectively). Mother degree (OR: 8.9; 3.1-25.5), duration of breast feeding>3th month (OR: 4.7; 1.8-12.6), father employment (OR: 4.1; 1.4-11.9), gluten introduction<6th month (OR: 3.7; 1.7-8.2), number of siblings<2 (OR: 3.2; 1.4-7) and family history of autoimmune diseases (OR: 2.5; 1-6.1) were significant risk factors for CD. Low adherence to Mediterranean diet (OR: 3.3; 1.8-6), gluten introduction<6th month (OR: 2.8; 1.6-4.7) and number of siblings<2 (OR: 1.9; 1-3.2) were significant risk factors for UC. Owning pets (OR: 0.2; 0.07-0.4) and bed sharing (OR: 0.1; 0.05-0.4) were protective factors for CD, while owning pets (OR: 0.4; 0.2-0.8) and family parasitosis (OR: 0.07; 0.01-0.4) resulted protective factors for UC. **Conclusions:** Our study confirms that environmental factors are closely linked to IBD onset and may partly explain IBD rise in developed countries.

Introduction

Inflammatory bowel diseases (IBD) represent a group of inflammatory conditions involving the gastrointestinal tract, whose etiology remains still unknown. Ulcerative colitis (UC) and Crohn's disease (CD) are the most common forms of IBD, both associated with high morbidity [1, 2]. In the last years, epidemiological and molecular studies stressed the importance of genetic susceptibility in causing IBD onset [3, 4]. However, important features such as the significant geographical variation in disease incidence, the incomplete penetrance among monozygotic twins, and the striking rise of IBD incidence over the course of the past century, can't be explained with genetic predisposition alone and reinforce the environmental hypothesis for IBD etiology [5-7]. So far, in addition to familial aggregation, which is the most important IBD risk factor, only tobacco smoking and appendectomy have been demonstrated to be strongly associated with IBD incidence [8-10]. The contribution of other potential environmental risk factors, proposed as important in IBD predisposition (including oral contraceptives and diet), has not yet been demonstrated because of discordant results [11, 12]. An emerging and interesting theory is the "hygiene hypothesis", which correlates the epidemiological rise in IBD incidence over the 20th century, both in developed and developing countries, with the improvement in general hygienic conditions (i.e. free access to clean water, smaller family size, etc.)[13-15]. This theory asserts that exposure to poor hygiene or infections during childhood can protect from developing IBD or other kind of autoimmune and allergic diseases later in life [16, 17]. Moreover, several epidemiological studies suggested a role of perinatal or early life events in the etiology of IBD [5, 7] such as non-specific (gastroenteritis and other non-specific infections) and specific (vaccines, passive smoking) exposures. A possible explanation is that a decreased prevalence of infections during childhood could lead to a major individual susceptibility in developing IBD later in life. However, there are no definitive data demonstrating a final role of hygiene in IBD development. Understanding the role of environmental factors is important not only for the possible preventive interventions in genetically predisposed

individuals, but also to offer a better disease care to those already suffering with IBD. Therefore, the purpose of our work was to investigate the relation between the exposure to some environmental factors and the risk to develop UC and CD in a cohort of pediatric patients in Southern Italy.

Materials and Methods

Case identification

A case-control study was undertaken at the Department of Translational Medical Science, Section of Pediatrics, University of Naples Federico II, in Campania, Southern Italy. Cases corresponded to IBD pediatric patients attending our Department for clinical management from 2000 to 2014 and with an age <18 years at the time of IBD diagnosis. The diagnosis of CD and UC was based on clinical, endoscopic, radiological and histo-pathological criteria [18].

Controls

Controls were chosen both from local public schools (n=111; 54.6%) in Campania, Italy, representatives of the general population, and from the outpatient clinic (n=92; 45.4%) at the Department of Translational Medical Science, Section of Pediatrics, University of Naples Federico II, Campania, Italy. Only children with functional gastrointestinal disorders, gastrointestinal reflux or constipation were enrolled.

Definition of Questionnaire

Investigators personally interviewed both the study subjects and their mothers (if the subject was <10 years old). A copy of the questionnaire *(Supplemental file)* was given to each subject to carefully think about each answer and, if necessary, to discuss it with other family members. Patients and their parents filled the questionnaire between January and June 2014. Questions covered the period from birth to the date of diagnosis for cases and the corresponding period for control (from birth to age of the enrollment). The questionnaire was a multi-item-questionnaire

related to five different areas: 1) family history of IBD and of autoimmune diseases; 2) perinatal period (gestational age at birth, mother diseases during pregnancy, infections or hospitalization during the first month of life); 3) home amenities (presence of bathrooms, hot water), water consumption (tap or bottle water), type of housing (flat, house or other), personal hygiene, presence of animals (pets, other animals), number of siblings, sharing the bed or the room with other family members, active and passive smoking, mother and father degree and employment, stressing events susceptibility (generalized anxiety, social anxiety, panic disorders, bereavement, divorce); 4) childhood infections (measles, mumps, rubella, chicken pox, whooping cough, croup, gastroenteritis, respiratory infections during the first years of life), other childhood diseases (atopy), use of antibiotics, appendectomy and vaccines (both compulsory such as measles-mumps-rubella vaccine, diphtheria, tetanus, poliomyelitis, pertussis and optional such as pneumococcus and meningococcal vaccines); in the latter category positive history for other infective diseases, both in the patients or in the family components, [such as Helicobacter Pylori (H. Pylori), parasites] were also included; 5) infants and children diet (breast-feeding, age of introduction of gluten). In addition, a separate validated questionnaire was designed to assess the adherence to Mediterranean diet, as previously described [19] (Table 2). The total duration of the interview was approximately one hour.

Ethical Considerations

The Institutional Review Board of the University of Naples "Federico II" approved the study protocol and questionnaire, and all participants gave informed written consent.

Statistics

The association between potential risk factors and IBD, CD or UC, was explored by Pearson's Chi-Square test for categorical variables. A p value of <0.05 (p^*) was considered to denote a statistically significant difference. A Bonferroni correction was applied to correct for multiple comparisons. A

multivariate model was performed in order to consider the effect (Odds Ratio) of each single factor, after having considered the contribution of all other factors. We selected a stepwise forward conditional procedure to identify the strongest risk factor and then we added the factors that contribute independently to further risk. We compared CD and UC cases versus controls to estimate the odds of being a control (not affected) by a Logistic Regression Model. Since environmental risk factors might be related to the socio-economic level of the family, we included in the first step of the multivariate model, mother's education and father's occupation, considered as the best markers of the socio-economic status and positive IBD family history and autoimmune diseases, the two strongest IBD-related risk factors. For a case-control study, a p value<0.05, a power of 80%, a mean risk factor prevalence of 30% and expected relative risk of 2.5 (selected for biological significance), the required sample size for cases and controls separately was estimated around 80 [20].

Results

Description of IBD cohort and controls

All IBD cases and controls agreed to participate in the study. A total of 467 subjects aged between 1 and 18 years, were enrolled. Among these 102 were affected by CD (median age: 11 years; range: 4-17 years; M/F: 58/44) and 162 by UC (median age: 10 years; range: 2-17 years; M/F: 80/82). In the IBD groups no case of indeterminate colitis was present. In addition, 203 controls (median age: 12 years; range: 5-17; M/F: 118/85) were also enrolled. Baseline characteristics of enrolled patients were not significantly different among cases and controls, as reported in Table 1.

Univariate Analysis (Table 3, Table 4)

Family history of IBD and autoimmune diseases

A family history of IBD was significantly associated with both CD (CD: 16.2% versus (vs) Controls: 2%; χ^2: 21.4; p<0.001) and UC (UC: 29.6% vs Controls: 2%; χ^2: 55.5; p<0.001). The presence of concomitant autoimmune diseases was associated to the risk of developing IBD, with a significant difference compared to controls both for CD (CD: 37% vs Controls: 19.4%; χ^2: 10.85; p=0.002) and UC (UC: 40%; Controls: 19.4%; χ^2: 19.7; p<0.001).

Perinatal period

Gestational age, mothers' diseases during pregnancy and infections or hospitalization during the first month of life were similar between cases and controls.

Home amenities, domestic and personal hygiene

A higher percentage of stressing events in the family was found in both group of cases compared with controls (CD: 46.1% vs Controls: 34.5%; χ^2: 3.9; p=0.05; UC: 54.7% vs Controls: 34.5%; χ^2: 14.9; P<0.001). Bed sharing was significantly more frequent in controls patients when compared with CD patients (Controls: 37.1% vs CD: 17.6%; χ^2: 12.1; p<0.001); this finding was not confirmed in UC patients (UC: 30.8% vs controls: 37.1%; χ^2: 1.6; p=0.1). The number of siblings resulted to be lower (< 2 siblings) in the CD group (CD: 69.3% vs Controls: 50.7%; χ^2: 9.5; p=0.002) and UC group (UC: 67.7% vs Controls: 50.7%; χ^2: 10.6; p=0.001) when compared to controls. A higher (>1) number of toilets was found in cases' houses compared to the controls both for CD (CD: 73.5% vs Controls: 61.6%; χ^2: 4.3; p=0.04) and UC (UC: 77.6% vs Controls: 61.6%; χ^2:10.8; p=0.001). Furthermore, owning a pet was more frequent in controls compared to both group of cases (CD: 17.6% vs Controls: 34.7%; χ^2: 9.5; p=0.001; UC: 23.5% vs Controls: 34.7%; χ^2: 5.4; p=0.02). In CD families tobacco smoking was statistically more frequent than in control's families (CD: 52.5% vs Controls: 41.9%; χ^2: 3.1; p=0.05).

Childhood diseases, vaccinations

No statistical significant difference was found in the exposure to vaccination or incidence of childhood exanthema (measles, mumps and rubella) between cases and controls. A positive family history for intestinal parasitosis and H.Pylori infection resulted more frequent in controls than in CD and UC cases (Parasitosis: CD: 3.1% vs Controls: 16%; χ^2: 10.6; p=0.001; UC: 1.3% vs Controls: 16%; χ^2: 22.136; p<0.001; H.Pylori: CD: 9.2% vs Controls: 17.9%; χ^2: 3.8; p=0.03; UC: 8.9% vs Controls: 17.9%; χ^2: 5.8; p=0.02). A higher use of antibiotic therapy was found in the CD group compared to controls (CD: 30.4% vs Controls: 21.2%; χ^2: 3.1; p=0.05). Recurrence of intestinal parasitosis and H.Pylori infection was not significantly different in cases (both CD and UC) compared to the control group. No difference was found regarding atopy (eczema, asthma), appendectomy and consumption of non-steroidal-anti-inflammatory drugs.

Infants and children diet

The number of subjects who showed a low adherence to Mediterranean diet was higher for CD (CD: 29.4% vs Controls: 17.3%; χ^2: 5.9; p=0.01) as well as for UC (UC: 37.7% vs Controls: 17.3%; χ^2: 19; p<0.001) when compared to controls. CD patients (CD: 78.2% vs Controls: 57.1%; χ^2: 10.2; p=0.001) showed a higher frequency of prolonged breastfeeding (>3 months) compared to controls. Gluten was introduced in child's diet more frequently before the 6th month of age in both CD (CD: 54% vs Controls: 29.3%; χ^2: 17.3; p<0.001) and UC (UC: 55.3% vs Controls: 29.3%; χ^2: 24.77; p<0.001) compared to controls.

Multivariate logistic regression analysis

IBD versus Controls

In a multivariate logistic regression model being affected by CD or UC were used as dependent variables. IBD family history resulted the most significant factor associated with the risk of being

affected by CD and UC (OR: 79; 95% CI 6.6-946.3; p=0.001; OR: 22.7; 95% CI 6.8-76; p<0.001, respectively). Mother degree was among the most significant variables associated with CD (OR: 8.9; 95% CI 3.1-25.5; p=<0.001), while a low adherence to Mediterranean diet resulted significantly associated with UC (OR: 3.3; 95% CI 1.8-6; p<0.001). Owning pets and bed sharing were independent protective factors for CD development (OR: 0.2; 95% CI 0.07-0.4; p<0.001; OR: 0.1; 95% CI 0.05-0.4; p<0.001). Owning pets and family parasitosis resulted significant protective factors for UC (OR: 0.4; 95% CI 0.2-0.8; p=0.01; OR: 0.07; 95% CI 0.01-0.4; p=0.002). All variables independently associated with the risk of being affected by CD and UC after multivariate analysis are showed in Table 5.

Discussion

Our study suggests numerous evidences in favor of the Hygiene Hypothesis as possible explanation for the significant increase of IBD incidence in the last decades [21]. To the best of our knowledge this is the first pediatric study addressing the hypothesis that the development of IBD may be related to a lower adherence to Mediterranean diet, assessed with a validated questionnaire.

As previously reported, having a first degree relative with IBD represents the single most important factor determining an individual's risk for developing the disease [22], confirming the importance of genetic background. However the fact that "hygiene hypothesis" has a major role in IBD development is corroborated by many of our results. We confirmed that antibiotics are associated with increased risk of CD [23, 24]. The use of antibiotics may alter the composition of the gut microbiome with the loss of potentially beneficial bacteria and the emergence of potentially pathogenic bacteria [23, 24]. Alternatively, the loss of immune interaction with potentially pathogenic bacteria at an early age fails to prime the immune system to harmful organisms that it may encounter later in life [23, 24]. Moreover, our study confirms an association between poor domestic hygiene and protection against the development of both IBD forms: owning pets, reduced

number of toilets at home and higher number of siblings were associated with a lower risk for both CD and UC. Family size can be used to indicate the level of overcrowding in a home, which has been associated with poor hygiene and potential exposure to infection. A small family size and thus a less propensity for exposure to infections have been associated with a higher risk for IBD [25]. Siblings may influence the development of IBD altering exposure patterns to microorganisms in early life, affecting acute manifestation of infections, or influencing age of transmission and severity [26]. Therefore, it is not surprising that H. Pylori infection and parasitosis were significantly more frequent in the families of the control group. In a recent meta-analysis H. Pylori infection was inversely associated with IBD [27, 28]. Also helminthes are thought to have an immunoregulatory role within the intestinal microbiome, and may have potentially protective effect in IBD development [28, 29], as demonstrated in a recent case control study conducted in South Africa [30]. Another strong finding supporting the hygiene hypothesis is that a higher educational level and a better social class according to mother degree and father occupation were significant risk factors for CD development, as previously reported by Lopez-Serrano et al. (31).

Interestingly, in our pediatric population we found a more frequent incidence of stressing events in life compared to controls. The exact mechanism behind the effect of stress on intestinal inflammation is unclear, however the evidence that stress can modulate the course of IBD is provided by clinical observations and by animal models of colitis and neuro-immune studies [32]. Depression, anxiety, and stress have also been associated with increased rates of relapse and surgery for IBD patients [33].

Above all, the striking difference in Mediterranean diet adherence, between cases and controls, has to be considered the most innovative finding of our study. Indeed, the Mediterranean diet is perhaps considered one of the healthiest dietary models currently existing. Numerous epidemiological and experimental nutrition studies have demonstrated how Mediterranean countries benefit from lower rates of chronic disease morbidity and higher life expectancy (19). There are

several proposed mechanisms of action to explain the association between IBD and dietary choices. These proposed mechanisms involve a direct effect of dietary antigens, alteration of gut permeability, and the auto inflammatory response of the mucosa due to changes in the microbiota [34]. In a case control study of children published in 2008, a positive association with CD was found in girls with a diet rich in meats, fatty foods, and desserts; whereas, a diet of vegetables, fruits, olive oil, fish, grains, and nuts was inversely associated with CD in both genders. As a matter of fact, we found that traditional Mediterranean dietary patterns characterized by elevated intakes of fruits and vegetables, olive oil, bread and cereals may exert a protective role in the development of IBD. However, diet is difficult to study as it is a multifactorial exposure, and patients may alter dietary habits based on symptom onset prior to diagnoses or as a result of increased disease activity [35]. Unfortunately, there is still a large gap in knowledge due to limitations of retrospective data collection and recall bias for dietary histories [36]. As reported from Baron et al [37] we found that breast-feeding was associated with an increased risk for CD. It is known that breast-feeding provides immunological protection to the newborn, therefore Baron et al speculated that when weaning occurs, delayed infections may lead to an inappropriate immune response and persistence of intestinal inflammation. However, the authors conclude that short and long term benefits of breast-feeding overrule by far the increased risk of IBD that was observed in the study [37]. We also explored the age of gluten introduction and we confirmed the results from Lopez-Serrano et al who found that the first exposure to wheat before 6 months of age was a significant risk factor for IBD in the univariate and multivariate analysis [31]. Lammers et al. demonstrated that gliadin is able to induce a direct increase in small intestinal permeability [38]. It is therefore hypothesizable that a longer exposure to gluten may be responsible of a detrimental effect on the gut barrier, participating in the chronic inflammatory process that leads to IBD onset. Moreover the duration of exposure to gluten has been related to an increase in the risk of other autoimmune disorders in patients with celiac disease [39]. As previously reported [40], we found that passive tobacco smoking contributes to an increased risk for CD, but we didn't observe a protective effect on UC.

On the other hand, we didn't find any association with appendectomy, which is considered an important and strong protective factor for UC and a risk factor for CD [41]. One of the reasons may be the relative small sample size of our patients.

The main limitations of this study are certainly related to the retrospective nature, and therefore, the possibility of recall biases has to be taken into account. Moreover, due to a lack of a pediatric validated questionnaire, we referred to the main risk factors previous analyzed.

Conclusions

Our study strongly supports the role of Hygiene Hypothesis in IBD pathogenesis. In addition, we reported for the first time the low adherence to Mediterranean diet as a possible risk factor for developing IBD. Considering the retrospective nature, there is a strong need for future prospective studies. Indeed, it is impossible to rule out the possibility, that the relationship between IBD and hygiene related factors, is not an indirect effect that may serve as a mechanism for other yet unknown lifestyle factors. Future research should also be targeted for the identification of potential mechanisms underlying associations with hygiene related factors in order to provide new clues for a better comprehension of IBD etiopathogenesis.

References

1. Love JR, Irvine EJ, Fedorak RN. Quality of life in inflammatory bowel disease. J Clin Gastroenterol. 1992; 14:15-19.
2. Sands BE. Therapy of inflammatory bowel disease. Gastroenterology. 2000; 118:S68-S82.
3. Ogura Y, Bonen DK, Inohara N et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001; 411:603-606.
4. Peyrin-Biroulet L, Parmentier-Decrucq E, Branche J et al. IL-23R, a novel susceptibility gene for inflammatory bowel disease. Med Sci (Paris). 2007; 23:250-252.
5. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology. 2004; 126:1504-17.
6. Loftus EV Jr, Silverstein MD, Sandborn WJ et al. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. Gastroenterology. 1998; 114:1161-1168.
7. Marshall JK, Hilsden RJ. Environment and epidemiology of inflammatory bowel diseases. In: Satsangi J, Sutherland RL, eds. Inflammatory bowel diseases. London: Churchill Livingston; 2003. pp. 17–28.
8. Peeters M, Nevens H, Baert F et al. Familial aggregation in Crohn's disease: Increased ageadjusted risk and concordance in clinical characteristics. Gastroenterology. 1996; 111:597-603.
9. Cosnes J, Carbonnel F, Carrat F et al. Effects of current and former cigarette smoking on the clinical course of Crohn's disease. Aliment Pharmacol Ther. 1999; 13:1403-1411.
10. Smithson JE, Radford-Smith G, Jewell GP. Appendectomy and tonsillectomy in patients with inflammatory bowel disease. J Clin Gastroenterol. 1995; 21:283-286.
11. Peeters M, Cortot A, Vermeire S et al. Familial and sporadic inflammatory bowel disease: different entities? Inflamm Bowel Dis. 2000; 6:314-20.
12. Andersson RE, Olaison G, Tysk C et al. Appendectomy and protection against ulcerative colitis. N Engl J Med. 2001; 344:808-14.
13. Askling J, Grahnquist L, Ekbom A et al. Incidence of pediatric Crohn's disease in Stockholm, Sweden. Lancet. 1999; 354:1179.
14. Yao T, Matsui T, Hiwatashi N. Crohn's disease in Japan: diagnostic criteria and epidemiology. Dis Colon Rectum. 2000; 43:S85-S93.
15. Kiloski N, Bret L, Radford-Smith G. Hygiene hypothesis in inflammatory bowel disease: a critical review of the literature. World J Gastroenterol. 2008; 14:165-73.
16. Bresciani M, Parisi C, Menghi G et al. The hygiene hypothesis: does it function worldwide? Curr Opin Allergy ClinImmunol. 2005; 5:147-51.
17. Forbes A, Kalantzis T. Crohn's disease: the cold chain hypothesis. Int J Colorectal Dis. 2006; 21:399-401.
18. IBD Working Group of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis – the Porto criteria. Journal of Pediatric Gastroenterology and Nutrition. 2005; 41:1-7.
19. Serra-Majem L, Ribas L, Ngo J et al. Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. Public Health Nutrition. 2004; 7:931–935.
20. Schlesselman. Case-control studies: Design, conduct, analysis. New York: Oxford University Press; 1982.
21. Bernstein CN, Rawsthorne P, Cheang M et al. A population-based case control study of potential risk factors for IBD. Am J Gastroenterol. 2006; 101:993-100.
22. Swidsinski A, Ladhoff A , Pernthaler A et al. Mucosal flora in inflammatory bowel disease. Gastroenterology. 2002; 122:44 – 54.

23. Ungaro R, Bernstein CN, Gearry R et al. Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. Am J Gastroenterol. 2014; 109:1728-38.
24. Kronman MP, Zaoutis TE, Haynes K et al. Antibiotic exposure and IBD development among children: a population-based cohort study. Pediatrics. 2012; 130:794-803.
25. Danese S, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. Autoimmun Rev. 2004; 3:394-400.
26. Montgomery SM, Lambe M, Wakefield AJ et al. Siblings and the risk of inflammatory bowel disease. Scand J Gastroenterol. 2002; 37:1301-8.
27. Luther J, Dave M, Higgins PD et al. Association between Helicobacter pylori infection and inflammatory bowel disease: a meta-analysis and systematic review of the literature. Inflamm Bowel Dis. 2010; 16:1077-8429.
28. L Saidel-Odesa, S Odesb. Hygiene hypothesis in inflammatory bowel disease. Annals of Gastroenterology. 2014; 27:189-190.
29. Hunter MM, McKay DM. Review article: helminths as therapeutic agents for inflammatory bowel disease. Aliment Pharmacol Ther. 2004; 19:167-77.
30. Chu KM, Watermeyer G, Shelly L et al. Childhood helminth exposure is protective against inflammatory bowel disease: a case control study in South Africa. Inflamm Bowel Dis. 2013; 19:614-20.
31. Lopez-Serrano P, Pérez-Calle, Pérez-Fernandez T et al. Environmental risk factors in inflammatory bowel diseases. Investigating the hygiene hypothesis: A Spanish case–control study. Scandinavian Journal of Gastroenterology. 2010; 45:1464-1471.
32. Collins SM. Stress and the Gastrointestinal Tract: IV. Modulation of intestinal inflammation by stress: basic mechanisms and clinical relevance. Am J Physiol Gastrointest Liver Physiol. 2001; 280:G315– 8.
33. Walker JR, Ediger JP, Graff LA et al. The Manitoba IBD cohort study: a population-based study of the prevalence of lifetime and 12-month anxiety and mood disorders. Am J Gastroenterol. 2008; 103:1989-97.
34. Chapman-Kiddel CA, Davies PS, Gillen L et al. Role of diet in the development of inflammatory bowel disease. Inflamm Bowel Dis. 2010; 16:137-51.
35. D'Souza S, Levy E, Mack D, et al. Dietary patterns and risk for Crohn's disease in children. Inflamm Bowel Dis. 2008; 14:367-373.
36. Ananthakrishnan AN. Environmental triggers for inflammatory bowel disease. Curr Gastroenterol Rep. 2013; doi: 10.1007/s11894-012-0302-4.
37. Baron S, Turck D, Leplat C et al. Environmental risk factors in pediatric inflammatory bowel diseases: a population based case control study. Gut. 2005; 54:357-363.
38. Lammers KM, Lu R, Brownley J, et al. Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. Gastroenterology. 2008; 135:194–204.
39. Ventura A, Magazzù G, Greco L. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. Gastroenterology. 1999; 117:297–303.
40. Lashner BA, Shaheen NJ, Hanauer SB et al. Passive smoking is associated with an increased risk of developing inflammatory bowel disease in children. Am J Gastroenterol. 1993; 88:356-9.
41. Duggan AE, Usmani I, Neal KR et al. Appendicectomy, childhood hygiene, Helicobacter pylori status, and risk of inflammatory bowel disease: a case control study. Gut. 1998; 43:494-8.

Table 1. Baseline characteristics of the enrolled patients

	IBD		*CONTROLS*		
VARIABLES (n,%)	**CD** *(n=102)*	**UC** *(n=162)*	**OP** *(n=92)*	**SC** *(n=111)*	**p**
Median age (y; range)	11 (4-17)	10 (2-17)	10.9 (5-17)	14.4 (5-17)	0.3
Median age at diagnosis (y; range)	9 (2-16)	10 (2-17)	-	-	NA
Male Gender (n, %)	58 (56.8)	80 (49.3)	56 (60.8)	62 (55.8)	0.22
Living area (n, %)					0.08
Urban	73 (71%)	106 (65%)	64 (69%)	89 (80%)	
Rural	29 (28%)	56 (34%)	28 (30%)	22 (20%)	

Legend:
CD: Crohn's disease
IBD: Inflammatory Bowel disease
OP: Outpatients
SC: School children
UC: Ulcerative Colitis

Table 2. Association of Crohn's disease cases with childhood factors

Variables (n/ %)	CD (n=102)	Controls (n=203)	OR	IC (95%)	p*	p**
CD Family history	3 (3)	0 (0)	3.0	2.6 – 3.6	0.03	-
Autoimmune diseases	37 (37.0)	38 (19.4)	2.4	1.4 - 4.1	0.002	+
Mother degree	90 (88.2)	121 (60.5)	4.8	2.5 - 9.5	<0.001	+
Father employment	87 (85.3)	150 (74.6)	1.9	1.0 - 3.7	0.02	-
Family Stress	47 (46.1)	70 (34.5)	1.6	1.0 - 2.6	0.05	-
Bed sharing	18 (17.6)	75 (37.1)	0.3	0.2 - 0.6	<0.001	+
N° Siblings<2	70 (69.3)	102 (50.7)	2.1	1.3 - 3.6	0.002	+
N° Toilets> 1	75 (73.5)	125 (61.6)	1.7	1.0 - 2.9	0.04	-
Pets	18 (17.6)	70 (34.7)	0.4	0.2 - 0.7	0.001	+
Family Smoke	53 (52.5)	85 (41.9)	1.5	0.9 - 2.4	0.05	-
Family Parasitosis	3 (3.1)	32 (16.0)	0.1	0.04 - 0.5	0.001	+
Family H.Pylori	9 (9.2)	35 (17.9)	0.4	0.2 - 1	0.03	-
Antibiotic therapy	31 (30.4)	43 (21.2)	1.6	0.9 - 2.7	0.05	-
Low adherence to Mediterranean diet	30 (29.4)	34 (17.3)	1.9	1.1 -3.5	0.01	-
Breast feeding >3 months	61 (78.2)	93 (57.1)	2.7	1.4 - 5	0.001	+
Gluten < 6th month	54 (54.0)	58 (29.3)	2.8	1.7 - 4.6	<0.001	+

Legend
*X^2 test
** Significance after Bonferroni correction
CD: Crohn's disease; HC: Healthy Controls

Table 3. Association of Ulcerative Colitis cases with childhood factors

Variables (n/ %)	UC (n=162)	Controls (n=203)	OR	IC (95%)	p*	p**
UC Family history	5 (3.1)	0 (0)	2.3	2.0 - 2.5	0.016	-
Autoimmune diseases	65 (40.0)	38 (19.4)	2.8	1.7 - 4.6	<0.001	+
Father employment	140 (87.5)	150 (74.6)	2.3	1.3 - 4.1	0.002	+
Family Stress	88 (54.7)	70 (34.5)	2.2	1.4 - 3.5	<0.001	+
N° Siblings< 2	109 (67.7)	102 (50.7)	2.0	1.3- 3.1	0.001	+
N° Toilets> 1	125 (77.6)	125 (61.6)	2.1	1.3 - 3.4	0.001	+
Pets	38 (23.5)	70 (34.7)	0.5	0.3 - 0.9	0.02	-
Family Parasitosis	2 (1.3)	32 (16.0)	0.06	0.01 – 0.2	<0.001	+
Family H.Pylori	14 (8.9)	35 (17.9)	0.4	0.2 - 0.8	0.02	-
Low Mediterranean diet adherence	61 (37.7)	34 (17.3)	2.8	1.7 - 4.7	<0.001	+
Gluten < 6 months	88 (55.3)	58 (29.3)	2.9	1.9 - 4.6	<0.001	+

Legend
*X^2 test
** Significance after Bonferroni correction
UC: Ulcerative Colitis; HC: Healthy Controls

Table 4-Multivariate Logistic regression analysis for IBD children

Variables	*OR*	*CI 95%*	*p*
CD			
Mother's degree	5.5	2.5-11.6	0.01
Breast feeding> 3th month	4.3	1.6-10.5	0.002
Father's employment	3.7	1.2-8.7	0.008
Gluten introduction < 6th month	2.8	1.5-5.0	0.001
N° Siblings<2	2.8	1.5-5.3	0.01
Autoimmune diseases	2.7	1.4-5.3	0.003
Pets	0.3	0.1-0.7	0.007
Bed sharing	0.2	0.1-0.6	0.001
UC			
Low adherence to Mediterranean diet	2.3	1.2-4.5	0.01
Gluten introduction < 6th month	2.8	1.6-4.9	<0.001
N° Siblings< 2	2.0	1.1-3.6	0.01
Pets	0.4	0.2-0.8	0.004
Family Parasitosis	0.07	0.01-0.4	0.01

Legend
IBD: Inflammatory Bowel Disease
CD: Crohn's Disease
UC: Ulcerative Colitis

2.4 Enterocytes and dendritic cells both contribute to the intestinal inflammation in pediatric inflammatory bowel diseases.

Gut inflammation occurring in patients with IBD is characterized by the infiltration and activation of either adaptive branch, as T and B lymphocytes, and innate system, as macrophages and dendritic cells, which in turn produce massive amounts of proinflammatory cytokines contributing to the typical mucosal lesions.[65-67] It is well known that in CD cytokines released by T helper (Th)-1 cells are dominant, as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin-12 (IL-12), whereas the Th2 cytokines, IL-5 and IL-13, are predominantly found in UC [68] However, in recent times several evidences have underlined the role of enterocytes asnon immune inflammatory cells in the pathogenesis of IBD.[69] Enterocytes have a pivotal role in maintaining the integrity of intestinal mucosa, and guarantee the gut homeostasis by sampling the luminal agents through the several receptors expressed on their surface.[70]

Therefore, the aim of our study was to evaluate the cytokine production profile and the activation status of both lamina propria and epithelium cell compartments through an ex vivo analysis of colon biopsies obtained from pediatric patients with IBD.

Our ex vivo analysis of biopsies mainly obtained from the unaffected colonic area revealed an increased densities of cells producing TNFα in CD mucosa and IFN-γ in UC mucosa compared to control gut, although in both cases the percentage of positive cells was low and the differences were slight. Of note, we found a marked increase of cells expressing IL-15 in biopsies from both forms of IBD. Interestingly, when we determined the cell source of these pro-inflammatory cytokines by a multiparametric flow cytometry analysis, we found that both immune and nonimmune cells displayed an inflammatory phenotype in pediatric IBD intestine. EpCam+ enterocytes expressing IL-15, HLA class I and TNF-α were found more frequent in IBD compared to control biopsies. Moreover, in fresh intestinal tissues from both CD and UC young patients we observed a higher infiltrates of $CD11c^+$ dendritic cells co-expressing TNF-α and the activation marker HLA-DR than the healthy mucosa, whilst $CD11c^+$ DC producing INF-γ were more abundant in the gut of UC subjects. In steady state the biopsies from UC patients had an increased density of $INF\text{-}\gamma^+$ T lymphocytes, whilst T cells producing TNF-α were prevalently found in the mucosal explants of both CD and UC patients.

In conclusion, we demonstrated that in young subjects either immune competent cells, as T lymphocytes and dendritic cells, and nonimmune cells as enterocytes, have a pro-inflammatory phenotype in intestinal mucosa of pediatric cohort of patients with IBD. Our study underlines the relevance of gut epithelial cells as one of central mediators of mucosal inflammation in IBD. It is

becoming clearer that enterocyte has a role in intestinal mucosa much more active than it has been considered so far. Taking into account that intestinal epithelium cells represent a central node of mucosal cell networks, and that their dysfunction has been related with IBD pathogenesis, these cells could be a new therapeutic target for IBD. Indeed many studied reported that epithelial cells respond to TNF-α [71], and are a target of TNF-α inhibitors [72,73]As prominent source of intestinal IL-15 in IBD, monoclonal anti-IL-15 antibodies[74],or pharmacologic agents that can selectively block IL-15 signal transduction pathways, could be considered a new strategic target for biological therapies directed to these gastrointestinal diseases.[75] However further studies are needed to improve the knowledge of the role of epithelial cells in IBD pathogenesis in order to better state the possible efficacy of epithelial cells-based therapeutic opportunities and their application. On the other hand, the marked rising in the incidence of IBD in childhood[76], renders of particular interest such studies, evaluating ex vivo the phenotype and the cytokine profile of innate and adaptive immune cells in young IBD patients. Pediatric patients with IBD seem to be a distinctive population with specificities requiring highly skilled and specialized approach for diagnosis and treatment.

Enterocytes and dendritic cells both contribute to the intestinal inflammation in pediatric inflammatory bowel diseases

Short title: **Mucosal immunity in pediatric IBD**

Serena Vitale[1*], Caterina Strisciuglio[2,3*], Laura Pisapia[4], Erasmo Miele[3], Pasquale Barba[4], Alessandra Vitale[3], Sabrina Cenni[3], Giovanna Del Pozzo[4], Riccardo Troncone[3], Annamaria Staiano[3], Carmen Gianfrani[1,3,**]

[1]Institute of Protein Biochemistry, CNR, Naples, Italy; [2] Department of Woman, Child and General and Specialized Surgery, Second University of Naples, Naples, Italy; [3]Department of Translational Medical Science (Section of Pediatrics), and European Laboratory for the Investigation of Food-Induced Diseases, University Federico II, Naples, Italy; [4]Institute of Genetics and Biophysics Adriano Buzzati Traverso, CNR, Naples, Italy.

Original articles

*__These authors equally contributed to the work__

**__Corresponding author__

Dr Carmen Gianfrani, Dr Biol. Sc.

Institute of Protein Biochemistry-CNR

Via Pietro Castellino 111, 80131, Naples Italy

Tel: +39-081-6132265

Fax: +39-081-6132277

e-mail: c.gianfrani@ibp.cnr.it

Word count:

Guarantor of the article: Carmen Gianfrani declares that all authors had access to the study data, reviewed and approved the final version of the manuscript.

Specific author contributions:

Serena Vitale: performed and analyzed FACS experiments and drafted the manuscript

Caterina Strisciuglio: enrolled the patients, analyzed FACS data, and drafted the manuscript

Laura Pisapia: performed and analyzed FACS experiments

Erasmo Miele: enrolled patients and collected clinical data

Pasquale Barba: provided critical evaluation of all FACS data

Alessandra Vitale: performed biopsy experiments

Sabrina Cenni: performed ELISA experiments

Giovanna Del Pozzo: provided critical evaluation of all FACS data and revised the manuscript

Riccardo Troncone: provided critical revision of the study design and drafted the manuscritpt

Annamaria Staiano: supported the study, enrolled patients, and collected clinical data

Carmen Gianfrani: conceived the study, planned the experiments, interpreted data and revised the manuscript.

Financial support: This work has been supported by funding from CNR-DSB Progetto Bandiera "InterOmics" (MIUR PNR 2011-2013).

Potential competing interests: Annamaria Staiano is a speaker for Valeas S.p.A.(Milan, Italy), Angelini, Menarini, Milte S.p.A.; and consultant for D.M.G. (Italy). For the remaining authors no conflict of interests were declared .

ABSTRACT

Introduction: Both innate and adaptive immunity contribute to the mucosal lesions in inflammatory bowel diseases (IBD). Recent evidences have also underlined the role of enterocytes in the IBD inflammatory cascade. IBD is dramatically increasing in young subjects, however pediatric IBD pathogenesis is poorly investigated.

Methods: The phenotype and cytokine profile of intestinal cells from uninflamed area of pediatric patients with Crohn's disease (CD), ulcerative colitis (UC), and non-IBD controls (HC) were investigated through an ex-vivo analysis. IL-15, TNF-α and INF-γ production was evaluated in enterocytes (EpCam), dendritic cells (CD11c), and T lymphocytes (CD3) subsets, either in unstimulated (basal) or stimulated (polyclonal mitogens) conditions by flow cytometry and ELISA.

Results: A higher frequency of IL-15 and TNF-α expressing enterocytes, as well as of TNF-α and INF-γ producing dendritic cells was observed in IBD mucosa. Enhanced density of enterocytes was detected in UC biopsies, whilst no differences were found in dendritic cells(DC) or T cell infiltrates among the three groups. A few T cells producing TNF-α and IFN-γ were detected in HC mucosa, in the steady state condition, although the PMA/ionomycin stimulation induced a marked expansion of these T cells. By contrast, TNF-α and IFN-γ were found highly secreted by intestinal IBD cells after the phytohaemagglutinin polyclonal stimulus.

Conclusions: Our study demonstrates a pro-inflammatory phenotype mainly in intestinal epithelial and dendritic cell subsets in pediatric IBD. A pathogenic function of IL-15 in addition to TNF-α is also reported, thus suggesting that IL-15 is a potential therapeutic target in pediatric IBD.

Study Highlights:

WHAT IS THE CURRENT KNOWLEDGE

- The adaptive immune response has a central role in IBD intestinal inflammation.
- Emerging role of enterocytes as non immune pathogenic cells in the intestinal inflammatory disorders.
- In Crohn's Disease the Th1-mediated response is dominant, whereas Ulcerative colitis has been mainly associated to Th2 cells.
- IL-15 is a pleiotropic cytokine involved in mucosal inflammation of chronic and autoimmune intestinal disorders.
- Very little is known concerning the cellular and cytokine pathways occurring in pediatric IBD.

WHAT IS NEW HERE

- Analysis of activation and cytokine profiles of different cellular subsets in ex-vivo intestinal mucosa from pediatric IBD.
- Enhanced number of enterocytes producing IL-15 and TNF-α in IBD mucosa.
- Increased infiltration of TNF-α and IFN-γ secreting dendritic cell in IBD mucosa.
- Innate immune pathways mediated by epithelial and dendritic cells contribute to the intestinal mucosa inflammation in pediatric IBD.
- IL-15 could be a target of new therapeutic strategy for IBD.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) in which an abnormal immune response against the luminal microflora is thought to be the main causative factor [1]. Gut inflammation occurring in patients with IBD is characterized by the infiltration and activation of both adaptive branch, as T and B lymphocytes, and innate system, as macrophages and dendritic cells, which in turn produce massive amounts of proinflammatory cytokines contributing to the typical mucosal lesions [2, 3, 4]. It is well known that in CD cytokines released by T helper (Th)-1 cells are dominant, as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin-12 (IL-12), whereas the Th2 cytokines, IL-5 and IL-13, are predominantly found in UC [5], the great majority of them being produced by lamina propria mononuclear cells [6].

Dendritic cells (DC) are the most potent professional antigen-presenting cells, and in mucosal immunity DC have an important role in maintaining the fragile equilibrium between tolerance and inflammatory response to mucosal antigens [7]. The involvement of mucosal DC in IBD pathogenesis has been also documented [8], though very little is known on their functions in pediatric IBD.

Enterocytes have a pivotal role in maintaining the integrity of intestinal mucosa, and they guarantee gut homeostasis by sampling luminal agents through several receptors, such as the pathogen recognition receptors (PPRs) expressed on their surface [9]. Given the prominent role of enterocytes in the intestinal immune homeostasis, dysfunctions within the epithelial layer can be associated with IBD pathogenesis. Interestingly, very recent evidences underlined a prominent role of enterocytes as non immune inflammatory cells in the IBD mucosal lesions [10]. IL-15 is a pleiotropic cytokine that is expressed on the surface of monocyte, macrophages, dendritic cells and is markedly expressed on intestinal epithelial cells [11] in response to inflammatory stimuli.. IL-15 is involved in several inflammatory mechanisms mediated by both adaptive and innate immune systems [12, 13], and , furthermore , it is a key mediator of intestinal mucosa damage in celiac

disease [14]. IL-15 has various functions due to its complex mechanisms of action: it binds different IL-15 receptors (IL-15R) with specific affinities and signal transduction pathways [15]. IL-15 is an important growth and activator factor for mucosal intraepithelial lymphocytes [16], and it has also a marked anti-apoptotic function, as previously shown [17]. Quite recently, it has been demonstrated a higher expression of IL-15 in IBD colonic mucosa, thus suggesting a role of this cytokine in the IBD pathogenesis [18, 19].

The contribution of the adaptive, T cell-mediated response against luminal components in IBD intestinal lesions has been observed in many studies [20]. Particularly, the role of cytokines released by bacterial-primed T cells is undoubted, and several therapeutic approaches to block cytokine-mediated inflammatory cascades are currently under investigation [21]. While it has been proved the crucial function of T cells in IBD, many aspects of the inflammatory process in IBD mucosa still need to be clarified, in order to develop more appropriate and disease-specific therapeutic strategies. A deeper dissection of the cross-talk between the different mucosal compartments, such as enterocytes, dendritic cells and T lymphocytes, especially with regard to the cytokines mediating the interaction of both innate and adaptive immune cells, will represent a step forward in the knowledge of IBD intestinal inflammation [9].

Therefore, the aim of our study was to evaluate in pediatric patients with IBD the cytokine production profile and the activation status of both lamina propria and epithelial cells compartments, through an *ex vivo* analysis of intestinal mucosal explants.

PATIENTS AND METHODS

Study population

The study population included a total of 59 young subjects divided into 3 groups on the basis of the clinical diagnosis: 21 (mean age 13.1 yr; range 7.8-18 yr) were affected by CD, 19 (mean age 14 yr; range 8.6-18.4 yr) affected by UC, and 19 (mean age 10.4 yr; range 3.4-18.5 yr) were non-IBD controls (HC). HC were subjects affected by functional gastrointestinal disorders, who performed blood analysis to exclude any organic disease or an inflammatory condition. For the IBD diagnosis, all children underwent ileo-colonoscopy, upper GI endoscopy and imaging studies, including abdominal ultrasound and entero-MRI, or a small bowel follow-through. The diagnosis of CD and UC was based upon conventional clinical, radiological and endoscopic features and was confirmed by histopathological examination of the resection specimens [22]. Demographic and clinical characteristics of enrolled subjects are described in **Table1**. Written informed consent was obtained from participants' parents, or from patients themselves if older than 10 years of age. The study was approved by the Institutional Review Board of the University of Naples "Federico II".

Intestinal cell isolation and stimulation

Intestinal biopsies were taken from macroscopically uninflamed areas of ileum and colon traits. Biopsy samples were washed in saline solution and digested with collagenase A of *Clostridium hystolyticum* (1 mg/mL; Roche, Mannheim, Germany) in 2 mL of culture medium, RPMI-1640 (Lonza Group Ltd, Basel, Switzerland), supplemented with 1% penicillin/streptomycin antibiotics for 1 hour and 30 minutes at 37°C and 5% CO_2 stirring the plate every 15 minutes. The cellular suspension was then passed through a 40-μm cell strainer filter (BD Falcon, Durham, USA), the median value of intestinal cells isolated was $5.73x10^6$ cells and the range was $1.8\text{-}13x10^6$ cells.

To ensure that all the samples had the same treatment, intestinal cells (from 13 CD, 12 UC and 11 HC individuals) were cultured for 16 hours at the cellular density of $1x10^6$- 1.4 $x10^6$ cells in 24-wells plates (Sarstedt; Newton, NC, USA) in complete medium (RPMI-1640 with 10% fetal calf serum and supplements), with 100 ng/mL GM-CSF and 50U/mL of IL-2 (both from R&D System, Minneapolis, MN, USA). In order to analyze the spontaneous cytokine production (in a steady state condition), brefeldin A (5 µg/ml; Sigma-Aldrich) was added to cell culture for 5 hours of incubation, whilst the mitogen-induced cytokine production was analyzed by adding a mixture of phorbol 12-myristate 13-acetate (stock 40.5 µM), ionomycin (stock 670 µM), brefeldin A (stock 5.3 mM), monesin (stock 1 mM) at the final dilution 1:500, as indicated by the manufacturers' instruction (Cell Stimulation Cocktail, eBioscience Inc. SanDiego, CA) for 5 hours. Then, stimulated and unstimulated cells were harvested and treated for flow cytometry analysis.

In the experiments, to measure cytokine production in the culture supernatants, intestinal cells ($2x10^5$ cells/well) obtained from 8 CD, 7 UC and 8 HC, were plated in triplicates in 96-wells plates (BD Bioscience, Oxford, UK) and stimulated with medium alone or with phytohaemagglutinin (PHA, 2 µg/ml; Sigma-Aldrich, Stockholm, Sweden). After 48 hours of incubation, cell supernatants were stored at 20°C until the use.

Flow Cytometry

Intestinal cells were stained with fluorochrome-labeled monoclonal antibodies against the following surface markers (-specific fluorochrome): CD3-PerCP-Vio700, CD11c-PE, HLA-DR-PerCP, CD326 (EpCam)-APC, HLA Class I-PE-Cy5, IL-15-PE. Intracellular cytokine staining was performed with the fluorochrome-conjugated monoclonal antibodies IFN-γ-APC and TNF-α-APC/Cy7. Appropriate isotype-matched control monoclonal antibodies were included in all staining experiments. All antibodies were purchased from BD Biosciences (San Jose, CA), or Miltenyi Biotec (Bologna, Italy), and used at concentration according to the manufacturer's instructions. At least $1x10^5$ viable cells (assessed at microscope by trypan blue dye exclusion) were used for each

staining done in phosphate saline (PBS)/0.5% bovine serum albumin (BSA) buffer. Surface-staining of cells was carried-out at 4°C for 30 minutes; then the cells were fixed with 2% paraformaldehyde and intracellular staining was performed in permeabilization buffer (PBS/BSA 0,5% with 0,5% saponin). All multi-color flow cytometry analyses included the appropriate fluorescence-minus-one control (FMOC) sample. Samples were acquired with FACS Canto II flow cytometer (BD Biosciences) and results analyzed with FlowJo software (Miltenyi Biotec). All analyses of cytokine-stained cells were done within a gate based on their forward-scatter/side-scatter characteristics that excluded dead cells, as assessed by propidium iodide staining run in parallel.

Cytokine quantification in culture supernatants

The concentrations of TNF-α and IFN-γ were analyzed in cell supernatants, collected after 48 hours of incubation with the mitogen. The cytokines production was measured using commercially available sandwich ELISA kits provided by Mabtech, (Nacka Strand, Sweden), according to the manufacturer's instructions. The sensitivity of ELISA kit was 12 pg/mL for TNF-α and 2 pg/mL for IFN-γ.

Statistical Analysis

Statistical analysis was performed using SPSS software package for Windows (version 13.0; SPSS, Chicago, IL). The Mann Whitney U test/Wilcoxon test for normally distributed variables were used where appropriate. A p value < 0.05 was considered statistical significant.

RESULTS

Cytokine production profile in intestinal IBD mucosa

By an *ex-vivo* approach, we have analyzed the frequency of intestinal mucosal cells that spontaneously produced cytokines in uninflamed area of young patients with IBD or non-disease controls. We detected the frequency of cells spontaneously producing IL-15, IFN-γ or TNF-α by flow cytometry in colonic biopsies from 13 CD, 12 UC, and 11 HC pediatric patients, without further *in vitro* stimulation with mitogen.

In particular, the evaluation of IL-15-positive cells was determined by a membrane surface staining, whilst the number of IFN-γ- and TNF-α-positive cells was assessed by intracellular staining with specific monoclonal antibodies. The percentage of IL-15-cells resulted significantly higher in the mucosa of CD and UC patients compared to non-IBD controls (CD: median 3.41, range 1.4 - 9.9%; UC: 6.14, 2.3 - 11.8%; HC: 2.3, 0 - 6.6%; $p<0.03$ UC vs HC, $p< 0.05$ CD vs HC), **Figures 1A&B**. An increase of cells producing IFN-γ was observed in UC though no statistical significant differences were found compared to other two groups, **Figure 1C**. Surprisingly, when we looked at the intracellular TNF-α positive cells, no marked differences were observed between the IBD and non-inflamed mucosa, **Figure 1D.**

Levels of TNF-α and IFN-γ secreted by intestinal mucosa cells

To further investigate the cytokine production profile in intestinal mucosa of IBD pediatric patients, we measured the IFN-γ and TNF-α secreted by intestinal cells either at basal condition (unstimulated) or after a mitogen stimulation (PHA) by enzyme-linked immunosorbent assay (ELISA). In agreement with FACS analysis, we found no differences in the levels of IFN-γ in unstimulated conditions among the three groups (CD: 204.4, 0 - 285 pg/mL; UC: 101.6, 0 - 406.3 pg/mL; HC: 151.1, 0 - 376.3 pg/mL; p=ns). Notably, upon the activation with PHA mitogen, a

marked IFN-γ production was detected in cell supernatants from IBD patients (CD: 1194.8, 188 - 3991.2 pg/mL; UC: 2062.4, 89.7 - 3767.5 pg/mL, $p<0.01$ CD stimulated vs CD unstimulated, $p<0.03$ UC stimulated vs UC unstimulated), whilst the IFN-γ level measured in cultures from HC intestinal mucosa (HC: 221.8, 65.8 - 405.1 pg/mL) resulted much lower compared to IBD mucosa ($p<0.02$, CD vs HC; $p<0.04$, UC vs HC), **Figure 2A**. A sustained production of TNF-α upon PHA stimulation was observed in both CD and UC mucosal cells (CD: 584.5, 346.7 - 691.2 pg/mL; UC: 316, 91.7 - 677.8 pg/mL) compared to unstimulated cells (CD: 255.2, 0 - 461.2 pg/mL; UC: 58.7, 0 - 577.8 pg/mL), though the statistical significance was only reached in CD intestinal cells ($p\leq0.05$). Unexpectedly, high levels of TNF-α were found in cell supernatants from HC biopsies despite the experimental conditions (348.4, 0 - 712 pg/mL; 423.4, 105.3 - 751.7 pg/mL, respectively in baseline or PHA-stimulated cultures), **Figure 2B**.

IL-15 was not measured in cell supernatants, since this cytokine is biologically active when bound to its specific receptor on the cell membrane surface [23].

Phenotype of inflammatory cytokine producing cells in colonic mucosa

To further dissect the phenotype and the activation status of various cell types producing cytokines in intestinal mucosa of young patients with IBD, we performed additional ex-vivo flow cytometry analysis. By using specific cell markers, we investigated enterocytes as EpCam (Epithelial Cell Adhesion Molecule) positive cells, dendritic cells as CD11c positive cells, and T lymphocytes defined as CD3 positive cells. More specifically, we performed the following fluorochrome multicolor staining combinations: i) EpCam, IL-15, TNF-α, HLA Class I; ii) CD11c, HLA DR, TNF-α, IFN-γ; iii) CD3, TNF-α, IFN-γ.

i) ***Enterocytes***

We first assessed the level of enterocyte expansion in uninflamed mucosal tissues, by detecting the percentage of EpCam+ cells. We found that the proportion of EpCam+ cells was

significantly higher in UC compared to the healthy mucosa (UC: 10.02, 5.1 - 16.5%; HC: 6.3, 1.9 - 10.3%, $p<0.05$). Interestingly, the number of EpCam stained cells isolated from UC mucosa resulted also greater than the level found in CD mucosa (CD: 6.7, 3.4 - 12.3%, $p<0.06$), **Figure 3A**. Several studies have reported that enterocytes are an important intestinal source of inflammatory IL-15 both in healthy and in food related disorders, as celiac disease [24, 25]. We next evaluated whether the enhanced levels of IL-15 expression we found in IBD mucosa (Figure 1A&B) could be specifically produced by enterocytes. Interestingly, a greater number of EpCam and IL-15 double positive cells was observed in intestinal samples from IBD patients, and particularly from those with UC (CD: 1.5, 0.5 - 3.2%; UC: 1.9, 1.2 - 5.4%; HC: 0.9, 0.2 - 2.7%; $p<0.02$ UC vs HC and $p<0.06$ CD vs HC), **Figures 3B&C**.

Because it has been shown that the intestinal epithelial cells are able to produce TNF-α [26], we next looked at TNF-α production in the EpCam+ cells subset. An increased density of EpCam+ cells producing TNF-α was found in IBD mucosa compared to healthy colonic tissues (CD: 0.7, 0.3 - 2.4%; UC: 0.8, 0.5 - 1.1%; HC: 0.3, 0.1 - 0.61%; $p<0.02$ UC vs HC and $p<0.05$ CD vs HC), indicating that enterocytes are a source of proinflammatory TNF-α in colonic tissues of young patients with IBD, **Figures 4A&B**.

Next, we looked at the Human Leukocyte Antigen (HLA) Class I expression on enterocytes, as this molecule is constitutively expressed on almost all cells either of immune or nonimmune systems, but strongly upregulated in response to infection or stress signals [10,27]. Consistently with above findings (Figure 3A), UC mucosa displayed an increased number of HLA Class I-expressing enterocytes in comparison to the non inflamed mucosa (UC: 6, 4.6 - 7.7%; HC: 3.9%, 2.1 - 5.1%; $p<0.04$), **Figures 4C&D.**

Dendritic cells

The majority of studies addressing the phenotype and function of mucosal DC have used the CD11c expression as specific surface marker of dendritic cell lineage [28], though macrophages, monocytes, neutrophils, and some B cells may express this molecule [29]. We found similar

percentages of CD11c positive dendritic cells (CD: 4.5, 3.1 - 10.4 %; UC: 3.9, 1.9 - 7.1%; HC: 3.5, 2.1 - 7.4%, p=ns) in mucosal samples from the IBD and control mucosa, **Figures 5A&B**.

Nevertheless, when we focused the analysis to the specific region of CD11c positive dendritic cell subsets, we observed that DC were more activated in IBD than in healthy mucosa, as they expressed the activation marker HLA-DR, as well as the pro-inflammatory cytokines TNF-α and IFN-γ. More specifically, we found within the CD11c+ subset a higher density of cells expressing HLA-DR in IBD mucosa than in control mucosa at the basal, "steady" state, condition (CD: 45, 27.2 - 57.5%; UC: 47.7, 26.3 - 62.4%; HC: 38.7, 24.3 - 47.2%) with a statistical significant increment in UC versus HC ($p<0.04$), **Figure 5C,** as well as an enhanced frequency of activated cells producing TNF-α (CD: 9.2, 7.8 - 19.5%; UC: 9.2, 6.7 - 24.8%; HC: 5.1, 2.6 - 18.9%; $p<0.03$ CD vs HC and $p<0.06$ UC vs HC) and IFN-γ (CD: 8.5, 2.7 - 20.1%; UC: 12.6, 4.1 - 15.6%; HC: 6.1, 3.5 - 7.2; $p<0.03$ UC vs HC), **Figures 5D&E.** Notably, these findings are in accordance with our previous report on peripheral blood monocyte-derived DC, showing a pro-inflammatory phenotype of dendritic cells in pediatric IBD [30].

iii) ***T cells***

Finally we expanded the ex vivo analysis to T (CD3+) cells. As observed in mucosal DC subset, we did not find differences in the percentage of T lymphocytes among the three pediatric groups (CD: 55.4, 49.6 - 73%; UC: 53.7, 33.1 - 74.8%; HC: 55.7, 42.6 - 68.5%), **Figure 6A**. Similarly, no differences in the frequency of CD3+ cells spontaneously producing TNF-α (CD: 2.87, 0.2 - 6.2%; UC: 3.2, 1.1 - 7.9%; HC: 2.2, 0.5 - 5.6%), and IFN-γ (CD: 0.9, 0.1 - 2.7%; UC: 1.5, 0.4 - 4.3%; HC: 0.9, 0.2 - 2.1%), were detected among the three groups (**Figures 6B&C)**.

To further analyze the production of these two inflammatory cytokines in T lymphocytes infiltrating the pediatric IBD mucosa, we did additional experiments to examine the cytokine production in CD3+ cells induced by a strong mitogenic stimulus PMA/Ion as described in M&M. Unexpectedly, we observed an increased percentage of CD3+ cells positive for both TNF-α

(**Figures D&E**) and IFN-γ (**Figures F&G**) in control mucosa with a significant difference in the frequency of CD3+ TNF-α+ cells between HC and UC patients (TNF-α, CD: 18.4, 6.0 - 27%; UC: 17.2 , 6.5 - 25.4 %; HC: 24.6, 15.9 - 32.1%, p<0.04 HC vs UC; IFN-γ, CD: 5.4, 1.7 - 22.8%; UC: 8.2, 2.9 - 25.5%; HC: 9.6, 7.7 - 15.7%, p=ns).

DISCUSSION

Through an ex-vivo analysis we have investigated the cytokine profile and the phenotype of cells producing cytokines in the intestinal mucosa of pediatric subjects with Crohns' disease and ulcerative colitis, the main forms of inflammatory bowel diseases. The cell subsets, such as enterocytes, dendritic cells, and T cells, were observed in fresh mucosal explants by an experimental approach based on flow cytometry analysis and enzyme-linked immunosorbent assay. Furthermore, we investigated the density and the pattern of cell cytokines release of both lamina propria and epithelium cell subsets, and we specifically compared the unaffected areas of IBD intestinal tissues to healthy mucosa of non-IBD young subjects. We found a marked increase of cells expressing IL-15 in both CD and UC in comparison with healthy mucosa. *In addition, TNFα and IFN-γ producing cells were slightly enhanced in IBD compared to control gut. By using specific cell markers and a multiparametric flow cytometry analysis, we revealed that both immune and non-immune cells were source of these pro-inflammatory cytokines in pediatric IBD intestine. EpCam+ enterocytes expressing IL-15, HLA class I and TNF-α were more frequent in IBD compared to non-IBD controls biopsies, as well as CD11c+ dendritic cells co-expressing the activation marker HLA-DR and producing TNF-α or INF-γ, that were more abundant in the IBD mucosa. Interestingly, these findings reflected the steady state cytokine production most likely induced by in vivo intestinal microenvironment stimuli. Of note, in our experimental basal condition, no marked differences were observed in TNF-α and INF-γ producing T cells subset among the three groups.

It is known that there is an enhanced expression of pro-inflammatory cytokines in gut mucosa from both UC (mostly Th2 cytokines:, IL-5, IL-9, IL-13) and CD (mostly Th1 cytokines: IFN-γ, TNF-β) [5], and it has been postulated these cytokines are directly responsible of tissue injury [6, 31, 32]. Most studies have investigated the pathological events occurring in inflamed areas of IBD intestinal mucosa, however, it is unclear the status of cells activation and the cytokine profile in unaffected IBD tissues, though high levels of pro-inflammatory cytokines in the unaffected areas of IBD intestine were also reported [33, 34]. In particular, Leon et al found that several cytokines, such as IFNγ, TNFα, IL-6, IL-15, IL-18 and IL-23, were increased in both affected and unaffected areas from IBD gut specimens compared to healthy controls, measured by ELISA and mRNA expression [34].

It has been documented that in IBD patients, the intestinal homeostasis system is disrupted and an innate immune response occurs at the level of the enterocytes barrier [35], with tight junction weakening as well as oxidative and immune stress, and inhibition of homeostatic signals secretion[36]. Recent data have highlighted the role of IL-15 as a key player in IBD mucosa lesions, in similar extent to TNF-α and INF-γ [19]. Herein, we demonstrated that there is a higher density of cells producing IL-15 in both CD and UC pediatric intestine. Furthermore, a sustained fraction of these IL-15+ cells is represented by EpCAM+ enterocytes. To the best of our knowledge, this is the first study reporting a IL-15+ cells infiltration in the gut mucosa of young subjects with IBD. IL-15 is a type I cytokine [37, 38] expressed by non- lymphoid cells and that shares the β chain receptor with IL-2 (IL-15/IL-2Rβ) [39]. A large body of findings have indicated that IL-15 is an important mediator of intestinal immune homeostasis, as it exerts an important growth and anti-apoptotic function on intraepithelial TCRγδ+ T cells [40], NK/NKT [41], and cytotoxic CD8+ T cells [42, 43]. However, an IL-15 overexpression has been reported in several pathological gut conditions, as celiac disease [14, 24, 44, 45] and chronic inflammatory disorders [18, 19, 34, 46, 47]. In these latter cases the vast majority of studies, investigating the pathogenic role of mucosal IL-15, has looked at the total amount of mRNA transcripts, protein levels by ELISA and western blot in

intestinal specimens from adult IBD patients. More specifically, Nishiwaki and co-workers found an increased expression of mRNA of both IL-15 and IL-15Rα receptor in the mucosal tissues of IBD patients, especially in subjects with ulcerative colitis [19]. Interestingly, more recently, Leon et al. showed higher levels of IL-15 in intestinal biopsies from UC and CD patients [34], thus in line with our current findings.

Along the intestinal tract, the enterocytes are one of the main source of cells expressing IL-15, [11]. By taking advantage of the specific epithelial cell marker EpCam, we monitored the density and the phenotype of intestinal epithelial cells in mucosal biopsies of pediatric IBD patients. Both in CD and UC the enterocytes produced TNF-α and expressed IL-15, and furthermore, a higher number of Epcam+ cells was found in the gut mucosa of UC patients in comparison to CD and control subjects. The increased frequency of enterocytes in UC mucosa could be due to an intestinal epithelium regeneration, as reported in UC patients [48]. Indeed, our results are in accordance to previous studies suggesting that the intestinal epithelium has a strategic role in gut inflammation, not only as a protective physical barrier to luminal microbiota, but it actively contributes to the activation and proliferation of different cellular components of mucosal immune systems [10].

Within the gut mucosa, DC have a principal role to guarantee the tissue integrity, the protection from pathogens, as well as the tolerance to dietary components [49]. In IBD, DC recruited at the lamina propria have an activated phenotype, they capture and present mucosal antigens to proinflammatory T lymphocytes. These latest cells secrete cytokines and are involved in tissue injury and in the loss of immune tolerance [50]. In the gut mucosa of pediatric IBD patients the DC, identified as CD11c positive cells, had a pro-inflammatory phenotype, indeed they expressed HLA-DR and produced TNF-α and INF-γ. Of note, we did not find a DC subset expansion in both CD and UC mucosa. One possible explanation is that we analyzed the phenotype and cytokine profile of DC taken from uninflamed mucosal areas and in steady state condition. The peculiar changes in gut bacterial profiles associated with these diseases in contrast to the “healthy

microbiota" presented in controls, may trigger the DC activation observed in pediatric IBD mucosa [51].

As T lymphocytes are key player in the IBD inflammatory process, by the ex-vivo analytical approach we looked at the cytokine production profile of T lymphocyte infiltrates in IBD mucosa. Similarly to the DC findings, we did not observe an enhanced density of CD3+ T cells in IBD compared to healthy samples. By contrast, we found a slight increase of T cells from uninflamed areas of IBD gut spontaneously producing TNF-α and INF-γ. Quite unexpectedly, after a strong stimulation, such as with PMA/ionomycin, a higher number of activated T cells producing TNF-α and INF-γ was found in control compared to IBD patients.. These data, in evident contrast with the baseline findings, markedly highlighted that pro-inflammatory T cells are also present in healthy mucosa and they are prone to release TNF-α and /or INF-γ upon strong intestinal stimuli, which overcome the regulatory pathways occurring in healthy mucosa. Therefore, the presence of T regulatory cells controlling the immune responses in healthy gut, or alternatively the intestinal microenvironment favoring the dysbiosis and the inflammatory reaction in IBD could explain our results.

However, the secretion of T cells cytokines can be influenced by the mitogens, chosen for T cell activation, and the different production profile may depend on the methods and the conditions used in the experimental approaches [52].

The importance of the specific stimuli influence in determining the cytokine profile of intestinal cells was further confirmed, when we measured the levels of TNF-α and INF-γ secreted upon PHA incubation, which is a stimulus less powerful than PMA/ionomycin for the Th1 cytokine secretion, as showed by previous studies [53, 54]. A higher production of these two cytokines were detected both in CD and UC cells, but not in healthy controls. The latest results re-marked a dysregulated activation of mucosal cells related to a specific local stimuli, that occurs in IBD patients.

In conclusion, we demonstrated that both immune competent cells, such as T lymphocytes and dendritic cells, and nonimmune cells such as enterocytes, have a pro-inflammatory phenotype in intestinal mucosa of pediatric patients with IBD. Our study underlines the relevance of gut epithelial cells as one of the central mediator of mucosal inflammation in IBD. It is becoming clearer that the enterocytes have a role in intestinal mucosa much more active than it has been considered so far. Taking into account that intestinal epithelium cells represent a central node of mucosal cell networks, and that their dysfunction has been related with IBD pathogenesis, these cells could be a new therapeutic target for IBD. Indeed many studied reported that epithelial cells respond to TNF-α [55], and they are a target of TNF-α inhibitors [56, 57]. Moreover since there is a prominent production ofIL-15 in IBD intestinal mucosa, monoclonal anti-IL-15 antibodies or pharmacologic agents, that can selectively block IL-15 signal transduction pathways, could be considered as an alternative biological therapy[58]. However, further studies are needed to improve the knowledge of the role of epithelial cells in IBD pathogenesis, in order to better state the possible efficacy of epithelial cells-based therapeutic opportunities and their applications. On the other hand, the marked rising in the incidence of IBD in childhood [59], renders of particular interest such studies, evaluating ex vivo the phenotype and the cytokine profile of innate and adaptive immune cells in young IBD patients. Indeed, pediatric patients with IBD seem to be a distinctive population with specificities requiring highly skilled and specialized approach for diagnosis and treatment.

Abbreviations:

List of abbreviations: IBD: inflammatory bowel disease, CD: Crohn's disease, UC: ulcerative colitis, HC: non-IBD children, PPRs: pathogen recognition receptors, IFN-γ: interferon-γ , TNF-α: tumor necrosis factor-α, IL-12: interleukin-12, PBS: phosphate buffered saline, BSA: bovine serum albumin, FMOC: fluorescence-minus-one control, ELISA: enzyme linked immunosorbent assay, PHA: phytohaemagglutinin , EpCam: Epithelial Cell Adhesion Molecule, HLA: Human Leukocyte Antigen, DC: dendritic cells.

Acknowledgment:

Authors would like to thank all of the participants in the study.

REFERENCES

1. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003; 3:521–33.

2. Cianci R, Pagliari D, Pietroni V, et al. Tissue infiltrating lymphocytes: the role of cytokines in their growth and differentiation. J Biol Regul Homeost Agents. 2010; 24:239-49.

3. Di Sabatino A, Calarota SA, Vidali F, et al. Role of IL-15 in immune-mediated and infectious diseases. Cytokine and Growth Factor Rev. 2011; 22:19-33.

4. Pandolfi F, Cianci R, Pagliari D, et al. Cellular mediators of inflammation: tregs and TH17 cells in gastrointestinal diseases. Mediators Inflamm. 2009; 2009:132028.

5. Neurath MF, Finotto S and Glimcher LH The role of Th1/Th2 polarization in mucosal immunity. Nat Med. 2002; 8:567-573.

6. Biancheri P, Di Sabatino A, Ammoscato F, et al. Absence of a role for interleukin-13 in inflammatory bowel disease. Eur J Immunol. 2014; 44:370-85.

7. Bernardo D. Human intestinal dendritic cells as controllers of mucosal immunity. Rev Esp Enferm Dig. 2013; 105:279-90.

8. Niess JH. Role of mucosal dendritic cells in inflammatory bowel disease. World J Gastroenterol. 2008; 14:5138-48.

9. Geremia A, Biancheri P, Allan P, et al. Innate and adaptive immunity in inflammatory bowel disease. Autoimmunity Rev. 2014; 13:3-10.

10. Danese S. Nonimmune cells in inflammatory bowel disease: from victim to villain. Trends Immunol. 2008; 29:555-64.

11. Reinecker HC, MacDermott RP, Mirau S, et al. Intestinal epithelial cells both express and respond to interleukin 15. Gastroenterology. 1996; 111:1706-13.

12. Pagliari D, Cianci R, Frosali S, et al. The role of IL-15 in gastrointestinal diseases: a bridge between innate and adaptive immune response. Cytokine Growth Factor Rev. 2013; 24:455-66.

13. Budagian V, Bulanova E, Paus R, et al. IL-15/IL-15 receptor biology: a guided tour through an expanding universe. Cytokine Growth Factor Rev. 2006; 17:259-80.

14. Maiuri L, Ciacci C, Auricchio S, et al. Interleukin 15 mediates epithelial changes in celiac disease. Gastroenterology 2000; 119:996-1006.

15. Anderson DM, Kumaki S, Ahdieh M, et al. Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. J Biol Chem. 1995; 270:29862-29869.

16. Fuchs A, Vermi W, Lee JS, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15- responsive IFN-gamma-producing cells. Immunity 2013; 38:769-81.

17. Malamut G, El Machhour R, Montcuquet N, et al. IL-15 triggers an antiapoptotic pathway in human intraepithelial lymphocytes that is a potential new target in celiac disease-associated inflammation and lymphomagenesis. J Clin Invest. 2010; 120:2131-43.

18. Vainer B, Nielsen OH, Hendel J, et al. Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease. Cytokine 2000; 12:1531-6.

19. Nishiwaki T, Ina K, Goto H, et al. Possible involvement of the interleukin-15 and interleukin-15 receptor system in a heightened state of lamina propria B cell activation and differentiation in patients with inflammatory bowel disease. J Gastroenterol. 2005; 40:128-36.

20. Fuss IJ, Neurath M, Boirivant M, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol. 1996; 157:1261-70.

21. Baumgart DC, Sandbor WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet. 2007; 369:1641-1657.

22. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. Inflamm Bowel Dis. 2011; 17:1314-21.

23. Imamura M, Shook D, Kamiya T, et al. Autonomous growth and increased cytotoxicity of natural killer cells expressing membrane-bound interleukin-15. Blood. 2014; 124:1081-8.

24. Ma LJ, Acero LF, Zal T, J Immunol. Trans-presentation of IL-15 by intestinal epithelial cells drives development of CD8αα IELs. J Immunol. 2009; 183: 1044-1054.

25. Abadie V and Jabri B. IL-15: a central regulator of celiac disease immunopathology. Immunol Rev. 2014; 260:221-234.

26. Tanahashi T, Kita M, Kodama T, et al. Cytokine expression and production by purified Helicobacter pylori urease in human gastric epithelial cells. Infect Immun. 2000; 68:664-71.

27. Garcia-Lora A, Algarra I and Garrido F. MHC class I antigens, immune surveillance, and tumor immune escape. J Cell Physiol. 2003; 195:346-55.

28. Raki M, Tollefsen S, Molberg Ø, et al. A unique dendritic cell subset accumulates in the celiac lesion and efficiently activates gluten-reactive T cells. Gastroenterology 2006; 131:428–438.

29. Rescigno M and Di Sabatino A. Dendritic cells in intestinal homeostasis and disease. J Clin Invest. 2009; 119:2441-50.

30. Strisciuglio C, Miele E, Giugliano FP, et al. Bifidobacteria Enhance Antigen Sampling and Processing by Dendritic Cells in Pediatric Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015; 21:1491-8.

31. Leòn A.J, Garrote J.A and Arranz E. Cytokines in the pathogenesis of inflammatory bowel diseases. Med Clin. 2006; 127:145-52.

32. MacDonald TT and Monteleone G. Immunity, inflammation, and allergy in the gut. Science. 2005; 307:1920-5.

33. Reimund JM, Wittersheim C, Dumont S, et al. Increased production of tumour necrosis factor-α, interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. Gut. 1996; 39: 684-689.

34. Leòn AJ, Gòmez E, Garrote JA, et al. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. Mediators Inflamm. 2009; 2009: 580450.

35. Coskun M. Intestinal epithelium in inflammatory bowel disease. Front Med (Lausanne). 2014; 1:24.

36. Su L, Nalle SC, Shen L, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. Gastroenterology. 2013; 145:407-15.

37. Fehniger TA and Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001; 97:14-32.

38. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol. 2006; 6:595-601.

39. Giri JG, Ahdieh M, Eisenman J, et al. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. 1994; 13:2822-30.

40. Maiuri L, Ciacci C, Vacca L, et al. IL-15 drives the specific migration of CD94+ and TCR-gammadelta+ intraepithelial lymphocytes in organ cultures of treated celiac patients. Am J Gastroenterol. 2001; 96:150-6.

41. Carson WE, Fehniger TA, Haldar S, et al. A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest. 1997; 99:937-43.

42. Mortier E, Advincula R, Kim L, et al. Macrophage- and dendritic cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8(+) T cell subsets. Immunity. 2009; 31:811-22.

43. Castillo EF, Schluns KS. Regulating the immune system via IL-15 transpresentation. Cytokine.

33. Reimund JM, Wittersheim C, Dumont S, et al. Increased production of tumour necrosis factor-α, interleukin-1β, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. Gut. 1996; 39: 684-689.

34. Leòn AJ, Gòmez E, Garrote JA, et al. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. Mediators Inflamm. 2009; 2009: 580450.

35. Coskun M. Intestinal epithelium in inflammatory bowel disease. Front Med (Lausanne). 2014; 1:24.

36. Su L, Nalle SC, Shen L, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. Gastroenterology. 2013; 145:407-15.

37. Fehniger TA and Caligiuri MA. Interleukin 15: biology and relevance to human disease. Blood. 2001; 97:14-32.

38. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol. 2006; 6:595-601.

39. Giri JG, Ahdieh M, Eisenman J, et al. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. 1994; 13:2822-30.

40. Maiuri L, Ciacci C, Vacca L, et al. IL-15 drives the specific migration of CD94+ and TCR-gammadelta+ intraepithelial lymphocytes in organ cultures of treated celiac patients. Am J Gastroenterol. 2001; 96:150-6.

41. Carson WE, Fehniger TA, Haldar S, et al. A potential role for interleukin-15 in the regulation of human natural killer cell survival. J Clin Invest. 1997; 99:937-43.

42. Mortier E, Advincula R, Kim L, et al. Macrophage- and dendritic cell-derived interleukin-15 receptor alpha supports homeostasis of distinct CD8(+) T cell subsets. Immunity. 2009; 31:811-22.

43. Castillo EF, Schluns KS. Regulating the immune system via IL-15 transpresentation. Cytokine.

2012; 59:479-90.

44. Mention JJ, Ben Ahmed M, Bègue B, et al. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. Gastroenterology. 2003 ;125:730-

45. Di Sabatino A, Ciccocioppo R, Cupelli F, et al. Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. Gut. 2006; 55:469-77.

46. Kirman I and Nielsen OH. Increased numbers of interleukin-15-expressing cells in active ulcerative colitis. Am J Gastroenterol. 1996; 91:1789-94.

47. Liu Z, Geboes K, Colpaert S, et al. IL-15 is highly expressed in inflammatory bowel disease and regulates local T cell-dependent cytokine production. J Immunol. 2000; 164:3608-15.

48. Dahan S, Roda G, Pinn D, et al. Epithelial: lamina propria lymphocyte interactions promote epithelial cell differentiation. Gastroenterology. 2008;134:192-203.

49. Kelsall BL and Rescigno M. Mucosal dendritic cells in immunity and inflammation. Nat Immunol. 2004; 5:1091-5.

50. Agace WW. T-cell recruitment to the intestinal mucosa. Trends Immunol. 2008; 29:514-22.

51. Burman S, Hoedt EC, Pottenger S, et al. An (Anti)-Inflammatory Microbiota: Defining the Role in Inflammatory Bowel Disease? Dig Dis. 2016; 34:64-71.

52. Olsen I and Sollid LM. Pitfalls in determining the cytokine profile of human T cells. J Immunol Methods. 2013; 390:106-12.

53. Rostaing L, Tkaczuk J, Durand M, et al. Kinetics of intracytoplasmic Th1 and Th2 cytokine production assessed by flow cytometry following in vitro activation of peripheral blood mononuclear cells. Cytometry. 1999; 35:318-328.

54. Baran J, Kowalczyk D, Ozóg M, et al. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. Clin Diagn Lab Immunol. 2001; 8:303-13.

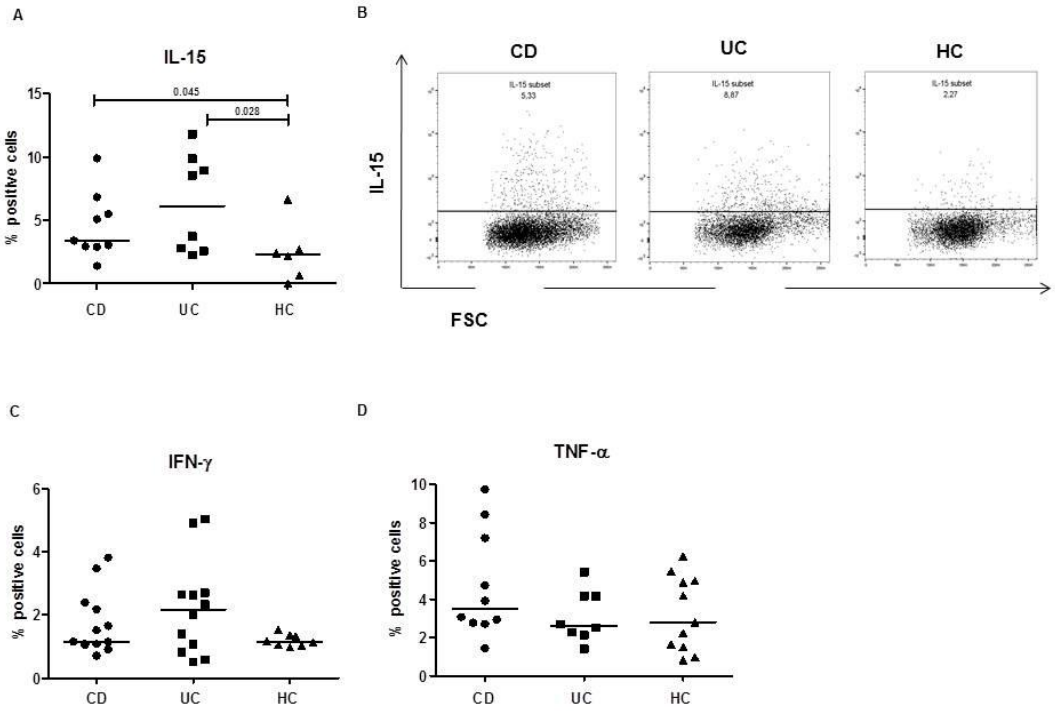
55. Bamias G, Dahman Ml, Arseneau KO, et al. Intestinal specific TNF alpha overexpression induces Crohn's-like ileitis in mice. PLoSOne. 2013; 8:e72594.

56. Hassan C, Ierardi E, Burattini O et al. Tumour necrosis factor alpha down-regulation parallels inflammatory regression in ulcerative colitis patients treated with infliximab. DigLiverDis. 2007; 39:811-7.

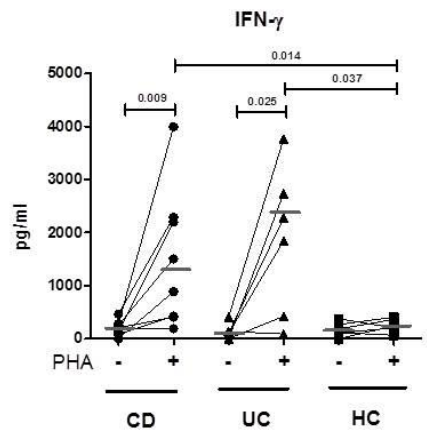
57. Fischer A, Gluth M, Pape UF, et al. Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF on tight junction proteins and signaling pathways in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol. 2013; 304:G970-9.

58. Sollid LM and Khosla C. Novel therapies for coeliac disease. J Intern Med. 2011; 269:604-13.

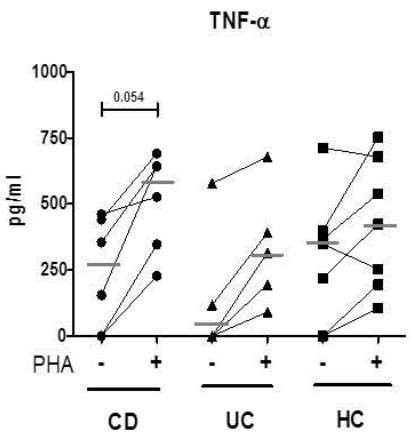
59. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. Gastroenterology. 2008; 135:1114-22.

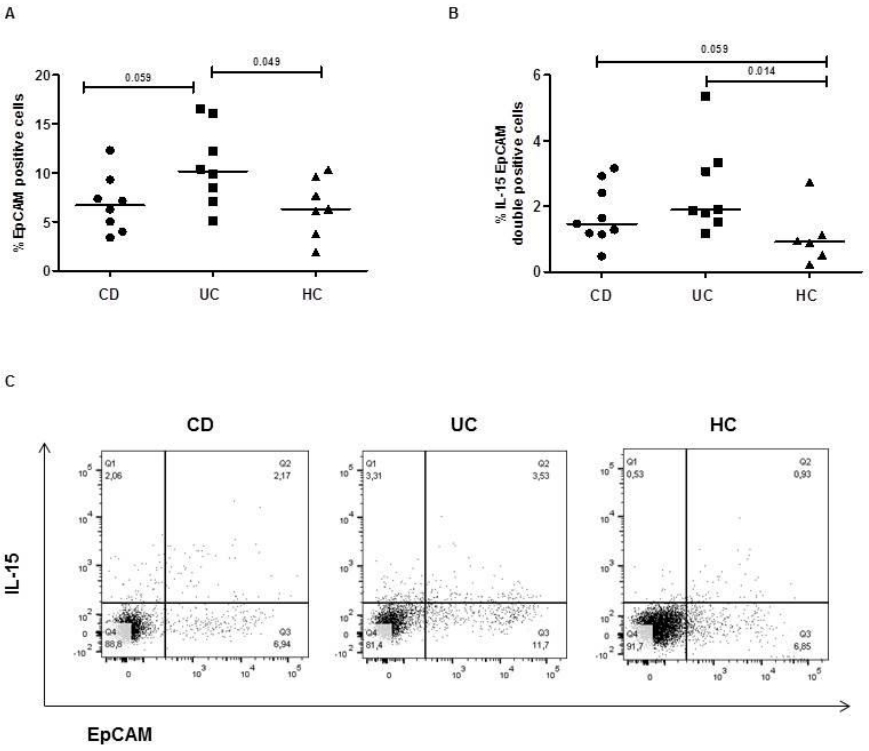

A
IL-15
0.045
0.028
% positive cells
15
10
5
0
CD
UC
HC
B
CD
UC
HC
IL-15
FSC
C
IFN-γ
% positive cells
6
4
2
0
CD
UC
HC
D
TNF-α
% positive cells
10
8
6
4
2
0
CD
UC
HC

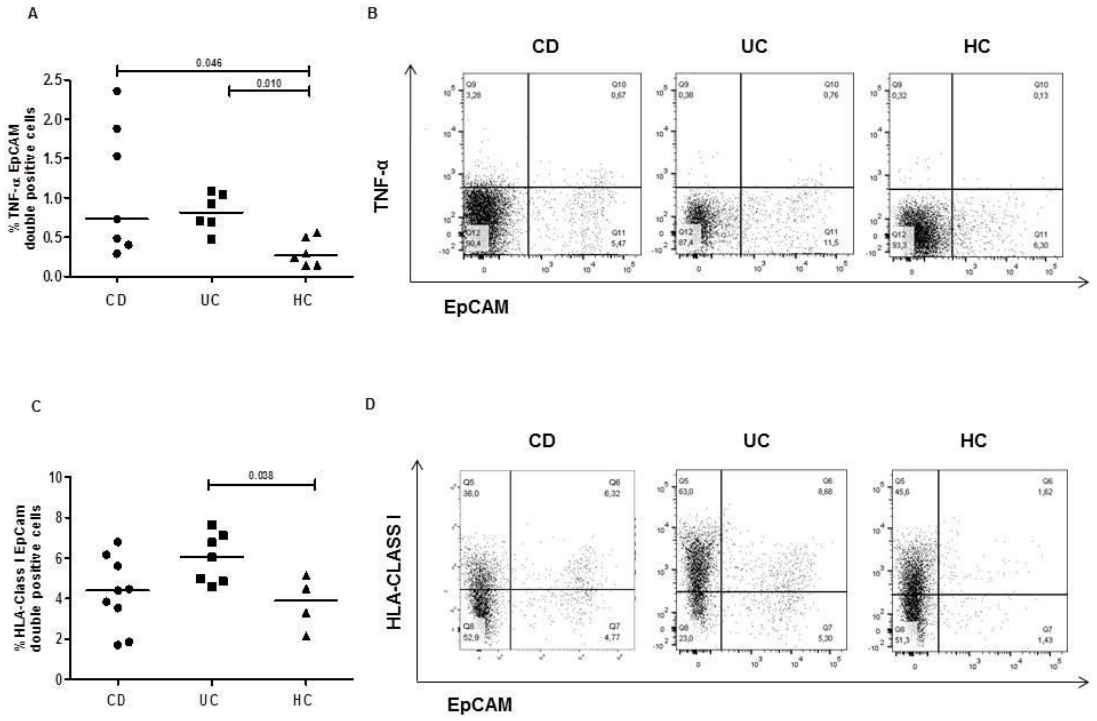
A

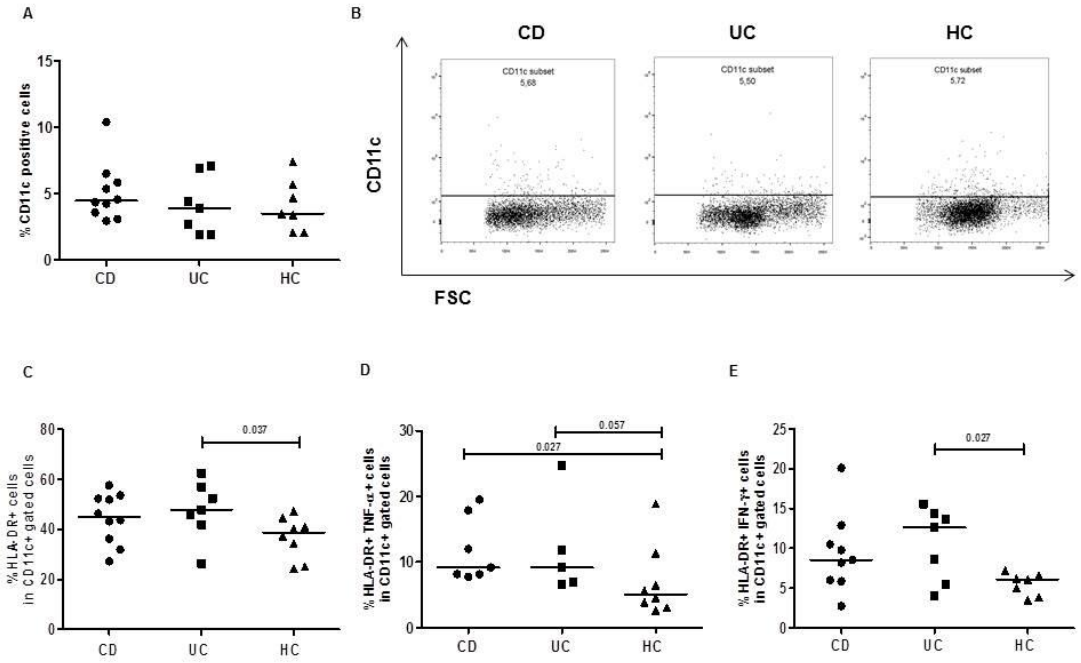

IFN-γ
0.014
0.037
0.009
0.025
5000
4000
3000
2000
1000
0
pg/ml
PHA - + - + - +
CD
UC
HC

B


TNF-α
0.054
1000
750
500
250
0
pg/ml
PHA - + - + - +
CD
UC
HC


A
% EpCAM positive cells
0.059
0.049
CD
UC
HC
B
% IL-15 EpCAM double positive cells
0.059
0.014
CD
UC
HC
C
CD
UC
HC
IL-15
EpCAM
Q1 2,06
Q2 2,17
Q4 88,8
Q3 6,94
Q1 3,31
Q2 3,53
Q4 81,4
Q3 11,7
Q1 0,53
Q2 0,93
Q4 91,7
Q3 6,85


A
B
C
D
0.046
0.010
0.008
% TNF-α EpCAM double positive cells
% HLA-Class I EpCam double positive cells
CD
UC
HC
TNF-α
HLA-CLASS I
EpCAM


A
% CD11c positive cells
15
10
5
0
CD
UC
HC
B
CD
UC
HC
CD11c subset
5,68
CD11c subset
5,50
CD11c subset
5,72
CD11c
FSC
C
% HLA-DR+ cells
in CD11c+ gated cells
80
60
40
20
0
0.037
CD
UC
HC
D
% HLA-DR+ TNF-α+ cells
in CD11c+ gated cells
30
20
10
0
0.057
0.027
CD
UC
HC
E
% HLA-DR+ IFN-γ+ cells
in CD11c+ gated cells
25
20
15
10
5
0
0.027
CD
UC
HC

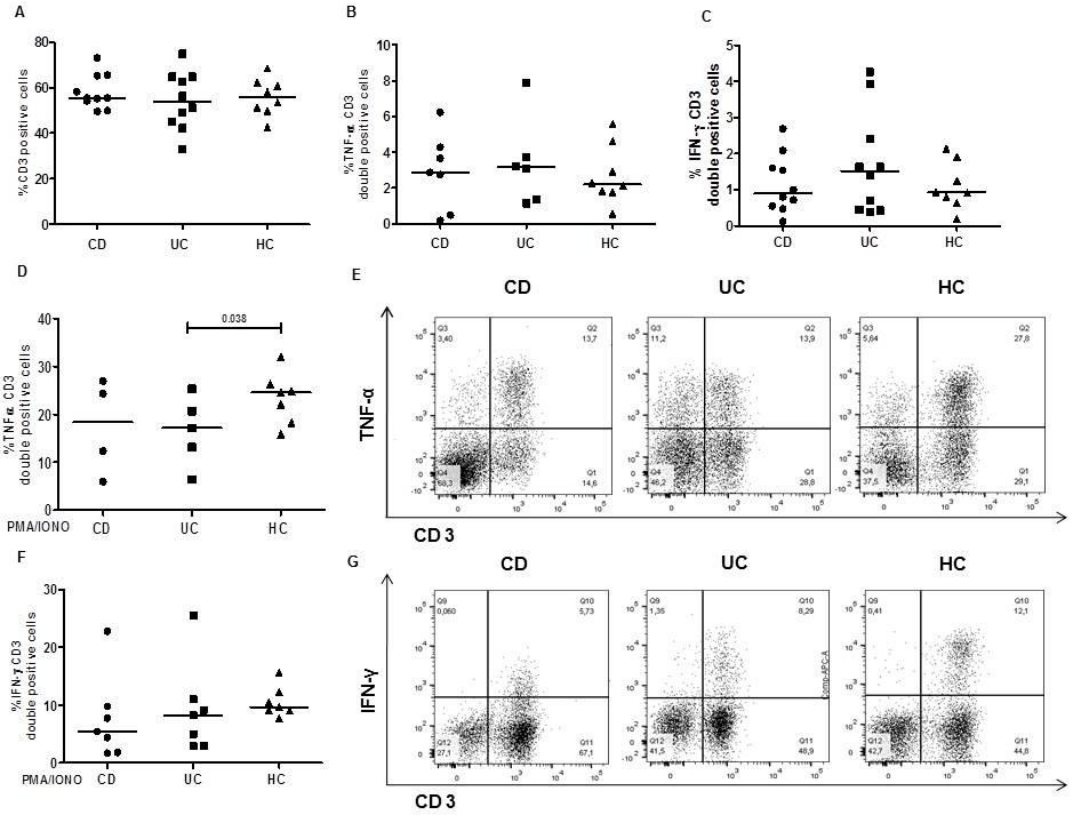

A
% CD3 positive cells
CD
UC
HC
B
% TNF-α CD3 double positive cells
C
% IFN-γ CD3 double positive cells
D
0.038
PMA/IONO
E
CD
UC
HC
TNF-α
CD 3
F
% IFN-γ CD3 double positive cells
G
IFN-γ

Table 1. Clinical features of 59 pediatric patients enrolled

Characteristics	CD	UC	HC
Patients, n	21	19	19
Gender, male/female	11/10	10/9	13/6
Mean age (range), yr	13,1 (7,8-18)	14 (8,6-18,4)	10,4 (3,4-18,5)
Mean age at diagnosis (range), yr	10,5 (2,9-18)	11,5 (5-15,8)	na
Mean disease duration (range), mo	2,30 (0-6,2)	2,6(0-11,5)	na
Therapy, n:			na
Aminosalicylates	5	7	
Immunosuppressants	5	7	
Aminosalicylates and immunosuppressants	4	0	
Monoclonal antibodies	1	0	
No therapy	6	5	

FIGURE LEGENDS

Figure 1. Increased densities of IL-15 producing cells and no marked differences in the frequencies of TNF-α and IFN-γ producing cells in the intestinal mucosa of pediatric patients with inflammatory bowel disease (IBD).

Ex-vivo analyses for the spontaneous cytokine production by the multi-color flow cytometry of colonic mucosal cells from pediatric patients with Crohn's disease (CD), ulcerative colitis (UC) and non-IBD healthy controls (HC).(**A**) The densities of IL-15+ intestinal cells were assessed by a surface staining. (**B**) Representative flow cytometry dot plots from each group of patients were shown. (**C**) The frequencies of intestinal cells producing TNF-α and (**D**) IFN-γ were evaluated by intracellular staining. Each point represented the percentage of positive cells in intestinal mucosa of one single subject. Horizontal bars were the median values. The Mann-Whitney U test was applied.

Figure 2. Enhanced levels of IFN-γ and TNF-α secreted by intestinal cells from young patients with inflammatory bowel disease (IBD) after in vitro phytohaemagglutinin (PHA) stimulation.

(**A**) IFN-γ and (**B**) TNF-α were measured by ELISA in the culture supernatants of intestinal cells isolated from IBD (CD, UC) intestinal or controls (HC). After the isolation from mucosal tissues, cells (2x105/well) were stimulated for 48 hours with phytohaemagglutinin (PHA) in triplicates. Each point represented the cytokine concentration (pg/ml) produced in absence or presence of PHA stimulation (2 µg/ml). Horizontal bars were median values. The Mann-Whitney U /Wilcoxon test was applied.

Figure 3. Higher number of enterocytes and enterocytes expressing IL-15 in gastrointestinal mucosa from young patients affected by inflammatory bowel disease (IBD).

Enterocytes were identified as intestinal cells EpCam expressing by ex–vivo flow cytometry analysis. (**A**) The percentage of EpCam positive cells and (**B**) double positive IL-15 EpCam cells were evaluated in basal condition. Each point represented the percentage of positive cells in intestinal mucosa of one single subject. Data were shown as median percentages (horizontal bars) of EpCam+ and EpCam+IL15+ cells detected in biopsies from each disease (CD, UC)/control group (HC).(**C**) Representative flow cytometry dot plots from double (IL-15, EpCam) stainings in intestinal cells of each group were illustrated. The Mann-Whitney U test was applied.

Figure 4. Increased densities of enterocytes producing TNF-α+ and expressing HLA Class I molecules in gut mucosa of pediatric patients with inflammatory bowel disease (IBD).

Enterocytes were identified as intestinal cells EpCam expressing by ex–vivo analysis. (**A**) Percentages of Epcam+ cells spontaneously producing TNF-α in biopsies from Crohn's disease (CD), ulcerative colitis (UC), or non-IBD healthy control(HC) young subjects were shown. Double positive cells were detected in freshly isolated intestinal cells by flow cytometry. Each point represented the percentage of positive cells in intestinal mucosa of one single subject. Horizontal bars were the median values. (**B**) Representative dot plots from double (EpCam TNF-α) stainings in intestinal cells from each group were shown. (**C**) EpCam and HLA-Class I double positive cells were detected in intestinal biopsies from CD, UC and HC patients. Horizontal bars were the median values. (**D**) Representative flow cytometry dot plots from double (EpCam HLA-Class I) stainings in intestinal cells from each group were shown. The Mann-Whitney U test was applied.

Figure 5. Dendritic cells infiltrating the intestinal mucosa of young patients with inflammatory bowel disease (IBD) produced TNF-α and IFN-γ.

Intestinal dendritic cells (DC) from Crohn's disease (CD), ulcerative colitis (UC), patients, and non-IBD controls were analyzed for the expression of HLA-DR and CD11c surface markers, and intracellularly for TNF-α and IFN-γ production by multicolor flow cytometry, as described in methods. (**A**) Percentages of CD11c positive dendritic cells infiltrating the mucosal tissue of each single subject were assessed. (**B**) Representative flow cytometry dot plots from CD11c staining in intestinal cells from each group were shown.

The densities of (**C**) HLA DR+ cells, (**D**) HLA DR+ producing TNF-α and(**E**) IFN-γ cells in CD11c+ DC at basal, steady state, condition were reported. Each point represented the percentage of positive cells in intestinal mucosa of one single subject. Horizontal bars were the median values. The Mann-Whitney U test was applied.

Figure 6. T lymphocytes producing TNF-α or IFN-γ were present in gut mucosa from both inflammatory bowel disease (IBD) and non-IBD pediatric subjects.

Intestinal cells were co-stained with anti-CD3 antibodies, anti-TNF-α and anti-IFN-γ monoclonal antibodies. (**A**)The proportions of T lymphocytes *ex-vivo* evaluated and (**B&C**) the percentage of T cells spontaneously producing TNF-α and IFN-γ were shown. (**D-G**) In a different set of experiments intestinal cells were stimulated *in vitro* for 5 hours with a cocktail of PMA/Ionomycin before the intracytoplasmic cytokine staining. Each point represented intestinal CD3+ and CD3 TNF-α or -IFN-γ double positive cells detected in intestinal biopsies from Crohn's disease (CD), ulcerative colitis (UC), and non-IBD controls (HC). Horizontal bars were median values.

Representative flow cytometry dot plots from double stainings CD3 TNF-α and CD3 IFN-γ in stimulated intestinal cells from each group were shown. The Mann-Whitney U test was applied.

28

2.5 References

1. Barrett JC, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008;40:955–62.
2. Franke A, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42:1118–25.
3. Latiano A, et al. Replication of interleukin 23 receptor and autophagy-related 16-like 1 association in adult and pediatric onset inflammatory bowel disease in Italy. World J Gastroenterol 2008;14:4643–51.
4. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 2010;22: 124–31.
5. Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 2006;281:11374–83.
6. Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 2006;313:1438–41.
7. Cadwell K, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008;456:259–63.
8. Hayee B, Rahman FZ, Sewell G, Smith AM, Segal AW. Crohn's disease as an immunodeficiency. Expert Rev Clin Immunol 2010;6:585–96.
9. Marks DJ, Rahman FZ, Sewell GW, Segal AW. Crohn's disease: an immune deficiency state. Clin Rev Allergy Immunol 2010;38:20–31.
10. Wildenberg ME, et al. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. Gastroenterology 2012;142:1493–503.
11. Niess JH, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005;307: 254–8.
12. Chieppa M, Rescigno M, Huang AY, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med 2006;203:2841–52.
13. Rescigno M, Rotta G, Valzasina B, Ricciardi-Castagnoli P. Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology 2001;204:572–81.
14. Jiang A, et al. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity 2007;27:610–24.
15. Wildenberg ME, Vos AC, Wolfkamp SC, Duijvestein M, Verhaar AP, Te Velde AA,

van den Brink GR, Hommes DW. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse. Gastroenterology. 2012 Jun;142(7):1493-1503.e6.

16. Strisciuglio C, Duijvestein M, Verhaar AP, Vos AC, van den Brink GR, Hommes DW, Wildenberg ME. Impaired autophagy leads to abnormal dendritic cell-epithelial cell interactions. J Crohns Colitis. 2013 Aug;7(7):534-541
17. Hyams JS, Ferry GD, Mandel FS, Gryboski JD, Kibort PM, Kirschner BS, Griffiths AM, Katz AJ, Grand RJ, Boyle JT, et al. Development and validation of a pediatric Crohn's disease activity index. J Pediatr Gastroenterol Nutr. 1991 May;12(4):439-447
18. Rescigno M, Rotta G, Valzasina B, Ricciardi-Castagnoli P. Dendritic cells shuttle microbes across gut epithelial monolayers. Immunobiology. 2001 Dec;204(5):572-581
19. Russell RK, Drummond HE, Nimmo EE, Anderson N, Smith L, Wilson DC, Gillett PM, McGrogan P, Hassan K, Weaver LT, Bisset M, Mahdi G, Satsangi J. Genotype-phenotype analysis in childhood-onset Crohn's disease: NOD2/CARD15 variants consistently predict phenotypic characteristics of severe disease. Inflamm Bowel Dis. 2005 Nov;11(11):955-964
20. Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science. 2006 Sep 8;313(5792):1438-1441. Epub 2006 Aug 3.
21. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW, Shugart YY, Griffiths AM, Targan SR, Ippoliti AF, Bernard EJ, Mei L, Nicolae DL, Regueiro M, Schumm LP, Steinhart AH, Rotter JI, Duerr RH, Cho JH, Daly MJ, Brant SR. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet. 2007 May;39(5):596-604.
22. Trinh TT, Rioux JD. Understanding association and causality in the genetic stud-ies of inflammatory bowel disease. Gastroenterology 2005;129:2106–10.
23. Mathew CG. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. Nature Reviews Genetics 2008;9:9–14.
24. Hugot JP, Laurent-Puig P, Gower-Rousseau C, et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. Nature 1996;379:821–3.
25. Van Limbergen J, Russell RK, Nimmo ER, et al. The genetics inflammatory bowel disease. American Journal of Gastroenterology 2007;102:2820–31.
26. Klionsky DJ. Crohn's disease, autophagy, and the Paneth cell. New England Journal of

Medicine 2009;360:1785–6.

27. Weiss B, Shamir R, Bujanover Y, et al. NOD2/CARD15 mutation analysis and genotype–phenotype correlation in Jewish pediatric patients compared with adults with Crohn's disease. Journal of Pediatrics 2004;145:208–12.
28. De Ridder L, Weersma RK, Dijkstra G, et al. Genetic susceptibility has a more important role in pediatric-onset Crohn's disease than in adult-onset Crohn's disease. Inflammatory Bowel Diseases 2007;13:1083–92.
29. Prescott NJ, Fisher SA, Franke A, et al. A nonsynonymus SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5.Gastroenterology 2007;132:1665–71.
30. Wagner J, Sim WH, Ellis JA, et al. Interaction of Crohn's disease susceptibility genes in an Australian paediatric cohort. PLoS ONE 2010;5:e15376,http://dx.doi.org/10.1371/journal.pone.0015376.
31. Lacher M, Schroepf S, Ballauf A, et al. Autophagy ATG16L1 rs2241880 allele is associated with Crohn's disease in German children. Acta Paediatrica2009;98:1835–40.
32. Fowler EV, Doecke J, Simms LA, et al. ATG16L1 T300A shows strong associations with disease subgroups in a large Australian IBD population: further support for significant disease heterogeneity Elizabeth V. American Journal ofGastroenterology 2008;103:2519–26.
33. Lewis JD. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. Gastroenterology 2011;140:1817–26.
34. Strisciuglio C, Miele E, Wildenberg ME, et al. T300A variant of autophagy ATG16L1 gene is associated with decreased antigen sampling and processingby dendritic cells in pediatric Crohn's disease. Inflammatory Bowel Diseases2013;19:2339–48.
35. Ahmad T, Armuzzi A, Bunce M, et al. The molecular classification of the clinical manifestations of Crohn's disease. Gastroenterology 2002;122:854–66.
36. Annese V, Lombardi G, Perri F, et al. Variants of CARD15 are associated withan aggressive clinical course of Crohn's disease – an IG-IBD study. American Journal of Gastroenterology 2005;100:84–92.
37. Gasche C, Lomer MCE, Cavill I, Weiss G. Iron, anemia, and inflammatory bowel diseases. *Gut* 2004;**53**:1190–7.
38. Wells CW, Lewis S, Barton JR, Corbett S. Effects of changes in hemoglobin level on quality of life and cognitive function in inflammatory bowel disease patients. *Inflamm*

Bowel Dis 2006;**12**:123–30.

39. Semrin G, Fishman DS, Bousvaros A, *et al.*. Impaired intestinal iron absorption in Crohn's disease correlates with disease activity and markers of inflammation. *Inflamm Bowel Dis* 2006;**12**:1101–6.
40. Oustamanolakis P, Koutroubakis IE, Messaritakis I, Malliaraki N, Sfiridaki A, Kouroumalis EA. Serum hepcidin and prohepcidin concentrations in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 2011;**23**:262–8.
41. De Vizia B, Poggi V, Conenna R, Fiorillo A, Scippa L. Iron absorption and iron deficiency in infants and children with gastrointestinal diseases. *J Pediatr Gastroenterol Nutr* 1992;**14**:21–6.
42. Ott C, Liebold A, Takses A, Strauch UG, Obermeier F. High prevalence but insufficient treatment of iron-deficiency anemia in patients with inflammatory bowel disease: results of a population-based cohort. *Gastroenterol Res Pract* 2012;**2012**:595970.
43. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 2001;**276**:7806–10.
44. Ganz T. Hepcidin. a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* 2003;**102**:783–8.
45. Goodhand JR, Kamperidis N, Rao A, *et al.* Prevalence and management of anemia in children, adolescents, and adults with inflammatory bowel disease. *Inflamm Bowel Dis* 2012;**18**:513–9.
46. Oustamanolakis P, Koutroubakis IE, Kouroumalis EA. Diagnosing anemia in inflammatory bowel disease: beyond the established markers. *J Crohns Colitis* 2011;**5**:381–91.
47. Ogura Y, Bonen DK, Inohara N et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001; 411:603-606.
48. Peyrin-Biroulet L, Parmentier-Decrucq E, Branche J et al. IL-23R, a novel susceptibility gene for inflammatory bowel disease. Med Sci (Paris). 2007; 23:250-252.
49. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. Gastroenterology. 2004; 126:1504-17.
50. Loftus EV Jr, Silverstein MD, Sandborn WJ et al. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. Gastroenterology. 1998; 114:1161-1168.

51. Marshall JK, Hilsden RJ. Environment and epidemiology of inflammatory bowel diseases. In: Satsangi J, Sutherland RL, eds. Inflammatory bowel diseases. London: Churchill Livingston; 2003. pp. 17–28.
52. Peeters M, Nevens H, Baert F et al. Familial aggregation in Crohn's disease: Increased ageadjusted risk and concordance in clinical characteristics. Gastroenterology. 1996; 111:597-603.
53. Cosnes J, Carbonnel F, Carrat F et al. Effects of current and former cigarette smoking on the clinical course of Crohn's disease. Aliment Pharmacol Ther. 1999; 13:1403-1411.
54. Smithson JE, Radford-Smith G, Jewell GP. Appendectomy and tonsillectomy in patients with inflammatory bowel disease. J Clin Gastroenterol. 1995;21:283-286.
55. Askling J, Grahnquist L, Ekbom A et al. Incidence of paediatric Crohn's disease in Stockholm, Sweden. Lancet. 1999; 354:1179.
56. Yao T, Matsui T, Hiwatashi N. Crohn's disease in Japan: diagnostic criteria and epidemiology. Dis Colon Rectum. 2000; 43:S85-S93.
57. Kiloski N, Bret L, Radford-Smith G. Hygiene hypothesis in inflammatory bowel disease: a critical review of the literature. World J Gastroenterol. 2008; 14:165-73.
58. Bernstein CN, Rawsthorne P, Cheang M et al. A population-based case control study of potential risk factors for IBD. Am J Gastroenterol. 2006; 101:993-100.
59. Swidsinski A, Ladhoff A , Pernthaler A et al. Mucosal flora in inflammatory bowel disease. Gastroenterology. 2002; 122:44 – 54 .
60. Ungaro R, Bernstein CN, Gearry R et al. Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. Am J Gastroenterol. 2014; 109:1728-38.
61. Kronman MP, Zaoutis TE, Haynes K et al. Antibiotic exposure and IBD development among children: a population-based cohort study. Pediatrics. 2012; 130:794-803.
62. Danese S, Sans M, Fiocchi C. Inflammatory bowel disease: the role of environmental factors. Autoimmun Rev. 2004; 3:394-400.
63. Montgomery SM, Lambe M, Wakefield AJ et al. Siblings and the risk of inflammatory bowel disease. Scand J Gastroenterol. 2002; 37:1301-8.
64. Serra-Majem L, Ribas L, Ngo J et al. Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. Public Health Nutrition. 2004; 7:931–935.
65. Cianci R, Pagliari D, Pietroni V, Landolfi R, Pandolfi F. Tissue infiltrating

lymphocytes: the role of cytokines in their growth and differentiation. Journal of Biological Regulators and Homeostatic Agents 2010;24:239–49.

66. Di Sabatino A, Calarota SA, Vidali F, Macdonald TT, Corazza GR. Role of IL-15 in immune-mediated and infectious diseases. Cytokine and Growth Factor Reviews 2011;22:19–33.
67. Pandolfi F, Cianci R, Pagliari D, Landolfi R, Cammarota G. Cellular mediators of inflammation: tregs and TH17 cells in gastrointestinal diseases. Mediators of Inflammation 2009;132028. 2009.
68. Neurath M. F, Finotto S and Glimcher L.H, The role of Th1/Th2 polarization in mucosal immunity. Nature Medicine 2002; 8:567-573
69. Geremia A, Biancheri P, Allan P, Corazza G. R, Di Sabatino A, Innate and adaptiveimmunity in inflammatory bowel disease. Autoimmunity Reviews 13 (2014) 3–10.
70. Danese S.Nonimmune cells in inflammatory bowel disease: from victim to villain.Trends Immunol. 2008 Nov;29(11):555-64. doi: 10.1016/j.it.2008.07.009.
71. BamiasG,Dahman M I,Arseneau K O et al.Intestinal specific TNF alpha over expression induces Crohn's-like ileitis in mice.*PLoSOne*(2013) 8:e72594.doi:10.1371/journal.pone.0072594
72. Hassan C, Ierardi E, Burattini O et al. Tumour necrosis factor alpha down-regulation parallels inflammatory regression in ulcerative colitis patients treated with infliximab. *DigLiverDis*(2007) 39:811–7.doi:10.1016/j.dld.2007.06.003
73. Fischer A, Gluth M, Pape U F. et al. Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF on tight junction proteins and signaling pathways in intestinal epithelial cells. *Am J Physiol Gastro intest Liver Physiol*(2013) 304:G970–9.doi:10.1152/ajpgi.00183.
74. Schuppan D1, Junker Y, Barisani D. Celiac disease: from pathogenesis to novel therapies. Gastroenterology. 2009 Dec;137(6):1912-33. doi: 10.1053/j.gastro.2009.09.008. Epub 2009 Sep 18.
75. Sollid LM1, Khosla C. Novel therapies for coeliac disease.J Intern Med. 2011 Jun;269(6):604-13. doi: 10.1111/j.1365-2796.2011.02376.x.
76. Van Limbergen J, Russell RK, Drummond HE et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. Gastroenterology. 2008 Oct;135(4):1114-22. doi: 10.1053/j.gastro.2008.06.081.

Chapter 3.

3.1 Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis.

A recent study has demonstrated that IBD with an early onset can be monogenic. Mutations in IL10 or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children.[1]

IL10R consists of two α (IL10RA) and two beta (IL10RB) molecules. IL10RA and IL10RB genes have been mapped on chromosomes 11q23.3 and 21q22, respectively, and many single-nucleotide polymorphisms (SNPs) have been identified.[2] Recently, Moran et al identified IL10Rs polymorphisms that confer risk for developing very early-onset IBD.[3]

The aims of this work were to clarify the molecular basis of UC in an 18-mo-old affected child. To this aim, we investigated the pathogenetic mechanisms of IL10 pathway alteration in the onset of UC in the proband, and we clarified the molecular changes associated with them. Moreover, we propose β-catenin and tumour necrosis factor α receptors-I (TNFRI) as molecular bio-markers of subclinical disease among apparently healthy family members of the index case. Finally, we have investigated the effect of mesalazine and azathioprine, the main pharmacological therapy used for IBD treatment, on the expression of IL10 receptors, TNFα and TNFα receptors.

As suggested by Moran et al [3] and also described for other human diseases [4], our results confirm that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding IL10 receptors. These variants, alone, could only give rise to a sub-clinical manifestation of the disease. In fact, the proband's father and his brother, both carriers of homozygous A/A polymorphism E47K for the IL10RB gene but without the -413G->T promoter mutation in the IL10RA gene, were apparently not affected. The proband's mother shows a genotype very similar to the proband. In fact, they are both heterozygous for the E47K IL10RB gene polymorphism and for the -413G->T promoter mutation in the IL10RA gene. Possibly due to the different IL10RA mRNA expression, the proband's mother has not developed the disease.

Unexpectedly, we observed β-catenin and TNFRI protein over-expression in the peripheral blood cells of the proband's apparently healthy relatives more than in the proband himself. Therefore, we suggest that these proteins could represent a good candidate for molecular markers of sub-clinical disease in relatives of patients with UC.

Because no therapeutic approach was successful in patients who are carriers of IL10 pathway alterations, we investigated the effect of mesalazine and azathioprine on the expression of IL10 receptors, TNFα and TNFα receptors. In agreement with our hypothesis, we found TNFRI under-expression and TNFRII and IL10RB over-expression in primary fibroblasts incubated with mesalazine and azathioprine, in both the UC and FAP patients. In the UC patient only, azathioprine, but not mesalazine, induces a TNFα decrease.

These observations could suggest that these drugs are only able to partially restore IL10 pathway function in UC, by activation of IL10RB, but not IL10RA, transcription. On the other hand, under-expression of TNFRI and over-expression of TNFRII could increase the risk of colorectal cancer-associated colitis in UC patients. As described by Chang et al [5], TNFRI has tumour suppressor activity in the context of colitis-associated cancer, and the role of TNFRII in cell proliferation is well known.

In conclusion, our results, in agreement with data from recently published literature[6,3,4], indicate that early-onset UC could be caused by a synergistic effect of more variant alleles of the IL10 receptors gene, resulting in alteration of the IL10 pathway. In our opinion, a dosage model of nonallelic non-complementation fits well with this case, whereby mutations in two different genes can behave as alleles of the same locus by causing or exacerbating the same phenotype. However, we cannot exclude, as described for others syndromes, that different mechanisms, such as alternative splicing mechanisms[7,8]or allelic variants of modifier genes, could contribute to the observed phenotypic variability.[4]

In addition, we suggest that the expression of β-catenin and TNFRI protein could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients. Recent findings suggest that chronic inflammation in IL10-/- mice increased P-β-catenin expression. Moreover, TNFRI exerts its tumour suppressor activity by modulating activation of β-catenin and controlling epithelial proliferation.[9] It clearly appears that classical therapeutic approaches do not seem adequate for IBD patients who are carriers of IL10 pathway alterations because under-expression of TNFRI signalling would confer increased risk of developing colitis associated-carcinoma. Allogenic hematopoietic stem cell transplantation could represent a causal therapeutic approach for IL10R-deficient patients, useful for the treatment of the intractable ulcerating enterocolitis of the infant, as recently suggested.[1,2,10,4]

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i46.8659

World J Gastroenterol 2013 December 14; 19(46): 8659-8670
ISSN 1007-9327 (print) ISSN 2219-2840 (online)
© 2013 Baishideng Publishing Group Co., Limited. All rights reserved.

BRIEF ARTICLE

Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis

Martina Galatola, Erasmo Miele, Caterina Strisciuglio, Lorella Paparo, Daniela Rega, Paolo Delrio, Francesca Duraturo, Massimo Martinelli, Giovanni Battista Rossi, Annamaria Staiano, Paola Izzo, Marina De Rosa

Martina Galatola, Lorella Paparo, Francesca Duraturo, Paola Izzo, Marina De Rosa, Department of Molecular Medicine and Medical Biotechnology and CEINGE Biotecnologie Avanzate, University of Naples "Federico Ⅱ", 80131 Naples, Italy
Martina Galatola, Erasmo Miele, Caterina Strisciuglio, Massimo Martinelli, Annamaria Staiano, Department of Translational Medical Sciences, Section of Pediatrics, University of Naples "Federico Ⅱ", 80131 Naples, Italy
Daniela Rega, Paolo Delrio, Colorectal Surgical Oncology - Abdominal Oncology Department, Istituto Nazionale per lo studio e la cura dei tumori, "Fondazione Giovanni Pascale" IRCCS-80131 Naples, Italy
Giovanni Battista Rossi, Endoscopy Unit, Istituto Nazionale per lo studio e la cura dei tumori, "Fondazione Giovanni Pascale" IRCCS-80131 Naples, Italy
Author contributions: Izzo P and De Rosa M contributed equally to this work; Galatola M, Duraturo F and Paparo L performed the majority of the experiments; Miele E, Strisciuglio C, Martinelli M, Rega D, Delrio P and Rossi GB provided the collection of all the human material and vital reagents and were also involved in editing the manuscript; Staiano A provided the collection of all the human material and the financial support for this work; Izzo P and De Rosa M coordinated and provided the financial support for this work, designed the study and wrote the manuscript.
Supported by A grant from Ministero Salute - Ricerca Oncologica - RECAM-2006-353005; PRIN 2007-prot. 2007EN8F7T-004; Convenzione CEINGE-Regione Campania. POR Campania FSE 2007-2013, Project CREME; PRIN 2010-2011-prot. 2010K34C45_006
Correspondence to: Marina De Rosa, PhD, Department of Molecular Medicine and Medical Biotechnology and CEINGE Biotecnologie Avanzate, University of Naples "Federico Ⅱ", 80131 Naples, Italy. marina.derosa@unina.it
Telephone: +39-81-7463136 Fax: +39-81-7464359
Received: May 8, 2013 Revised: July 26, 2013
Accepted: August 17, 2013
Published online: December 14, 2013

Abstract

AIM: To investigated the molecular cause of very early-onset ulcerative colitis (UC) in an 18-mo-old affected child.

METHODS: We analysed the interleukin-10 (*IL10*) receptor genes at the DNA and RNA level in the proband and his relatives. Beta catenin and tumor necrosis factor-α (TNFα) receptors were analysed in the proteins extracted from peripheral blood cells of the proband, his relatives and familial adenomatous polyposis (FAP) and PTEN hamartoma tumor syndrome (PHTS) patients. Samples were also collected from the proband's inflamed colorectal mucosa and compared to healthy and tumour mucosa collected from a FAP patient and patients affected by sporadic colorectal cancer (CRC). Finally, we examined mesalazine and azathioprine effects on primary fibroblasts stabilised from UC and FAP patients.

RESULTS: Our patient was a compound heterozygote for the *IL10RB* E47K polymorphism, inherited from his father, and for a novel point mutation within the *IL10RA* promoter (the -413G->T), inherited from his mother. Beta catenin and tumour necrosis factor α receptors-1 (TNFRI) protein were both over-expressed in peripheral blood cells of the proband's relatives more than the proband. However, TNFRII was over-expressed only in the proband. Finally, both TNFα-receptors were shown to be under-expressed in the inflamed colon mucosa and colorectal cancer tissue compared to healthy colon mucosa. Consistent with this observation, mesalazine and azathioprine induced, in primary fibroblasts, *IL10RB* and TNFRII over-expression and TNFRI and TNFα under-expression. We suggest that β-catenin and TNFRI protein expression in peripheral blood cells could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

CONCLUSION: A synergistic effect of several variant alleles of the *IL10* receptor genes, inherited in a Mende-

lian manner, is involved in UC onset in this young child.

© 2013 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Inflammatory bowel disease; Ulcerative colitis; Interleukin 10 receptors; Tumour necrosis factor α receptors; Beta catenin

Core tip: We identified a novel point mutation within the interleukin-10 (*IL10*) receptor genes promoter (the -413G->T), associated with mRNA under-expression. We propose that this mutation has a synergistic effect with other variant alleles of *IL10* receptor genes in very-early ulcerative colitis (UC) onset in this young child. β-catenin and tumour necrosis factor α receptors-I (TNFRI) protein were both over-expressed in peripheral blood cells of proband relatives, whereas TNFRII was over-expressed only in the proband. We suggest that β-catenin and TNFRI protein expression could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

Galatola M, Miele E, Strisciuglio C, Paparo L, Rega D, Delrio P, Duraturo F, Martinelli M, Rossi GB, Staiano A, Izzo P, De Rosa M. Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis. *World J Gastroenterol* 2013; 19(46): 8659-8670 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8659.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8659

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host[1]. Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinicopathological subtypes of IBD, common in developed countries, affecting the quality of life of approximately 1.4 million individuals in the United States and 2.2 million people in Europe[2-4].

Accumulating data suggest that these disorders result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host[5]. Active IBD is defined as an infiltration of the lamina propria by innate immune cells (neutrophils, macrophages, dendritic and natural killer T cells) and adaptive immune cells (B and T cells). Increased numbers and activation of these cells in the intestinal mucosa enhance local levels of tumour necrosis factor-α (TNFα) and several pro-inflammatory interleukins (IL)[5-8].

Genome-wide association studies (GWAS) have been successful in IBD, identifying 99 non-overlapping genetic risk loci, including 28 that are shared between CD and UC[9,10]. Analyses of the genes and genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species generation, autophagy, adaptive immunity regulation, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis. Early studies have suggested the existence of both protective and predisposing alleles[11]. Again, many genetic changes might affect genetic regions other than coding regions, indicating that allele-specific gene-expression changes contribute to the disease risk[12].

The relative importance of each individual pathway in the pathogenesis of IBD has not been determined. There is enthusiasm for a model in which mucosal inflammation results from defective activity of Treg cells. In this model, effector T cells that react to the microbial flora or other GI antigens are kept in check by a population of regulatory cells; defects in these cells lead to GI inflammation. IL10 production by Treg cells appears to be required for suppression of colitis[13].

A recent study has demonstrated that IBD with an early onset can be monogenic. Mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children[14].

*IL10*R consists of two α (*IL10RA*) and two beta (*IL10RB*) molecules. *IL10RA* and *IL10RB* genes have been mapped on chromosomes 11q23.3 and 21q22, respectively, and many single-nucleotide polymorphisms (SNPs) have been identified[15]. Recently, Moran *et al*[16] identified *IL10Rs* polymorphisms that confer risk for developing very early-onset IBD. Each novel, nonsynonymous SNP was identified only in the heterozygous state, and none of the resulting amino acid changes were predicted to be deleterious by SIFT or Polyphen.

The aims of this work were to clarify the molecular basis of UC in an 18-mo-old affected child. To this aim, we investigated the pathogenetic mechanisms of IL10 pathway alteration in the onset of UC in the proband, and we clarified the molecular changes associated with them. Moreover, we propose β-catenin and tumour necrosis factor α receptors- I (TNFRI) as molecular biomarkers of subclinical disease among apparently healthy family members of the index case. Finally, we have investigated the effect of mesalazine and azathioprine, the main pharmacological therapy used for IBD treatment, on the expression of IL10 receptors, TNFα and TNFα receptors.

MATERIALS AND METHODS

Patients

The proband, exhibiting UC, was referred by paediatric gastroenterologists to the laboratory for genetic analysis. He was admitted to the hospital for bloody diarrhoea, asthenia, fever and a severe anaemia (haemoglobin 3.7 g/dL). He underwent upper and lower GI endoscopy. The upper GI endoscopy did not reveal any macroscopic and/or microscopic sign of disease. Ileocolo-

noscopy showed a severe ulcerative pancolitis, (E4-S1) according to the Paris classification[17]. The colonoscopic grade of inflammation was characterised by the presence of marked erythema, absent vascular pattern, friability erosions, associated with spontaneous bleeding and ulcerations, suggesting a grade 3 according to the Mayo endoscopic score[18]. A severe grade of inflammation was confirmed histologically by the diffuse presence of a large number of neutrophilic leukocytes (> 50/HPF) with crypt abscesses and significant acute inflammation with ulcerations in lamina propria. The presence of granulomas was excluded at any colonic levels, as well as at level of the distal ileum.

The child was treated with blood transfusions, antibiotics and steroid therapy without improvement. A rescue therapy with cyclosporine followed by mesalazine and azathioprine was then started. His following clinical history was characterised by relapsing-remitting symptoms and by the lack of response to drugs. The proband's mother referred episodes of bloody diarrhoea, but she refused colonoscopy.

Blood samples from proband and healthy family members were collected at the same hospital as the patient. Normal colorectal mucosa and colorectal cancer tissues were sampled from patients with FAP or sporadic colon cancer operated on the "Istituto Nazionale dei Tumori" in Naples.

Samples from all subjects who participated in the study were collected after being granted authorisation from the "Comitato etico per le attività Biomediche - Carlo Romano" of the University of Naples Federico II, with protocol number 120/10. Such authorisation is given only once the study has received ethical approval, and participants' informed and written consent has been obtained.

Molecular analysis of IL10RA and IL10RB messenger

Reverse transcription polymerase chain reaction of *IL10RA* and *IL10RB* of full length coding regions: Total RNA was extracted from 3 mL of peripheral blood cells of the UC patient and his healthy family members, using Trizol reagent (Invitrogen, Life Technologies, CA), cDNA was synthesised and 1 μL of the cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) as previously described[19], using the following pairs of oligonucleotides: *IL10RA*-5'UTR-FP/*IL10RA*-3'UTR-RP; *IL10RB*-5'UTR-FP/*IL10RB*-3'UTR-RP. Two fragments of 2023 bp and 1197 bp, respectively, were produced. The PCR products were analysed on a 1% agarose gel in a tris-acetic acid (TAE)-EDTA standard buffer, and visualised by ethidium bromide staining (Table 1).

Sequence analysis of *IL10RA* and *IL10RB* mRNA: Sequence analysis of *IL10RA* and *IL10RB* full length coding regions was performed on amplified fragments from the cDNA of the proband and his healthy family members, using the following primer pairs, localised inside these regions: *IL10RA*-5'UTRb-FP; *IL10RA*-3'UTRb-RP; *IL10RA*-3cFP; *IL10RA*-4cRP; *IL10RA*-6cFP; *IL10RA*-7cRP; *IL10RB*-5'UTRb-FP; *IL10RB*-3'UTRb-RP; *IL10RB*-4cFP; *IL10RB*-5cRP (Table 1). The analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of *IL10RA* and *IL10RB* mRNA sequences [GenBank Accession numbers: NM_001558.3 and NM_000628.3, respectively]; all oligonucleotides were obtained with primer-BLAST Software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Real time RT-PCR quantification analysis: Real time PCR quantification analysis was performed for *IL10RA* and *IL10RB* messengers. The relative expression was calculated with the comparative Ct method. Patient numbering corresponds to that adopted in Figure 1A. Three millilitres of peripheral blood cells from the UC patient, his healthy family members and 8 healthy subjects were pelleted after erythrocyte lysis and resuspended in Trizol reagent. The mean value across all of the healthy samples ($H_{1\text{-}8}$) was used as a calibrator to measure the relative expression. *IL10RA* and *IL10RB* mRNA quantification was carried out by amplifying fragments spanning the junctions between exons 3-4, for *IL10RA* messenger and exons 4-5 for *IL10RB* messenger, compared to the glucuronidase transcript fragment, using the oligonucleotides described above: *IL10RA*-3cFP/*IL10RA*-4cRP; *IL10RB*-4cFP/*IL10RB*-5cRP (Table 1). The quantitative real time assays were performed using the iCycler iQ Real Time Detection System BIO-RAD as previously described[19].

Molecular analysis of IL10RA gene

Genomic PCR and sequencing: Genomic DNA was extracted from 3 mL of peripheral blood cells of UC patient, using Nucleon BACC2 Kit (Amersham Biosciences). Genomic PCR and sequencing of all exons was performed for *IL10RA* gene, using oligonucleotides complementary to intronic neighbouring boundary regions of each exon, described in Table 1. The GenBank Accession number of *IL10RA* genomic sequence is: (NC_ 000011.9/gi:224589802). Mutational analysis of *IL10RA* promoter region, from bp -2159 to bp +1, was performed by PCR and sequencing. This region was amplified into three overlapping fragments of 788, 782 and 788 bp in molecular weight, respectively, using the following primer pairs: *IL10RA*p1-FP/*IL10RA*p1-RP; *IL10RA*p2-FP/*IL10RA*p2-RP; *IL10RA*p3-FP/*IL10RA*p3-RP (Table 1).

Amplification refractory mutation-PCR of the -413G->T IL10-RA promoter mutation: We set up an amplification refractory mutation-PCR (ARMS-PCR) reaction to analyse 200 DNA extracted from blood samples of control subjects apparently healthy, for the -413G->T promoter mutation identified in the UC proband and his mother.

Table 1 Oligonucleotide sequences

	RT-PCR of IL10RA and IL10RB of full length coding regions	
IL10RA-5'UTR-FP:	GTCCCAGCCCAAGGGTAG	[NM_001558.3; start: + 5]
IL10RA-3'UTR-RP:	CACCCACATACCCTGCACTA	[NM_001558.3; start: + 2027]
IL10RB-5'UTR-FP:	GTCGTGTGCTTGGAGGAAG	[NM_000628.3; start: + 57]
IL10RB-3'UTR-RP:	GTGGCTAAGTCCAGGGTCTG	[NM_000628.3; start: + 1223]
	Sequence analysis of IL10RA and IL10RB messenger/real time RT-PCR quantification analysis	
IL10RA-5'UTRb-FP:	TCAGACGCTCATGGGACA	[NM_001558.3; start: + 132]
IL10RA-3'UTRb-RP:	CCCAGTGGACTTGCAGAAA	[NM_001558.3; start: + 1938]
IL10RA-3cFP:	AACTGGACCGTCACCAACAC	[NM_001558.3; start: + 405]
IL10RA-4cRP:	AATCTTCCCGAGGATGAAGC	[NM_001558.3; start: + 506]
IL10RA-6cFP:	AGCTACCCAGTGTCCTGCTC	[NM_001558.3; start: + 871]
IL10RA-7cRP:	CAAAAAGGCCTCCTCATCAA	[NM_001558.3; start: + 983]
IL10RB-5'UTRb-FP:	CATGGCGTGGAGCCTT	[NM_000628.3; start: + 99]
IL10RB-3'UTRb-RP:	GATGGTCTTGGCCCTTGTT	[NM_000628.3; start: + 1177]
IL10RB-4cFP:	GTGCAATACTGGAAAAACGGT	[NM_000628.3; start: + 565]
IL10RB-5cRP:	CCCTCGAACTTGAACACAATAA	[NM_000628.3; start: + 678]
	Genomic PCR and sequencing	
*IL10RA*p1-FP:	GCGGTTTGAGGCTCAGC	[NC_000011.9; start: + 117856447]
*IL10RA*p1-RP:	CAAGACGGAGGCTGAGGA	[NC_000011.9; start: + 117857234]
*IL10RA*p2-FP:	CTAGCAGGGGAAGAGCAGC	[NC_000011.9; start: + 117855574]
*IL10RA*p2-RP:	AACCTTCGTCTCCCAGGTTC	[NC_000011.9; start: + 117856355]
*IL10RA*p3-FP:	TGAGCCAAGTGACACAGAGG	[NC_000011.9; start: + 117855023]
*IL10RA*p3-RP:	TTGAACATATACCCTGCTGAAGAG	[NC_000011.9; start: + 117855810]
IL10RA-1FP:	CTGTCAGTCCCAGCCCAA	[NC_000011.9; start: + 17857104]
IL10RA-1RP:	TCTCCACTGGATGGAGAACTTTA	[NC_000011.9; start: + 117857327]
IL10RA-2FP:	TTGGTAAAATTGGGGTCATCA	[NC_000011.9; start: + 117859029]
IL10RA-2RP:	GCCCTCAGGCACTCACTTC	[NC_000011.9; start: + 117859328]
IL10RA-3FP:	AAGCTCGTTTCCAGTGCCTA	[NC_000011.9; start: + 117860120]
IL10RA-3RP:	GGCAGACATGGTGAGCTATG	[NC_000011.9; start: + 117860439]
IL10RA-4FP:	ACAAACCTGTGGCCAAGTTT	[NC_000011.9; start: + 117863822]
IL10RA-4RP:	CACACAAGGGTGCTTCCAG	[NC_000011.9; start: + 117864202]
IL10RA-5FP:	ATCACCTCTAAAGGCCCACC	[NC_000011.9; start: + 117864629]
IL10RA-5RP:	GGATGCAGAGCTATGTGAAGC	[NC_000011.9; start: + 117864993]
IL10RA-6FP:	TTTCATGGGACCAGAGTCCT	[NC_000011.9; start: + 117866223]
IL10RA-6RP:	CTGGCTGGGAGGAAAAGAG	[NC_000011.9; start: + 117864993]
IL10RA-7.1FP:	GCTCTCCTCCTGGGCCT	[NC_000011.9; start: + 117869338]
IL10RA-7.1RP:	CGGCCCTCAGAGTTTTGA	[NC_000011.9; start: + 117869854]
IL10RA-7.2FP:	ACCTGGGAGCAACAGGTG	[NC_000011.9; start: + 117869775]
IL10RA-7.2RP:	CGTGCCTAACTTCTGCCC	[NC_000011.9; start: + 117870445]
	ARMS PCR of the -413G->T IL10-RA promoter mutation	
IL10RA-ARMS-FP-N:	CCGGCACGCCAGGCAAAAGCGGCTCGGTCG	[NC_000011.9; start: + 117856738]
IL10RA-ARMS-FP-M:	CCGGCACGCCAGGCAAAAGCGGCTCGGTCT	[NC_000011.9; start: + 117856738]
IL10RA-ARMS-RP:	GCCTCCAGTGCCTTCGGATCAA	[NC_000011.9; start: + 117856897]
	Gene copy number quantification of IL10RA gene	
IL10RA-4cFP:	TCCTCGGGAAGATTCAGCTA	[NM_001558.3; start: + 493]
IL10RA-4c2RP:	TGCGAATGGCAATCTCATAC	[NM_001558.3; start: + 594]
IL10RA-7cFP:	ACTGAAGAGCCCCAGTTCCT	[NM_001558.3; start: + 1065]
IL10RA-7c2RP:	GCTGTCTGTGCTATTGCTGC	[NM_001558.3; start: + 1187]

RT-PCR: Reverse transcription polymerase chain reaction; IL10: Interleukin-10.

This ARMS reaction was performed with following oligonucleotide primers: *IL10RA*-ARMS-FP-N; *IL10RA*-ARMS-FP-M; *IL10RA*-ARMS-R (Table 1).

Gene copy number quantification of *IL10RA* gene: For the genomic quantification of *IL10RA* gene, specific amplified fragments were compared to a fragment of the exon 15 of *MUTYH* gene. For *IL10RA* specific quantification, two short fragments, one inside exon 4 and the other inside exon 7, were amplified, using the following primer pairs: *IL10RA*-4cFP/*IL10RA*-4c2RP; *IL10RA*-7cFP/*IL10RA*-7c2RP (Table 1). Patient numbering corresponds to that adopted in Figure 1A.

In silico analysis

In silico analysis of the -413G->T point mutation was performed using the Patch 1.0 software. Patch is a pattern-based program for predicting transcription factor binding sites (TFBS) in DNA sequences. It uses the set of binding sites from TRANSFAC® Public 6.0 and is free online available at the web site: http://www.biobase-international.com/.

β-catenin, TNFRI and TNFRII protein analysis in peripheral blood cells of UC patients

Western blotting assay of β-catenin, TNFRI and TNFRII proteins: Total protein was extracted from 3 mL

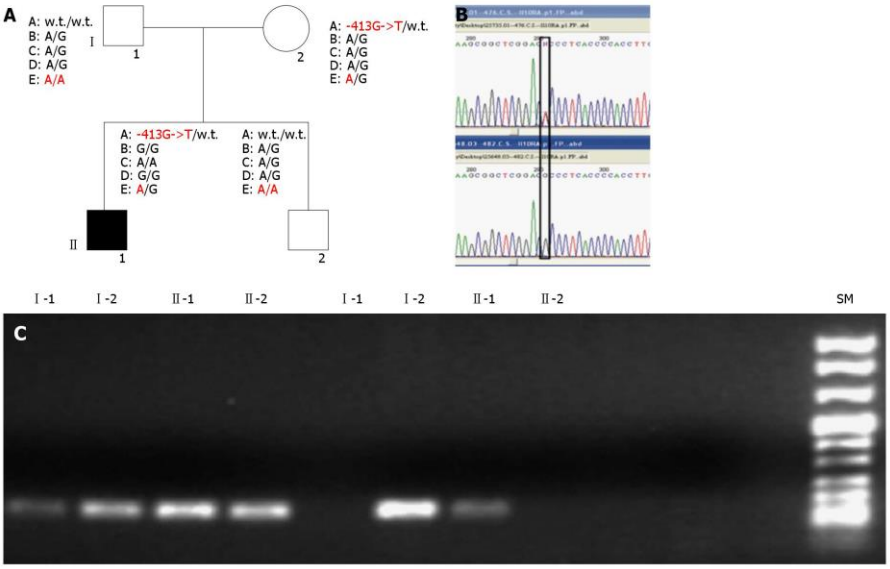


Figure 1 Molecular characterisation of variant alleles within *interleukin-10* receptor genes in the inflammatory bowel diseases family members. A: Pedigree of the inflammatory bowel diseases (IBD) family and genomic single-nucleotide polymorphisms identified: interleukin-10 (*IL10*) *RA*: - 413G->T (A); *IL10RA*-rs.: 2256111 Esone 4 c.549A->G (p.153Ala->Ala) (B); *IL10RA*-rs.:2229113 Esone 7 c.1051A->G (p.351Arg->Gly) (C); *IL10RA*-rs.:9610 3'UTR c.2543G->A (D); *IL10RB*-rs.: 2834167 Esone 1 c.139G->A (p.47 Lys ->Glu) (E); B: Sequence analysis of *IL10RA* promoter region. Sequence analysis was performed on amplified fragments from gDNA of the patients. Reported here are the electropherogram around the identified mutation - 413G->T. The specific mutated nucleotide is shown within the black box; C: Gel-electrophoresis of the amplification refractory mutation-polymerase chain reaction performed for the - 413G->T *IL10RA* promoter mutations. Patient numbering corresponds to that adopted in the shown above pedigree.

of peripheral blood cells (approximately 5-7 × 10^3/mL cells) using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Concentrations were determined and Western blotting assay was performed as previously described[19]. The primary antibody against amino-terminal β-catenin was from Cell Signaling Technology (Beverly, MA). Primary antibodies against TNFRI and TNFRII were from R&D System (R and D System, Minneapolis). The antibody against actin was from Santa Cruz (Santa Cruz, CA). H1-5 and H6-10 are mixes of healthy subjects. PHTS and FAP are two patients affected by PTEN hamartoma tumour syndrome and adenomatous polyposis coli syndrome, respectively. I -1, I -2, II -1 and II -2 are UC family members as reported in Figure 1A.

Real time PCR quantification analysis of *COX2* mRNA: Real time PCR quantification analysis was performed for *COX2* messengers. Relative expression was calculated with the comparative Ct method and normalised against the Ct of Glucuronidase (GUS) mRNA. The quantitative RNA real time assays were performed as described before. To better normalise the healthy values, we used three blood mixes as controls, each containing five samples collected from healthy subjects, for a total of fifteen controls. H1-5, H6-10, H11-15 are mixes of healthy subjects. Hm is the mean value among all healthy samples used as calibrator to measure the relative expression. Patient numbering corresponds to that adopted in Figure 1A.

β-catenin, TNFRI and TNFRII proteins expression in colorectal mucosa

Western blotting assay of β-catenin, TNFRI and TNFRII proteins: Total protein was extracted from the injured colorectal mucosa of the IBD proband and from healthy and tumour mucosa collected from patients affected by FAP and sporadic colorectal cancer using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Western blotting analysis of β-catenin (amino-terminal antigen), TNFRI and TNFRII was performed as previously described.

Incubation with mesalazine and azathioprine of established colon fibroblast culture: Samples of colorectal mucosa from IBD proband and one FAP patient were washed three times in PBS containing 300 U/mL penicillin, 300 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany), finely minced with scissors (tissue pieces of approximately 30 mm^3) and digested in 2 mL 0.1% collagenase II (Boehringer Man-

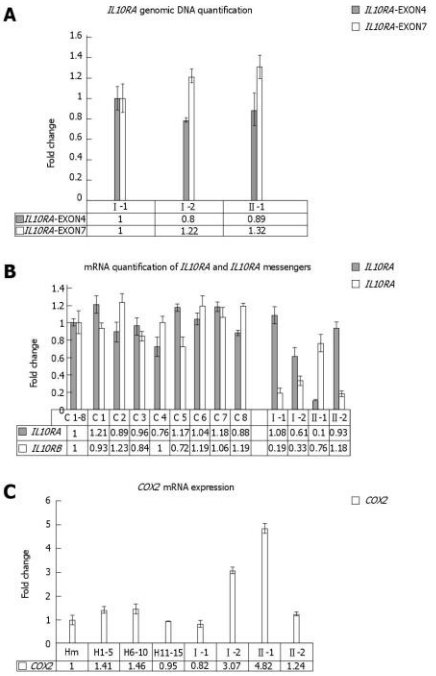


Figure 2 Real time polymerase chain reaction analysis of *interleukin-10 receptors* and *COX2* performed on peripheral blood cells. A: Copy number quantification of interleukin-10 *(IL10)* gene. Real time polymerase chain reaction (PCR) quantification analysis was performed for *IL10RA*. *IL10RA*-exon4: Amplified fragment at the boundaries of exon 4 and IVS4 of the gene; *IL10RA*-exon7: Amplified fragment at the boundaries of exon 7 and IVS7 of the gene; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. B: Real time PCR quantification analysis of *IL10RA* and *IL10RB* mRNA. Real time RT-PCR quantification analysis was performed for *IL10RA* and *IL10RB* mRNA. C1-8: Mean value between all healthy samples used as calibrator to measure the relative expression; C1 to C8: Healthy subjects. Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. C: Real Time PCR quantification analysis of *COX2 messenger*. H1-5, H6-10, H11-15: Mixes of healthy subjects; Hm: Mean value between all healthy samples used as calibrator to measure the relative expression; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A.

nheim, Mannheim, Germany) in DMEM-15% FBS for 2 h at 37 ℃, 5% CO_2. The cell suspension was then collected by centrifugation, washed twice with serum-free DMEM medium, and subsequently cultured for 7 d in DMEM-15% FBS/CHANG C medium (1:1), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany). Primary fibroblasts from IBD and FAP patients were stabilised, cultured on plates, and incubated with mesalazine (30 mmol/L) and azathioprine (30 mmol/L) for 12 h, alternatively. A combination of real time PCR of *IL10* receptors and Western blotting analysis of TNFα and TNFα receptors were performed as previously described.

RESULTS

Variant alleles of the IL10 receptor genes act in a synergistic manner in the onset of UC

Molecular screening of *IL10RA* and *IL10RB*, performed on the proband and his relatives, revealed the presence of multiple SNPs in the patient, inherited from his parents, as shown in Figure 1A.

Specifically, the proband was heterozygous for the *IL10RB* E47K polymorphism (rs2834167, A/G genotype), inherited from his father, described to be associated with a low level of specific mRNA expression (to the A allele). As shown in Figure 1, he was also carrier of an *IL10RA* promoter point mutation (the -413G->T point mutation), inherited from his mother and not previously described in literature. In silico analysis of this mutation, performed using the Patch 1.0 software, shows that it alters a binding site for the Sp1 transcription factor. This genomic variant represents a specific mutation of this IBD family because it was not identified in 200 healthy subjects. The proband's father and his brother were both homozygous for *IL10RB* E47K polymorphism (rs rs.:2834167 A/A genotype; 47K/K), whereas his mother was heterozygous A/G. Only the proband and his mother were carriers of the -413G->T point mutation identified in the promoter region of the *IL10RA* gene. For the following SNPs of *IL10RA*, the rs2256111, localised in the exon 4 (c.549A->G; p.153Ala->Ala), the rs.:2229113, localised in the exon 7 (c.1051A->G; p.351Arg->Gly) and the rs.:9610, localised in the 3'UTR (c.2543G->A), the proband was homozygous G/G, G/G and A/A, respectively. These tree polymorphisms were A/G heterozygous in all other family members (Figure 1A). Using DNA real-time PCR for gene dosage of *IL10RA* gene, we ruled out the presence of intragenic or whole gene deletion (Figure 2A).

IL10 receptor variants are associated with mRNA under-expression

Associated with these genomic variants, we observed a under-expression of *IL10RA* and *IL10RB* mRNA in the proband compared to the average values of 8 healthy subjects, which segregates with each specific variant among the family members. In fact, as revealed by mRNA real-time quantification of both mRNAs of *IL10* receptors shown in Figure 2B, only the proband and his mother, carriers of the -413G->T promoter point mutation, showed a decrease in *IL10RA* mRNA. In contrast, the proband's father and his brother, both homozygous A/A for the *IL10RB* E47K polymorphism, show very low levels of *IL10RB* mRNA expression (fold change of approximately 0.19 and 0.18, respectively), whereas the proband and his mother, who were heterozygous A/G for this polymorphism, showed approximately 50% mRNA expression of the *IL10RB* compared to the mean value across eight healthy samples used as a calibrator (fold change of approximately 0.5 and 0.7 for the proband's mother and the proband himself, respectively). Furthermore, only the proband and his mother showed

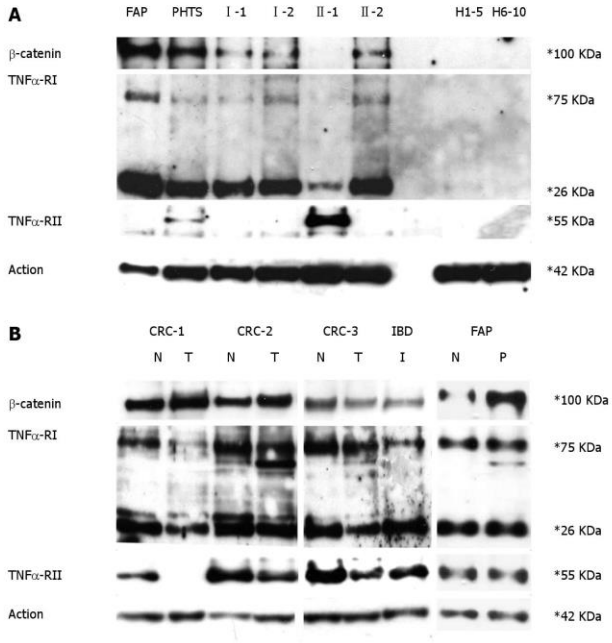


Figure 3 β-catenin, tumour necrosis factor α receptors-I and II protein expression performed on peripheral blood cells and colon mucosa. A: Western blotting assay of β-catenin tumour necrosis factor α receptors-I (TNFRI) and TNFRII performed on protein extracts from peripheral blood cells. Familial adenomatous polyposis (FAP): Patient affected by adenomatous polyposis coli; PHTS: Patient affected by PTEN hamartoma tumour syndrome; I-1, I-2, II-1, II-2: Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. H1-5, H6-10: mixes of healthy subjects; B: Western blotting assay of β-catenin TNFRI and TNFRII performed on protein extracts from colon mucosa. FAP: Patient affected by adenomatous polyposis coli; colorectal cancer (CRC)1, CRC2, CRC3: Patients affected by sporadic colorectal mucosa; inflammatory bowel diseases (IBD): Affected proband; N: Healthy colon mucosa; T: Colon tumour; P: Colon polyp; I: Inflamed colon mucosa.

COX2 overexpression, analysed in peripheral blood cells (Figure 2C).

Alteration of WNT/β-catenin pathway and TNFα receptors expression in the UC patient

As shown in Figure 3A, β-catenin and TNFRI protein were both over-expressed in the peripheral blood cells of the proband's relatives more than the proband. In contrast, TNFRII was over-expressed only in the proband. None of these proteins were detectable in healthy controls. When investigated in colon mucosa, both TNFα receptors were observed to be under-expressed in the inflamed colon mucosa and colorectal cancer compared to healthy colon mucosa. In the FAP patient, normal colon mucosa and polyps express TNFα receptors at the same level. Furthermore, as expected, β-catenin expression is much higher in the polyp than in normal mucosa. (Figure 3B)

Effects of mesalazine and azathioprine on primary fibroblasts

Finally, we show that after incubation with mesalazine and azathioprine of primary fibroblasts of the proband and of a FAP patient, drugs induce *IL10RB* mRNA and TNFRII protein over-expression, whereas TNFRI protein was under-expressed. A decrease of TNFα expression was also observed after incubation with azathioprine but not with mesalazine only in the IBD patient. Fibroblasts isolated from an FAP patient did not show any signal for TNFα hybridisation in our experimental conditions (Figure 4).

DISCUSSION

A recent study demonstrated that mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children[20,21]. In another approach to determining the genetic basis for these disorders, Moran *et al*[16] identified risk SNPs for very early onset IBD. Two SNPs, rs2228054 and rs2228055, were frequently found in the heterozygous state among IBD patients and inherited as a haplotype. The authors propose that the conferred risk may be due to one or both SNPs. Alternatively, the increased

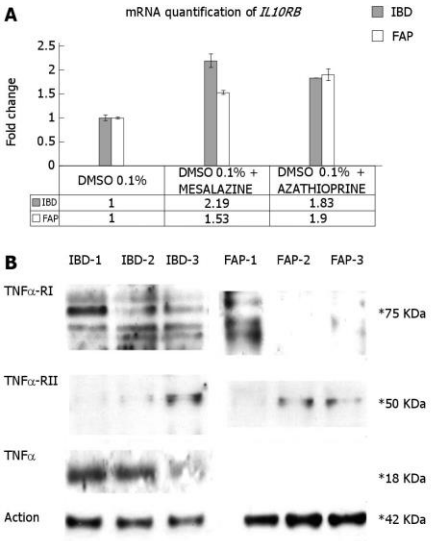


Figure 4 Effects of mesalazine and azathioprine on inflammatory bowel diseases and familial adenomatous polyposis primary fibroblasts. A: Real time polymerase chain reaction (PCR) quantification analysis of interleukin-10 (*IL10*) mRNA; Real time RT-PCR quantification analysis was performed for *IL10RB* mRNA on primary fibroblasts extracted from an inflammatory bowel diseases (IBD) and a familial adenomatous polyposis (FAP) patient and incubated with mesalazine and azathioprine; B: Western blotting assay of tumour necrosis factor α receptors-I (TNFRI) and TNFRII and tumour necrosis factor α (TNFα) performed on protein extracts from primary fibroblasts of an IBD and of a FAP patient. IBD-1: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO only; IBD-2: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and mesalazine; IBD-3: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and azathioprine; FAP-1: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO only; FAP-2: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and mesalazine; FAP-3: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and azathioprine.

risk may reside in a regulatory region (*e.g.*, promoter) in linkage disequilibrium with these SNPs and suggest that this risk haplotype exerts a mild phenotype in the general population resulting in disease only in the presence of other genetic variants or environmental triggers[16].

As suggested by Moran *et al*[16] and also described for other human diseases[22], our results confirm that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding *IL10* receptors. These variants, alone, could only give rise to a sub-clinical manifestation of the disease. In fact, the proband's father and his brother, both carriers of homozygous A/A polymorphism E47K for the *IL10RB* gene but without the -413G->T promoter mutation in the *IL-10RA* gene, were apparently not affected. The proband's mother shows a genotype very similar to the proband. In fact, they are both heterozygous for the E47K *IL10RB* gene polymorphism and for the -413G->T promoter mutation in the *IL10RA* gene. They show different mRNA expression for the *IL10RA* gene and quantitative real-time PCR revealed a 0.1 and 0.6-fold change for the *IL10RA* mRNA in the proband and his mother, respectively. This different gene expression could be due to other intragenic SNPs in the *IL10RA* gene whose alleles are different, such as, the rs.:2256111, localised in exon 4 (c.549A->G; p.153Ala->Ala), the rs.:2229113, localised in exon 7 (c.1051A->G; p.351Arg->Gly) and the rs.:9610, localised in the 3'UTR (c.2543G->A), that were homozygous G/G, G/G and A/A in the proband but A/G heterozygous in all other family members. However, we cannot rule out other gene expression regulatory mechanisms. Possibly due to the different *IL10RA* mRNA expression, the proband's mother has not developed the disease. However, she referred to an episode of rectal bleeding and shows increased levels of *COX2* mRNA expression in peripheral blood cells.

In a recent study, 66 early onset IBD patients were analysed. The authors identified 16 patients with loss-of-function mutations in the *IL10* or *IL10R* genes. A variety of mutations were discovered. Most patients were born from consanguineous parents and they carried homozygous biallelic mutations (point mutations or deletions). However, some patients also presented compound heterozygous mutations. Genotype/phenotype correlations were not clearly observed. In fact, siblings sharing the same homozygous *IL10RB* mutation showed a remarkably distinct level of disease severity, suggesting that the phenotypic manifestation is dependent on other intrinsic or extrinsic factors that remain presently unknown[21,23].

Non-coding single nucleotide polymorphisms (SNPs) can be associated with qualitative and quantitative changes. Furthermore, genetic changes may affect transcription-factor-binding sequences, locus accessibility, translational efficiency and trans-regulators such as noncoding RNAs and microRNAs[12]. Cis- or trans-expression quantitative trait loci are detected for approximately half of the IBD risk regions, indicating that allele-specific gene-expression changes contribute to disease risk[24].

Unexpectedly, we observed β-catenin and TNFRI protein over-expression in the peripheral blood cells of the proband's apparently healthy relatives more than in the proband himself. FAP and PHTS patients, but not healthy subjects, also expressed this protein, as previously described[19]. Therefore, we suggest that these proteins could represent a good candidate for molecular markers of sub-clinical disease in relatives of patients with UC. Previous studies showed that faecal calprotectin concentration in patients with CD and relatives differed significantly from controls, suggesting that there is a high prevalence of subclinical disease in first-degree relatives of these patients. This result conforms to an additive inheritance pattern in which the genetic basis for this abnormality may represent a risk factor for CD and UC[25,26].

Because no therapeutic approach was successful in patients who are carriers of IL10 pathway alterations, we investigated the effect of mesalazine and azathioprine on the expression of IL10 receptors, TNFα and TNFα receptors. In agreement with our hypothesis, we found TNFRI under-expression and TNFRII and *IL10RB* over-expression in primary fibroblasts incubated with mesalazine and azathioprine, in both the UC and FAP patients. In the UC patient only, azathioprine, but not mesalazine, induces a TNFα decrease.

These observations could suggest that these drugs are only able to partially restore IL10 pathway function in UC, by activation of *IL10RB*, but not *IL10RA*, transcription. On the other hand, under-expression of TNFRI and over-expression of TNFRII could increase the risk of colorectal cancer-associated colitis in UC patients. As described by Chang *et al*[27], TNFRI has tumour suppressor activity in the context of colitis-associated cancer, and the role of TNFRII in cell proliferation is well known.

Current therapeutic strategies for paediatric IBD include the use of exclusive enteral nutrition, corticosteroids, mesalamine, sulfasalazine, immunomodulators (azathioprine, 6-mercaptopurine, methotrexate) and anti-TNFα-antibodies[22,28]. Aminosalicylates are the undisputed first-line option for treating and maintaining remission in UC[29]. However, the role that these drugs may play in the management of Crohn's disease has been controversial. Thiopurine drugs, azathioprine and mercaptopurine, have been shown to be effective in inducing and maintaining remission in IBD[30]. Most epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC[14,31], although some studies failed to show this, as described by Velayos *et al*[32].

TNF signals *via* two cell surface receptors, TNFRI and TNFRII, resulted in several, sometimes opposing, cellular responses that vary by context and cell nature[33,34]. In the colonic mucosa, TNF is involved in both cell survival and cell death[35]. Additionally, increased levels of TNF have been found in the setting of cancers, including those of the pancreas, skin, and ovaries[36]. With specific regard to colon carcinogenesis, TNF activity has been shown both to promote and to protect from neoplastic transformation[37-39] and there are case studies of development of cancer in other organ systems (lymphatic and skin) following the use of anti-TNF for IBD or rheumatological disease[40]. For this reason, we investigated protein expression of TNF receptors in colon mucosa of the UC patient compared to that of normal and cancer colon mucosa from patients affected by FAP and sporadic colorectal cancer. In agreement with the hypothesis suggested by Chang *et al*[27] about the tumour suppressor activity of TNFRI in the context of colitis-associated carcinogenesis, we found not only a decrease in the expression of TNFRI but also of TNFRII in colorectal cancer when compared to normal colon mucosa for each patient. The expression of TNF receptor proteins in colon mucosa of our UC patient was at an intermediate level between that observed in colorectal tumour tissue and normal mucosa of CRC patients.

In conclusion, our results, in agreement with data from recently published literature[5,16,22], indicate that early-onset UC could be caused by a synergistic effect of more variant alleles of the *IL10* receptors gene, resulting in alteration of the IL10 pathway. In our opinion, a dosage model of nonallelic non-complementation fits well with this case, whereby mutations in two different genes can behave as alleles of the same locus by causing or exacerbating the same phenotype. However, we cannot exclude, as described for others syndromes, that different mechanisms, such as alternative splicing mechanisms[41,42] or allelic variants of modifier genes, could contribute to the observed phenotypic variability[22].

In addition, we suggest that the expression of β-catenin and TNFRI protein could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients. Recent findings suggest that chronic inflammation in IL10-/- mice increased P-β-catenin552 expression. Moreover, TNFRI exerts its tumour suppressor activity by modulating activation of β-catenin and controlling epithelial proliferation[43]. It clearly appears that classical therapeutic approaches do not seem adequate for IBD patients who are carriers of IL10 pathway alterations because under-expression of TNFRI signalling would confer increased risk of developing colitis associated-carcinoma. Allogenic hematopoietic stem cell transplantation could represent a causal therapeutic approach for IL10R-deficient patients, useful for the treatment of the intractable ulcerating enterocolitis of the infant, as recently suggested[14,15,20-22].

COMMENTS

Background

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. Mutations in interleukin-10 (*IL10*) or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children.

Research frontiers

Increased numbers and activation of immune cells in the intestinal mucosa enhance local levels of tumour necrosis factor-α (TNFα) and several proinflammatory IL. Recent work has demonstrated that IBD with an early onset can be monogenic and *IL10* polymorphisms have been associated with IBD in genome-wide association studies. The aims of this work were to clarify the molecular basis of disease in this young child, shedding light on a synergistic effect of *IL10RA* and *IL10RB* polymorphisms. The authors also assessed the possible presence and inheritance of subclinical intestinal inflammation in apparently healthy relatives of this patient with ulcerative colitis (UC).

Innovations and breakthroughs

Recent studies have shown that loss-of-function mutations in *IL10RA*, *IL10RB* and *IL10* genes, in immunodeficient patients, are associated with severe, infantile-onset IBD. In particular, literature reports have highlighted the role of *IL10RA* polymorphisms in the risk for developing very early onset UC. This is the first study reporting that *IL10RA* polymorphisms could have synergistic effect with those of *IL10RB*. The authors propose that these risk polymorphisms exert a mild phenotype in the general population resulting in disease only in the presence of other genetic variants in the *IL10RA* or *IL10RB*. Furthermore, these observations would suggest an inherited abnormality of beta catenin and TNFRI in the proband's relatives.

Applications

This work expands the understanding of the complex inheritance pattern of very early onset ulcerative colitis. It seems possible that the subclinical phenotypic manifestations identified in the first-degree relatives of the proband represents the consequence of inherited defects of *IL10R* genes, which then represent one of the risk factors for the disease. This study could contribute to identifying at-risk families for very early onset UC allowing clinicians to perform genetic tests and appropriate care.

Terminology

IL10 is an anti-inflammatory cytokine secreted by a variety of cell types and is critical for maintaining immune homeostasis in the gastrointestinal tract. IL10 activates downstream signalling by binding to IL10R, comprised of two α subunits (encoded by *IL10RA*) and two beta subunits (encoded by *IL10RB*).

Peer review

The authors investigated the molecular cause of very early-onset inflammatory bowel disease in an 18-mo-old child as well as his relatives. They concluded that a synergistic effect of several variant alleles of the *IL10 receptor* genes, inherited in a Mendelian manner, is involved in IBD onset in this young child. This study supports a special enthusiasm about the potential power of genomics to define the aetiology and/or phenotype of diseases. When a single specific case or family is studied, the discovery of new functional polymorphisms and the functional consequences of these mutations deserves attention even if the functional characterisation and the real pathogenic contribution of susceptible genes are hard to assess in complex disorders such as IBD.

REFERENCES

1 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]

2 **Abraham C**, Medzhitov R. Interactions between the host innate immune system and microbes in inflammatory bowel disease. *Gastroenterology* 2011; **140**: 1729-1737 [PMID: 21530739 DOI: 10.1053/j.gastro.2011.02.012]

3 **Matricon J**, Barnich N, Ardid D. Immunopathogenesis of inflammatory bowel disease. *Self Nonself* 2010; **1**: 299-309 [PMID: 21487504 DOI: 10.4161/self.1.4.13560]

4 **Bouguen G**, Chevaux JB, Peyrin-Biroulet L. Recent advances in cytokines: therapeutic implications for inflammatory bowel diseases. *World J Gastroenterol* 2011; **17**: 547-556 [PMID: 21350703 DOI: 10.3748/wjg.v17.i5.547]

5 **Abraham C**, Cho JH. Inflammatory bowel disease. *N Engl J Med* 2009; **361**: 2066-2078 [PMID: 19923578 DOI: 10.1056/NEJMra0804647]

6 **Bamias G**, Nyce MR, De La Rue SA, Cominelli F. New concepts in the pathophysiology of inflammatory bowel disease. *Ann Intern Med* 2005; **143**: 895-904 [PMID: 16365470 DOI: 10.7326/0003-4819-143-12-200512200-00007]

7 **Andoh A**, Yagi Y, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y. Mucosal cytokine network in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 5154-5161 [PMID: 18777592 DOI: 10.3748/wjg.14.5154]

8 **Fantini MC**, Monteleone G, Macdonald TT. New players in the cytokine orchestra of inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 1419-1423 [PMID: 17712836 DOI: 10.1002/ibd.20212]

9 **Franke A**, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, Anderson CA, Bis JC, Bumpstead S, Ellinghaus D, Festen EM, Georges M, Green T, Haritunians T, Jostins L, Latiano A, Mathew CG, Montgomery GW, Prescott NJ, Raychaudhuri S, Rotter JI, Schumm P, Sharma Y, Simms LA, Taylor KD, Whiteman D, Wijmenga C, Baldassano RN, Barclay M, Bayless TM, Brand S, Büning C, Cohen A, Colombel JF, Cottone M, Stronati L, Denson T, De Vos M, D'Inca R, Dubinsky M, Edwards C, Florin T, Franchimont D, Gearry R, Glas J, Van Gossum A, Guthery SL, Halfvarson J, Verspaget HW, Hugot JP, Karban A, Laukens D, Lawrance I, Lemann M, Levine A, Libioulle C, Louis E, Mowat C, Newman W, Panés J, Phillips A, Proctor DD, Regueiro M, Russell R, Rutgeerts P, Sanderson J, Sans M, Seibold F, Steinhart AH, Stokkers PC, Torkvist L, Kullak-Ublick G, Wilson D, Walters T, Targan SR, Brant SR, Rioux JD, D'Amato M, Weersma RK, Kugathasan S, Griffiths AM, Mansfield JC, Vermeire S, Duerr RH, Silverberg MS, Satsangi J, Schreiber S, Cho JH, Annese V, Hakonarson H, Daly MJ, Parkes M. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010; **42**: 1118-1125 [PMID: 21102463 DOI: 10.1038/ng.717]

10 **Anderson CA**, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD, Lee JC, Goyette P, Imielinski M, Latiano A, Lagacé C, Scott R, Amininejad L, Bumpstead S, Baidoo L, Baldassano RN, Barclay M, Bayless TM, Brand S, Büning C, Colombel JF, Denson LA, De Vos M, Dubinsky M, Edwards C, Ellinghaus D, Fehrmann RS, Floyd JA, Florin T, Franchimont D, Franke L, Georges M, Glas J, Glazer NL, Guthery SL, Haritunians T, Hayward NK, Hugot JP, Jobin G, Laukens D, Lawrance I, Lémann M, Levine A, Libioulle C, Louis E, McGovern DP, Milla M, Montgomery GW, Morley KI, Mowat C, Ng A, Newman W, Ophoff RA, Papi L, Palmieri O, Peyrin-Biroulet L, Panés J, Phillips A, Prescott NJ, Proctor DD, Roberts R, Russell R, Rutgeerts P, Sanderson J, Sans M, Schumm P, Seibold F, Sharma Y, Simms LA, Seielstad M, Steinhart AH, Targan SR, van den Berg LH, Vatn M, Verspaget H, Walters T, Wijmenga C, Wilson DC, Westra HJ, Xavier RJ, Zhao ZZ, Ponsioen CY, Andersen V, Torkvist L, Gazouli M, Anagnou NP, Karlsen TH, Kupcinskas L, Sventoraityte J, Mansfield JC, Kugathasan S, Silverberg MS, Halfvarson J, Rotter JI, Mathew CG, Griffiths AM, Gearry R, Ahmad T, Brant SR, Chamaillard M, Satsangi J, Cho JH, Schreiber S, Daly MJ, Barrett JC, Parkes M, Annese V, Hakonarson H, Radford-Smith G, Duerr RH, Vermeire S, Weersma RK, Rioux JD. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat Genet* 2011; **43**: 246-252 [PMID: 21297633 DOI: 10.1038/ng.764]

11 **Momozawa Y**, Mni M, Nakamura K, Coppieters W, Almer S, Amininejad L, Cleynen I, Colombel JF, de Rijk P, Dewit O, Finkel Y, Gassull MA, Goossens D, Laukens D, Lémann M, Libioulle C, O'Morain C, Reenaers C, Rutgeerts P, Tysk C, Zelenika D, Lathrop M, Del-Favero J, Hugot JP, de Vos M, Franchimont D, Vermeire S, Louis E, Georges M. Resequencing of positional candidates identifies low frequency IL23R coding variants protecting against inflammatory bowel disease. *Nat Genet* 2011; **43**: 43-47 [PMID: 21151126 DOI: 10.1038/ng.733]

12 **Khalil AM**, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci USA* 2009; **106**: 11667-11672 [PMID: 19571010 DOI: 10.1073/pnas.0904715106]

13 **MacDonald TT**, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. *Gastroenterology* 2011; **140**: 1768-1775 [PMID: 21530743 DOI: 10.1053/j.gastro.2011.02.047]

14 **Glocker EO**, Kotlarz D, Boztug K, Gertz EM, Schäffer AA, Noyan F, Perro M, Diestelhorst J, Allroth A, Murugan D, Hätscher N, Pfeifer D, Sykora KW, Sauer M, Kreipe H, Lacher M, Nustede R, Woellner C, Baumann U, Salzer U, Koletzko S, Shah N, Segal AW, Sauerbrey A, Buderus S, Snapper SB, Grimbacher B, Klein C. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009; **361**: 2033-2045 [PMID: 19890111 DOI: 10.1056/NEJMoa0907206]

15 **Kotenko SV**, Krause CD, Izotova LS, Pollack BP, Wu W, Pestka S. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO*

J 1997; **16**: 5894-5903 [PMID: 9312047 DOI: 10.1093/emboj/16.19.5894]
16 **Moran CJ**, Walters TD, Guo CH, Kugathasan S, Klein C, Turner D, Wolters VM, Bandsma RH, Mouzaki M, Zachos M, Langer JC, Cutz E, Benseler SM, Roifman CM, Silverberg MS, Griffiths AM, Snapper SB, Muise AM. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* 2013; **19**: 115-123 [PMID: 22550014 DOI: 10.1002/ibd.22974]
17 **Levine A**, Griffiths A, Markowitz J, Wilson DC, Turner D, Russell RK, Fell J, Ruemmele FM, Walters T, Sherlock M, Dubinsky M, Hyams JS. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis* 2011; **17**: 1314-1321 [PMID: 21560194 DOI: 10.1002/ibd.21493]
18 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629 [PMID: 3317057 DOI: 10.1056/NEJM198712243172603]
19 **Galatola M**, Paparo L, Duraturo F, Turano M, Rossi GB, Izzo P, De Rosa M. Beta catenin and cytokine pathway dysregulation in patients with manifestations of the "PTEN hamartoma tumor syndrome". *BMC Med Genet* 2012; **13**: 28 [PMID: 22520842 DOI: 10.1186/1471-2350-13-28]
20 **Glocker EO**, Kotlarz D, Klein C, Shah N, Grimbacher B. IL-10 and IL-10 receptor defects in humans. *Ann N Y Acad Sci* 2011; **1246**: 102-107 [PMID: 22236434 DOI: 10.1111/j.1749-6632.2011.06339.x]
21 **Kotlarz D**, Beier R, Murugan D, Diestelhorst J, Jensen O, Boztug K, Pfeifer D, Kreipe H, Pfister ED, Baumann U, Puchalka J, Bohne J, Egritas O, Dalgic B, Kolho KL, Sauerbrey A, Buderus S, Güngör T, Enninger A, Koda YK, Guariso G, Weiss B, Corbacioglu S, Socha P, Uslu N, Metin A, Wahbeh GT, Husain K, Ramadan D, Al-Herz W, Grimbacher B, Sauer M, Sykora KW, Koletzko S, Klein C. Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. *Gastroenterology* 2012; **143**: 347-355 [PMID: 22549091 DOI: 10.1053/j.gastro.2012.04.045]
22 **Duraturo F**, Liccardo R, Cavallo A, De Rosa M, Grosso M, Izzo P. Association of low-risk MSH3 and MSH2 variant alleles with Lynch syndrome: probability of synergistic effects. *Int J Cancer* 2011; **129**: 1643-1650 [PMID: 21128252 DOI: 10.1002/ijc.25824]
23 **Spehlmann ME**, Begun AZ, Burghardt J, Lepage P, Raedler A, Schreiber S. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. *Inflamm Bowel Dis* 2008; **14**: 968-976 [PMID: 18253950 DOI: 10.1002/ibd.20380]
24 **He X**, Fuller CK, Song Y, Meng Q, Zhang B, Yang X, Li H. Sherlock: detecting gene-disease associations by matching patterns of expression QTL and GWAS. *Am J Hum Genet* 2013; **92**: 667-680 [PMID: 23643380 DOI: 10.1016/j.ajhg.2013.03.022]
25 **Thjodleifsson B**, Sigthorsson G, Cariglia N, Reynisdottir I, Gudbjartsson DF, Kristjansson K, Meddings JB, Gudnason V, Wandall JH, Andersen LP, Sherwood R, Kjeld M, Oddsson E, Gudjonsson H, Bjarnason I. Subclinical intestinal inflammation: an inherited abnormality in Crohn's disease relatives? *Gastroenterology* 2003; **124**: 1728-1737 [PMID: 12806605 DOI: 10.1016/S0016-5085(03)00383-4]
26 **Montalto M**, Curigliano V, Santoro L, Armuzzi A, Cammarota G, Covino M, Mentella MC, Ancarani F, Manna R, Gasbarrini A, Gasbarrini G. Fecal calprotectin in first-degree relatives of patients with ulcerative colitis. *Am J Gastroenterol* 2007; **102**: 132-136 [PMID: 17100982 DOI: 10.1111/j.1572-0241.2006.00884.x]
27 **Chang F**, Lacey MR, Bouljihad M, Höner Zu Bentrup K, Fortgang IS. Tumor necrosis factor receptor 1 functions as a tumor suppressor. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G195-G206 [PMID: 22052015 DOI: 10.1152/ajpgi.00209.2011]
28 **Van Assche G**, Dignass A, Reinisch W, van der Woude CJ, Sturm A, De Vos M, Guslandi M, Oldenburg B, Dotan I, Marteau P, Ardizzone A, Baumgart DC, D'Haens G, Gionchetti P, Portela F, Vucelic B, Söderholm J, Escher J, Koletzko S, Kolho KL, Lukas M, Mottet C, Tilg H, Vermeire S, Carbonnel F, Cole A, Novacek G, Reinshagen M, Tsianos E, Herrlinger K, Oldenburg B, Bouhnik Y, Kiesslich R, Stange E, Travis S, Lindsay J. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Special situations. *J Crohns Colitis* 2010; **4**: 63-101 [PMID: 21122490 DOI: 10.1016/j.crohns.2009.09.009]
29 **Kornbluth A**, Sachar DB. Ulcerative colitis practice guidelines in adults: American College Of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2010; **105**: 501-523; quiz 524 [PMID: 20068560 DOI: 10.1038/ajg.2009.727]
30 **Gisbert JP**, Chaparro M, Gomollón F. Common misconceptions about 5-aminosalicylates and thiopurines in inflammatory bowel disease. *World J Gastroenterol* 2011; **17**: 3467-3478 [PMID: 21941413 DOI: 10.3748/wjg.v17.i30.3467]
31 **Koelink PJ**, Robanus-Maandag EC, Devilee P, Hommes DW, Lamers CB, Verspaget HW. 5-Aminosalicylic acid inhibits colitis-associated but not sporadic colorectal neoplasia in a novel conditional Apc mouse model. *Carcinogenesis* 2009; **30**: 1217-1224 [PMID: 19420017 DOI: 10.1093/carcin/bgp113]
32 **Velayos FS**, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005; **100**: 1345-1353 [PMID: 15929768 DOI: 10.1111/j.1572-0241.2005.41442.x]
33 **Ebach DR**, Newberry R, Stenson WF. Differential role of tumor necrosis factor receptors in TNBS colitis. *Inflamm Bowel Dis* 2005; **11**: 533-540 [PMID: 15905700 DOI: 10.1097/01]
34 **Ebach DR**, Riehl TE, Stenson WF. Opposing effects of tumor necrosis factor receptor 1 and 2 in sepsis due to cecal ligation and puncture. *Shock* 2005; **23**: 311-318 [PMID: 15803053 DOI: 10.1152/ajpgi.00142.2011]
35 **Corredor J**, Yan F, Shen CC, Tong W, John SK, Wilson G, Whitehead R, Polk DB. Tumor necrosis factor regulates intestinal epithelial cell migration by receptor-dependent mechanisms. *Am J Physiol Cell Physiol* 2003; **284**: C953-C961 [PMID: 12466150 DOI: 10.1152/ajpcell.00309.2002]
36 **Pezzilli R**, Corsi MM, Barassi A, Morselli-Labate AM, Dogliotti G, Casadei R, Corinaldesi R, D'Eril GM. The role of inflammation in patients with intraductal mucinous neoplasm of the pancreas and in those with pancreatic adenocarcinoma. *Anticancer Res* 2010; **30**: 3801-3805 [PMID: 20944173 DOI: 10.1158/0008-5472]
37 **Balkwill F**. Tumor necrosis factor or tumor promoting factor? *Cytokine Growth Factor Rev* 2002; **13**: 135-141 [PMID: 11900989 DOI: 10.1016/S1359-6101(01)00020-X]
38 **Grimm M**, Lazariotou M, Kircher S, Höfelmayr A, Germer CT, von Rahden BH, Waaga-Gasser AM, Gasser M. Tumor necrosis factor-α is associated with positive lymph node status in patients with recurrence of colorectal cancer-indications for anti-TNF-α agents in cancer treatment. *Cell Oncol* (Dordr) 2011; **34**: 315-326 [PMID: 21573932 DOI: 10.1007/s13402-011-0027-7]
39 **Popivanova BK**, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* 2008; **118**: 560-570 [PMID: 18219394 DOI: 10.1172/JCI32453]
40 **Ochenrider MG**, Patterson DJ, Aboulafia DM. Hepatosplenic T-cell lymphoma in a young man with Crohn's disease: case report and literature review. *Clin Lymphoma Myeloma Leuk* 2010; **10**: 144-148 [PMID: 20371449 DOI: 10.3816/CLML.2010.n.021]
41 **De Rosa M**, Galatola M, Borriello S, Duraturo F, Masone S, Izzo P. Implication of adenomatous polyposis coli and MUTYH mutations in familial colorectal polyposis. *Dis Colon*

Rectum 2009; **52**: 268-274 [PMID: 19279422 DOI: 10.1007/DCR.0b013e318197d15c]

42 **De Rosa M**, Morelli G, Cesaro E, Duraturo F, Turano M, Rossi GB, Delrio P, Izzo P. Alternative splicing and nonsense-mediated mRNA decay in the regulation of a new adenomatous polyposis coli transcript. *Gene* 2007; **395**: 8-14 [PMID: 17360132 DOI: 10.1016/j.gene.2006.10.027]

43 **Lee G**, Goretsky T, Managlia E, Dirisina R, Singh AP, Brown JB, May R, Yang GY, Ragheb JW, Evers BM, Weber CR, Turner JR, He XC, Katzman RB, Li L, Barrett TA. Phosphoinositide 3-kinase signaling mediates beta-catenin activation in intestinal epithelial stem and progenitor cells in colitis. *Gastroenterology* 2010; **139**: 869-881, 881.e1-9 [PMID: 20580720 DOI: 10.1053/j.gastro.2010.05.037]

P- Reviewers: Corleto VD, Yamakawa M
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Liu XM

3.2 Cannabinoid receptor 2 functional variant contributes to the risk of pediatric inflammatory bowel disease

The endocannabinoid (EC) system has been recently indicated as a possible therapeutic target in IBD. [11,12]

The EC system includes the EC endogenous lipid transmitters, their G-protein-coupled cannabinoid receptors type 1 and 2 (CB1 and CB2), and the enzymes for EC metabolism. ECs are synthesized and immediately released "on demand", although they can also accumulate in intracellular adiposomes .[13]

Common variant, rs35761398 (CAA/CGG), of the CNR2 gene, encoding for the CB2 receptor, is associated to autoimmunity unbalance. [14,15]

In particular, the presence of the arginine (R) at codon 63 significantly reduces the EC-induced inhibition of lymphocyte proliferation, suggesting the R variant as the less functional.[14]

This less functional variant CB2-R63 has been associated with increased risk of chronic immune thrombocytopenic purpura and celiac disease, in childhood. [16,17]

We conducteda case-control association analysis between the common CB2-Q63R functional variant and IBD in a cohort of 221 Italian children with IBD comparedto 600 controls previously recruited from the same geographic area. Moreover, we evaluated possible correlations between the CB2-Q63R variant and the clinical features of our pediatric IBD patients.

We describe, for the first time, the association between the CNR2 rs35761398 polymorphism (CB2-Q63R variant) and the risk of pediatric UC and CD. Of note, we also found an association between the aforementioned SNP and specific clinical CD and UC sub-phenotypes. Taken together, our data highlight the involvement of CB2 in the pathogenesis of pediatric IBD and provide insight into its possible role inthe clinical features of chronic intestinal inflammation. We found that the GG/GG genotype (RR homozygous subjects) was highly prevalent in IBD children. Interestingly, when CD and UC patients were analyzed separately in comparison with controls, a significant over-representation of the RR genotype and of the R allele was observed for the CD children.

This finding is in line with the fact that ECs produce a bias in the balance between the two types of Th cells, suppressing Th1 and enhancing Th2, and that the CB2-Q63R variant isinvolved in this process exerting a different inhibition of these cells according to the presence of Q or R 20. This bias has several mechanisms. It has been partly explained by

different expression of cannabinoid receptors on subpopulations and on antigen-presenting cells. It could be due to modulation of cytokines generated by dendritic cells. [18]

In addition, the induction of Th2 associated cytokines can after wards inhibit Th1cells. [19]

Recentdata show that ECs, via CB2 receptor, can directly induceB-cell class switching from IgM to IgE in favor of a Th2 type immunity. [20]

Therefore it is reasonable that this CB2 variant, which has been related to decreased immunomodulatory response and the development of autoimmune disease[14], is mainly associated with a Th1-mediated disease, such as CD.

Indeed, in many previous studies, cytokine profiles of CD patients displayed Th1 dominance characterized by higher levels of IFN-γ and TNF-α and lower amounts of IL-4 in the intestinal mucosa and serum of CD patients compared with healthy controls. [21,22]

Finally, it has been suggested that a shift towards the Th1 profile may contribute to the onset of CD by activating macrophages, which produce pro-inflammatory cytokines causing intestinal damage. [23]

In both CD and UC children, the R63 variant was strongly associated with moderate to severe activity of the disease calculated with PCDAI and PUCAI score, respectively. Moreover, in UC patients, the CB2-R63 variant was associated also to a higher incidence of early clinical relapse, further suggesting an association with a more severe phenotype of disease. Indeed, an impaired functionality of CB2 receptor could determine a decreased suppression of activated macrophages and mast cells and stimulate the secretion of pro-inflammatory cytokines such as TNF-α [24,25], causing an exacerbation of intestinal inflammation and worsening disease course.

The marked rising in the incidence of IBD in childhood[17], renders of particular interest our study since in a paediatric population with IBD the genetic susceptibility could be greater and influence a more severe disease course.The present results also support the concept that the CB2 receptor on immune effector cells may represent a potential molecular target for selective CB2 agonist therapies that could suppress autoreactive, pro-inflammatory innate and adaptive immune responses.Further investigation as well as targeted functional studies are needed to understand how Q63R genotype contributes to disease susceptibility in pediatric IBD, and whether presence of these polymorphic markers might have clinical therapeutic implications.

\ **Cannabinoid receptor 2 functional variant contributes to the risk of pediatric inflammatory bowel disease**

Caterina Strisciuglio [1, *], Giulia Bellini [2], Erasmo Miele [3], Massimo Martinelli [3], Sabina Cenni [3], Chiara Tortora [2], Carlo Tolone [1], Annamaria Staiano, MD [3], Emanuele Miraglia del Giudice [1], Francesca Rossi [1]

[1] Department of Women, Child and General and Specialist Surgery, The Second University of Naples, Naples, Italy

[2] Department of Experimental Medicine, Division of Pharmacology "Leonardo Donatelli", The Second University of Naples, Naples, Italy

[3] Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Naples, Italy

Running Head: IBD and CB2

[*]**Corresponding Author:**

Caterina Strisciuglio, MD

Department of Women, Child and General and Specialist Surgery, SUN

via Luigi De Crecchio, 4 – 80138 Napoli, Italy

telephone: +39-081-5665464; **fax:** +39-081-5665430;

e-mail address:caterina.strisciuglio@unina2.it

Abstract

Background: Endocannabinoids may limit intestinal inflammation via cannabinoid receptor 1 and/or 2 (CB_1, CB_2). We conducted a case-control association analysis to establish the role of a common CB2 functional variant, Q63R, in the susceptibility to inflammatory bowel disease (IBD).

Methods: We genotyped 217 pediatric IBD patients (112 Crohn's disease (CD), 105 ulcerative colitis, UC), and 600 controls for the CB2-Q63R variant by Taqman assay. The disease activity was measured with Pediatric Crohn Disease Activity Index or Pediatric Ulcerative Colitis Activity Index. Additional data were collected from clinical records on age at diagnosis, disease duration and location, extraintestinal manifestations, therapy, clinical relapses, and need for surgery.

Results: We found a significant association of the CB2-R63variant with IBD (allele frequencies p=0.04; genotype distributions p=0.0006), in particular with CD (allele frequencies p=0.002; genotype distributions p=0.00005) and with UC only for genotype distributions (p=0.03). RR-carriers showed an increased risk to develop a kind of IBD (OR=1.82; CI 95%; p=0.0002 for IBD; OR=2.02; CI 95%; $p=10^{-3}$ for CD; OR=1.63; CI 95%; p=0.02 for UC). Upon genotype-phenotype evaluation, RR patients showed an increased frequency of moderate-severe disease activity in both CD and UC (p=0.01 and p=0.02, respectively) and also an earlier clinical relapse in UC (p=0.04).

Conclusions: The CB2-Q63R variant contributes to the risk of pediatric IBD, in particular CD. The R63 variant is associated with a more severe phenotype in both UC and CD. Taken together, our data suggest the involvement of CB2 receptor in the pathogenesis and in the clinical features of pediatric IBD.

Keywords: inflammation; Th1/Th2 balance; Crohn's disease; Ulcerative Colitis; rs35761398

Introduction

Inflammatory bowel disease (IBD) is an inflammatory condition of the gastrointestinal tract that comprises two main forms: Crohn's disease (CD) and ulcerative colitis (UC). IBD are characterized by chronic inflammation, remitting and relapsing episodes, and a progressive course at diagnosis.

IBD prevalence has been increasing among children and adolescents [1].

The etiology and pathogenesis of IBD remain elusive, although accumulating evidence indicate that sustained intestinal infections, mucosal barrier defects, immune dysregulation, genetic and environmental factors are involved. Among these factors, the dysfunction of the mucosal immune system plays a pivotal role in the pathogenesis of IBD [2]. Innate and adaptive immunity contribute to trigger and maintain inflammatory events, resulting in IBD [3]. Furthermore, the production and release of cytokines and chemokines represent an important underlying mechanism, able to drive inflammatory events at local and systemic levels [4,5].

Despite the therapeutic advances related to an improved knowledge of the disease pathogenesis, alternative therapeutic approaches are necessary since IBD patients are often resistant to treatment. The endocannabinoid (EC) system has been recently indicated as a possible therapeutic target in IBD [6,7]. The EC system includes the EC endogenous lipid transmitters, their G-protein-coupled cannabinoid receptors type 1 and 2 (CB1 and CB2), and the enzymes for EC metabolism. ECs are synthesized and immediately released "on demand", although they can also accumulate in intracellular adiposomes [8].Common variant, rs35761398 (CAA/CGG), of the *CNR2* gene, encoding for the CB2 receptor, is associated to autoimmunity unbalance [9,10]. The relative glutamine-arginine substitution at codon 63, Q63R, affects the response of CB2 to ECs, resulting in a different modulation of its effectors cells [11]. In particular, the presence of the arginine (R) at codon 63 significantly reduces the EC-induced inhibition of lymphocyte proliferation, suggesting the R variant as the less functional [9]. This less functional variant CB2-R63 has been associated with increased risk of chronic immune thrombocytopenic purpura and celiac disease, in childhood [12,13].

We conducted a case-control association analysis between the common CB2-Q63R functional variant and IBD in a cohort of 221 Italian children with IBD compared to 600 controls previously recruited from the same geographic area. Moreover, we evaluated possible correlations between the CB2-Q63R variant and the clinical features of our pediatric IBD patients.

Materials and methods

Study population

The study population included 221consecutive children diagnosed with IBD between January 2001 and August 2015. Moreover 600 Italian healthy controls, previously recruited and genotyped for the CB2-Q63R variant were included [13]. Demographic and clinical data of each patient were retrospectively collected from medical records (Table 1). Only children with at least 1 year of follow-up were included in this study.

Diagnosis of IBD was based on clinical history, physical examination, radiologic studies, endoscopic appearance, and histologic findings, according to Porto criteria[14].

Physical examination for each patient and control included: body weight, measured by a balance beam scale; height, measured by a Harpenden stadiometer ; BMI, calculated by dividing the weight for the height square; z-score-BMI, calculated by using the LMS method; pubertal stage, assessed according to Tanner criteria. All measurements were taken by the same operator.

A blood sample was drawn from each patient at 8 a.m. after an overnight fast. Erythrocyte sedimentation rate and C-reactive protein were measured. Fecal samples were collected for Calprotectin levels measurements. Analyses were all performed in the same laboratory.

Ethical Considerations

The Institutional Review Board of the University of Naples "Federico II" approved the study protocol and questionnaire with the registration number 58/16. All participants gave informed written consent.

Phenotype analysis

CD and UC disease location were categorized using the Paris classification.

For CD: L1 for cecal and distal ileum involvement; L2 for colonic disease; L3 for ileocolonic disease; L4a for upper disease proximal to the ligament of Treitz; L4b for upper disease distal to the ligament of Treitz.

For UC: E1, ulcerative proctitis; E2, Left-sided UC (distal to splenic flexure); E3, Extensive (hepatic flexure distally); E4, Pancolitis (proximal to hepatic flexure). Perianal disease was defined by the presence of fissures, perianal ulcers, abscesses or fistulae, and skin tags. Disease behaviour based on clinical history, was categorized as fistulising, stricturing, or inflammatory using the Paris classification guidelines criteria [15]. Stricturing disease was referred to the presence of a constant luminal narrowing radiologically, endoscopically, or surgically diagnosed. Penetrating disease was referred to radio- graphic, endoscopic, surgical, or clinical evidence of an abscess or fistula in any location. Patients who had neither stricturing nor fistulising disease at diagnosis and throughout follow- up were classified as having inflammatory disease behaviour. Moreover, informations regarding changes from the inflammatory to the fistulising or stricturing patterns, as well as any clinical relapses, were collected. Disease activity at the diagnosis was scored by the Paediatric Crohn's Disease Activity Index (PCDAI) [16] or the Paediatric Ulcerative Colitis Activity Index (PUCAI) [17] for CD and UC, respectively. Clinical remission was defined as an activity score of the disease ≤ 10, while clinical response to the induction treatment was identified by a change in the score of at least 15 points from the baseline. Clinical relapse was defined as the occurrence or worsening of symptoms accompanied by a clinical score of >10 points, sufficient to require rescue

treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery [18]. Family history was defined as positive if at least one first or second- degree relative was diagnosed with IBD. We also considered clinical therapy at disease onset and during the follow-up. Two expert pediatric gastroenterologists (AS and EM) made all decisions regarding therapeutic interventions, in line with the validated international guidelines [19]. Extraintestinal manifestations included eye, joint, skin, or liver involvement and persistent fever, defined as temperature ≥38 °C for 3 days during the week before the treatment. The laboratory tests used for inflammation parameters included: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), hemoglobin concentration, and fecal calprotectin. Written, informed consent was obtained from the participants' parents, and assent was obtained for all patients older than 10 years of age. The study was approved by the Institutional Review Board of the University of Naples "Federico II".

Molecular screening

After informed consent from parents and assent from children were obtained, genomic DNA was extracted from 10 ml peripheral whole blood anticoagulated with EDTA (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA).

Genotype for the rs35761398 polymorphism was assigned for each patient by using a Taqman Assay (Real Master Mix Probe, Eppendorf, Italy). Primers and probes were the following: Sense primer 5'-GTGCTCTATCTGATCCTGTC-3'/Anti-sense primer 5'-TAGTCACGCTGCCAATC-3';AA-Probe 5'-CCCACCAACTCCGC-3'/GG-Probe 5'-CCCACCGGCTCCG-3'(PRIMM, Milan, Italy). Random samples were genotyped by polymerase chain reaction followed by direct sequencing. The PCR, consisted of an initial denaturation at 94 °C for 4 min followed by 31 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and generated a 280 bp amplimer (forward primer 5'-GAGTGGTCCCCAGAAGACAG-3'; reverse 5'-CACAGAGGCTGTGAAGGTCA-3'). PCR products were analyzed by using an ABI PRISM 310 automated sequencer (Applied

Biosystem, Foster City, CA). All primers were picked by using Primer3 software.

Statistics

The patients' allele frequencies were tested for Hardy-Weinberg equilibrium (HWE) by comparing expected and observed genotypes with the Fisher's exact test.

A linear logistic regression or a cross tabulation was performed to analyze clinical data with respect to the CB2 Q63R variant. The Tukey post-hoc test and the Mood's Median test were used for comparison of continuous variables, and the chi-square test for categorical variables, as appropriate. Data were normalized for age, sex, z-score BMI and pubertal stage. A p value < 0.05 was considered to be statistically significant. All of the analyses were performed using StatGraphics CENTURION XV.II (Adalta, Arezzo, Italy; STATPOINT TECHNOLOGIES INC., Virginia, USA).

Results

Association between CB2 polymorphism and IBD

We analyzed a cohort of 217 consecutive children with IBD (males 50%; median age 13.1 years, range 6-16). Clinical features of the study population are summarized in Table 1.

By using the control frequencies of 600 previously genotyped controls (males 46%; median age 12.5 years, range 4-16.8) [13] (allele frequency of the minor AA-allele, encoding for Q, was 0.42), we estimated that genotype frequencies of IBD patients were not distributed according to Hardy–Weinberg (p =0.009). Stratifying with respect to the type of IBD (CD or UC) the observed frequencies of CD patients significantly differed from those expected (p=0.0004), whereas UC were distributed in equilibrium (p=0.24) (Table S1).

Accordingly, the allelic frequency of the AA-allele was significantly decreased in CD patients respect to controls (χ^2=9.484; df 1; p=0.002) and comparison between patient and control genotype

distributions revealed a statistically significant difference both in total IBD and CD patients (p=0.0006 and p=0.00005, respectively). Interestingly, also when comparing CD to UC the frequency of CB2-R63 variant was significantly higher both for allelic and genotype distribution (Table 2). Indeed, in UC the allelic frequency of the AA-allele was not significantly different respect to control, although when considering the genotype frequencies a statistically significant difference compared to control was observed (Table 2). In particular, the GG/GG genotype (RR homozygous subjects) was highly prevalent in IBD children resulting in a frequency of about 50% compared to the 32% in controls with an increased susceptibility for RR homozygous subjects to develop a kind of IBD, in particular CD (OR=1.82 at CI 95%, p = 0.0002 for general IBD; OR=2.02 at CI 95%, p=0.001 for CD; OR=1.63 at CI 95%, p=0.02 for UC). Moreover, considering the presence of the GG allele alone (homozygous RR plus heterozygous QR subjects *vs* QQ subjects) the risk for developing CD was significantly higher (OR = 6.92 at C.I. 95%; p = 0.0002) (Table 3).

Association between CB2 polymorphism and disease phenotype

Analysis of genotype-phenotype was performed, investigating, in each patient, the following clinical features: gender, age at diagnosis, IBD family history, severity and disease localization, presence of perianal fistulas, extra intestinal manifestations, previous abdominal surgery, use and response to medical therapy (mesalamine, corticosteroids, immunosuppressive drugs, azathioprine, 6-mercaptopurine, methotrexate, cyclosporine and infliximab).

Stratifying clinical features with respect to CB2-Q63R we found in both IBD forms an increase of disease activity index in RR homozygous patients. In particular, an increased frequency of moderate to severe PCDAI was observed in CD patients carrying the risk genotype (p=0.01) compared with QQ and QR patients (Table 4). Similarly, UC patients homozygous for the R63 variant showed a more active disease (PUCAI>35, p = 0.02) compared with Q63 carriers (Table 5). Moreover, in UC RR patients a significant anticipation of disease relapse was observed (p=0.04).

No other significant correlations of the CB2 genotype with either clinical features of patients with CD and UC or efficacy of medical therapy, were found.

Discussion

We describe, for the first time, the association between the *CNR2*rs35761398 polymorphism (CB2-Q63R variant) and the risk of pediatric UC and CD. Of note, we also found an association between the aforementioned SNP and specific clinical CD and UC sub-phenotypes. Taken together, our data highlight the involvement of CB2 in the pathogenesis of pediatric IBD and provide insight into its possible role in the clinical features of chronic intestinal inflammation.

The EC system is involved in immune regulation by suppression of cell activation, modulation of balance of T helper cell types 1 and 2 (Th1 and Th2 balance) [20], inhibition of pro-inflammatory cytokine production [21], and nuclear factor- B-dependent apoptosis [22]. CB2 receptor, which is expressed at higher levels than CB1 receptors on many types of immune cells, is thought to be the principal cannabinoid receptor that mediates immune modulation by endocannabinoids [23]. In the field of gastroenterology, the CB2-Q63R variant has been shown to modulate hepatic inflammation and the risk of liver damage in obese children [24]. Moreover, the Q63R polymorphism seems to increase more than six-fold the risk for developing celiac disease [13]. Regarding IBD, a recent association study on Turkish patients, failed to find any association between the CB2-Q63R polymorphism and susceptibility to IBD or disease phenotypes [25]. A different allele frequencies and genotype distributions of the CB2-Q63R variant among Turkish and Italian populations could account for these discrepancies. Indeed, an impact of the race and the ethnicity on the incidence and the phenotype of IBD has been reported [26]. Moreover, the Turkish group analyzed an adult population whereas it has been pointed out that Pediatric patients with IBD seem to be a distinctive population with specificities requiring highly skilled and specialized approach also for diagnosis and treatment [27].

Numerous evidences based on rodents models of IBD [i.e. tri-nitro-benzene-sulphonic acid (TNBS)] suggest that ECs may limit intestinal inflammation via CB_1 and/or CB_2 receptor activation [28-37]. Moreover, human preclinical experiments show an up-regulation of both CB receptors expression and EC levels in intestinal biopsies of patients with inflammatory diseases of the gastrointestinal tract, such as UC, CD, diverticulitis and coeliac disease [38,32,34,36].

Finally, the importance of both CB_1 and CB_2 receptors in modulating inflammatory processes has been highlighted also by in-vitro studies demonstrating that ECs promote epithelial wound healing in the human colon by the activation of CB_1 receptor [32] and inhibit the release of interleukin (IL)-8 in human colonic epithelial cells by the activation of CB_2 receptor [39].

We found that the GG/GG genotype (RR homozygous subjects) was highly prevalent in IBD children. Interestingly, when CD and UC patients were analyzed separately in comparison with controls, a significant over-representation of the RR genotype and of the R allele was observed for the CD children.

These findings are in line with the fact that ECs produce a bias in the balance between the two types of Th-cells, suppressing Th1and enhancing Th2, and that the CB2-Q63R variant is involved in this process exerting a different inhibition of these cells according to the presence of Q or R [20]. This bias has several mechanisms. It has been partly explained by different expression of cannabinoid receptors on Th subpopulations and on antigen-presenting cells. It could be due to modulation of cytokines generated by dendritic cells [40]. In addition, the induction of Th2 associated cytokines can afterwards inhibit Th1 cells [41]. Recent data show that ECs, via CB2 receptor, can directly induce B-cell classs witching from IgM to IgE in favor of a Th2 type immunity [42].

Therefore it is reasonable that this CB2 variant, which has been related to decreased immunomodulatory response and the development of autoimmune disease [9], is mainly associated with a Th1-mediated disease, such as CD.

Indeed, in many previous studies, cytokine profiles of CD patients displayed Th1 dominance characterized by higher levels of IFN-γand TNF-α and lower amounts of IL-4 in the intestinal mucosa and serum of CD patients compared with healthy controls [43-47]. Finally, it has been suggested that a shift towards the Th1 profile may contribute to the onset of CD by activating macrophages which produce pro-inflammatory cytokines causing intestinal damage [48].

In both CD and UC children, the R63 variant was strongly associated with moderate to severe activity of the disease evaluated with PCDAI and PUCAI score, respectively. Moreover, in UC patients, the CB2-R63 variant was associated also to a higher incidence of early clinical relapse, further suggesting an association with a more severe phenotype of disease. Indeed, an impaired functionality of CB2 receptor could determine a decreased suppression of activated macrophages and mast cells and stimulate the secretion of pro-inflammatory cytokines such as TNF-α [49-55], causing an exacerbation of intestinal inflammation and worsening disease course.

This study has some limitations. Firstly, we enrolled only patients from Southern Italy, thus our result cannot be generalized to the whole Italian population or to other ethnicity. Another limitation is that this study cannot provide data about the disease progress with respect to the CB2-Q63R genotype. Moreover, the genotype distribution in CD cohort, that resulted in very few QQ patients, could have masked further genotype-phenotype correlations.

Although these limitations, the marked rising in the incidence of IBD in childhood [27], gives particular interest to our study since in a paediatric population with IBD the genetic susceptibility could be greater and influence a more severe disease course.

The present results also support the concept that the CB2 receptor on immune effector cells may represent a potential molecular target for selective CB2 agonist therapies that could suppress autoreactive, pro-inflammatory innate and adaptive immune responses.

Nevertheless, further investigation as well as targeted functional studies are needed to understand how Q63R genotype contributes to disease susceptibility in pediatric IBD, and whether this genetic

variant might represent a disease marker and have therapeutic implications.

Acknowledgements

Authors would like to thank all of the participants in the study

Authorship Statement

- **Guarantor of article**:

  CS

- **Specific author contributions**:

  ChT and SC performed the research,

  CT, MM, ChT and SC collected the data,

  GB, EM, MM, AS analysed the data,

  EM, FR, CS and AS designed the research study,

  CS, GB, EMdG and FR wrote the paper,

  EMdG and CT contributed to the design of the study.

- **ALL authors approved the final version of the article, including authorship list.**

Statement of Interests

1. **Authors' declaration of personal interests:**

The authors have no conflict of interest to disclose.

2. **Declaration of funding interests:**

   No specific funding has been received.

References

1. Malaty HM, Fan X, Opekun AR, *et al.* Rising incidence of inflammatory bowel disease among children: a 12-year study. *J Pediatr Gastroenterol Nutr* 2010; **50**: 27-31. doi:10.1097/MPG.0b013e3181b99baa.
2. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-17. doi:10.1038/nature10209.
3. Kaistha A, Levine J. Inflammatory bowel disease: the classic gastrointestinal autoimmune disease. *Curr Probl Pediatr Adolesc Health Care*. 2014; **44**: 328-334. doi:10.1016/j.cppeds.2014.10.003.
4. Ueno A, Ghosh A, Hung D, *et al.* Th17 plasticity and its changes associated with inflammatory bowel disease. *World J Gastroenterol* 2015; **21**: 12283-12295. doi:10.3748/wjg.v21.i43.12283.
5. Singh UP, Singh NP, Murphy EA, *et al.* Chemokine and cytokine levels in inflammatory bowel disease patients. *Cytokine* 2016; **77**: 44-49. doi:10.1016/j.cyto.2015.10.008
6. Naftali T, Mechulam R, Lev LB, *et al.* Cannabis for inflammatory bowel disease. *Dig Dis* 2014; **32**: 468-474. doi:10.1159/000358155.
7. Alhouayek M, Muccioli GG. The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. *Trends Mol Med* 2012; **18**: 615-25. doi: 10.1016/j.molmed.2012.07.009.
8. Maccarrone M, Bab I, Bíró T, *et al.* Endocannabinoid signaling at the periphery: 50 years after THC. *Trends Pharmacol Sci* 2015; **36**: 277-296. doi: 10.1016/j.tips.2015.02.008.
9. Sipe JC, Arbour N, Gerber A, *et al.* Reduced endocannabinoid immune modulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders. *J Leukoc Biol* 2005; **78**: 231-238.

10. Bellini G, Olivieri AN, Grandone A, *et al.* Association between cannabinoid receptor type 2 Q63R variant and oligo/polyarticular juvenile idiopathic arthritis. *Scand J Rheumatol* 2015; 14: 1–4.

11. Carrasquer A, Nebane NM, Williams WM, *et al.* Functional consequences of non synonymous single nucleotide polymorphisms in the CB2 cannabinoid receptor. *Pharmacogenet Genomics* 2010; **20**: 157-166. doi: 10.1097/FPC.0b013e3283367c6b.

12. Rossi F, Mancusi S, Bellini G, *et al.* CNR2 functional variant (Q63R) influences childhood immune thrombocytopenic purpura. *Haematologica* 2011; **96**: 1883-1885. doi: 10.3324/haematol.2011.045732.

13. Rossi F, Bellini G, Tolone C, *et al.* The cannabinoid receptor type 2 Q63R variant increases the risk of celiac disease: implication for a novel molecular biomarker and future therapeutic intervention. *Pharmacol Res* 2012; **66**: 88-94. doi: 10.1016/j.phrs.2012.03.011.

14. IBD Working Group of the European Society for Paediatric Gastroenterology Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis – the Porto criteria. *Journal of Pediatric Gastroenterology and Nutrition* 2005; **41**: 1-7.

15. Levine A, Griffiths A, Markowitz J, *et al.* Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflammatory Bowel Diseases* 2011; **17**: 1314-1321.

16. Hyams JS, Ferry GD, Mandel FS, *et al.* Development and validation of a pediatric Crohn's disease activity index. *Journal of Pediatric Gastroenterology and Nutrition* 1991; **12**: 439-447.

17. Turner D, Otley AR, Mack D, *et al.* Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multi center study. *Gastroenterology* 2007; **133**: 423-432.

18. Hyams J, Crandall W, Kugathasan S, *et al.* Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. *Gastroenterology* 2007; **132**: 863-873.

19. Biancone L, Michetti P, Travis S, *et al.* European evidence-based consensus on the management of ulcerative colitis: special situations. *Journal of Crohn'sandColits* 2008; **2**: 63-92.

20. Yuan M, Kiertscher SM, Cheng Q, *et al.* Δ-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *J Neuroimmunol* 2002; **133**:124-131.

21. Klein TW, Lane B, Newton CA, *et al.* The cannabinoid system and cytokine network. *Proc Soc Exp Biol Med* 2000; **225**: 1-8.

22. Do Y, McKallip R, Nagarkatti M, *et al.* Activation through cannabinoid receptors 1 and 2 on dendritic cells triggers NF-kB-dependent apoptosis: novel role for endogenous and exogneous cannabinoids in immunoregulation. *J Immunol* 2004; **173**: 2373-2382.

23. Parolaro D, Massi P, Rubino T, *et al.* Endocannabinoids in the immune system and cancer. *Prostaglandins Leukot Essent Fatty Acids* 2002; **66**: 319-332.

24. Bellini G, Grandone A, Torella M, *et al.* The Cannabinoid Receptor 2 Q63R Variant Modulates the Relationship between Childhood Obesity and Age at Menarche. *PLoS One* 2015; **10**: e0140142. doi: 10.1371/journal.pone.0140142.

25. Yonal O, Eren F, Yılmaz Y, *et al.* No association between the functional cannabinoid receptor type 2Q63R variants and inflammatory bowel disease in Turkish subjects. *Turk J Gastroenterol* 2014; **25**: 639-643.

26. Basu D, Lopez I, Kulkarni A, *et al.* Impact of race and ethnicity on inflammatory bowel disease. *Am J Gastroenterol* 2005; **100**: 2254-2261.

27. Benchimol EI, Fortinsky KJ, Gozdyra P, *et al.* Epidemiology of pediatric inflammatory bowel disease: a systematic review of international trends. *Inflamm Bowel Dis* 2011; **17**: 423-439.

28. Izzo A A, Fezza F, Capasso R, *et al.* Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. *Br J Pharmacol* 2001; **134**: 563-570.

29. Mascolo N, Izzo AA, Ligresti A, *et al.* The endocannabinoid system and the molecular basis of paralytic ileus in mice. *FASEB J* 2002; **16**: 1973-1975.

30. McVey DC, Schmid PC, Schmid HH, *et al.* Endocannabinoids induce ileitis in rats via the capsaicin receptor (VR1). *J Pharmacol Exp Ther* 2003; **304**: 713-722.

31. Massa F, Marsicano G, Hermann H, *et al.* The endogenous cannabinoid system protects against colonic inflammation. *J Clin Invest* 2004; **113**: 1202–1209.

32. Wright KL, Rooney N, Feeney M, *et al.* Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. *Gastroenterology* 2005; **129**: 437-453.

33. D'Argenio G, Valenti M, Scaglione G, *et al.* Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. *FASEB J* 2006; **20**: 568-570.

34. Guagnini F, Valenti M, Mukenge S, *et al.* Neural contractions in colonic strips from patients with diverticular disease: role of endocannabinoids and substance P. *Gut* 2006; **55**: 946-953.

35. Kimball ES, Schneider CR, Wallace, NH, *et al.* Agonists of cannabinoid receptor 1 and 2 inhibit experimental colitis induced by oil of mustard and by dextran sulfate sodium. *Am J Physiol Gastro intest Liver Physiol* 2006; **291**: G364-G371.

36. D'Argenio G, Petrosino S, Gianfrani C, *et al.* Overactivity of the intestinal endocannabinoid system in celiac disease and in methotrexate-treated rats. *J Mol Med* 2007; **85**: 523-530.

37. Borrelli F, Izzo AA. Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. *Best Pract Res Clin Endocrinol Metab* 2009; **23**: 33-49.

38. Ligresti A, Bisogno T, Matias I, *et al.* Possibile endocannabinoid control of colorectal cancer growth. *Gastroenterology* 2003; **125**: 677-687.

39. Ihenetu K, Molleman A, Parsons ME, *et al.* Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. *Eur J Pharmacol* 2003; **458**: 207-215.

40. Lu T, Newton C, Perkins I, *et al.* Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with Legionella pneumophila. *Eur J Pharmaco*l 2006; **532**: 170-177.

41. Croxford J L, Yamamura T. Cannabinoids and the immune system: potential for the treatment of inflammatory diseases? *J Neuroimmunol* 2005; **166**: 3-18.

42. Agudelo M, Newton C, Widen R, *et al.* Cannabinoid receptor 2 (CB2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. *J Neuroimmune Pharmacol* 2008; **3**: 35-42. doi: 10.1007/s11481-007-9088-9.

43. Parronchi P, Romagnani P, Annunziato F, *et al.* Type 1 T-helper cell predominance and interleukin-12 in the gut of patients with Crohn's disease. *Am J Clin Pathol* 1997; **150**: 823-832.

44. Niessner M, Volk BA. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). *Clin Exp Immunol* 1995; **101**: 428-435.

45. Karttunnen R, Breese EJ, Walker-Smith JA, *et al.* Decreased mucosal interleukin-4 (IL-4) production in gut inflammation. *J Clin Pathol* 1994; **47**: 1015-1018.

46. Reimund JM, Wittersheim C, Dumont S, *et al.* Increased production of tumour necrosis factor-a, interleukin-1B, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* 1996; **39**: 684-689.

47. Murch SH, Lamkin VA, Savage MO, *et al.* Serum concentrations of tumor necrosis factor a in childhood chronic inflammatory bowel disease. *Gut* 1991; **32**: 913-917.

48. Romagnani P, Annunziato F, Baccari MC, *et al.* T cells and cytokines in Crohn's disease. *Immunology* 1997; **9**: 793-799.

49. Klein T W, Cabral GA. Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. *J Neuroimmune Pharmacol* 2006; **1**: 50-64.

50. Bueb JL, Lambert DM, Tschirhart EJ. Receptor-independent effects of natural cannabinoids in rat peritoneal mast cells in vitro. *Biochim Biophys Acta* 2001; **1538**: 252-259.

51. Chang YH, Lee ST, Lin WW. Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrophages: involvement of eicosanoids. *J Cell Biochem* 2001; **81**: 715-723.

52. Facchinetti F, Del Giudice E, Furegato S, *et al.* Cannabinoids ablate release of TNF alpha in rat microglial cells stimulated with lypopolysaccharide. *Glia* 2003; **41**: 161-168.

53. Samson MT, Small-Howard A, Shimoda LM, *et al.* Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. *J Immunol* 2003; **170**: 4953-4962.

54. Vannacci A, Passani MB, Pierpaoli S, *et al.* Nitric oxide modulates the inhibitory effect of cannabinoids on the immunological activation of guinea pig mast cells. *Inflamm Res* 2003; **52**: S7-S8.

55. Small-Howard AL, Shimoda LM, Adra CN, *et al.* Anti-inflammatory potential of CB1-mediated cAMP elevation in mast cells. *Biochem J* 2005; **388**: 465-473.

Table 1

Clinical and demographic characteristic of 217 children and adolescents with IBD

	CD	UC
Patients, n	112	105
Males, n (%)	67 (59.8)	42 (40)
Median age at diagnosis, years (range)	9.9 (6-14)	12.5 (7-14)
Median disease duration, years (range)	2.7 (0.6-4.2)	6.4 (2-8)
Positive family history, n (%)	7 (6.2)	10 (9.5)
Extraintestinal manifestation, n (%)	19 (16.9)	11 (10.5)
Location of disease, CD, n (%)		
L1	13 (12)	
L2	24 (21)	-
L3	45 (40)	
L4	30 (27)	
Perianal disease, n (%)	23 (20.5)	-
Disease extent, UC, n (%)		
E1		24 (23)
E2		24 (23)
E3	-	24 (23)
E4		33 (31)
Disease behaviour, CD, n (%)		
Inflammatory, B1	99 (88)	-
Stricturing, B2	8 (7)	
Fistulizing, B3	5 (5)	
Change in clinical behaviour, n (%)	18 (16)	-
Need for surgery, n (%)	12 (10.7)	2 (1.8)

IBD = Inflammatory Bowel Disease; CD = Crohn's Disease; UC = Ulcerative Colitis

Table 2

Allelic and genotype distribution for CB2-Q63R variant in IBD Italian paediatric patients

	IBD	**CD**	**UC**	**Controls** [a]
Individuals, n	217	112	105	600
AAAA Homozygous (%)	22 (10)	3 (3)	19 (18)	96 (16)
AAGG Heterozygous (%)	94 (43)	54 (48)	40 (38)	310 (52)
GGGG Homozygous (%)	101 (47)	55 (49)	46 (44)	194 (32)
Minor allelic (AA) frequency, %	32	26.8	37	42
Allele frequencies				
IBD *vs* Controls	$\chi^2 = 4.105$; 1 df; $\boldsymbol{p = 0.04}$			
CD *vs* Controls	$\chi^2 = 9.484$; 1 df; $\boldsymbol{p = 0.002}$			
UC *vs* Controls	$\chi^2 = 1.026$; 1 df; $p = 0.31$			
CD *vs* UC	$\chi^2 = 4.463$; 1 df; $\boldsymbol{p = 0.03}$			
Genotype frequencies				
IBD *vs* Controls	$\chi^2 = 14.950$; 2 df; $\boldsymbol{p = 0.0006}$			
CD *vs* Controls	$\chi^2 = 19.859$; 2 df; $\boldsymbol{p = 0.00005}$			
UC *vs* Controls	$\chi^2 = 7.013$; 2 df; $\boldsymbol{p = 0.03}$			
CD *vs* UC	$\chi^2 = 14.152$; 2 df; $\boldsymbol{p = 0.00008}$			

[a] data from previously genotyped controls [13]

IBD = Inflammatory Bowel Disease; CD = Crohn's Disease; UC = Ulcerative Colitis

Table 3

Odd ratio for susceptibility to IBD for CB2-R63 carriers

	IBD		CD		UC		Controls [a]	
Genotype	*Frequency*	*Relative Frequency*	*Frequency*	*Relative Frequency*	*Frequency*	*Relative Frequency*	*Frequency*	*Relative Frequency*
QQ	22	0.1014	3	0.0268	19	0.1809	96	0.1600
QR	94	0.4332	54	0.4821	40	0.3809	310	0.5167
RR	101	0.4654	55	0.4911	46	0.4382	194	0.3233

RR *vs* (QQ+QR)	***Odds Ratio***	***CI 95%***	***p***
IBD *vs* Controls	1.822	1.311-2.533	**0.0002**
CD *vs* Controls	2.019	1.316-3.099	**0.001**
UC *vs* Controls	1.632	1.047-2.540	**0.026**
CD *vs* UC	1.238	0.700-2.190	0.496
(QR+RR) *vs* QQ	***Odds Ratio***	***CI 95%***	***p***
IBD *vs* Controls	1.723	1.029-2.906	**0.033**
CD *vs* Controls	6.921	2.072-27.857	**0.0002**
UC *vs* Controls	0.862	0.487-1.541	0.569
CD *vs* UC	8.027	2.149-35.330	**0.0002**

[a] data from previously genotyped controls [13]

IBD = Inflammatory Bowel Disease; CD = Crohn's Disease; UC = Ulcerative Colitis

Table 4

Clinical characteristic of 112 children and adolescents with CD stratified for CB2-Q63R variant

CD	QQ	QR	RR	$p^{a,b,c}$
Patients, n (%)	3 (3)	54 (48)	55 (49)	
Males, n (%)	2 (1.8)	31 (27.7)	34 (30.4)	0.61 [a]
Median age at diagnosis, years (range)	13.9 (13-14)	10.6 (6.2-12.6)	11.3 (9.9-13.1)	0.27 [b]
Median disease duration, years (range)	0.9 (0.6-1.0)	2.4 (1.8-4.2)	2.5 (1.9-3.8)	0.35 [b]
Positive family history, n (%)	0 (0)	5 (0.9)	2 (0.4)	0.43 [a]
Extraintestinal manifestation, n (%)	0 (0)	9 (1.7)	10 (1.8)	0.71 [a]
Disease Location, n				
L1	1	5	7	
L2	0	13	11	0.66 [a]
L3	2	20	23	
L4	0	16	14	
Perianal disease, n %	1 (33.3)	10 (18.5)	11 (20)	0.82 [a]
Disease behaviour, n				
Inflammatory, B1	3	48	48	0.96 [a]
Stricturing, B2	0	4	4	
Fistulizing, B3	0	2	3	
Change in clinical behaviour, n (%)	0 (0)	11 (2)	8 (1.4)	0.52 [a]
Relapse, n (%)	2 (66.6)	29 (53.7)	38 (69)	0.25 [a]
Relapse during the first year of disease, n (%)	1 (50)	18 (62)	18 (47.4)	0.27 [a]
First relapse, years, mean±SD	1.00±0.00	1.39±1.85	1.72±2.36	0.65 [c]
Immunosuppressant therapy, n (%)	2 (66.6)	24 (44.4)	34 (61.8)	0.17 [a]
Steroid therapy, n (%)	1 (33.3)	16 (3)	27 (4.9)	0.11 [a]
Biologic therapy, n (%)	1 (33.3)	4 (7.4)	9 (16.4)	0.20 [a]
Need for surgery, n (%)	1 (33.3)	6 (1.1)	5 (0.9)	0.41 [a]
Median PCDAI, (range)	25 (25-30)	27.5 (22.5-35.0)	30 (25-35)	0.30 [b]
PCDAI, n				
<10	1	0	3	**0.012** [a]
≥10<30	1	31	23	
≥30	1	23	31	
Median Calprotectin, ug/g, (range)	500 (500-500)	416.5 (350-459)	375 (299-438)	0.18 [b]
Median CRP, mg/dl (range)	13 (7-13)	5.75 (3.0-13.4)	7.75 (3.8-20.6)	0.72 [b]

CD = Crohn's Disease; PCDAI = Pediatric Crohn's Disease Activity Index; CRP = C Reactive Protein

[a] Chi-Square Test; [b] Mood's Median Test; [c] Tukey post-hoc Test

Table 5

Clinical characteristic of 105 children and adolescents with UC stratified for CB2-Q63R variant

UC	QQ	QR	RR	$p^{a,b,c}$
Patients, n (%)	19 (18)	40 (38)	46 (44)	
Males, n (%)	10 (52.6)	15 (37.5)	17 (37)	0.46[a]
Median age at diagnosis, years (range)	13 (7.3-14.3)	11 (8.6-13)	10 (8.3-12)	0.43[b]
Median disease duration, years (range)	4.0 (2.0-8.0)	6.5 (3.7-7.7)	5.2 (3-6.6)	0.61[b]
Positive family history, n (%)	3 (15.9)	3 (7.5)	4 (8.7)	0.58[a]
Extraintestinal manifestation, n (%)	1 (5.3)	4 (10)	6 (1.3)	0.64[a]
Disease extent, n				
E1	4	7	13	
E2	1	11	12	0.37[a]
E3	6	10	8	
E4	8	12	13	
Relapse, n (%)	14 (73.7)	29 (72.5)	32 (69.6)	0.93[a]
Relapse in the first year of disease, n (%)	8 (57.1)	12 (30)	21 (45.6)	0.31[a]
First relapse, years, mean±SD	2.21±2.65	1.86±2.25	1.07±0.90	**0.04**[c]
Immunosuppressant therapy, n (%)	11 (57.9)	21 (52.5)	20 (43.5)	0.51[a]
Steroid therapy, n (%)	15 (79)	24 (60)	33 (72)	0.28[a]
Biologic therapy, n (%)	1 (5.3)	1 (2.5)	0 (0)	0.35[a]
Need for surgery, n (%)	0 (0)	0 (0)	2 (4.4)	0.27[a]
Median PUCAI (range)	35 (30-40)	30 (25-40)	40 (34-40)	0.27[b]
PUCAI, n				
inactive/mild <35	6	24	15	**0.021**[a]
moderate/severe ≥35	13	16	31	
Median Calprotectin, ug/g (range)	300 (101-500)	336.5 (274-500)	418 (270-500)	0.45[b]
Median CRP, mg/dl (range)	0.6 (0.3- 6.0)	0.6 (0.3-2.6)	0.6 (0.3-3.0)	1.00[b]

UC = Ulcerative Colitis; PUCAI = Pediatric Ulcerative Colitis Activity Index; CRP = C Reactive Protein

[a]Chi-Square Test; [b]Mood's Median Test; [c]Tukey post-hoc Test

Table S1

Hardy-Weinberg for CB2 Q63R distribution in IBD Italian paediatric patients

	Observed			Expected *		
Genotype	**IBD**	**CD**	**UC**	**IBD**	**CD**	**UC**
QQ	22	3	19	38	20	19
QR	94	54	40	106	54	51
RR	101	55	46	73	38	35
TOT	217	112	105	217	112	105

Hardy-Weinberg			
Observed *vs* Expected	*Chi-squared*	*df*	*p*
IBD *vs* **Controls**	9.492	2	**0.009**
CD *vs* **Controls**	15.673	2	**0.0004**
UC *vs* **Controls**	2.823	2	0.24

* with respect to data from previously genotyped controls (96 QQ, 310 QR, 194 RR; n=600)[13]

IBD = Inflammatory Bowel Disease; CD = Crohn's Disease; UC = Ulcerative Colitis

3.3 Genetic sharing and heritability of paediatric age of onset autoimmune diseases.

nature COMMUNICATIONS

ARTICLE

Received 14 Feb 2015 | Accepted 21 Aug 2015 | Published 9 Oct 2015

DOI: 10.1038/ncomms9442 OPEN

Genetic sharing and heritability of paediatric age of onset autoimmune diseases

Yun R. Li[1,2], Sihai D. Zhao[3], Jin Li[1], Jonathan P. Bradfield[1], Maede Mohebnasab[1], Laura Steel[1], Julie Kobie[4], Debra J. Abrams[1], Frank D. Mentch[1], Joseph T. Glessner[1], Yiran Guo[1], Zhi Wei[1,5], John J. Connolly[1], Christopher J. Cardinale[1], Marina Bakay[1], Dong Li[1], S. Melkorka Maggadottir[1,6], Kelly A. Thomas[1], Haijun Qui[1], Rosetta M. Chiavacci[1], Cecilia E. Kim[1], Fengxiang Wang[1], James Snyder[1], Berit Flatø[7], Øystein Førre[7], Lee A. Denson[8], Susan D. Thompson[9], Mara L. Becker[10], Stephen L. Guthery[11], Anna Latiano[12], Elena Perez[13], Elena Resnick[14], Caterina Strisciuglio[15], Annamaria Staiano[15], Erasmo Miele[15], Mark S. Silverberg[16], Benedicte A. Lie[17], Marilynn Punaro[18], Richard K. Russell[19], David C. Wilson[20], Marla C. Dubinsky[21], Dimitri S. Monos[22,23], Vito Annese[24], Jane E. Munro[25,26], Carol Wise[27], Helen Chapel[28], Charlotte Cunningham-Rundles[14], Jordan S. Orange[29], Edward M. Behrens[23,30], Kathleen E. Sullivan[6,23], Subra Kugathasan[31], Anne M. Griffiths[32], Jack Satsangi[33], Struan F.A. Grant[1,23], Patrick M.A. Sleiman[1,23], Terri H. Finkel[34], Constantin Polychronakos[35], Robert N. Baldassano[23,36], Eline T. Luning Prak[37], Justine A. Ellis[38,39], Hongzhe Li[4], Brendan J. Keating[1,23] & Hakon Hakonarson[1,23,40]

Autoimmune diseases (AIDs) are polygenic diseases affecting 7–10% of the population in the Western Hemisphere with few effective therapies. Here, we quantify the heritability of paediatric AIDs (pAIDs), including JIA, SLE, CEL, T1D, UC, CD, PS, SPA and CVID, attributable to common genomic variations (SNP-h^2). SNP-h^2 estimates are most significant for T1D (0.863 ± s.e. 0.07) and JIA (0.727 ± s.e. 0.037), more modest for UC (0.386 ± s.e. 0.04) and CD (0.454 ± 0.025), largely consistent with population estimates and are generally greater than that previously reported by adult GWAS. On pairwise analysis, we observed that the diseases UC-CD (0.69 ± s.e. 0.07) and JIA-CVID (0.343 ± s.e. 0.13) are the most strongly correlated. Variations across the *MHC* strongly contribute to SNP-h^2 in T1D and JIA, but does not significantly contribute to the pairwise *rG*. Together, our results partition contributions of shared versus disease-specific genomic variations to pAID heritability, identifying pAIDs with unexpected risk sharing, while recapitulating known associations between autoimmune diseases previously reported in adult cohorts.

[1] Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [2] Medical Scientist Training Program, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [3] Department of Statistics, University of Illinois at Urbana-Champaign, Champaign, Illinois 61820, USA. [4] Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [5] Department of Computer Science, New Jersey Institute of Technology, Newark, New Jersey 07103, USA. [6] Division of Allergy and Immunology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [7] Department of Rheumatology, Oslo University Hospital, Rikshospitalet, Oslo 0372, Norway. [8] Center for Inflammatory Bowel Disease, Division of Gastroenterology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA. [9] Divison of Rheumatology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA. [10] Division of Rheumatology and Division of Clinical Pharmacology, Toxicology, and Therapeutic Innovation, Children's Mercy-Kansas City, Kansas City, Missouri 64108, USA. [11] Department of Pediatrics, University of Utah School of Medicine and Primary Children's Medical Center, Salt Lake City, Utah 84113, USA. [12] RCCS 'Casa Sollievo della Sofferenza', Division of Gastroenterology, San Giovanni Rotondo 71013, Italy. [13] Division of Pediatric Allergy and Immunology, University of Miami Miller School of Medicine, Miami, Florida 33136, USA. [14] Institute of Immunology, Department of Medicine, Icahn School of Medicine at Mount Sinai, Mount Sinai Hospital, New York, New York 10029, USA. [15] Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Naples 80138, Italy. [16] IBD Centre, Mount Sinai Hospital, University of Toronto, 441-600 University Avenue, Toronto, Ontario, Canada M5G 1X5. [17] Department of Immunology, Oslo University Hospital, Rikshospitalet, 0027 Oslo 0372, Norway. [18] Texas Scottish Rite Hospital for Children, Dallas, Texas 750219, USA. [19] Yorkhill Hospital for Sick Children, Glasgow G38SJ, Scotland. [20] Paediatric Gastroenterology and Nutrition, Royal Hospital for Sick Children, Edinburgh and Child Life and Health, University of Edinburgh, Edinburgh EH9 1UW, UK. [21] Departments of Pediatrics and Common Disease Genetics, Cedars Sinai Medical Center, Los Angeles, California 90048, USA. [22] Department of Pathology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [23] Department of Pediatrics, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [24] Unit of Gastroenterology, Department of Medical and Surgical Specialties, Careggi University Hospital, Viale Pieraccini 18, Florence 50139, Italy. [25] Paediatric Rheumatology Unit, Royal Children's Hospital, Parkville, Victoria 3052, Australia. [26] Arthritis and Rheumatology Research, Murdoch Childrens Research Institute, Parkville, Victoria 3052, Australia. [27] Sarah M. and Charles E. Seay Center for Musculoskeletal Research, Texas Scottish Rite Hospital for Children, Dallas, Texas 750219, USA. [28] Department of Clinical Immunology, Nuffield Department of Medicine, University of Oxford, OX1 1NF, UK. [29] Section of Immunology, Allergy, and Rheumatology, Department of Pediatric Medicine, Texas Children's Hospital, Houston, Texas 77030, USA. [30] Division of Rheumatology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [31] Department of Pediatrics, Emory University School of Medicine and Children's Health Care of Atlanta, Atlanta, Georgia 30329, USA. [32] Hospital for Sick Children, University of Toronto, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. [33] Gastrointestinal Unit, Division of Medical Sciences, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK. [34] Department of Pediatrics, Nemours Children's Hospital, Orlando, Florida 32827, USA. [35] Departments of Pediatrics and Human Genetics, McGill University, Montreal, Quebec, Canada H3H 1P3. [36] Division of Gastroenterology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [37] Department of Pathology and Lab Medicine, Perelman School of Medicine University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. [38] Genes, Environment and Complex Disease, Murdoch Childrens Research Institute, Parkville, Victoria 3052, Australia. [39] Department of Paediatrics, University of Melbourne, Parkville, Victoria 3052, Australia. [40] Division of Pulmonary Medicine, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. Correspondence and requests for materials should be addressed to H.H. (email: hakonarson@email.chop.edu)

© 2015 Macmillan Publishers Limited. All rights reserved.

Autoimmune (AI) diseases affect approximately 1 in 12 individuals living in the Western Hemisphere, representing a significant cause of morbidity, chronic disability and health-care burden. High rates of sibling recurrence and twin–twin concordance, both within and across multiple independent AI diseases, coupled with recent results from genome-wide association studies (GWAS), suggest that a set of shared genetic risk factors underlie paediatric AI disease (pAID) aetiology[1–3]. Moreover, a number of AI diseases show clear familial clustering, such as inflammatory bowel disease (IBD)[4], whereas others (for example, type 1 diabetes (T1D), AI thyroiditis (THY) and celiac disease (CEL) may manifest as comorbid diseases in polyglandular AI syndromes[2]. Although the concept of genetic sharing among AIs is intriguing, it remains unclear if this is due to 'pleiotropic' risk factors that predispose to multiple AI diseases via shared mechanisms or if multiple, independent risk factors are responsible.

GWAS have identified single-nucleotide polymorphisms (SNPs) across hundreds of loci as being associated with an increased risk of developing AI[5–12]. These findings, coupled with those from epidemiological studies, strongly support the existence of (i) an overlapping 'AI disease genetic landscape'[13,14] and (ii), consequently, a shared heritability across these diseases. Heritability, in the broad-sense (H^2), is defined as the entirety of an individual's phenotypic variation explained by genetic variance, but in practicality, it can be difficult to quantify and partition precisely[15]. A major contribution to H^2 is the narrow-sense or additive heritability (h^2), which can be more accurately quantified.[15]. Recently, a new method was established to estimate the total phenotype variance attributable to additive genetic variations using genome-wide SNP genotyping data[16–19]. The method has been since applied to dozens of GWAS-examined traits and extended to examine jointly the co-heritability of related diseases[20].

We systematically quantified the narrow-sense heritability, h^2, as well as the pairwise joint heritability of pAIDs attributable to common genomic variation using a single-centre accrued cohort of over 5,000 unrelated cases composed of nine independent pAIDs and 36,000 shared, population-based healthy controls. We first report the genome-wide SNP genotype-derived heritability estimates (referred to as SNP-h^2) and then the genetic correlation (SNP-rG) across pairs of the nine investigated pAIDs. We contextualize these findings alongside a comprehensive review of available literature and epidemiological data sets, illustrate a method for quantifying genetic risk factor sharing across pAIDs, and provide considerations for how such genetic data can aid disease prediction.

Results

Quantifying the heritability of paediatric AI diseases. To quantify the SNP-h^2 of the nine pAIDs, we utilized genome-wide SNP genotypes ascertained from DNA samples of patients of each pAID cohort along with samples from population-based control subjects with no known diagnosis of autoimmunity or immunodeficiency. Following extensive quality control (QC), removing SNPs of lower minor allele frequency (MAF), missingness and differential missingness in cases and controls, and deviation from Hardy–Weinberg equilibrium (see Methods), we retained 461,301 SNPs. We excluded samples for low genotyping rates, cryptic relatedness and genetic outliers, leaving a cohort consisting of 4,956 cases distributed across nine pAIDs and 27,451 unrelated shared population-based controls (Table 1). We also included, for comparison, a non-immune-mediated dichotomous trait, paediatric-onset epilepsy (EPI); this cohort of ~800 case subjects was recruited and genotyped at our centre using the same platforms over the same time period.

Table 1 | Summary of cohorts included.

Disease	Full disease name	Cases	Controls	GIF*	Prevalence†
CD	Crohn's disease	1,848	27,457	1.086	3.00E − 03
CEL	Celiac disease	137	27,435	1.006	1.00E − 02
CVID	Common variable immunodeficiency disorder	304	27,492	1.010	1.00E − 04
EPI	Epilepsy	754	26,122	1.027	1.00E − 04
JIA	Juvenile idiopathic arthritis	1,112	27,131	1.000	2.00E − 03
PS	Psoriasis	85	27,474	1.012	1.00E − 03
SLE	Systemic lupus erythematosus	252	27,525	1.019	1.00E − 04
SPA	Spondyloarthropathy	98	27,483	1.020	1.00E − 04
T1D	Type 1 diabetes	664	27,395	1.062	5.00E − 03
UC	Ulcerative colitis	854	27,482	1.041	1.00E − 03

GIF, genomic inflation factor; SNP, single-nucleotide polymorphism.
*GIF is provided for each cohort and included all SNPs (including *ChrX* and the extended *MHC*).
†Prevalence estimates used here are those made based on observations at our center.

We used a previously described method for estimating disease variance explained by additive genetic factors using GWAS data (referred to as SNP-based heritability or SNP-h^2)[17]. We transformed the SNP-h^2 estimates from the observed to the liability-scale using respective observed disease prevalence. To assess if our SNP-h^2 estimates are consistent with previously published findings and other population-based heritability estimates (POP-h^2), we performed a systematic literature search followed by manual curation of prevalence and heritability estimates for each of the nine pAIDs (Fig. 1a and Supplementary Tables 1 and 2).

Among the pAIDs examined where the SNP-h^2 estimates were at least nominally significant ($P<0.05$), T1D and juvenile idiopathic arthritis (JIA) were the most highly heritable (Fig. 1b). Considerably lower estimates were observed for ulcerative colitis (UC) and Crohn's disease (CD; Supplementary Fig. 1A), suggesting that environmental factors may play a much larger role in IBD aetiology (Fig. 1d). We also observed relatively low SNP-h^2 estimates for systemic lupus erythematosus (SLE; $0.205 \pm$ s.e. 0.076).

Contribution of the MHC region and *ChrX* to SNP-h^2. Given the known association of variants across the *MHC* with AI diseases, we quantified their contribution to the SNP-h^2 for each of the nine pAIDs. We first performed HLA imputation[21], to identify the most strongly associated SNP, amino acid or HLA allele with each pAID (Supplementary Table 5) and we estimated POP-h^2 attributable to the extended *MHC* based on previous analyses (Supplementary Tables 6 and 7). The *MHC*-specific SNP-h^2 estimates correlated well with the strength of lead *MHC* *P*-value. For example, variations across the extended *MHC* region accounted for 32.7% of the total autosomal SNP-h^2 in T1D and 24.7% of that in CEL, with no significant contribution to the SNP-h^2 estimates in psoriasis (PS), SLE, CD or the non-pAID, EPI. Despite the pervasive association between SNPs within the *MHC* and both JIA and UC, contributions of the extended *MHC* to their total SNP-h^2 (10.7% and 5.8%, respectively) were limited (Fig. 1c and Table 2). Despite the known association with HLA-DRB1*0103 and HLA-B*52 in UC[13], we observed that removing the extended *MHC* did not significantly reduce the observed SNP-h^2 for either UC or, the related IBD phenotype, CD (Supplementary Table 8). As expected, the contribution of *ChrX* to the overall SNP-h^2 was small across all pAIDs (Supplementary Table 2). These estimates are consistent with expectations as

© 2015 Macmillan Publishers Limited. All rights reserved.

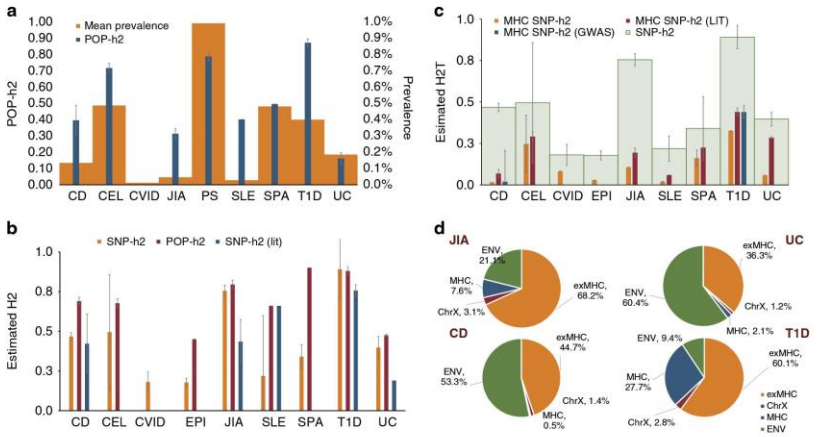


Figure 1 | Autoimmune disease prevalence and heritability estimates. (**a**) Mean population-based AI disease prevalence (orange) and heritability (blue) estimates (mean ± s.d.). Data are curated from epidemiological surveys among Caucasian populations in Europe or North America based on studies indexed in PubMed between 1975 and 2015. Where multiple sources of data are available for a given trait, we reported a simple non-weighted arithmetic mean and provided as error bars the standard deviation. Most heritability estimates were based on twin concordance rates. Raw data used and references can be found in Supplementary Tables 1 and 2. (**b**) Univariate SNP-heritability (SNP-h^2, orange) compared with estimates reported by prior studies. (SNP-h^2 (lit), blue) based on variations across the autosomes compared with population-based estimates (POP-h^2, red) as reported in the literature (lit). Raw data used from prior GWAS SNP-h^2 estimates are provided in Supplementary Table 3. Error bars denote standard error. (**c**) Univariate SNP-heritability (autosomal) estimates with (Light green, wide) and without the extended *MHC* (orange, narrow). Results are compared with corresponding heritability estimates reported using population-based (red, narrow) versus other published SNP-heritability estimates (blue, narrow), when available for a given disease. Literature data used and references can be found in Supplementary Table 2 and Supplementary Tables 6 and 7. Error bars denote standard error. (**d**) Partitioning phenotypic variance to genetic and non-genetic (ENV, green) components in the four largest pAID cohorts. Genetic components include contributions from the entire autosomal regions excluding the *MHC* (ex*MHC*, orange), the extended *MHC* (*MHC*, blue) alone as well as from the *X-chromosome* (*ChrX*, red).

Table 2 | Contribution of autosomal, autosomal with extended MHC removed (exMHC) and ChrX variations to pAID heritability (h^2).

Disease	h^2(auto)	s.e.	*P*	h^2(exMHC)	s.e.	*P*	%MHC*	ChrX	s.e.	*P*
CD	0.454	0.025	<1.00E-04	0.447	0.025	<1.00E-04	1.54	0.014	0.004	2.35E-04
CEL	0.447	0.362	1.06E-01	0.337	0.361	1.74E-01	24.72	0.048	0.058	1.89E-01
CVID†	0.181	0.063	1.72E-03	0.167	0.063	3.66E-03	8.12	NA	NA	NA
EPI	0.168	0.027	1.05E-10	0.163	0.027	3.90E-10	2.91	0.010	0.005	1.21E-02
JIA	0.727	0.037	<1.00E-04	0.650	0.037	<1.00E-04	10.66	0.027	0.007	4.91E-06
PS	0.949	0.381	5.90E-03	0.949	0.380	5.87E-03	−0.02	0.003	0.061	4.82E-01
SLE	0.206	0.076	3.16E-03	0.202	0.076	3.74E-03	1.89	0.013	0.013	1.60E-01
SPA	0.370	0.192	2.45E-02	0.310	0.191	4.91E-02	16.17	−0.029	0.028	1.74E-01
T1D	0.863	0.070	<1.00E-04	0.581	0.069	<1.00E-04	32.66	0.028	0.012	5.27E-03
UC	0.386	0.041	<1.00E-04	0.363	0.041	<1.00E-04	5.84	0.012	0.007	3.38E-02

CD, crohn's disease; CEL, celiac disease; CVID, common variable immunodeficiency disorder; EPI, epilepsy; JIA, juvenile idiopathic arthritis; NA, not applicable; pAID, paediatric autoimmune disease; PS, psoriasis; SLE, systemic lupus erythematosus; SPA, spondyloarthropathy; T1D, type 1 diabetes; UC, ulcerative colitis.
P-values (*P*) are based on results from the restricted maximum likelihood estimate (likelihood ratio test). Error bars represent standard error.
*Percentage contribution of the extended *MHC* to total autosomal SNP-h^2
†REML estimates could not be made due to limited common SNP variability among this cohort on the *X-chromosome*

ChrX makes up only about 5% of the total genome[22], has comparatively fewer coding bases and is less polymorphic[23].

Disease prediction using support vector machines (SVM)s. Given that we observed relatively high rates of heritability across many of the pAIDs, we evaluated the utility of common genomic variations in predicting pAID disease risk, using a SVM model-based approach. Using a tenfold cross-validation study design, we built a linear SVM model using the top GWAS signals observed using nine out of ten of the total samples and tested this SVM predictor in the remaining 10% of the samples. Based on previous analyses in both case–control[24] and quantitative traits[25], we expect that disease prediction accuracy to behave as function

© 2015 Macmillan Publishers Limited. All rights reserved.

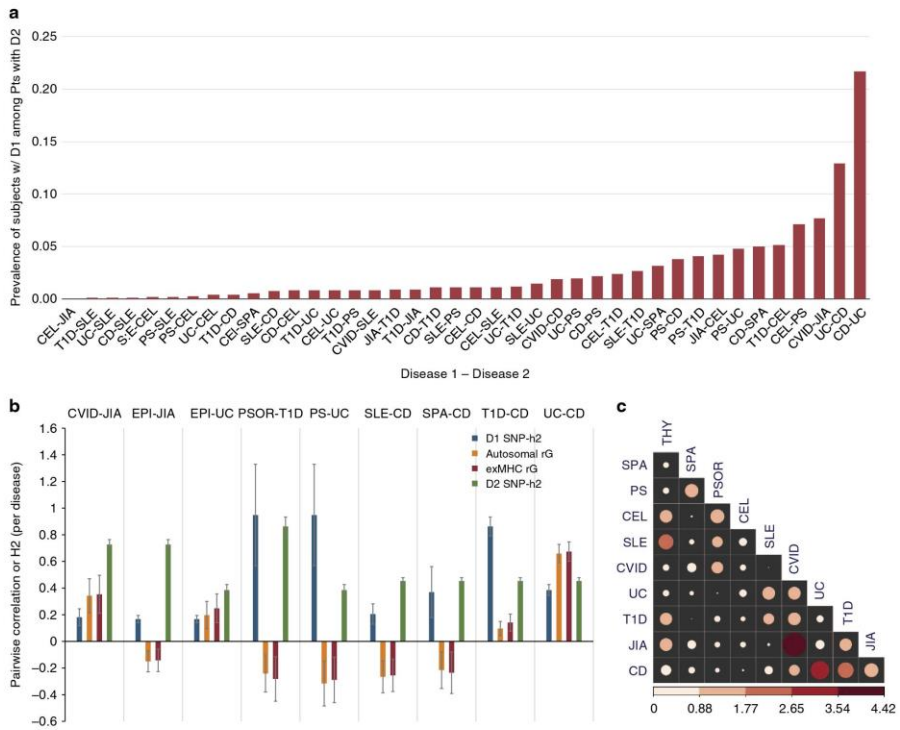


Figure 2 | Prevalence of AI disease co-morbidities and estimates of genetic correlation (co-heritability) across pAIDs. (**a**) Observed prevalence of pAID comorbidity observed in Caucasian populations in Europe and North America as curated from large-scale cohort studies. For each pairwise combination (for example, Disease 1–Disease 2), the rate (*y* axis) indicates the percentage of patients with Disease 2 who have also been diagnosed with Disease 1. Literature data used and references can be found in Supplementary Table 9. (**b**) Bivariate estimates of genetic correlation (pairwise co-heritability) across pAIDs. The heritability (SNP-h^2) for the first and second disease are shown for each pAID pair (blue and green bars, respectively) along with the genetic correlation (*rG*) for the pair estimated based on total autosomal common genetic variants (orange) and based on autosomal variants excluding the *MHC* (red). Displayed are those pairs for which the *rG* estimates reached nominal significance ($P<0.05$). *P*-values are based on restricted maximum likelihood ratio test. Error bars represent standard error. (**c**) Genetic sharing using the genome-wide pairwise sharing statistic (GPS). Correlation plot of the *P*-values obtained from the genome-wide pairwise shared analysis. Significant *P*-values support evidence of genetic sharing based on the correlation of significant association findings reported by GWAS for each pair of diseases.

of heritability, sample size and the number of causal variants. We assessed the mean and maximum area under the receiver operating characteristic curve (AUC) achieved, showing that our SVM predictor was most effective for JIA and T1D ($AUC_{max}>0.9$; $AUC_{mean}>0.85$), although satisfactory results was also seen in CEL ($AUC_{max}>0.8$ and $AUC_{mean}>0.7$). These findings are consistent with that recently reported by Speed *et al.* using an independent adult CEL cohort[26]. The predictability of all nine pAIDs was fairly robust to range of *P*-value thresholds used for selecting SNP predictors in building the SVM model (Fig. 3 and Supplementary Table 11).

Estimation of pairwise co-heritability across pAIDs. To investigate diseases with shared underlying genetic risk factors, we assessed the genetic correlation *(rG)* for each pair of pAIDs and between each of the nine pAIDs and EPI, which provided a comparative baseline for non-significant genetic correlation[20]. We used both a strict (P_{BS}) and a more relaxed Bonferroni correction (P_{BL}) to adjust for either 45 (all pairwise combinations) or 9 comparisons (combinations per pAID); (see Methods). We observed the highest *rG* between UC and CD ($rG=+0.66$; $P_{BS}<0.001$), consistent with the reported sharing of association loci by several published GWAS, immunochip and fine-mapping studies[11,27–29] (Supplementary Table 10). We also noted a positive *rG* between common variable immunodeficiency disorder (CVID) and JIA ($rG=+0.34$), although it was more modest ($P_{BL}<0.01$). While we did observe a marginally positive *rG* for CD and T1D consistent with results from published GWAS metanalysis[30], although it did not reach significance at a liberal Bonferroni threshold ($rG=+0.096$; $P_{BL}=0.17$). Of note, we did not observe a significant reduction in *rG* estimates when

© 2015 Macmillan Publishers Limited. All rights reserved.

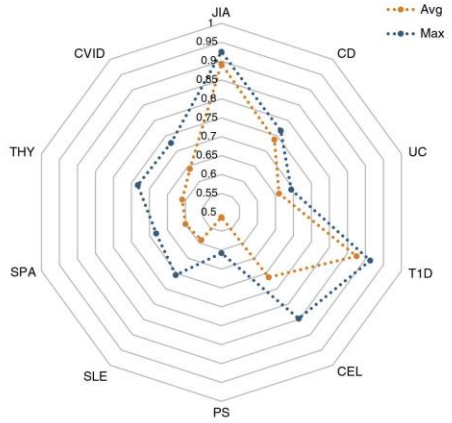


Figure 3 | Disease prediction using a support vector machine model. Shown are the mean (orange) and maximum (blue) areas under the curve (AUC) achieved in the validation set as obtained for each disease in the ten-fold cross-validation analysis. The mean and maxima refer to the best AUC's when testing a range of *P*-value thresholds from which to pick SNPs in training the linear SVM. SPA, spondyloarthropathy.

the extended *MHC* was entirely removed from the analysis across any of the pAID pairs, making it unlikely that the sharing of common *HLA* alleles could significantly account for the degree of co-heritability observed (Fig. 2b).

Discussion

To our knowledge, this is the most comprehensive assessment of heritability and disease prediction using genome-wide dense genotyping data across multiple pAIDs. The results show that SNP-h^2 estimates were significantly higher for the pAID cohorts as compared with those obtained for the non-immune-mediated disease EPI (Fig. 1a and Supplementary Tables 1 and 2). Among the pAIDs examined where the SNP-h^2 estimates were at least nominally significant ($P<0.05$), T1D and JIA were the most highly heritable (Fig. 1b). These results are in keeping with the SNP-h^2 estimates reported for T1D and Rheumatoid Factor Positive (RF+), Rheumatoid Arthritis (RA) in adults, using the Wellcome Trust Case Control Consortium data sets[17,26,31]. Considerably weaker SNP-h^2 estimates were observed for UC and CD, consistent with previous reports in adults[32] (Supplementary Fig. 1A). Although the sample size of CD was several fold greater than those of T1D and UC, and twice that for JIA, the SNP-h^2 estimates are lower in CD, suggesting that environmental factors play a much larger role in CD disease aetiology (Fig. 1d). This finding is in keeping with studies demonstrating a key role for the gut microbiome and faecal flora in disease-onset and severity in the IBDs[11,33,34].

As noted, the SNP-h^2 observed for JIA was high despite the known heterogeneous nature of this disease, including seven distinct JIA subtypes[35]. Little is known about the heritability of JIA as it is fairly uncommon. However, in RA, the more common JIA counterpart in adults, a range of SNP-h^2 estimates has been reported[17,26,31,36]. Some of the heterogeneity in SNP-h^2 estimates for RA may be attributable to the different ratios of RF+ vs RF− patients across different study cohorts, as recent analyses suggest that RF+ RA may be 'distinct' from RF− forms of RA in terms of genetic aetiology[37]. Moreover, the subphenotype of JIA that is most similar to RF+ RA (i.e. RF+ JIA) made up only a small component of our JIA cohort (4.9%). Thus, the high estimated heritability observed in JIA suggests that despite the heterogeneous clinical findings, there may be a strongly shared genetic component contributing to a common aetiology.

We observed relatively low SNP-h^2 estimates for SLE (Fig. 1b). Although these estimates are lower than those reported by *So et al.*,[38,39] they are higher than the POP-*h2* reported based on sibling-recurrence[40]. These observations are consistent with strong environmental and epigenetic components to SLE liability[41,42]. We included in our analysis a non-immune-mediated disease, early-onset EPI, as a comparator cohort. As expected, the SNP-h^2 estimates on the liability scale, albeit non-zero, was relatively low compared with any of the AI diseases. That we observed slightly higher heritability estimates across our paediatric cohorts than previously reported in adults is also in keeping with the notion that paediatric-onset diseases have been noted previously to reflect disease aetiologies with a stronger genetic component[29] and less confounding due to reduced timespan of environmental exposure(s). Adult or late-onset AI diseases can be associated with environmental precipitating factors such as viral infections or drug exposures, which have been implicated in a range of AI diseases including T1D, CEL and SLE[3,42].

Although estimates for JIA and T1D are higher than SNP-h^2 estimates reported previously, our estimates for RA and T1D are more consistent, although still falling short of, than those reported by population estimates from twin-based or familial studies (Supplementary Table 2). That these SNP-h2 based estimates are in general still falling behind estimates made from epidemiological studies illustrates the 'missing heritability' phenomenon. Disparities between POP-h^2 and SNP-h^2 estimates may be at least partially attributable to inflation of population-based estimates in the presence of ascertainment-bias and/or insufficient adjustment for confounding effects. The latter tends to occur if there are significant non-additive or shared environmental factors that contribute to phenotypic variation[36,43].

A number of previous epidemiological and genetic studies have suggested a significant degree of shared risk across AI diseases[44–47]. There are a number of reasons why our results may differ from these reports. In such population-based studies, observed sharing of risk in the population is inevitably confounded by common environmental factors or gene–environment interactions, neither of which would be parsed out from purely epidemiological observations. In addition, it can be challenging to perform these comparisons in heterogeneous populations because they may be composed of different underlying genetic backgrounds, and genetic ancestry is known to dramatically affect the risk for many AI diseases (for example, greater risk of CEL and JIA in Caucasians)[4,20].

Although there are several prior large-scale analyses of genetic sharing among AI diseases using GWAS data, these are based on somewhat different analytical approaches or study methodology than those employed here. A notable example comes from Cotsapas *et al.*, who derived a Cross-Phenotype Meta-Analysis test statistic that powerfully combines multiple independent AI data sets to analyse the likelihood that a SNP is shared across disease phenotypes. They applied this test statistic to the 140 top genetic risk variants reported previously by GWAS across seven AI diseases[47]. Although there is no doubt that findings from this study are informative, the targeted candidate approach has clear limitations and only summary statistics were available. Another concern, which is not unique to the study by Cotsapas *et al.*, but a concern in most large GWAS meta-analyses, is inter-study

© 2015 Macmillan Publishers Limited. All rights reserved.

heterogeneity these studies often combine summary data obtained from independent case–control study cohorts accrued and genotyped across North America and Europe using different genotyping platforms and QC/analysis steps, requiring *post-hoc* statistical adjustments for heterogeneity, genetic variation and the use of SNP proxies. Although single-institution study designs can have limited applicability, in our study, using a common shared control accrued in the same institution and genotyped on the same platform does limit the effect of inter-study heterogeneity in our analysis.

As expected, we found that variations across the extended *MHC* strongly contributed to both heritability estimates and disease risk predictability in T1D and CEL, and more modestly in UC and JIA. The contribution of the extended *MHC* to total phenotypic variance explained correlated with the strength of the strongest association signal within the extended *MHC*. However, as recent reports have shown, this method for estimating h^2 is sensitive to the variation in linkage-disequilibrium (LD) across the genome[18,31]. We therefore examined the effect of LD on the SNP-h^2 estimates by comparing the results with those obtained using non-correlated SNP markers (Supplementary Fig. 2B; see Methods for details). As anticipated, the effect of the pruning is mostly attributable to the strong role of the *MHC* in the heritability of these diseases, as pruning had little effect on the heritability estimates once the extended *MHC* was removed. Thus, the number and degree of LD for the input SNPs used for calculating h^2 can be important for diseases where the *MHC* plays a major role, consistent with previous studies[31,48,49].

The SNP-h^2 for T1D was most strongly affected by the removal of the extended *MHC*, emphasizing the importance of *MHC* polymorphisms in T1D pathogenesis. In addition, the estimates for SPA and CEL both fell significantly when markers across the *MHC* were excluded from further analysis. The relatively limited contribution of the genetic polymorphisms across the *MHC* to heritability in IBD was consistent with prior GWAS results, as the *MHC* SNP (rs1626392, $P<2.27\times10^{-7}$) most significantly associated with CD did not reach genome-wide significance, defined as $P<5\times10^{-8}$ (Supplementary Table 8). Aside from the MHC, recent work has examined the degree to which functional or coding loci, for example, DNAse I Hypersensitivity Sites[50], contribute to disease heritability. Such studies, currently underway, will help delineate biological functions and connect genetic associations with mechanistic roles of such functional variants.

A still unrealized, but much anticipated goal of personalized medicine is to utilize genomic data to accurately predict disease risk[26,51–53]. We found that for the three pAIDs (T1D, JIA and CEL) that were most predictable, a range of *P*-value thresholds ($P<1\times10^{-6}$ and $P<1\times10^{-8}$) could be used to identify the predictive SNPs without significantly impacting maximum or mean AUC achieved, suggesting that the SVM model was robust to this parameter (Fig. 3 and Supplementary Table 11). In comparison, we obtained fairly modest AUCs for CD, UC and CVID ($AUC_{max}>0.7$, $AUC_{mean}>0.65$). These are in keeping with our expectation that genetic prediction should rest on underlying genetic heritability and confirms the value of SNP heritability analysis.

Indeed, the above observations are perhaps not surprising, given recent findings that support a strong contribution for environmental factors in disease susceptibility. For example, host-microbial interactions have been implicated in the pathogenesis of IBD and RA[11,54]. Furthermore, in CVID, it is well-established that although genetic risk factors play a role in disease risk, there is significant within-disease heterogeneity in terms of aetiology. Patients with CVID are often diagnosed in late adolescence, suggesting that environmental risk factors play a greater role. Likewise, most cases of paediatric-onset IBD also have a post-pubescent age of onset. This is in contrast to T1D, JIA or CEL, which are commonly diagnosed by or before the age of 12 years, although some degree of variability is observed. This is consistent with the correlations noted above, in that the three diseases with more moderate SNP-h^2 estimates were also less predictable.

Among the three largest cohorts, namely JIA, UC and CD, CD was by far the largest. However, the heritability estimated for CD in our data set was the lowest of the three. As we know from prior studies that disease prediction is a function of heritability, sample size and the number of causal variants, we might expect the accuracy of disease prediction for CD to be relatively poor. This is exactly what we observed. In contrast, we had somewhat limited sample sizes for SPA, PS and CEL cohorts, and we caution against the interpretation of the high heritability estimates observed for PS. Another limitation of the present study is that we have not considered the role of rare, or potentially *de novo*, variants in the overall estimates of genetic heritability. As more sequencing data using either whole-exome or whole-genome approaches become available, future studies will help address this question.

A unique opportunity provided by our cohort was the ability to quantify pairwise pAID genetic correlations as numerous epidemiological analyses have shown that subsets of pAIDs co-cluster in families or exhibit high rates of comorbidity[55–57] (Table 3). As pAID co-heritability has not been systematically examined using genome-wide SNP data, we aimed to identify pAIDs showing significantly positive *rG* (that are consequently co-heritable) versus diseases that are either genetically unrelated or negatively correlated (and are consequently 'mutually-protective'). We calculated the *rG* for each pAID pair and between each of the nine diseases and EPI. This latter analysis provides a 'control' or contextual baseline, akin to the inclusion of

Table 3 | pAID joint heritabilities or genetic correlation (rG) reaching nominal significance.

pAID pair	rG (auto)	s.e.	Pval	rG (exMHC)	s.e.	P_nominal	P_adj
CVID-JIA	0.343	0.127	1.22E-03	0.354	0.142	2.47E-03	2.23E-02
EPI-JIA	−0.150	0.079	2.95E-02	−0.142	0.085	4.87E-02	0.44
EPI-UC	0.197	0.103	2.77E-02	0.248	0.108	1.06E-02	0.10
PS-T1D	−0.241	0.139	3.29E-02	−0.282	0.167	3.74E-02	0.34
PS-UC	−0.316	0.169	2.31E-02	−0.289	0.171	3.76E-02	0.34
SLE-CD	−0.266	0.120	8.25E-03	−0.255	0.121	1.15E-02	0.10
SPA-CD	−0.215	0.138	4.64E-02	−0.235	0.156	4.67E-02	0.42
T1D-CD	0.096	0.053	3.45E-02	0.142	0.064	1.33E-02	0.12
UC-CD	0.659	0.069	<1.00E-04	0.674	0.072	<1.00E-04	9.00E-04

Auto, autosomal; CD, crohn's disease; CEL, celiac disease; CVID, common variable immunodeficiency disorder; EPI, epilepsy; JIA, juvenile idiopathic arthritis; NA, not applicable; pAID, paediatric autoimmune disease; PS, psoriasis; exMHC, MHC excluded; SLE, systemic lupus erythematosus; SPA, spondyloarthropathy; T1D, type 1 diabetes; UC, ulcerative colitis.
P-values (*P*) are based on results from the restricted maximum likelihood estimate (likelihood ratio test). *P*_adj is made using a Bonferonni-adjustment for nine pairwise tests for each disease.

© 2015 Macmillan Publishers Limited. All rights reserved.

CD as a 'null comparator' phenotype by the Psychiatric Genomics Consortium[20]. We observed a strongly and moderately positive *rG* between two pAID pairs, namely UC-CD and JIA-CVID.

Although the *MHC* made major contributions to the disease-specific heritability, we found no evidence that variations across the *MHC* significantly contributed to the pAID co-heritability for any of the investigated disease pairs (Fig. 2b). For the pAID pairs with significantly positive *rG*'s (UC-CD and JIA-CVID), we did not observe a significant reduction in *rG* estimates when the SNPs within the *MHC* were removed from the analysis, making it unlikely that genetic sharing of *MHC* haplotypes can explain the genetic correlation observed among pAIDs in this data set (Fig. 2b). In addition, that the UC-CD and JIA-CVID pairs were the two with the largest positive rG is also consistent with results we obtained using an independent genome-wide pairwise sharing metric for genetic correlation, in which we considered all genome-wide SNP markers except those within the extended *MHC* locus (Fig. 2c, see Methods for details). Although it may appear to be surprising given the known association with the *MHC* across all pAIDs, these results are in keeping with our finding that the most significant *MHC* association signals identified for each pAID was disease-specific and did not overlap across the nine pAIDs (Supplementary Table 5).

Somewhat unexpectedly, we observed a negative marginal *rG* across several pAID pairs, including SLE-CD, SPA-CD, PS-UC and PS-T1D. Although none of these was significant following a Bonferroni correction, in each of the negatively correlated pAID pairs, one of the two diseases is considered a 'classic autoimmune' (that is, SLE, UC, and T1D), whereas the other pAID in the pair (that is, CD and PS) has been noted to have a strong 'inflammatory' component.

Taken together, we report genome-wide SNP genotype-derived heritability estimates and genetic correlations of disease liability across pairs of nine investigated pAIDs using common and low-frequency genetic variants. We contextualized these findings alongside a comprehensive review of available literature and epidemiological data sets, illustrate a method for quantifying genetic risk factor sharing across pAIDs and provide considerations for how such genetic data can aid in disease prediction. We observed that SNP-h^2 estimates in pediatric AI diseases tend to be greater in magnitude when compared to SNP-h^2 reported previously based on GWAS data from studies of adult AI disease cohorts, particularly for T1D, UC, JIA/RA. Moreover, we also observed that the 'co-heritability' across pAIDs was minimally attributable to shared *MHC* variations. While genomic screening in the general population on a large scale is not currently feasible, or of high utility (given the low disease prevalence and consequently, limited positive predictive value as well as the limitations in interpretability), our analysis suggests that there is a high heritability and disease predictability across the pAIDs. Future studies in larger sample sizes and in adult cohorts will be helpful in validating these results and developing new and improved methods for genome-based disease prediction and for the development of novel biomarkers that can be used to predict pAID risk.

Methods

Study population. Information regarding the patient cohorts have been published previously and are summarized briefly below.

Cases and controls were either directly ascertained as described in prior studies[29,53,58–65] or obtained from de-identified samples and associated electronic medical records (EMRs) residing in the genomics biorepository at the Children's Hospital of Philadelphia. EMR searches were conducted using previously described algorithms[58,59] based on phenotype mapping established using PheWAS ICD-9 code mapping tables[53,58,60] in consultation with qualified physician specialists for each disease cohort. All DNA samples were assessed for QC and genotyped on the Illumina HumanHap550 or HumanHap610 platforms at the Center for Applied Genomics (CAG) at the Children's Hospital of Philadelphia (CHOP, Philadelphia, Pennsylvania, USA). Note that the patient counts below refer to the total recruited sample size from which we excluded non-qualified samples/genotypes that did not pass QC criteria required for inclusion in the genetic analysis (for example, because of relatedness or poor genotyping rate; see details below).

The IBD cohort comprised 2,796 individuals aged 2–17 years of European ancestry with biopsy-proven disease, including 1,931 with CD and 865 with UC, excluding all patients with unclassified type (IBD-U). Affected individuals were recruited from multiple centres from four geographically discrete countries and diagnosed before their nineteenth birthday according to the standard IBD diagnostic criteria, as previously reported[3,29].

The T1D cohort consisted of 1,120 cases from nuclear family trios (one affected child and two parents), including 267 independent Canadian T1D cases collected in paediatric diabetes clinics in Montreal, Toronto, Ottawa and Winnipeg (Canada) and 203 T1D cases recruited at CHOP since September 2006. All patients were Caucasians by self-report and ranged in age between 3 and 17 years, with 7.9 years being the median age at onset. All patients have been treated with insulin since diagnosis. Disease diagnosis was based on these clinical criteria, rather than any laboratory tests.

The JIA cohort was recruited in the United States of America, Australia and Norway and comprised of a total of 1,123 patients with onset of arthritis at <16 years of age. JIA diagnosis and JIA subtype were determined according to the International League of Associations for Rheumatology revised criteria[35] and confirmed using the JIA Calculator software[66] (http://www.jra-research.org/JIAcalc/), an algorithm-based tool adapted from the International League of Associations for Rheumatology criteria. Before standard QC procedures and exclusion of non-European ancestry, the JIA cohort was comprised of 464 case subjects from Texas Scottish Rite Hospital for Children (Dallas, Texas, USA) and the Children's Mercy Hospitals and Clinics (Kansas City, Missouri, USA) of self-reported European ancestry; 196 subjects from the CHOP; 221 subjects from the Murdoch Childrens Research Institute (Royal Children's Hospital, Melbourne, Australia) and 504 subjects from the Oslo University Hospital (Oslo, Norway).

The CVID study population consisted of 223 patients from the Mount Sinai School of Medicine (New York City, New York, USA); 76 patients from the University of Oxford (London, England); 47 patients from the CHOP and 27 patients from the University of South Florida (Tampa, Florida). The diagnosis in each case was validated against the ESID/PAGID diagnostic criteria, as previously described[67]. Although the diagnosis of CVID is most commonly made in young adults (aged 20–40 years), all of the CHOP and University of South Florida cases had paediatric age of onset disease, whereas the majority of the cases from the Mount Sinai School of Medicine and Oxford had onset in young adulthood. We note that as the number of individuals with adult-onset CVID disease is so small (less than 5% of all cases presented), and all ten diseases have paediatric age of onset disease, we have elected to refer to the cohort material as pAIDs.

The balance of the paediatric AI disease subjects' (SPA, PS, CEL and SLE) samples were accrued by our biorepository at the CHOP, which includes over 60,000 paediatric patients recruited and enrolled by the Center for Applied Genomics at CHOP. These individuals were ascertained for having a confirmed diagnosis of SPA, PS, CEL and SLE in the age range of 1–17 years during time of diagnosis and were required to fulfill clinical criteria for these respective disorders, as confirmed by a specialist. Only cases that upon EMR search were confirmed to have at least two or more in-person visits, at least one of which is with the specified ICD9 diagnosis code(s) were pursued for clinical confirmation (see Supplementary Table 12 for ICD-9 inclusion and exclusion codes). We used ICD9 codes previously identified and utilized for PheWAS or EMR-based GWAS[59,60] and agreed upon by board-certified physicians.

Age- and gender-matched control subjects, including the EPI cohort of both generalized and focal idiopathic EPI (ICD-9 345.9 and 345.4, respectively), were identified from the CHOP-CAG biobank and ascertained by exclusion of any patient with any ICD-9 codes for disorders of autoimmunity or immunodeficiency[58] (http://eicd9.com/). Research Ethics Boards at the CHOP and each of the collaborating centre, including: the Mount Sinai School of Medicine, University of Oxford, University of South Florida, the Children's Mercy Hospitals and Clinics, Texas Scottish Rite Hospital for Children, Murdoch Children's Research Institute, Oslo University Hospital, Cincinnati Children's Hospital Medical Center, McGill University, RCCS 'Casa Sollievo della Sofferenza', University of Toronto, University of Edinburgh, Emory University, University of Naples 'Federico II', Cedars Sinai Medical Center, Yorkhill Hospital for Sick Children, University of Miami Miller School of Medicine, Careggi University Hospital, University of Utah School of Medicine and Primary Children's Medical Center, approved this study.

Written informed consent was obtained from all subjects (or their legal guardians). Genomic DNA extraction and sample QC before and following genotyping were performed using standard methods[61]. To minimize confounding because of population stratification, we focused on only individuals of European ancestry, as determined by both self-reported ancestry and principle component analysis, PCA) in the present study (see below and Supplementary Fig. 4).

Genotyping and QC. All samples were genotyped at the CAG on the Human-Hap550 or 610 BeadChip arrays. Although some published analyses using GWAS data to derive heritability estimates have applied whole-genome imputation

© 2015 Macmillan Publishers Limited. All rights reserved.

because of the presence of samples with non-matching platforms, this is not ideal given (i) added risk of artefacts and (ii) consequent variations in coverage (genotyping density) across the genome. Without adding significant additional information, this can result in biased heritability estimates unless careful corrections are made to apply additional down-sampling/weighting of more densely imputed regions[31,48,49]. As over 90% of the markers on the two arrays are shared, whole-genome imputation was not necessary and we utilized only the set of directly overlapping genotyped SNPs in the analysis (~500,000).

After extracting the overlapping SNPs from the two platforms, SNPs with a low genotyping rate <95%, low MAF (<0.01) or significantly departing from the expected Hardy–Weinberg equilibrium ($P<0.01$) were excluded. Samples with low average genotyping call rate (<95%) or determined to be of outliers of European ancestry by PCA (Any of the top ten principal components (PCs) >6.0 standard deviations as reported by SMARTPCA/EIGENSTRAT[68]) were removed. In addition, one of each pair of distantly related individuals, as determined by Identify-by-State analysis (>0.05), was excluded, such that the largest sample size would be retained in the final cohort.

Web-based access to all novel data included in this manuscript is available through our website at http://www.caglab.org.

Population stratification correction. The final cohort, following all above-noted QC, included a total of 4,956 pAID cases inclusive of 9 pAIDs and 27,451 population-matched controls, as well as a cohort consisting of 819 cases of paediatric-onset EPI. To avoid confounding, we assigned individuals fitting the diagnosis criteria for two or more pAIDs to the smaller disease cohort by sample size. No individual was included twice. To ensure that the markers tested across the cohorts were consistent, we included only SNPs that passed all QC criteria (461,301 SNPs). The filtered SNPs were tested in cases and controls for association with disease and used for the estimation of the genetic relationship matrix (see below). We used a logistic regression equation to estimate ORs/betas, 95% confidence intervals and *P*-values for trend, using additive coding for genotypes (0,1,2 minor alleles). We adjusted for gender and population stratification by including the binary gender and the first ten PCs (*GCTA)* from the PCA calculated from a set of 100,000 pruned SNPs as covariates in the logistic regression analyses[69]. From the results of the association testing, we determined the genomic inflation per disease-common control cohort. All disease-specific, case–control GWAS had λ_{GC} values at or below 1.04 with the exception of CD (1.09), consistent with that previously reported for this data set[29]. Final counts from each pAID cohort, included controls and genomic inflation calculated from median χ^2 association test statistics are reported in Table 1.

Estimation of the variance components for each pAID. Only individuals and SNPs that passed all QC metrics were used to estimate the variance components for the ten diseases (nine pAIDs and one non-pAID condition EPI). For disease-specific analysis, the common set of controls were used for each case–control analysis cohort, after excluding individuals who are relatives up to within the 5th degree. The genetic relationship between individuals was estimated using (i) all autosomal SNPs, (ii) all autosomal SNPs excluding the extended *MHC* (chr6:26.5–34 Mb) and (iii) SNPs only found on the *X-chromosome* (*ChrX*).

We applied the previously described linear mixed model method for estimating whole-genome SNP-based heritability using both common and low-frequency variants, which is implemented in the software *GCTA*. We estimated the genetic variance associated with genome-wide SNPs on the observed scale (SNP-heritability or SNP-h^2)[70], conditioning on the top 20 ancestry PCs derived from a pruned set of ~100,000 independent SNPs across the same data set (that is, *PLINK* --indep-pairwise 50 10 0.2) obtained also using *GCTA*. As our phenotypes are dichotomous traits, we subsequently transformed these results to the liability scale based on approximately observed disease prevalence at our centre for each trait (Table 1). Note that the total control sample size utilized varied slightly as we optimized our analysis to maximize the retained sample size when conservatively removing distantly related individuals during QC. As we excluded rare variants (MAF<0.01), these variants are therefore not included in the heritability estimates attributable to genetic variation.

Joint heritability across pAID pairwise combinations. We estimated the genetic correlation in disease risk for each of other pAID pairs using a bivariate linear mixed model, as described previously[17]. For each pairwise analysis, the pooled control samples passing QC were randomly allocated to the two diseases evenly and the top 20 PCs were again included as covariates. By jointly analysing a pair of cohorts, these results estimate both the SNP-h^2 of liability to both diseases and an estimate of the SNP-genetic correlation between these liabilities. We determined the significance of the *rG* using a likelihood ratio test by fixing the genetic correlation at zero[17]. Significantly positive (or negative) *rG's* should reflect a shared (or disparate) genetic background, as a positive (negative) *rG* means that the correlation in the genetic variance components are higher (lower) between case subjects than between the case subjects and the respective control cohorts.

Genome-wide pairwise sharing analysis. We applied a novel test to detect the presence of SNPs anywhere in the genome that are simultaneously associated with each of two diseases; these SNPs are the genetic risk factors shared by that pair of pAIDs. Most existing tests require choosing a significance threshold to determine which SNPs are associated with which disease, but it is unknown how best to choose this threshold. Our method is threshold-free and requires no tuning parameters. Specifically, for any two diseases, we converted the *P*-values for all SNPs in the genome into *Z*-scores, such that for example:

$$X_1, \ldots, X_n \text{ are the } Z \text{ scores for } D_1 \text{ across } n \text{ SNPs,} \quad (1)$$

$$Y_1, \ldots, Y_n \text{ are the } Z \text{ scores for } D_2 \text{ across } n \text{ SNPs.} \quad (2)$$

The test statistic, γ, used to detect genetic sharing between two diseases is

$$\gamma = \max_{1 \le j \le n} \left(\min \left(|x_j|, |y_j| \right) \right), \quad (3)$$

which is the maximum of the pairwise minima of the signals across all of the n SNPs. The rationale is that if SNP j is associated with both D_1 and D_2, the magnitudes of both X_j and Y_j should be large. The more shared SNPs there are, the greater the likelihood that the maximum of the pairwise minimal values will be large. Under the null hypothesis that any genetic sharing is due only to chance, γ should be relatively small. We can obtain the *P*-value of this statistic by permuting the labels of the *Z*-scores relative to each other in order to simulate the null hypothesis. In fact, these *P*-values can be calculated analytically using a hypergeometric distribution, and no actual permutation is needed. Note that no significance threshold is required. This test was performed for all 45 pairwise pAID combinations (hence, the reported *P*-values are Bonferroni-adjusted for 45 independent tests).

Disease prediction using a linear SVM. Given that we observed relatively high rates of heritability across many of pAIDs, we sought to evaluate the utility of genome-wide SNP data in predicting pAID disease liability, using a previously described SVM pipeline that can be applied to GWAS results for a dichotomous trait[52].

We identified SNPs to be used as predictors based on the strength of association with a given disease in a training set, testing graded *P*-value thresholds ($P<1\times10^{-5}$, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}) for selecting SNP predictors, where the *P*-value is derived from the case–control association testing using samples in the training data set. We used each set of SNPs passing the tested threshold to then train the linear SVM model.

We then validated the SVM model by testing the accuracy of disease liability predictions for each of the nine pAIDs in the remaining independent sample set. We reported the prediction performance as the mean and maximum AUC achieved in both the training and validation sets (Fig. 3 and Supplementary Table 11).

References

1. Anaya, J.-M., Gómez, L. & Castiblanco, J. Is there a common genetic basis for autoimmune diseases? *Clin. Dev. Immunol.* **13,** 185–195 (2006).
2. Rojas-Villarraga, A., Amaya-Amaya, J., Rodriguez-Rodriguez, A., Mantilla, R. D. & Anaya, J.-M. Introducing polyautoimmunity: secondary autoimmune diseases no longer exist. *Autoimmune Dis.* **2012,** 254319 (2012).
3. Lettre, G. & Rioux, J. D. Autoimmune diseases: insights from genome-wide association studies. *Hum. Mol. Genet* **17,** R116–R121 (2008).
4. Nunes, T., Fiorino, G., Danese, S. & Sans, M. Familial aggregation in inflammatory bowel disease: is it genes or environment? *World J. Gastroenterol.* **17,** 2715–2722 (2011).
5. Cooper, J. D. *et al.* Seven newly identified loci for autoimmune thyroid disease. *Hum. Mol. Genet.* **21,** 5202–5208 (2012).
6. Tsoi, L. C. *et al.* Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nat. Genet.* **44,** 1341 (2012).
7. Hinks, A. *et al.* Dense genotyping of immune-related disease regions identifies 14 new susceptibility loci for juvenile idiopathic arthritis. *Nat. Genet.* **45,** 664–669 (2013).
8. L, J. *et al.* Dense fine-mapping study identifies new susceptibility loci for primary biliary cirrhosis. *Nat. Genet.* **44,** 1137 (2012).
9. Liu, J. Z. *et al.* Dense genotyping of immune-related disease regions identifies nine new risk loci for primary sclerosing cholangitis. *Nat. Genet.* **45,** 670–675 (2013).
10. Eyre, S. *et al.* High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. *Nat. Genet.* **44,** 1336 (2012).
11. DP, M. *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491,** 119 (2012).
12. Beecham, A. H. *et al.* Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat. Genet.* **45,** 1353–1360 (2013).
13. Zhernakova, A. *et al.* Detecting shared pathogenesis from the shared genetics of immune-related diseases. *Nat. Rev. Genet.* **10,** 43–55 (2009).
14. Parkes, M., Cortes, A., van Heel, D. A. & Brown, M. A. Genetic insights into common pathways and complex relationships among immune-mediated diseases. *Nat. Rev. Genet.* **14,** 661–673 (2013).

© 2015 Macmillan Publishers Limited. All rights reserved.

15. Visscher, P. M., Hill, W. G. & Wray, N. R. Heritability in the genomics era--concepts and misconceptions. *Nat. Rev. Genet.* **9,** 255–266 (2008).
16. Yang, J., Lee, S. H., Goddard, M. E. & Visscher, P. M. GCTA: a tool for genome-wide complex trait analysis. *Am. J. Hum. Genet.* **88,** 76–82 (2011).
17. Lee, S. H., Yang, J., Goddard, M. E., Visscher, P. M. & Wray, N. R. Estimation of pleiotropy between complex diseases using single-nucleotide polymorphism-derived genomic relationships and restricted maximum likelihood. *Bioinformatics* **28,** 2540–2542 (2012).
18. Lee, S. H., Wray, N. R., Goddard, M. E. & Visscher, P. M. Estimating missing heritability for disease from genome-wide association studies. *Am. J. Hum. Genet.* **88,** 294–305 (2011).
19. Yang, J. *et al.* Common SNPs explain a large proportion of the heritability for human height. *Nat. Genet.* **42,** 565–569 (2010).
20. Lee, S. H. *et al.* Genetic relationship between five psychiatric disorders estimated from genome-wide SNPs. *Nat. Genet.* **45,** 984–994 (2013).
21. Jia, X. *et al.* Imputing amino acid polymorphisms in human leukocyte antigens. *PLoS ONE* **8,** e64683 ((2013).
22. Schaffner, S. F. The X chromosome in population genetics. *Nat. Rev. Genet.* **5,** 43–51 (2004).
23. Gottipati, S., Arbiza, L., Siepel, A., Clark, A. G. & Keinan, A. Analyses of X-linked and autosomal genetic variation in population-scale whole genome sequencing. *Nat. Genet.* **43,** 741–743 (2011).
24. Daetwyler, H. D., Villanueva, B. & Woolliams, J. A. Accuracy of predicting the genetic risk of disease using a genome-wide approach. *PLoS ONE* **3,** e3395 (2008).
25. Lee, S. H. & Wray, N. R. Novel genetic analysis for case-control genome-wide association studies: quantification of power and genomic prediction accuracy. *PLoS ONE* **8,** e71494 (2013).
26. Speed, D. & Balding, D. J. MultiBLUP: improved SNP-based prediction for complex traits. *Genome Res* **24,** 1550–1557 (2014).
27. Franke, A. *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42,** 1118–1125 (2010).
28. Barrett, J. C. *et al.* Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. *Nat. Genet.* **41,** 1330–1334 (2009).
29. Imielinski, M. *et al.* Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat. Genet.* **41,** 1335–1340 (2009).
30. Wang, K. *et al.* Comparative genetic analysis of inflammatory bowel disease and type 1 diabetes implicates multiple loci with opposite effects. *Hum Mol Genet* **19,** 2059–2067 (2010).
31. Speed, D., Hemani, G., Johnson, M. R. & Balding, D. J. Improved heritability estimation from genome-wide SNPs. *Am. J. Hum. Genet.* **91,** 1011–1021 (2012).
32. Chen, G.-B. *et al.* Estimation and partitioning of (co)heritability of inflammatory bowel disease from GWAS and immunochip data. *Hum. Mol. Genet* **23,** 4710–4720 (2014).
33. Cho, I. & Blaser, M. J. The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* **13,** 260–270 (2012).
34. Márquez, A. *et al.* Specific association of a CLEC16A/KIAA0350 polymorphism with NOD2/CARD15(-) Crohn's disease patients. *Eur. J. Hum. Genet.* **17,** 1304–1308 (2009).
35. Petty, R. E. *et al.* International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J. Rheumatol.* **31,** 390–392 (2004).
36. Zaitlen, N. *et al.* Using extended genealogy to estimate components of heritability for 23 quantitative and dichotomous traits. *PLoS Genet.* **9,** e1003520 (2013).
37. Han, B. *et al.* Fine mapping seronegative and seropositive rheumatoid arthritis to shared and distinct HLA alleles by adjusting for the effects of heterogeneity. *Am. J. Hum. Genet.* **94,** 522–532 (2014).
38. So, H.-C., Gui, A. H. S., Cherny, S. S. & Sham, P. C. Evaluating the heritability explained by known susceptibility variants: a survey of ten complex diseases. *Genet. Epidemiol.* **35,** 310–317 (2011).
39. So, H.-C., Yip, B. H. K. & Sham, P. C. Estimating the total number of susceptibility variants underlying complex diseases from genome-wide association studies. *PLoS ONE* **5,** e13898 (2010).
40. Harley, J. B. *et al.* Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat. Genet.* **40,** 204–210 (2008).
41. Kamen, D. L. Environmental influences on systemic lupus erythematosus expression. *Rheum. Dis. Clin. North Am* **40,** 401–412 vii (2014).
42. Mok, C. C. & Lau, C. S. Pathogenesis of systemic lupus erythematosus. *J. Clin. Pathol.* **56,** 481–490 (2003).
43. Manolio, T. A. *et al.* Finding the missing heritability of complex diseases. *Nature* **461,** 747–753 (2009).
44. Rzhetsky, A., Wajngurt, D., Park, N. & Zheng, T. Probing genetic overlap among complex human phenotypes. *Proc. Natl Acad. Sci. USA* **104,** 11694–11699 (2007).
45. Eaton, W. W., Rose, N. R., Kalaydjian, A., Pedersen, M. G. & Mortensen, P. B. Epidemiology of autoimmune diseases in Denmark. *J. Autoimmun.* **29,** 1–9 (2007).
46. EC, S., Cooper, G. S., Bynum, M. L. K. & Somers, E. C. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J. Autoimmun.* **33,** 197–207 (2009).
47. Cotsapas, C. *et al.* Pervasive sharing of genetic effects in autoimmune disease. *PLoS Genet.* **7,** e1002254 (2011).
48. Speed, D. *et al.* SNP-based heritability analysis with dense data. *Am. J. Hum. Genet.* **93,** 1155–1157 (2013).
49. Lee, S. H. *et al.* Estimation of SNP heritability from dense genotype data. *Am. J. Hum. Genet.* **93,** 1151–1155 (2013).
50. Gusev, A. *et al.* Partitioning heritability of regulatory and cell-type-specific variants across 11 common diseases. *Am. J. Hum. Genet.* **95,** 535–552 (2014).
51. Kang, J., Kugathasan, S., Georges, M., Zhao, H. & Cho, J. H. Improved risk prediction for Crohn's disease with a multi-locus approach. *Hum. Mol. Genet* **20,** 2435–2442 (2011).
52. Mittag, F. *et al.* Use of support vector machines for disease risk prediction in genome-wide association studies: Concerns and opportunities. *Hum. Mutat.* **33,** 1708–1718 (2012).
53. Liao, K. P. *et al.* Electronic medical records for discovery research in rheumatoid arthritis. *Arthritis Care Res. (Hoboken)* **62,** 1120–1127 (2010).
54. Scher, J. U. *et al.* Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis. *Elife* **2,** e01202 (2013).
55. Ramos, P. S. *et al.* A comprehensive analysis of shared loci between systemic lupus erythematosus (SLE) and sixteen autoimmune diseases reveals limited genetic overlap. *PLoS Genet.* **7,** e1002406 (2011).
56. Tait, K. F. *et al.* Clustering of autoimmune disease in parents of siblings from the Type 1 diabetes Warren repository. *Diabet. Med.* **21,** 358–362 (2004).
57. Lin, J.-P. *et al.* Familial clustering of rheumatoid arthritis with other autoimmune diseases. *Hum. Genet.* **103,** 475–482 (1998).
58. Denny, J. C. *et al.* PheWAS: demonstrating the feasibility of a phenome-wide scan to discover gene-disease associations. *Bioinformatics* **26,** 1205–1210 (2010).
59. Ritchie, M. D. *et al.* Robust replication of genotype-phenotype associations across multiple diseases in an electronic medical record. *Am. J. Hum. Genet.* **86,** 560–572 (2010).
60. Liao, K. P. *et al.* Associations of autoantibodies, autoimmune risk alleles, and clinical diagnoses from the electronic medical records in rheumatoid arthritis cases and non-rheumatoid arthritis controls. *Arthritis Rheum.* **65,** 571–581 (2013).
61. Hakonarson, H. *et al.* A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene. *Nature* **448,** 591 (2007).
62. Kugathasan, S. *et al.* Loci on 20q13 and 21q22 are associated with pediatric-onset inflammatory bowel disease. *Nat. Genet.* **40,** 1211–1215 (2008).
63. Orange, J. S. *et al.* Genome-wide association identifies diverse causes of common variable immunodeficiency. *J. Allergy Clin. Immunol.* **127,** 1360–1367 e6 (2011).
64. Behrens, E. M. *et al.* Association of the TRAF1-C5 locus on chromosome 9 with juvenile idiopathic arthritis. *Arthritis Rheum.* **58,** 2206–2207 (2008).
65. Grant, S. F. *et al.* Association of the BANK 1 R61H variant with systemic lupus erythematosus in Americans of European and African ancestry. *Appl. Clin. Genet.* **2,** 1–5 (2009).
66. Behrens, E. M. *et al.* Evaluation of the presentation of systemic onset juvenile rheumatoid arthritis: data from the Pennsylvania Systemic Onset Juvenile Arthritis Registry (PASOJAR). *J. Rheumatol.* **35,** 343–348 (2008).
67. Conley, M. E., Notarangelo, L. D. & Etzioni, A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin. Immunol.* **93,** 190–197 (1999).
68. Price, A. L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38,** 904 (2006).
69. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81,** 559–575 (2007).
70. NASU, Y. *et al.* Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. *Osteoarthr. Cartil.* **16,** 723–732 (2008).

Acknowledgements

We thank the patients and their families for their participation in the genotyping studies and in the Biobank Repository at the Center for Applied Genomics. We are also thankful for the contributions of the Italian IBD Group, including Cucchiara S (Roma), Lionetti P (Firenze), Barabino G (Genova), de Angelis GL (Parma), Guariso G (Padova), Catassi C

© 2015 Macmillan Publishers Limited. All rights reserved.

(Ancona), Lombardi G (Pescara), Staiano AM (Napoli), De Venuto D (Bari), Romano C (Messina), D'incà R (Padova), Vecchi M (Milano), Andriulli A and Bossa F (S. Giovanni Rotondo). Y.R.L. is supported by the Paul and Daisy Soros Fellowship for New Americans and an NIH F30 Individual NRSA Training Grant (1F30AR066486). This study was supported by Institutional Development Funds from the Children's Hospital of Philadelphia, and by DP3DK085708, RC1AR058606, U01HG006830, the Crohn's and Colitis Foundation, the Juvenile Diabetes Research Foundation and a grant from the LRI to E.L.P.

Author contributions

Y.R.L. and H.H. were leading contributors in the design, analysis and writing of this study. D.J.A., M.M. and L.S. contributed to data collection and literature review. B.F., Ø.F., L.A.D., S.D.T., M.L.B., S.L.G., A.L., E.P., E.R., C.S., A.S., E.M., M.S.S., B.A.L., M.P., R.K.R., D.C.W., H.C., C.C.-R., J.S.O., E.M.B., K.E.S., S.K., A.M.G., J.S., T.F., C.P., R.N.B. and J.A.E. contributed samples and phenotypes. F.D.M., K.A.T., H.Q., R.M.C., C.E.K., F.W. and J.S. provided assistance with samples genotyping, and data processing. J.K., S.D.Z., J.P.B., J.L. and H.L. contributed to, advised and supervised statistical analysis. E.T.L.P., J.A.E. and B.J.K. assisted in composing and revising the manuscript. All authors read, edited and approved of the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Li, Y. R. *et al.* Genetic sharing and heritability of paediatric age of onset autoimmune diseases. *Nat. Commun.* 6:8442 doi: 10.1038/ncomms9442 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© 2015 Macmillan Publishers Limited. All rights reserved.

3.4 Meta-analysis of shared genetic architecture across ten pediatric autoimmune diseases.

ARTICLES

nature medicine

Meta-analysis of shared genetic architecture across ten pediatric autoimmune diseases

Yun R Li[1,2], Jin Li[1], Sihai D Zhao[3,43], Jonathan P Bradfield[1], Frank D Mentch[1], S Melkorka Maggadottir[1,4], Cuiping Hou[1], Debra J Abrams[1], Diana Chang[5,6], Feng Gao[5], Yiran Guo[1], Zhi Wei[7], John J Connolly[1], Christopher J Cardinale[1], Marina Bakay[1], Joseph T Glessner[1], Dong Li[1], Charlly Kao[1], Kelly A Thomas[1], Haijun Qiu[1], Rosetta M Chiavacci[1], Cecilia E Kim[1], Fengxiang Wang[1], James Snyder[1], Marylyn D Richie[8], Berit Flatø[9], Øystein Førre[9], Lee A Denson[10], Susan D Thompson[11], Mara L Becker[12], Stephen L Guthery[13], Anna Latiano[14], Elena Perez[15], Elena Resnick[16], Richard K Russell[17], David C Wilson[18], Mark S Silverberg[19], Vito Annese[20], Benedicte A Lie[21], Marilynn Punaro[22], Marla C Dubinsky[23], Dimitri S Monos[24,25], Caterina Strisciuglio[26], Annamaria Staiano[26], Erasmo Miele[26], Subra Kugathasan[27], Justine A Ellis[28,29], Jane E Munro[30,31], Kathleen E Sullivan[4,25], Carol A Wise[32], Helen Chapel[33], Charlotte Cunningham-Rundles[16], Struan F A Grant[1,25], Jordan S Orange[34], Patrick M A Sleiman[1,25], Edward M Behrens[25,35], Anne M Griffiths[36], Jack Satsangi[37], Terri H Finkel[38], Alon Keinan[5,6], Eline T Luning Prak[39], Constantin Polychronakos[40], Robert N Baldassano[25,41], Hongzhe Li[39], Brendan J Keating[1,25] & Hakon Hakonarson[1,25,42]

© 2015 Nature America, Inc. All rights reserved.

Genome-wide association studies (GWASs) have identified hundreds of susceptibility genes, including shared associations across clinically distinct autoimmune diseases. We performed an inverse χ^2 meta-analysis across ten pediatric-age-of-onset autoimmune diseases (pAIDs) in a case-control study including more than 6,035 cases and 10,718 shared population-based controls. We identified 27 genome-wide significant loci associated with one or more pAIDs, mapping to *in silico*–replicated autoimmune-associated genes (including *IL2RA*) and new candidate loci with established immunoregulatory functions such as *ADGRL2*, *TENM3*, *ANKRD30A*, *ADCY7* and *CD40LG*. The pAID-associated single-nucleotide polymorphisms (SNPs) were functionally enriched for deoxyribonuclease (DNase)-hypersensitivity sites, expression quantitative trait loci (eQTLs), microRNA (miRNA)-binding sites and coding variants. We also identified biologically correlated, pAID-associated candidate gene sets on the basis of immune cell expression profiling and found evidence of genetic sharing. Network and protein-interaction analyses demonstrated converging roles for the signaling pathways of type 1, 2 and 17 helper T cells (T_H1, T_H2 and T_H17), JAK-STAT, interferon and interleukin in multiple autoimmune diseases.

Autoimmune diseases affect 7–10% of individuals living in Europe and North America[1] and represent a significant cause of chronic morbidity and disability. High rates of familial clustering and comorbidity across autoimmune diseases suggest that genetic predisposition underlies disease susceptibility. GWASs and immune-focused fine-mapping studies of autoimmune thyroiditis (THY)[2], psoriasis (PSOR)[3], juvenile idiopathic arthritis (JIA)[4], primary biliary cirrhosis (PBC)[5], primary sclerosing cholangitis (PSC)[6], rheumatoid arthritis (RA)[7], celiac disease (CEL)[8], inflammatory bowel disease (IBD, which includes Crohn's disease (CD) and ulcerative colitis (UC)[9]), and multiple sclerosis (MS)[10,11] have identified hundreds of autoimmune disease–associated SNPs across the genome[12–14]. SNP associations in certain pan-autoimmune loci, such as *PTPN22* c.1858C>T (rs2476601), are evident in independent GWASs across multiple autoimmune diseases[15–18], whereas others have been uncovered through large-scale meta-analyses (for example, CEL-RA and type 1 diabetes (T1D)-CD) or by searches for known loci from one disease in another (for example, systemic lupus erythematosus (SLE))[19]. These studies demonstrate that more than half of genome-wide significant (GWS) autoimmune disease associations are shared by at least two distinct autoimmune diseases[20,21]. However, the degree to which common, shared genetic variations may similarly affect the risk of different pAIDs and whether these effects are heterogeneous have not been systematically examined at the genotype level across multiple diseases simultaneously.

RESULTS

Shared genetic risk associations across ten pediatric autoimmune diseases

We performed whole-genome imputation on a combined cohort of more than 6,035 pediatric subjects across ten clinically distinct pAIDs (**Supplementary Table 1**) and 10,718 population-based control

A full list of affiliations appears at the end of the paper.

Received 22 February; accepted 23 July; published online 24 August 2015; doi:10.1038/nm.3933

subjects without prior history of autoimmune or immune-mediated disorders. We performed whole-chromosome phasing and used the 1,000 Genomes Project Phase I Integrated cosmopolitan reference panel (1KGP-RP) for imputation as previously described (SHAPEIT and IMPUTE2)[22,23]. Only individuals of self-reported European ancestry and confirmed by principal-component analysis (**Supplementary Figs. 1** and **2**) were included (Online Methods). Rare (minor allele frequency (MAF) < 1%) and poorly imputed (INFO score < 0.8) SNPs were removed, leaving a total of 7,347,414 variants.

Whole-genome case-control association testing was done using case samples from each of the ten pAIDs and the shared controls, and additive logistic regression was applied with SNPTESTv2.5 (ref. 24). There was no evidence of genomic inflation. To identify shared pAID-association loci, we performed an inverse χ^2 meta-analysis, accounting for sample-size variation and the use of a shared control across the ten pAIDs[25]. We identified 27 linkage disequilibrium (LD)-independent loci, consisting of associated SNPs with $r^2 > 0.05$ within a 1-Mb window where at least one lead SNP reached a conventionally defined GWS threshold ($P < 5 \times 10^{-8}$; **Fig. 1** and **Supplementary Fig. 1b**). An additional 19 loci reached a genome-wide marginally significant (GWM) threshold at or below $P_{META} < 1 \times 10^{-6}$, of which 12 mapped to previously reported autoimmune loci and 7 mapped to putatively novel autoimmune loci (**Fig. 1** and **Supplementary Table 2a**).

We identified five putatively novel GWS loci: *CD40LG* ($P_{META} < 8.38 \times 10^{-11}$), *ADGRL2* ($P_{META} < 8.38 \times 10^{-11}$), *TENM3* ($P_{META} < 8.38 \times 10^{-11}$), *ANKRD30A* ($P_{META} < 8.38 \times 10^{-11}$) and *ADCY7* ($P_{META} < 5.99 \times 10^{-9}$). For each lead association locus, we identified the corresponding combination of pAIDs contributing to the association signal by enumerating all 1,023 unique disease combinations (for example, one disease, T1D; two diseases, T1D and SLE; or four diseases, UC, CD, CEL and SLE) and performing association testing to identify the disease combination that yielded the maximum logistic regression *Z*-score (Online Methods)[26]. With the exception of *ANKRD30A*, the loci were jointly associated with at least two or more pAIDs; for example, *CD40LG* was shared by CEL, CD and UC (**Fig. 1** and **Table 1**). Among the 27 GWS lead SNPs, 22 had been reported previously as GWS for at least one of the associated pAIDs (specifically, for the corresponding adult phenotypes) identified by our analysis (**Supplementary Tables 1b** and **2b**)[12,27]. The most widely shared locus, chr4q27:rs62324212, mapping to an intronic SNP in *IL21-AS1* and residing just upstream of *IL21*, was shared across all ten diseases, and three of these associations were novel (THY, ankylosing spondylitis (AS) and common variable immunodeficiency (CVID)). For more than 50% of previously known GWS loci in adult-onset or generalized autoimmune disease, we identified at least one previously unrecognized pAID association (**Supplementary Table 2c**,**d**).

A number of the pAIDs were significantly associated with disease-specific signals mapping to or near the locus encoding HLA-DRB1. However, even the two most significant LD-independent variants that mapped to this locus and were associated with T1D and JIA, respectively, were disease specific (**Supplementary Fig. 3**), which suggests that the variants associated with a given disease are distinct. Although some of these associations were shared by at least two diseases, in no instance was a single signal associated with any of the diseases shared across all other diseases, which further underscores the complexity of signal sharing across the major histocompatibility complex (MHC) (**Supplementary Fig. 3b**).

Disease-specific and cross-autoimmune replication support for pAID-associated loci

We performed *in silico* analysis to test whether the reported associations could be replicated in an independent data set. We observed nominally significant replication support for four of the five putatively novel GWS loci, including three instances of disease-specific replication (**Supplementary Table 1d**). Among the replicated loci, chrXq26.3 (rs2807264), mapping within 70 Kb upstream of *CD40LG*, was notable, as we observed disease-specific replication in both UC ($P < 4.66 \times 10^{-5}$) and CD ($P < 5.81 \times 10^{-4}$), as well as cross-autoimmune replication in AS ($P < 9.54 \times 10^{-3}$). Although rs2807264 was not identified in our analysis as associated with pediatric AS, it is well documented that adult-onset AS and pediatric AS may be biologically different diseases with independent genetic etiologies[28,29]. A third disease-specific replication ($P < 5.99 \times 10^{-6}$) was identified in CD for the chr16q12.1 (rs77150043) signal mapping to an intronic position in *ADCY7*. This third instance and the replication of the *CD40LG* locus in UC were both significant, even after a very conservative Bonferonni adjustment for 156 tests ($P < 3.21 \times 10^{-4}$). A nominally significant pan-autoimmune replication signal ($P < 1.69 \times 10^{-2}$) was also observed at chr1p31.1 (rs2066363) near *LPHN2* in UC, and a replication signal ($P < 3.65 \times 10^{-3}$) was also observed at the chr4q35.1 locus (rs77150043) in PSOR (**Supplementary Tables 1d** and **2e**).

Sharing of pAID-associated SNPs and bidirectional effects of some SNPs on disease-specific risk

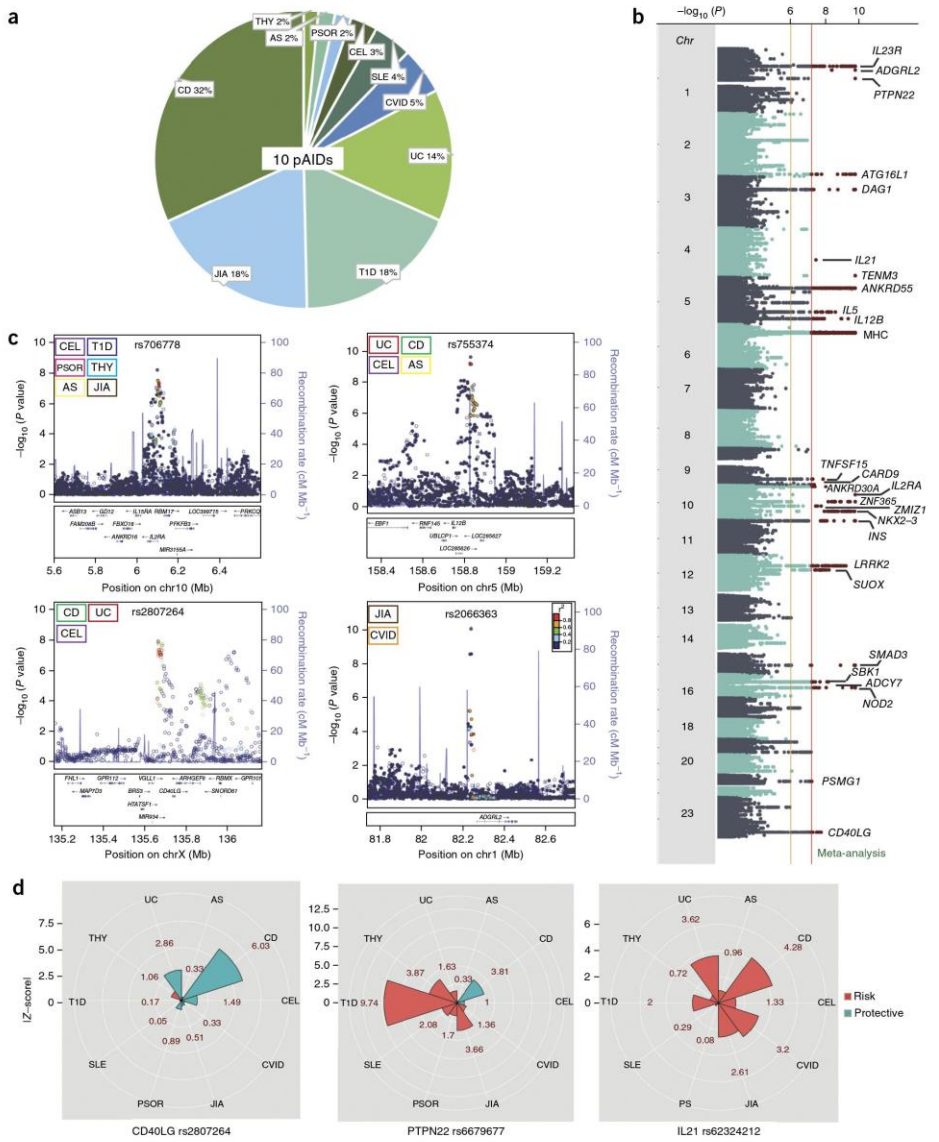
Of the 27 GWS loci, 81% (22) showed evidence of being shared among multiple pAIDs. These mapped to 77 different SNP-pAID combinations, 44 of which had been previously reported at or near genome-wide significance ($P < 1 \times 10^{-6}$), whereas 33 represented potentially novel disease-association signals (**Table 1** and **Supplementary Table 1**). Although *PTPN22* c.1858C>T (rs2476601) increases the risk for T1D, the variant is protective against CD[17,30–32]. We identified eight other instances ($P < 0.05$) where the risk allele shared by the model pAID combination was associated with protection against another pAID (**Fig. 2** and **Supplementary Fig. 4**).

Figure 1 The ten pAID case cohorts and top pAID-association loci identified. (**a**) Percentage and relative contribution of cases for the ten pediatric autoimmune diseases studied. (**b**) Top pAID-association signals identified by inverse χ^2 meta-analysis. The top 27 loci (where at least one lead SNP reached genome-wide significance: $P_{META} < 5 \times 10^{-8}$) are annotated with the candidate gene symbol. (**c**) Novel and established pAID-association loci. Top left: rs706778 (chr10p15.1) is a known DNase I peak and an intronic SNP in *IL2RA* and was associated with THY, AS, PSOR, CEL, T1D and JIA. Top right: rs755374 (chr5q33.3) is an intergenic SNP upstream of *IL12B* and was associated with AS, CEL, UC and CD. Bottom left: rs2807264 (chrXq26.3), mapping near *CD40LG*, was associated with CEL, UC and CD, and chr15q22.33 (rs72743477), also mapping to an intronic position in *SMAD3*, was associated with UC, CD and AS. Bottom right: SNPs are colored according to pairwise LD (r^2) with respect to the most strongly associated lead SNP in the locus. Associated pAIDs are indicated at the upper left. pAID associations are color-coded according to the key in each plot. (**d**) Pleiotropic candidate genes have pleiotropic effect sizes and directions across pAIDs. Although a few pleiotropic SNPs had consistent effect directions across diseases (e.g., *IL21*), for many loci (e.g., *PTPN22* and *CLEC16A*), the candidate SNP had variable effect directions across diseases. The radii of the wedges correspond to the absolute values of the *Z*-scores (beta/s.e.) for each pAID, and the color indicates whether the SNP is protective (green) or risk-associated (red) for each disease.

© 2015 Nature America, Inc. All rights reserved.

Biological support of associated loci from the public domain

To integrate our results with experimental and predictive biological data, we curated four categories of SNP annotations: (1) functional: variants that are exonic, affect transcription, are miRNA targets or tag copy-number polymorphic regions; (2) regulatory: transcription factor (TF)-binding sites and DNase-hypersensitivity sites or eQTL SNPs; (3) conserved: variants with evolutionarily constrained positions or CpG islands; or (4) prior literature support: a gene or locus previously



© 2015 Nature America, Inc. All rights reserved.

Table 1 Twenty-seven independent loci reaching genome-wide significance ($P_{META} < 5 \times 10^{-8}$) after adjustment for the use of shared controls using an inverse χ^2 meta-analysis across the pAIDs

Chr	Pos (Mb)	SNP	Region	Gene	A1	MAF	P_{META}	Known P^*	pAIDs
1	67.7	rs11580078	1p31.3	*IL23R*	G	0.43	8.4×10^{-11}	1.0×10^{-146}	CD#
1	**82.2**	**rs2066363**	**1p31.1**	***ADGRL2***	**C**	**0.34**	**8.4×10^{-11}**	**Novel**	**CVID, JIA**
1	114.3	rs6679677	1p13.2	*PTPN22*	A	0.09	8.4×10^{-11}	1.1×10^{-88}	THY#, PSOR, T1D#, JIA#
2	234.2	rs36001488	2q37.1	*ATG16L1*	C	0.48	8.4×10^{-11}	1.0×10^{-12}	PSOR, CD#
3	49.6	rs4625	3p21.31	*DAG1*	G	0.31	8.4×10^{-11}	1.0×10^{-47}	PSOR#, CEL, UC#, CD#
4	123.6	rs62324212	4q27	*IL21*	A	0.42	2.6×10^{-8}	1.0×10^{-9}	THY, AS, CEL#, CVID, UC#, T1D#, JIA#, CD#
4	**183.7**	**rs7660520**	**4q35.1**	***TENM3***	**A**	**0.26**	**8.4×10^{-11}**	**Novel**	**THY, AS, CEL, SLE, CVID, JIA**
5	40.5	rs7725052	5p13.1	*PTGER4*	C	0.43	8.4×10^{-11}	1.4×10^{-10}	CD#
5	55.4	rs7731626	5q11.2	*ANKRD55*	A	0.39	1.4×10^{-10}	2.7×10^{-11}	JIA#, CD#
5	131.8	rs11741255	5q31.1	*IL5*	A	0.42	1.6×10^{-9}	1.4×10^{-52}	PSOR#, CEL, CD#
5	158.8	rs755374	5q33.3	*IL12B*	T	0.32	2.3×10^{-10}	1.4×10^{-42}	AS#, CEL, UC#, CD#
9	117.6	rs4246905	9q32	*TNFSF15*	T	0.28	9.5×10^{-9}	1.2×10^{-17}	UC#, CD#
9	139.3	rs11145763	9q34.3	*CARD9*	C	0.40	3.3×10^{-8}	1.0×10^{-6}	AS#, UC#, CD#
10	6.1	rs706778	10p15.1	*IL2RA*	T	0.41	6.3×10^{-9}	1.7×10^{-12}	THY, AS, PSOR#, CEL, T1D#, JIA#
10	**37.6**	**rs7100025**	**10p11.21**	***ANKRD30A***	**G**	**0.34**	**8.4×10^{-11}**	**Novel**	**JIA**
10	64.4	rs10822050	10q21.2	*ZNF365*	C	0.39	8.4×10^{-11}	5.0×10^{-17}	SLE, CD#
10	81.0	rs1250563	10q22.3	*ZMIZ1*	C	0.29	1.3×10^{-8}	1.1×10^{-30}	PSOR#, CD#
10	101.3	rs1332099	10q24.2	*NKX2-3*	T	0.46	9.1×10^{-11}	1.0×10^{-54}	UC#, CD#
11	2.2	rs17885785	11p15.5	*INS*	T	0.20	8.4×10^{-11}	4.4×10^{-48}	T1D#
12	40.8	rs17466626	12q12	*LRRK2*	G	0.02	3.2×10^{-10}	3.0×10^{-10}	AS, CD#
12	56.4	rs1689510	12q13.2	*SUOX*	C	0.31	4.0×10^{-9}	1.1×10^{-10}	PSOR#, T1D#
15	67.5	rs72743477	15q22.33	*SMAD3*	G	0.21	8.4×10^{-11}	2.7×10^{-19}	AS, UC, CD#
16	28.3	rs12598357	16p11.2	*SBK1*	G	0.39	4.4×10^{-9}	1.0×10^{-8}	THY, AS#, PSOR, CEL, UC, CD#
16	**50.3**	**rs77150043**	**16q12.1**	***ADCY7***	**T**	**0.23**	**6.0×10^{-9}**	**Novel**	**PSOR, CD**
16	50.7	rs117372389	16q12.1	*NOD2*	T	0.02	8.4×10^{-11}	2.9×10^{-69}	CD#
21	40.5	rs2836882	21q22.2	*PSMG1*	A	0.27	4.8×10^{-8}	2.8×10^{-14}	UC#, CD#
23	**135.7**	**rs2807264**	**Xq26.3**	***CD40LG***	**C**	**0.21**	**1.3×10^{-8}**	**Novel**	**CEL, UC, CD**

Chr, chromosome; Pos (Mb), position in *hg19*; Region, cytogenetic band; A1, alternative allele; MAF, minor allele frequency (controls); Known P^*, lowest P value from published association studies. Pound symbols (#) denote previously reported disease-associated SNPs. "Novel" denotes new loci (bolded) that reached genome-wide significance for the first time in the present study (to our knowledge).

© 2015 Nature America, Inc. All rights reserved.

reported to be associated with autoimmune diseases or immune function. Indeed, 100% of the GWS lead SNPs or their nearby LD proxies ($r^2 > 0.8$ on the basis of 1KGP-RP within 500 Kb up- or downstream) belonged to one or more of these categories (**Fig. 3a**). Nevertheless, the majority of the 27 GWS SNPs did not confer direct transcriptional consequences (51% were intronic variants and 28% were intergenic

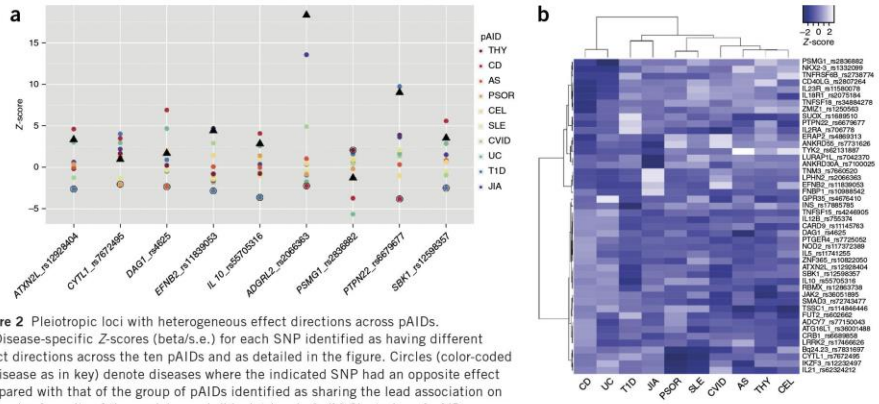


Figure 2 Pleiotropic loci with heterogeneous effect directions across pAIDs. (**a**) Disease-specific *Z*-scores (beta/s.e.) for each SNP identified as having different effect directions across the ten pAIDs and as detailed in the figure. Circles (color-coded by disease as in key) denote diseases where the indicated SNP had an opposite effect compared with that of the group of pAIDs identified as sharing the lead association on the basis of results of the model search (black triangles). (**b**) Clustering of pAIDs across the lead loci on the basis of disease-specific effect sizes. Agglomerative hierarchical clustering across ten pAIDs on the basis of normalized directional *Z*-scores (beta/s.e.) resulting from logistic regression analysis in each disease for the 27 lead loci based on those disease combinations identified by the model search analysis as producing the strongest association-test statistics.

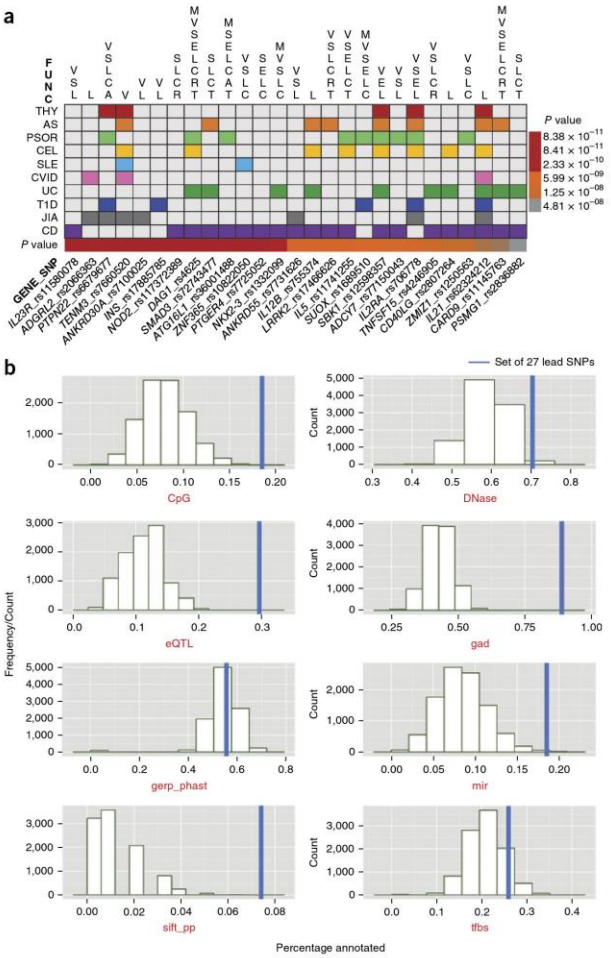


Figure 3 Integrated annotation of pAID-association loci using existing predictive and experimental data sets. (**a**) Biological, functional and literature annotations for the 27 loci reaching genome-wide significance in meta-analysis. Loci (identified by the lead SNPs and candidate genes) are organized by column; the colors in the table denote the associated pAIDs, functional annotations are presented at the top of the table, and the color bar at the bottom represents the meta-analysis P_{meta} values (according to key at right). For each locus, the lead SNP and proxy SNPs ($r^2 > 0.8$) were included in the annotation protocol (Online Methods). (**b**) Distribution and enrichment of experimental and predicted annotations for the top 27 GWS SNPs. The annotation frequencies were used to calculate the relative enrichment of pAID SNPs (blue bars) as compared with that of 10,000 random 100-SNP sets drawn from the genome in each annotation category. CpG, CpG islands; DNase, DNase-hypersensitivity I sites; gad, known genetic association; gerp_phast, conserved positions; mir, miRNAs; sift_pp, functional mutations in SIFT; tfbs, TF–binding sites.

© 2015 Nature America, Inc. All rights reserved.

npg

or up- or downstream gene variants), which suggests that many of these SNPs either tag the true causal variants or affect disease risk through regulatory and/or epigenetic mechanisms (**Fig. 3b**).

To determine whether the set of pAID-associated SNPs was enriched for specific annotation categories, we compared its annotation percentage with the percentages of 10,000 simulated sets of SNPs with MAF > 0.01 drawn from 1KGP-RP for each category. We found that pAID-associated SNPs were enriched for CpG islands ($P_{perm} < 1.0 \times 10^{-4}$), TF-binding sites ($P_{perm} < 3.4 \times 10^{-3}$) and miRNA-binding sites ($P_{perm} < 1.0 \times 10^{-4}$), among other findings of biological disease relevance (**Supplementary Fig. 1d,e**).

Candidate pAID genes share expression profiles across immune cell types and tissues

Recent studies show that gene-based association testing (GBAT) may boost the power of genetic discovery[33–35]. We performed GBAT (with VEGAS[33]) using genome-wide summary-level P_{META} values. We identified 182 significant pAID-associated genes (simulation-based $P_{sim} < 2.80 \times 10^{-6}$) on the basis of a Bonferonni adjustment for ~17,500 protein-coding genes in the genome (**Supplementary Table 3a**). To illustrate the biological relevance of this set of genes, we examined their transcript levels in a human gene expression microarray data set consisting of 12,000 genes and 126 tissue and/or cell types[36]. pAID-associated gene expression across immune tissues or cell types (ES-I, 4.05) was notably higher than that across non-immune types (ES-NI, 2.10) on the basis of a one-tailed Wilcoxon rank-sum test ($P < 1.66 \times 10^{-10}$). When all extended MHC genes were excluded, the average expression of pAID-associated genes remained significantly higher ($P < 1.27 \times 10^{-7}$) for immune (1.043) than for non-immune (0.648) tissues and cell types. The immune-specific enrichment of pAID-associated gene transcripts was comparable to that observed in adult cohorts[12]; comparatively, schizophrenia-associated genes showed no such enrichment (**Fig. 4a** and **Supplementary Table 3b**). We observed similar results when we used the Kolmogorov-Smirnov test (**Supplementary Fig. 5**).

We examined the expression of pAID genes across a whole-transcriptome data set comprising more than 200 murine immune cell types isolated by flow cytometry (ImmGen[37]; Online Methods and **Supplementary Table 3c**). Genes associated with pAIDs demonstrated differential expression across immune cell types (**Supplementary Fig. 6**) and showed higher expression than genes associated with non-immune traits, similar to results observed from human tissue data (**Fig. 4b**).

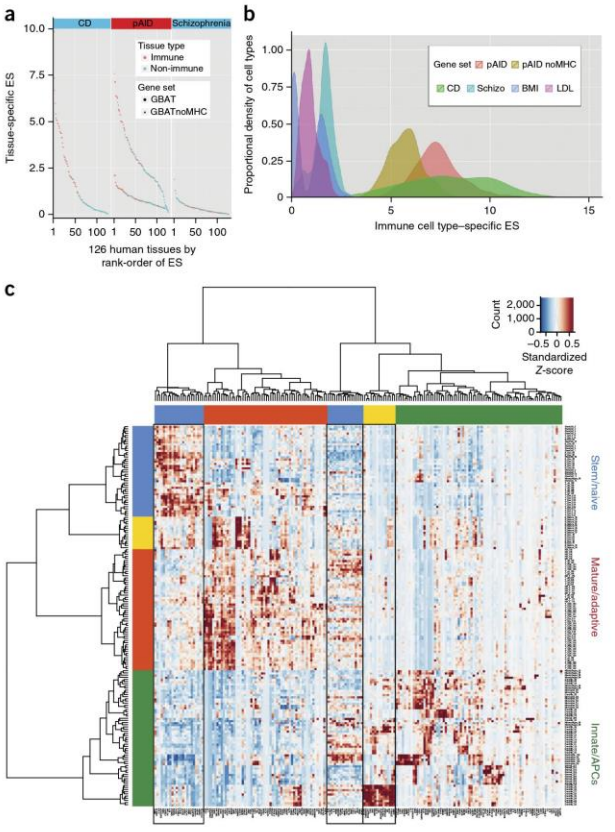


Figure 4 Tissue-specific gene set enrichment analysis (TGSEA) of pediatric and adult autoimmune data sets identifies autoimmune-associated gene expression patterns across immune cells and tissues. (**a**) Expression enrichment of autoimmune-associated genes across human tissues. Distribution of TGSEA enrichment score (ES) values across 126 tissues for pAID-associated genes (center) either with (circles, top curve) or without (triangles, bottom curve) the extended MHC. Results for the pAID gene set are compared with those obtained for known genes associated with CD (left) and schizophrenia (right). Tissue and cell types are classified as immune (red) or non-immune (blue) and are ranked left to right on the basis of the magnitude of the ES test statistic. (**b**) Enrichment of pAID-associated gene expression across diverse murine immune cell types. Distribution of pAID-associated gene ES values across murine immune cell types either including (red) or excluding the genes within the MHC (gold); results are compared with those for genes associated with CD, schizophrenia (Schizo, turquoise), LDL cholesterol (LDL, magenta) or body mass index (BMI, blue) abstracted from the National Human Genome Research Institute (NHGRI) GWAS Catalog. (**c**) Hierarchical clustering based on the expression of pleiotropic candidate genes associated with three or more autoimmune diseases across the murine immune cells. Boxes outlined in black denote gene clusters enriched for specific disease associations discussed in the text. An enlarged version of **c** is presented in **Supplementary Figure 8**.

© 2015 Nature America, Inc. All rights reserved.

npg

As the expression levels of these 'pleiotropic' genes varied diversely across immune cell types, we performed agglomerative hierarchical clustering to identify sets of genes sharing similar profiles. Genes that belonged to the same cluster (and thus shared similar expression profiles) were found to be enriched for association with specific individual or multiple autoimmune diseases (**Fig. 4c**). For example, cluster 1 genes, such as *ICAM1*, *CD40*, *JAK2*, *TYK2* and *IL12B*, with known roles in immune effector cell activation and proliferation, were enriched for association with PSC and UC and were associated with both diseases ($P < 6.82 \times 10^{-4}$, one-tailed Fisher's exact test), and the expression of these genes was highest in a small subset of CD11b^{+} dendritic cells[6]. These findings are consistent with the clinical observation that as many as 80% of patients diagnosed with PSC have been diagnosed with UC, and that the risk of PSC is approximately 600-fold higher in patients with UC[38,39]. Cluster 2 genes included genes encoding a number of cytokines and cytokine-response factors, such as *IL19*, *IL20*, *STAT5A* and *IL2RA*, the products of which regulate effector T cell activation, differentiation and proliferation. All of these were more broadly expressed across mature natural killer (NK) cells, NK T cells and T cells, as well as neutrophils. This cluster of genes was enriched for association with MS ($P < 9.8 \times 10^{-4}$), with CEL (marginally) ($P < 0.062$) and with both diseases ($P < 3.41 \times 10^{-4}$). Genes encoding nucleic acid–binding proteins, such as *ILF3*, *CENPO*, *MED1* and *NCOA3*, were enriched in cluster 3. Genes in this cluster were jointly associated with SLE and PSOR ($P < 0.03$), which is consistent with experimental and clinical data demonstrating that early defects in B cell[40,41] and T cell[42–44] clonal selection, respectively, may have important roles in the etiology of these diseases.

Quantification of genetic risk factors shared across pAIDs

We developed a novel method to specifically examine genome-wide pairwise-association signal sharing (referred to as a GPS test) across the pAIDs (Online Methods). Only data from the genotyped pAID cohort were used for this analysis. After Bonferroni adjustment for 45 pairwise combinations, the GPS test identified evidence of sharing between a number of pAID pairs at marginal levels of significance, as reported previously, including T1D-CEL ($P_{gps} < 3.44 \times 10^{-5}$), T1D-THY ($P_{gps} < 2.03 \times 10^{-3}$) UC-CD ($P_{gps} < 2.36 \times 10^{-3}$) and AS-PS ($P_{gps} < 8.15 \times 10^{-3}$). We also identified a strong GPS score for JIA-CVID ($P_{gps} < 6.88 \times 10^{-5}$). The correlations between JIA-CVID ($P_{gps} < 7.30 \times 10^{-5}$) and UC-CD ($P_{gps} < 7.32 \times 10^{-4}$) were more significant after the exclusion of markers from within the MHC region (**Supplementary Fig. 4b**).

Finally, we examined evidence of sharing across the full range of autoimmune diseases using ImmunoBase[27]. We identified significant

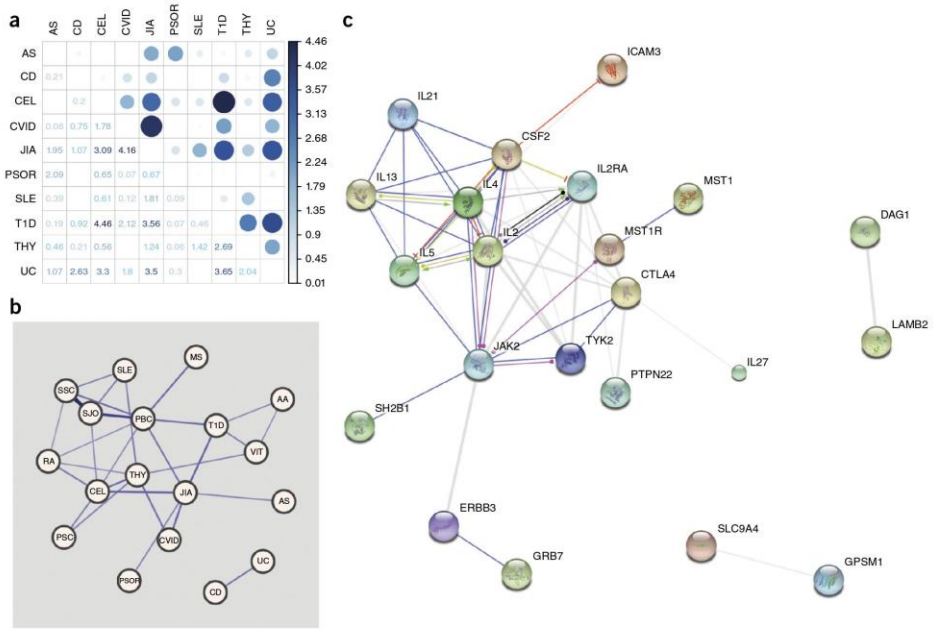


Figure 5 Genetic variants shared across the ten pAIDs reveal autoimmune disease networks. (**a**) Quantification of pAID genetic sharing by GPS test including SNPs within the extended MHC. Correlation plot of results of the pairwise pAID GPS test; the color intensity and the size of each circle are proportional to the strength of the correlation as the negative base ten logarithms of the GPS test *P* values (color-coded numbers in squares). (**b**) Quantification of autoimmune disease genetic sharing by locus-specific pairwise sharing. Undirected weighted network graph depicting results from the LPS test. Edge size represents the magnitude of the LPS test statistic; labeled nodes for each of the 17 autoimmune diseases are positioned on the basis of a force-directed layout. Edges represent significant pairs after Bonferroni adjustment ($P_{adj} < 0.05$). (**c**) Protein-protein interaction network analysis of the top pAID-associated protein candidates in STRING; action view of protein interactions observed across the top 46 GWM ($P < 1 \times 10^{-6}$) signals, of which 44 could be mapped to corresponding proteins. Views were generated on the basis of results for known and predicted protein interactions produced by the STRING DB *Homo sapiens* database. The plots shown are results of the 'action' view, where the molecular actions (stimulatory, repressive or binding) are illustrated by arrows.

© 2015 Nature America, Inc. All rights reserved.

associations between UC-CD ($P < 2.15 \times 10^{-4}$) and JIA-CVID ($P < 1.44 \times 10^{-6}$), along with a number of novel pairwise relationships that included autoimmune diseases other than the ten in this study, such as that between Sjogren's disease (SJO)–systemic sclerosis (SS) ($P < 1.30 \times 10^{-28}$) and PBC-SJO ($P < 3.86 \times 10^{-12}$). We plotted those relationships that were significant after Bonferroni adjustment for 153 pairwise tests using an undirected weighted network (**Fig. 5** and **Supplementary Table 4**). Collectively, these results support genetic sharing between the various autoimmune diseases and allow for further refinement of the shared signals, potentially enabling the application of targeted therapeutic interventions at multiple levels, such as along the CD40L-CD40, JAK-STAT and T_H1/T_H2-T_H17-interleukin signaling pathways.

DISCUSSION

A major goal of this study was to identify shared genetic etiologies across pAIDs and illustrate how they jointly and disparately affect pAID susceptibility. Knowledge of shared genetic etiologies may help pinpoint common therapeutic mechanisms, especially since certain pAIDs (for example, THY, CEL and T1D) exhibit high rates of comorbidity and concordance in twins, and others (for example, CD and UC) cluster in families[9,19,45,46].

Of the 27 GWS pAID-association loci identified, 81% were shared by at least two pAIDs (**Table 1** and **Supplementary Table 1**). Moreover, 5 of the 27 loci were novel signals not previously reported at GWS levels in association with autoimmune diseases, including chr1p31.1 (rs2066363), mapping near *ADGRL2*, a gene that encodes a member of the latrophilin subfamily of G protein–coupled receptors that regulates exocytosis. Although this signal was associated with JIA and CVID, a microsatellite study of PBC in a Japanese cohort localized an association signal to a 100-Kb region enclosing *ADGRL2* (ref. 47). Nominally significant replication support at this locus was identified in the adult UC cohort from the International IBD Genetics Consortium. Both JIA and CVID are among the six pAIDs (THY, AS, CEL, SLE, CVID and JIA) associated with the chr4q35.1 locus (rs7660520), which resides just downstream of *TENM3*. The observed association with a broad range of pAIDs may be related to eQTL signals in *TENM3* SNPs that correlate with

serum eosinophil counts[48] and immunoglobulin G (IgG) glycosylation rates; the latter was referenced in a study showing a pleiotropic role for IgG glycosylation–associated SNPs in autoimmune-disease risk susceptibility[49]. The third novel association was identified near chr10p11.21 (rs7100025), mapping to TF gene *ANKRD30A*, which encodes an antigen recognized by $CD8^+$ T cell clones[50]. The fourth signal was associated with the inflammatory diseases PSOR and CD near chr16q12.1 (rs77150043), an intronic SNP in *ADCY7*. *ADCY7* encodes a member of the adenylate cyclase enzyme family; is strongly expressed in peripheral leukocytes, spleen, thymus and lung tissues[51]; and it is supported by data from studies in mice[52]. The fifth novel signal, rs34030418, mapping near *CD40LG* and associated with CEL, UC and CD, is the ligand of the prominent TNF superfamily receptor CD40 (refs. 53,54). The CD40 ligand is a particularly compelling candidate, as the locus encoding the CD40 receptor is an established GWAS locus in RA and MS, has been functionally studied in cell culture and animal models, and was the focus of a recent large-scale RA drug-screening effort[55].

A set of GWS candidate SNPs were enriched for miRNA and TF-binding sites. We performed a gene-set enrichment analysis[56] using GBAT and identified 39 significant ($P_{BH} < 0.05$ (BH, Benjamini-Hochberg)) miRNAs, including as top candidates two well-known miRNA families, miR-22 and miR-135a (**Supplementary Table 5a**). miR-135a has been shown to target IRS2, a regulator of insulin signaling and glucose uptake, in model systems[57]. Our candidate genes were enriched for targets of dozens of TFs, with the most prominent being SP1 ($P_{BH} < 2.30 \times 10^{-12}$), NFAT ($P_{BH} < 8.54 \times 10^{-9}$) and NFKB ($P_{BH} < 1.03 \times 10^{-8}$) (**Supplementary Table 5b**).

Using GBAT with DAVID[58], GSEA[36], IPA[59] and Pathway Commons[60], among others, we identified strong enrichment for proteins that act in cytokine signaling; antigen processing and presentation; T cell activation; JAK-STAT activation; and T_H1-, T_H2- and T_H17-associated cytokine signaling (**Supplementary Tables 6** and **7**). Of these pathways, JAK2 signaling was particularly compelling ($P_{BH} < 6.93 \times 10^{-5}$), consistent with the enrichment of known protein-protein interactions ($P_{STRING} < 1 \times 10^{-20}$) (**Supplementary Fig. 7**). We also uncovered evidence supporting shared genetic susceptibility for disease pairs that have not yet been well established (for example, JIA-CVID). The association between JIA and CVID is noteworthy, given that CVID actually represents a group of complex immunodeficiencies rather than a classic autoimmune disease. When we examined the overlap between CVID and each of the other pAIDs using both GPS ($P_{adj} < 3.10 \times 10^{-3}$) and locus-specific pairwise sharing (LPS) ($P_{adj} < 1.47 \times 10^{-8}$) network analysis tests, we consistently observed overrepresentation of interaction between CVID and JIA (**Fig. 5** and **Supplementary Fig. 4b**). Our results show that more than 70% (19) of the 27 GWS loci we identified were shared by at least three autoimmune diseases (**Table 1**), including both previously reported (for example, *IL2RA* (six diseases) and *IL12B* (four diseases)) and novel (for example, *TENM3* (six diseases) and *CD40LG* (three diseases)) signals. Moreover, using tissue-specific gene set enrichment analysis, we not only highlighted the expected enrichment of genes associated with CEL and SLE in γδ T cells, $CD4^+$ T cells and NK T cells but also identified interesting joint enrichment of genes associated with PSC and UC in a set of $CD11b^+$ dendritic cells (**Fig. 4c**).

Many of the shared risk variants in pAIDs affect genes encoding proteins that are established therapeutic targets (for example, CD40L and CD40 (refs. 54,55)), and a number of the genes identified here have diverse biological effects and are currently being explored for clinical uses. Consequently, drug-repurposing approaches may present feasible options in pAIDs, where these gene networks and pathways could be targeted in an expedited manner.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank the subjects and their families for their participation in genotyping studies and the Biobank Repository at the Center for Applied Genomics at the Children's Hospital of Philadelphia. We acknowledge M.V. Holmes, H. Matsunami, L. Steel and E. Carrigan for their technical assistance and review of the manuscript. We are also thankful for the contributions of the Italian IBD Group, including S. Cucchiara (Roma), P. Lionetti (Firenze), G. Barabino (Genova), G.L. de Angelis (Parma), G. Guariso (Padova), C. Catassi (Ancona), G. Lombardi (Pescara), A.M. Staiano (Napoli), D. De Venuto (Bari), C. Romano (Messina), R. D'incà (Padova), M. Vecchi (Milano), A. Andriulli and F. Bossa (S. Giovanni Rotondo). The data sets used for the replication analyses were obtained through dbGaP accession numbers phs000344, phs000127, phs000274, phs000171, phs000224, phs000130, phs000019, phs000091, phs000206, phs000168, phs000138, phs000125 and phs000092. We thank the NIH data repository, the investigators who contributed the phenotype data and DNA samples from their original studies, and the primary funding organizations that supported these contributing investigators. This study made use of data generated by the Wellcome Trust Case Control Consortium. A full list of the investigators who contributed to the generation of the data is available from http://www.wtccc.org.uk. Funding for the project was provided by the Wellcome Trust under award 076113. Y.R.L. is supported by the Paul and Daisy Soros Fellowship for New Americans and the NIH F30 Individual NRSA Training Grant. This study was supported by Institutional Development Funds from The Children's Hospital of Philadelphia and by DP3DK085708, RC1AR058606, U01HG006830, the Crohn's & Colitis Foundation of America, the Juvenile Diabetes Research Foundation, NIH grant CA127334 (to H.L. and S.D.Z.), the UK National Institutes of Healthcare Research (to H.C.) and a grant from the Lupus Research Institute (to E.T.L.P.). This work was supported in part by the NIH (grant R01-HG006849 to A.K.). F.G. is a Howard Hughes Medical Institute International Student Research fellow.

AUTHOR CONTRIBUTIONS

Y.R.L. and H.H. were leading contributors in the design, analysis and writing of this study. D.J.A. contributed to data collection and literature review. B.F., Ø.F., L.A.D., S.D.T., M.L.B., S.L.G., A.L., E.P., E.R., C.S., A.S., E.M., M.S.S., B.A.L., M.P., R.K.R., D.C.W., H.C., C.C.-R., J.S.O., E.M.B., K.E.S., S.K., A.M.G., J. Snyder, T.H.F., C.P., R.N.B., J.E.M. and J.A.E. contributed samples and phenotypes. F.D.M., K.A.T., H.Q., R.M.C., C.E.K., F.W. and J. Satsangi provided assistance with samples, genotyping and data processing. S.D.Z., J.P.B., J.L. and H.L. contributed to, advised on and supervised statistical analysis. E.T.L.P., J.A.E. and B.J.K. assisted in composing and revising the manuscript. A.K., C.A.W., C.H., C.J.C., C.K., D.C., D.L., D.S.M., F.G., J.J.C., J.T.G., M.B., M.C.D., M.D.R., P.M.A.S., S.F.A.G., S.M.M., V.A., Y.G. and Z.W. read, edited and approved of the manuscript, along with all other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Cooper, G.S., Bynum, M.L. & Somers, E.C. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J. Autoimmun.* **33**, 197–207 (2009).
2. Cooper, J.D. *et al.* Seven newly identified loci for autoimmune thyroid disease. *Hum. Mol. Genet.* **21**, 5202–5208 (2012).
3. Tsoi, L.C. *et al.* Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nat. Genet.* **44**, 1341–1348 (2012).
4. Hinks, A. *et al.* Dense genotyping of immune-related disease regions identifies 14 new susceptibility loci for juvenile idiopathic arthritis. *Nat. Genet.* **45**, 664–669 (2013).
5. Liu, J.Z. *et al.* Dense fine-mapping study identifies new susceptibility loci for primary biliary cirrhosis. *Nat. Genet.* **44**, 1137–1141 (2012).
6. Liu, J.Z. *et al.* Dense genotyping of immune-related disease regions identifies nine new risk loci for primary sclerosing cholangitis. *Nat. Genet.* **45**, 670–675 (2013).
7. Eyre, S. *et al.* High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. *Nat. Genet.* **44**, 1336–1340 (2012).

© 2015 Nature America, Inc. All rights reserved.

8. Zhernakova, A. *et al.* Meta-analysis of genome-wide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. *PLoS Genet.* **7**, e1002004 (2011).
9. Jostins, L. *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124 (2012).
10. International Multiple Sclerosis Genetics Consortium. *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214–219 (2011).
11. Beecham, A.H. *et al.* Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat. Genet.* **45**, 1353–1360 (2013).
12. National Human Genome Research Institute Published Genome-Wide Associations through 08/01/2014. *NHGRI GWAS Catalog* https://www.genome.gov/26525384 (2014).
13. Welter, D. *et al.* The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. *Nucleic Acids Res.* **42**, D1001–D1006 (2014).
14. Cortes, A. & Brown, M.A. Promise and pitfalls of the Immunochip. *Arthritis Res. Ther.* **13**, 101 (2011).
15. Hakonarson, H. *et al.* A genome-wide association study identifies *KIAA0350* as a type 1 diabetes gene. *Nature* **448**, 591–594 (2007).
16. Hinks, A. *et al.* Association between the *PTPN22* gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that *PTPN22* is an autoimmunity gene. *Arthritis Rheum.* **52**, 1694–1699 (2005).
17. Smyth, D.J. *et al.* Shared and distinct genetic variants in type 1 diabetes and celiac disease. *N. Engl. J. Med.* **359**, 2767–2777 (2008).
18. Harley, J.B. *et al.* Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in *ITGAM, PXK, KIAA1542* and other loci. *Nat. Genet.* **40**, 204–210 (2008).
19. Ramos, P.S. *et al.* A comprehensive analysis of shared loci between systemic lupus erythematosus (SLE) and sixteen autoimmune diseases reveals limited genetic overlap. *PLoS Genet.* **7**, e1002406 (2011).
20. Cotsapas, C. *et al.* Pervasive sharing of genetic effects in autoimmune disease. *PLoS Genet.* **7**, e1002254 (2011).
21. Cotsapas, C. & Hafler, D.A. Immune-mediated disease genetics: the shared basis of pathogenesis. *Trends Immunol.* **34**, 22–26 (2013).
22. Howie, B., Fuchsberger, C., Stephens, M., Marchini, J. & Abecasis, G.R. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat. Genet.* **44**, 955–959 (2012).
23. Delaneau, O., Coulonges, C. & Zagury, J.-F. Shape-IT: new rapid and accurate algorithm for haplotype inference. *BMC Bioinformatics* **9**, 540 (2008).
24. Marchini, J. SNPTEST (v2.5) https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html (2007).
25. Zaykin, D.V. & Kozbur, D.O. *P*-value based analysis for shared controls design in genome-wide association studies. *Genet. Epidemiol.* **34**, 725–738 (2010).
26. Bhattacharjee, S. *et al.* A subset-based approach improves power and interpretation for the combined analysis of genetic association studies of heterogeneous traits. *Am. J. Hum. Genet.* **90**, 821–835 (2012).
27. Institute for Systems Biology and Juvenile Diabetes Research Foundation–Wellcome Trust Diabetes and Inflammation Laboratory. ImmunoBase http://www.immunobase.org (2013).
28. Gensler, L.S. *et al.* Clinical, radiographic and functional differences between juvenile-onset and adult-onset ankylosing spondylitis: results from the PSOAS cohort. *Ann. Rheum. Dis.* **67**, 233–237 (2008).
29. Lin, Y.-C., Liang, T.-H., Chen, W.-S. & Lin, H.-Y. Differences between juvenile-onset ankylosing spondylitis and adult-onset ankylosing spondylitis. *J. Chin. Med. Assoc.* **72**, 573–580 (2009).
30. Anaya, J.-M., Gómez, L. & Castiblanco, J. Is there a common genetic basis for autoimmune diseases? *Clin. Dev. Immunol.* **13**, 185–195 (2006).
31. De Jager, P.L. *et al.* Evaluating the role of the 620W allele of protein tyrosine phosphatase PTPN22 in Crohn's disease and multiple sclerosis. *Eur. J. Hum. Genet.* **14**, 317–321 (2006).
32. Zhernakova, A. *et al.* Differential association of the PTPN22 coding variant with autoimmune diseases in a Dutch population. *Genes Immun.* **6**, 459–461 (2005).
33. Liu, J.Z. *et al.* A versatile gene-based test for genome-wide association studies. *Am. J. Hum. Genet.* **87**, 139–145 (2010).
34. Li, M.-X., Gui, H.-S., Kwan, J.S.H. & Sham, P.C. GATES: a rapid and powerful gene-based association test using extended Simes procedure. *Am. J. Hum. Genet.* **88**, 283–293 (2011).
35. Huang, H., Chanda, P., Alonso, A., Bader, J.S. & Arking, D.E. Gene-based tests of association. *PLoS Genet.* **7**, e1002177 (2011).
36. Benita, Y. *et al.* Gene enrichment profiles reveal T-cell development, differentiation, and lineage-specific transcription factors including ZBTB25 as a novel NF-AT repressor. *Blood* **115**, 5376–5384 (2010).
37. Heng, T.S.P. & Painter, M.W. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* **9**, 1091–1094 (2008).
38. Olsson, R. *et al.* Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. *Gastroenterology* **100**, 1319–1323 (1991).
39. Feld, J.J. & Heathcote, E.J. Epidemiology of autoimmune liver disease. *J. Gastroenterol. Hepatol.* **18**, 1118–1128 (2003).
40. Yurasov, S. *et al.* Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J. Exp. Med.* **201**, 703–711 (2005).
41. Cappione, A. *et al.* Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J. Clin. Invest.* **115**, 3205–3216 (2005).
42. Evenou, J.-P. *et al.* The potent protein kinase C-selective inhibitor AEB071 (sotrastaurin) represents a new class of immunosuppressive agents affecting early T-cell activation. *J. Pharmacol. Exp. Ther.* **330**, 792–801 (2009).
43. Jegasothy, B.V. Tacrolimus (FK 506)—a new therapeutic agent for severe recalcitrant psoriasis. *Arch. Dermatol.* **128**, 781–785 (1992).
44. Nograles, K.E. & Krueger, J.G. Anti-cytokine therapies for psoriasis. *Exp. Cell Res.* **317**, 1293–1300 (2011).
45. Ergür, A.T. *et al.* Celiac disease and autoimmune thyroid disease in children with type 1 diabetes mellitus: clinical and HLA-genotyping results. *J. Clin. Res. Pediatr. Endocrinol.* **2**, 151–154 (2010).
46. Eyre, S. *et al.* Overlapping genetic susceptibility variants between three autoimmune disorders: rheumatoid arthritis, type 1 diabetes and coeliac disease. *Arthritis Res. Ther.* **12**, R175 (2010).
47. Joshita, S. *et al. A2BP1* as a novel susceptible gene for primary biliary cirrhosis in Japanese patients. *Hum. Immunol.* **71**, 520–524 (2010).
48. Pruitt, K., Brown, G., Tatusova, T. & Maglott, D. The Reference Sequence (RefSeq) database http://www.ncbi.nlm.nih.gov/books/NBK21091/ (2012).
49. Lauc, G. *et al.* Loci associated with N-glycosylation of human immunoglobulin G show pleiotropy with autoimmune diseases and haematological cancers. *PLoS Genet.* **9**, e1003225 (2013).
50. Jäger, D. *et al.* Humoral and cellular immune responses against the breast cancer antigen NY-BR-1: definition of two HLA-A2 restricted peptide epitopes. *Cancer Immun.* **5**, 11 (2005).
51. Ludwig, M.-G. & Seuwen, K. Characterization of the human adenylyl cyclase gene family: cDNA, gene structure, and tissue distribution of the nine isoforms. *J. Recept. Signal Transduct. Res.* **22**, 79–110 (2002).
52. Jiang, L.I., Sternweis, P.C. & Wang, J.E. Zymosan activates protein kinase A via adenylyl cyclase VII to modulate innate immune responses during inflammation. *Mol. Immunol.* **54**, 14–22 (2013).
53. Anderson, D.M. *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390**, 175–179 (1997).
54. Miyashita, T. *et al.* Bidirectional regulation of human B cell responses by CD40–CD40 ligand interactions. *J. Immunol.* **158**, 4620–4633 (1997).
55. Li, G. *et al.* Human genetics in rheumatoid arthritis guides a high-throughput drug screen of the CD40 signaling pathway. *PLoS Genet.* **9**, e1003487 (2013).
56. Wang, J., Duncan, D., Shi, Z. & Zhang, B. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res.* **41**, W77–W83 (2013).
57. Agarwal, P., Srivastava, R., Srivastava, A.K., Ali, S. & Datta, M. miR-135a targets IRS2 and regulates insulin signaling and glucose uptake in the diabetic gastrocnemius skeletal muscle. *Biochim. Biophys. Acta* **1832**, 1294–1303 (2013).
58. Huang, D.W. *et al.* The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol.* **8**, R183 (2007).
59. Ingenuity Systems. *Ingenuity Pathway Analysis* http://www.ingenuity.com/products/ipa (2015).
60. Cerami, E.G. *et al.* Pathway Commons, a web resource for biological pathway data. *Nucleic Acids Res.* **39**, D685–D690 (2011).

[1]The Center for Applied Genomics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [2]Medical Scientist Training Program, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. [3]Department of Biostatistics, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. [4]Division of Allergy and Immunology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [5]Department of Biological Statistics and Computational Biology, Cornell University, Ithaca, New York, USA. [6]Program in Computational Biology and Medicine, Cornell University, Ithaca, New York, USA. [7]Department of Computer Science, New Jersey Institute of Technology, Newark, New Jersey, USA. [8]Department of Biochemistry and Molecular Biology, Eberly College of Science, The Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, Pennsylvania, USA. [9]Department of Rheumatology, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [10]Division of Gastroenterology, The Center for Inflammatory Bowel Disease, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. [11]Divison of Rheumatology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. [12]Division of Rheumatology, Children's Mercy Hospitals and Clinics, Kansas City, Missouri, USA. [13]Department of Pediatrics, University of Utah School of Medicine and Primary Children's Medical Center, Salt Lake City, Utah, USA. [14]Division of Gastroenterology, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy. [15]Division of Pediatric Allergy and Immunology, University of Miami Miller School of Medicine, Miami, Florida, USA. [16]Institute of Immunology and Department of Medicine, Mount Sinai School of Medicine, New York, New York, USA. [17]Department of Paediatric Gastroenterology, Yorkhill Hospital for Sick Children, Glasgow, Scotland, UK. [18]Paediatric Gastroenterology and Nutrition, Royal Hospital for Sick Children, University of Edinburgh, Ediburgh, UK. [19]Mount Sinai Hospital IBD Centre, University of Toronto, Toronto, Ontario, Canada. [20]Unit of Gastroenterology, Department of Medical and Surgical Specialties, Careggi University Hospital, Florence, Italy. [21]Department of

© 2015 Nature America, Inc. All rights reserved.

Immunology, Oslo University Hospital, Rikshospitalet, Oslo, Norway. [22]Department of Rheumatology, Texas Scottish Rite Hospital for Children, Dallas, Texas, USA. [23]Department of Pediatrics, Pediatric IBD Center, Cedars Sinai Medical Center, Los Angeles, California, USA. [24]Department of Pathology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [25]Department of Pediatrics, The Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. [26]Department of Translational Medical Science, Section of Pediatrics, University of Naples Federico II, Naples, Italy. [27]Department of Pediatrics, Emory University School of Medicine and Children's Health Care of Atlanta, Atlanta, Georgia, USA. [28]Genes, Environment and Complex Disease, Murdoch Children's Research Institute, Parkville, Victoria, Australia. [29]Department of Pediatrics, University of Melbourne, Parkville, Victoria, Australia. [30]Pediatric Rheumatology Unit, Royal Children's Hospital, Parkville, Victoria, Australia. [31]Arthritis and Rheumatology Research, Murdoch Children's Research Institute, Parkville, Victoria, Australia. [32]Sarah M. and Charles E. Seay Center for Musculoskeletal Research, Texas Scottish Rite Hospital for Children, Dallas, Texas, USA. [33]Department of Clinical Immunology, Nuffield Department of Medicine, University of Oxford, Oxford, UK. [34]Section of Immunology, Allergy, and Rheumatology, Department of Pediatric Medicine, Texas Children's Hospital, Houston, Texas, USA. [35]Division of Rheumatology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [36]The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada. [37]Gastrointestinal Unit, Division of Medical Sciences, School of Molecular and Clinical Medicine, University of Edinburgh, Edinburgh, UK. [38]Department of Pediatrics, Nemours Children's Hospital, Orlando, Florida, USA. [39]Department of Pathology and Lab Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. [40]Departments of Pediatrics and Human Genetics, McGill University Health Centre Research Institute, Montréal, Québec, Canada. [41]Division of Gastroenterology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [42]Division of Pulmonary Medicine, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [43]Present address: Department of Statistics, University of Illinois at Urbana-Champaign, Champaign, Illinois, USA. Correspondence should be addressed to H.H. (hakonarson@email.chop.edu).

© 2015 Nature America, Inc. All rights reserved.

ONLINE METHODS

Study population. Affected subjects and controls were identified either directly as described in prior studies[61–70] or from de-identified samples and associated electronic medical records (EMRs) in the genomics biorepository at The Children's Hospital of Philadelphia (CHOP). The predominant majority (>80%) of the included cases for IBD, T1D and CVID have been described in previous publications.

Details of each study population are outlined below. EMR searches were conducted with previously described algorithms based on phenotype mapping established using phenome-wide association study (PheWAS) ICD-9 code mapping tables[61–63,70] in consultation with qualified physician specialists for each disease cohort. All DNA samples were assessed for quality control (QC) and genotyped on the Illumina HumanHap550 or HumanHap610 platform at the Center for Applied Genomics (CAG) at CHOP. Note that the patient counts below refer to the total recruited sample size from which we excluded non-qualified samples or genotypes that did not pass QC criteria required for inclusion in the genetic analysis (for example, because of relatedness or poor genotyping rate).

The IBD cohort comprised 2,796 individuals between the ages of 2 and 17, of European ancestry, and with biopsy-proven disease, including 1,931 with CD and 865 with UC and excluding all patients with unclassified IBD. Affected individuals were recruited from multiple centers from four geographically discrete countries and were diagnosed before their 19th birthday according to standard IBD diagnostic criteria, as previously reported[63,65].

The T1D cohort consisted of 1,120 subjects from nuclear family trios (one affected child and two parents), including 267 independent Canadian T1D patients collected in pediatric diabetes clinics in Montreal, Toronto, Ottawa and Winnipeg and 203 T1D patients recruited at CHOP since September 2006. All patients were Caucasian by self-report and between 3 and 17 years of age, with a median age at onset of 7.9 years. All patients had been treated with insulin since diagnosis. Disease diagnosis was based on these clinical criteria, rather than on any laboratory tests.

The JIA cohort was recruited in the United States, Australia and Norway and comprised a total of 1,123 patients with onset of arthritis at less than 16 years of age. JIA diagnosis and JIA subtype were determined according to the International League of Associations for Rheumatology (ILAR) revised criteria[71] and confirmed using the JIA Calculator[72] (http://www.jra-research.org/JIAcalc/), an algorithm-based tool adapted from the ILAR criteria. Prior to standard QC procedures and exclusion of non-European ancestry, the JIA cohort comprised 464 subjects of self-reported European ancestry from Texas Scottish Rite Hospital for Children (Dallas, Texas, USA) and the Children's Mercy Hospitals and Clinics (Kansas City, Missouri, USA); 196 subjects from CHOP; 221 subjects from the Murdoch Children's Research Institute (Royal Children's Hospital, Melbourne, Australia); and 504 subjects from Oslo University Hospital (Oslo, Norway).

The CVID study population consisted of 223 patients from Mount Sinai School of Medicine (MSSM; New York, New York, USA), 76 patients from University of Oxford, (London, England), 47 patients from CHOP, and 27 patients from University of South Florida (USF; Tampa, Florida, USA). The diagnosis in each case was validated against the ESID-PAGID diagnostic criteria, as previously described[73]. Although the diagnosis of CVID is most commonly made in young adults (ages 20–40), all of the CHOP and USF subjects had pediatric-age-of-onset disease, whereas the majority of the subjects from MSSM and Oxford had onset in young adulthood. We note that as the number of individuals with adult-onset CVID is so small (less than 5% of all cases presented) and all ten diseases studied here can present with pediatric age of onset, we elected to refer to the cohort material as pAID.

The balance of the pediatric subjects' (THY, AS, PSOR, CEL and SLE ; a full list of phenotype abbreviations is provided in **Supplementary Table 8**) samples were derived from our biorepository at CHOP, which includes more than 50,000 pediatric patients recruited and enrolled by CAG at CHOP (**Supplementary Table 9a** includes details of genotyped subjects within the CAG pediatric biobank). These individuals were confirmed for diagnosis of THY, SPA, PSOR, CEL and SLE in the age range of 1–17 years at the time of diagnosis and were required to fulfill the clinical criteria for these respective disorders, as confirmed by a specialist. Only patients that upon EMR search were confirmed to have at least two or more in-person visits, at least one of which was with the specified ICD-9 diagnosis code(s), were pursued for clinical confirmation (**Supplementary Table 9b** presents ICD-9 inclusion and exclusion codes). We used ICD-9 codes previously identified and used for PheWASs or EMR-based GWASs and agreed upon by board-certified physicians[62,63].

Age- and gender-matched control subjects were identified from the CHOP-CAG biobank and selected by exclusion of any patient with any ICD-9 codes for disorders of autoimmunity or immunodeficiency[61] (http://icd9.chrisendres.com/). Research ethics boards of CHOP and other collaborating centers approved this study, and written informed consent was obtained from all subjects (or their legal guardians). Genomic DNA extraction and sample QC before and after genotyping were performed using standard methods as described previously[64]. All samples were genotyped at CAG on HumanHap550 and 610 BeadChip arrays (Illumina, CA). To minimize confounding due to population stratification, we included only individuals of European ancestry (as determined by both self-reported ancestry and principal-component analysis (PCA)) for the present study. Details of the PCA are provided below.

Genotyping, imputation, association testing and QC. *Disease-specific QC.* We merged the genotyping results from each disease-specific cohort with data from the shared controls before extracting the genotyping results from SNPs common to both Infinium HumanHap550 and 610 BeadChip array platforms and performing genotyping QC. SNPs with a low genotyping rate (<95%) or low MAF (<0.01) or those significantly departing from the expected Hardy-Weinberg equilibrium (HWE; $P < 1 \times 10^{-6}$) were excluded. Samples with low overall genotyping call rates (<95%) or determined to be of outliers of European ancestry by PCA (>6.0 s.d. as identified by EIGENSTRAT[74]) were removed. In addition, one of each pair of related individuals as determined by identity-by-state analysis (PI_HAT > 0.1875) was excluded, with cases preferentially retained where possible.

Merged-cohort QC. To prepare for whole-genome imputation across the entire study cohort, we combined case samples across the 10 pAIDs with the shared control samples. We repeated the genotyping and sample QC with the same criteria as described above, leaving a final set of ~486,000 common SNPs passing individual-cohort and merged-cohort QC. We again performed identity-by-state analysis and removed related samples (in order to remove related subjects that may have been recruited for different disease studies). We also repeated the PCA and removed population outliers. The final cohort, after the application of all QC metrics mentioned above, included a total of 6,035 patients representing ten pAIDs and 10,718 population-matched controls.

Note that because of the merged QC, compared with the sum of all ten disease-specific GWASs, the final case and control counts in the merged cohort were smaller than the "sum of all cases and controls" (**Supplementary Table 1a**). In addition, to avoid the potential for confounding due to the presence of duplicated samples, we assigned individuals fitting the diagnostic criteria for two or more pAIDs to whichever disease cohort had the smaller (or smallest) sample size. No subject was included twice. A total of 160 subjects in the study cohort fulfilled criteria for two or more diseases but were counted only once in our reported total of 6,035 unique subjects.

Whole-genome phasing and imputation. We used SHAPEIT[75] for whole-chromosome prephasing and IMPUTE2 (ref. 76) for imputation to the 1KGP-RP (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html, June 2014 haplotype release). For both, we used parameters suggested by the developers of the software and described elsewhere[75–77]. Imputation was done for each 5-Mb regional chunk across the genome, and data were subsequently merged for association testing. Prior to imputation, all SNPs were filtered using the criteria described above.

To verify the imputation accuracy, we validated randomly selected SNPs that reached a nominally significant *P* value after imputation. Because commercially designed genotyping probes were not readily available, we performed Sanger sequencing by designing primers to amplify and sequence the 200-bp region around the imputed SNP markers for two separate 96-well plates. We manually visualized and examined sequences and chromatograms using SeqTrace[78]. Results from this are presented in **Supplementary Table 1e**, showing >99% mean imputation accuracy.

doi:10.1038/nm.3933

© 2015 Nature America, Inc. All rights reserved.

In addition, a subset of the IBD and CVID subjects were subsequently genotyped on the Immunochip (Illumina) platform. We compared the genotype concordance of all pAID GWAS imputed SNPs that were directly genotyped on the Immunochip after performing sample and marker QC as described above. Results are shown in **Supplementary Table 1f.**

Disease-specific association testing. We performed whole-genome association testing using post-imputation genotype probabilities with the software SNPTEST (v2.5)[24]. We used logistic regression to estimate odds ratios and betas, 95% confidence intervals and *P* values for trend, using additive coding for genotypes (0, 1 or 2 minor alleles). For autosomal regions, we used a score test, whereas for regions on ChrX we used the ChrX-specific SNPTEST method Newml. QC was performed directly after association testing, excluding any SNPs with an INFO score of <0.80, HWE $P < 1 \times 10^{-6}$, and MAF < 0.01 (overall).

In all analyses, we adjusted for both gender and ancestry by conditioning on gender and the first ten principal components derived from EIGENSTRAT PCA[79]. The λ_{GC} values for all cohorts were within acceptable limits; the highest was observed for the cohort with the largest case sample size, namely, CD ($\lambda_{GC} < 1.07$), consistent with what was previously reported for this data set[65]. In fact, we have previously reported on all the non-CHOP cases included in the present analysis in individual studies using CHOP controls and shown that these individual case-control analyses were well controlled for genomic inflation[61–70]. A QQ plot is provided for each independent cohort in **Supplementary Figure 2a**.

Meta-analysis to identify shared pAID association loci. To identify association loci shared across pAIDs, we meta-analyzed the summary-level test statistics from each of the study cohorts after extracting those markers that passed post-association testing QC for all ten individual disease-specific analyses. To adjust for confounding due to the use of a shared or pooled control population, we applied a previously published method to perform an inverse weighted χ^2 meta-analysis[80].

We LD-clumped the results of the meta-analysis (PLINK) and identified 27 LD-independent associations ($r^2 < 0.05$ within 500 kB up- or downstream of the lead or most strongly associated SNP) reaching a conventional genome-wide significance threshold of $P_{META} < 5 \times 10^{-8}$. We observed that the calculated meta-analysis λ_{GC} was less than 1.09. As recently discussed by de Bakker and colleagues and shown in a number of large-scale GWAS publications, λ_{GC} is related to sample size[81]. As discussed by Yang *et al.*, λ_{GC} depends on the relative contribution of variance due to population structure and true associations versus sampling variance: with no population structure or systematic error, inflation would still depend on heritability, genetic architecture and study sample size[82]. On the basis of de Bakker *et al.*'s recommendations, we also calculated a sample-size-adjusted λ_{1000} by interpolating the λ_{GC} that would have been expected if this study had included only 1,000 cases and 1,000 controls. We performed this only for the meta-analysis results, as the case and control counts for the meta-analysis were both significantly greater than 1,000 (**Supplementary Table 1a**).

Model search to identify pAIDs associated with the lead signals. The meta-analysis identified SNPs significantly associated with at least one pAID. To determine which pAIDs each SNP was most strongly associated with, we performed a model or 'disease-combination' search. For the lead SNP in each pAID-association locus, we searched for the pAID disease combination that, when the corresponding cases were merged in a mega-analysis, yielded the largest association test statistic.

To identify the disease phenotypes most likely contributing to each identified association signal, we applied the "h.types" method as implemented in the R statistical software package ASSET[83] to perform an exhaustive disease-subtype model search. Note that ASSET provides both a method for genotype-level association testing (h.types used in this study) and a summary-level modified fixed-effect meta-analysis approach ("h.traits") that allows for heterogeneity of SNP effects across different phenotypes. Both methods exhaustively enumerate each combination of phenotypes that are jointly considered, and therefore test a total of

$$\text{pAID disease model combinations} = \sum_{i=1}^{r} \frac{n!}{(n-i)!(i)}$$

where r is the total number of disease subtypes assigned to cases (for example, ranging from one to ten pAIDs) and n is the total number of disease subtypes (i.e., ten pAIDs). Note that this reduces to $2^n - 1$ (or 1,023 unique combinations here), as in this case we considered all possibilities of r across n of ten diseases. The ASSET algorithm iteratively tests each pAID case combination using logistic regression to determine whether there is an association between genotype counts and case status. For each SNP tested, the 'optimal' subtype model is the combination of pAIDs that, when tested against the shared controls in the logistic regression analysis produced the best test statistic after the DLM method had been used to correct for multiple testing across all subtype combinations.

Identification of lead associated variants showing opposite direction of effect. For each of the top 46 associating loci ($P_{META} < 1 \times 10^{-6}$), we identified those loci for which the lead SNP had an effect direction (on the basis of logistic regression betas) opposite that reported for the disease combination identified by the subtype model search and whose corresponding association *P* value reached at least nominal significance ($P < 0.05$). We identified nine instances.

Candidate gene prioritization. To annotate the lead SNPs to candidate genes, we prioritized the mapping to candidate genes systematically in the following manner:

1. If the SNP or locus was previously reported in autoimmune diseases at genome-wide significance, we provided the candidate gene symbol, where available, as identified in the GWAS Catalog[84] or ImmunoBase[83].
2. If an SNP was annotated as coding or fell within the coding DNA sequence (i.e., intronic or in the UTRs), we reported that gene as identified by the variant effect predictor (VEP)[85].
3. If the SNP was upstream, downstream, or intergenic, we prioritized the gene by using the best candidate gene identified with the network tool DAPPLE[86].
4. If none of the above was feasible, we manually curated the most 'likely' gene on the basis of the observed LD block and evidence of prior association signals with autoimmune diseases or other immune-related phenotypes as presented in the dbSNP or GWAS catalog.

Functional or biological annotations and enrichment analysis using publicly accessible resources. We annotated the lead pAID-associated SNPs using publicly available functional and biological databases and resources. We considered the top imputed lead SNP for each locus and, in addition, any of its near-perfect proxies (defined as $r^2 > 0.8$ within 500 kB up- or downstream) on the basis of the 1KGP-RP.

We included annotation, expression, interaction and network data from the following resources:

1. Genomic mapping and annotation: SNAP[87], SNP-Nexus[88], Ensemble[89] and UCSC[90].
2. Regulatory annotations: EnCODE (TF-binding sites and DNase-hypersensitivity sites)[91], GTex[92] (eQTLs), and a published lymphoblastoid cell line eQTL data set[93].
3. Functional annotations: SIFT[94], Polyphen[95], miRNA target site polymorphisms[96,97].
4. Conservational or evolutionary predictions: GERP[98], PHAST++[99], CpG islands[100].
5. Literature search: GAD[101], NHGRI GWAS catalog[102], dbGAP[103], or published Immunochip studies[104] (http://www.immunobase.org) for literature support.
6. Gene expression and enrichment analysis: ImmGen[102] (murine) and whole-transcriptome analysis across 126 tissues[104] (human).
7. Protein-protein interaction (PPI) database: DAPPLE[86], STRING[105].
8. Pathway-based and gene set enrichment analysis: Gene Ontogeny[106], Webgestalt[107], Wikipathways[108], IPA[109], DAVID[110], GSEA[111], and Pathways Commons[112].
9. Gene network analysis and visualization: DAPPLE[86] and VEP[85] to prioritize candidate causal genes and Grail[113] for text-mining of PubMed database for coassociations.

© 2015 Nature America, Inc. All rights reserved.

Functional and biological annotations (categories 1–5) for the 27 lead SNPs are illustrated in **Figure 3a**; annotations are also provided for the 46 GWM loci in **Supplementary Figure 5**. The following annotation types were used:

1. Regulatory: EnCODE consensus TF-binding sites (T), DNase I hypersensitivity sites (S), or published eQTL signals (E)
2. Functional: known mutations in PolyPhen or SIFT (A), experimentally validated (miRBASE 18.0) and predicted (mirSNP) miRNA target sites (R), or SNPs that tag regions containing common copy-number variation regions reported by the database of genomic variants (DGV) (V)
3. Conserved: conserved nucleotide sequences based on GERP++/phastCon (C) or known CpG islands that correlate with epigenetic methylation patterns (M)
4. Literature-supported: published association with immune or inflammatory diseases or immune-related endophenotypes from candidate studies or GWASs catalogued in the Genetic Association Database, NHGRI GWAS catalog, dbGAP, or Immunochip studies (L)

In addition to determining whether the 27 GWS pAID-associated SNPs were enriched for a given annotation type, we performed Monte Carlo simulations to resample 10,000 times the SNPs (MAF > 0.01 in Europeans) from all SNPs in 1KGP-RP. As for the 27 lead SNPs, for each set of 100 randomly sampled SNPs, we expanded the list by first identifying all nearby SNPs in strong LD (i.e., LD proxies with $r^2 > 0.8$ within 500 kB up- or downstream) within the 1KGP-RP data set filtered for only SNPs with MAF > 0.01 in the European population. We then annotated each original and any proxy SNPs as above for each major annotation category. We collapsed the information for all proxies identified for a given lead such that for any given category, if the lead SNP or any of its proxies were annotated, the lead SNP was marked as annotated. We then calculated the frequency of annotation for the 100 SNPs in each set. After sampling and annotating 100-SNP sets 10,000 times, we use the permutation-derived distribution of annotation percentages for each annotation type to calculate an enrichment *P* value such that

$$P_{\text{enrich}} = 1 - \frac{N(f_{\text{pAID}} > F_{\text{conclusive}})}{10{,}000}$$

where *N* is the number of permutations, *f* is the percentage of SNPs in the pAID set that are annotated and *F* is the distribution of the percentage of SNPs annotated across 10,000 sets of 100 SNPs resampled from the 1KGP-RP using only markers with MAF > 0.01 in Europeans.

Hierarchical clustering based on effect size and direction of association. We performed agglomerative hierarchical clustering across the top 27 independent loci using the directional *Z*-score obtained from logistic regression analysis in each of the ten disease-specific GWASs, defined as

$$Z = \frac{\text{beta}}{\text{s.e.}}$$

where beta is the effect size. The standardized and normalized *Z*-scores were used as inputs to the agglomerative hierarchical clustering. We used Ward's minimal-variance method to identify relatively consistent gene and locus cluster sizes.

Gene-based association testing. Given our interest in genetic overlap across pAIDs, we sought to identify genes associated with pAIDs in a disease-agnostic manner that was insensitive to locus and phenotypic heterogeneity. We used VEGAS[114], a set-based method, to perform GBAT.

As input, we used the nominal P_{META} values from the pooled, inverse χ^2 meta-analysis for the ten pAIDs across the genome as the input summary statistics for VEGAS, without considering which specific diseases were identified in the model search analysis. We assigned SNPs to gene regions and performed 10^7 simulations to estimate the gene-based *P* value as described in VEGAS's documentation. We used two thresholds: $P_{\text{sim}} < 2.8 \times 10^{-6}$ to identify significant candidate genes, on the basis of a Bonferroni adjustment for approximately 17,500 genes tested, and a false discovery rate (FDR) of <2%, which corresponds to a *q* value of <0.0205, which was used only for pathway and gene set enrichment analysis.

Tissue-specific gene set enrichment analysis. With few exceptions, most genes that are known to have a causative role in autoimmune disease have been shown to regulate molecular or subcellular processes in immune or immune-related tissues. If candidate pAID-associated genes are relevant to autoimmune-disease biology, then expression of these genes would be expected to be, on average, higher across immune or immune-related tissues (as compared with expression in non-immune-related tissues). Thus, we compared the expression of candidate pAID-associated genes identified by GBAT with that of non-candidate genes in a variety of tissues.

We curated the expression of the transcriptome in a broad spectrum of human tissues using a publicly available data set consisting of summary-level, normalized gene expression levels for more than 12,000 unique genes across 126 tissues and/or cell types, including a large number of immune tissues and cells[104]. We downloaded the processed data set "mean expression data matrix."

Across the 126 unique tissues, we tested whether the median or cumulative distribution of expression of pAID-associated gene transcripts as identified by GBAT was higher than that of the remaining transcripts in the data set using a one-sided Wilcoxon rank test or a one-sided Kolmogorov-Smirnov (KS) test, respectively. We calculated a tissue-specific gene expression ES value, which is the $-\log_{10}$ (*P* value) obtained from comparing the relative enrichment in transcript expression of pAID-associated genes versus the transcripts of the remaining genes in the data set. The tests were done on a per-tissue basis to derive a set of KS and a set of Wilcoxon ES values. We performed this per tissue analysis (1) for the total set of pAID-associated genes from GBAT and (2) when genes across the extended MHC (chr6: 25–34 Mb) were excluded.

We performed the secondary immune–versus–non-immune comparative analysis by plotting the ES values obtained from either Wilcoxon or KS tests in descending rank order of the respective test statistics, as shown in **Figure 4a** and **Supplementary Figure 6a** for all 126 tissue types. In those figures each point represents a single tissue and is colored according to its classification as either immune (red) or non-immune (blue), as described previously[86]. To formally test whether the overall ES values were higher among immune tissues than among non-immune tissues, we performed both the Wilcoxon rank sum test and the KS test on the vector of per-tissue ES values, comparing those derived from immune and non-immune tissues. We found that the enrichment observed across immune tissues was specific and not general to any GWAS-identified signals. We repeated this analysis in two sets of candidate genes, one for CD and another for schizophrenia, by identifying all associated genes for the two phenotypes from the NHGRI GWAS Catalog.

Immune cell gene set enrichment analysis. Cells of the immune system are extremely diverse in function and gene expression. To more precisely assess the expression of pAID-associated genes, we examined the mRNA expression of pAID candidate genes across specific immune cell subtypes, as well as during different developmental time points.

ImmGen provides a publicly available, high-quality murine gene expression data set. The ImmGen data set consists of 226 murine immune cell types across different lineages at multiple developmental stages, sorted by FACS and assayed at least in triplicate. Standard QC and quantile-normalization methods were applied to the data set as described by ImmGen[102]. The total set of transcripts mapped to 14,624 homologs in the human transcriptome on the basis of genes annotated in the hg18/build36 of the human reference genome, which were used to query the gene expression data.

Some of the cell types were derived from genetically altered animals, and the results from analysis of those cell types would have been difficult to interpret, so we removed those cell lines from the analysis. The complete list of cell types used in the analysis and the category to which we assigned each cell type for the categorical analysis are presented in **Supplementary Table 3c**. A total of 176 unique cell lines remained for subsequent analyses using this data set.

As with the human data set, we calculated the ES values by comparing the expression of the pAID-associated candidate gene transcripts to that of the remaining transcripts assayed in the data set for each immune cell type examined. We plotted the distribution of relative gene expression ES values as a density plot across the range of ES values from all of the examined cell types available. We compared the results obtained using the full set of candidate pAID genes identified by GBAT or obtained when we excluded the genes within the extended MHC. To ensure that this was not simply a result of selection bias

© 2015 Nature America, Inc. All rights reserved.

(as GWASs may be biased toward regions or genes across the genome that are better sampled or more densely genotyped), we compared the results to those obtained with the curated gene lists from the GWAS catalog (as above) for CD, schizophrenia, body mass index and LDL cholesterol.

To determine whether pAID-associated candidate genes are expressed at higher levels (relative to the rest of the genes in the transcriptome) in some immune cell types than in others, we defined immune cell types according to surface marker expression and tissue isolation details provided by ImmGen. Some categories were further divided into subcategories (for example, B and T cells) on the basis of developmental stage or lineage into a total of 16 non-overlapping cell-type categories. To compare the results across the cell-type categories, we plotted the distribution of ES value ranks for each cell type, binning the results according to the category each cell type belonged to (again, we performed the analysis either with or without the extended MHC region).

Expression profiling of pleiotropic autoimmune disease–associated genes across specific immune cell types. We profiled the expression of genes that had been identified in at least three autoimmune diseases in our subtype model search, previously published Immunochip fine-mapping studies, or a combination thereof (for example, identified as associated with JIA and UC in our analysis but previously identified as a candidate gene from an Immunochip analysis of alopecia areata). We identified 217 candidate pleiotropic genes, of which 191 could be mapped to unique gene transcripts within the ImmGen data sets.

We performed agglomerative hierarchical clustering with the matrix of gene expression levels from the 191 candidate gene transcripts using Ward's minimal-variance method across all 176 immune cell types. The genes and cell types shown in dendrograms are based on the results of unsupervised hierarchical clustering analysis and represent four major groups of cells and six major groups of genes.

We examined whether genes that were clustered on the basis of similar immune cell–expression profiles were likely to be associated with the same disease(s). Specifically, given a set of genes associated with one or more autoimmune diseases grouped in cluster i (C_i), we asked whether there is an increased likelihood (i.e., more so than expected by chance as compared with genes not found within this cluster) that these genes are also associated with disease j (D_j), such that

	C_i (yes)	C_i (no)
D_j (yes)	a	b
D_j (no)	c	d

where the expected probability of the values observed under the null is given by the hypergeometric distribution. As some of the cell counts were small and we were interested only in identifying instances where $a >> b$, c or d, we used a one-sided Fisher's exact test. We first tested each of the 18 autoimmune diseases across all identified clusters, declaring nominal and Bonferroni-adjusted significance at $P < 0.05$ and $P < 5.6 \times 10^{-4}$, respectively. For any clusters where at least two diseases reached nominal or marginal significance, we also tested whether there was an overrepresentation of genes associated with both diseases at $P < 0.05$.

PPI and network analysis. DAPPLE[86]: PPIs among the set of either 27 GWS or 46 GWM candidate regions were identified; the input seeds were defined as the 100-kB sequences up- and downstream of the most significantly associated SNP (based on hg19) in each candidate region. Other input parameters included 50-kB regulatory region length, a common interactor binding degree cutoff of 2, and the following specified known genes: *IL23R*, *PTPN22*, *INS*, *NOD2*, *DAG1*, *SMAD3*, *ATG16L1*, *ZNF365*, *PTGER4*, *NKX2-3*, *ANKRD55* and *IL12B*. We performed 10,000 permutations to accurately calculate enrichment network statistics. Seed scores P_{dapple} were used to color the protein nodes in the network plot.

STRING[105]: We used the *Homo sapiens* PPI database to query one of three lists: (1) the GWS loci, (2) GWS and GWM loci or (3) the list of genes identified by GBAT shown to be enriched for key proteins in the JAK-STAT pathway. We assessed and reported the evidence of PPI enrichment on the basis of these queries as compared to the results expected for the rest of the genes in the human genome. We generated network plots for the directly connected protein candidates (**Supplementary Fig. 7a–c** represents the "evidence" plot option).

Pathway and gene set enrichment analysis. Webgestalt[107]: For pathway and gene set analysis, we used the web-based tool Webgestalt to examine evidence of shared TF binding, miRNA target–binding sites, and enrichment in specific Gene Ontology and Pathway Commons categories. The inputs for this analysis included all lead genes (FDR < 2%) from the GBAT (similar to that for the other pathway annotation databases below for consistency).

DAVID[110]: We used the bioinformatics web tool DAVID (v6.7, available at http://david.abcc.ncifcrf.gov) for functional-annotation analysis of the significant genes. Significant genes with FDR < 2% in VEGAS, the gene-based association analysis, were used as input for DAVID. DAVID performed overrepresentation analysis of functional-annotation terms on the basis of hypergeometric testing and adjusted for multiple testing. To compare the results of this analysis with results obtained via other methods, we used BioCarta, KEGG pathways and GO_BP_FAT as gene set definition files.

IPA[109]: We used IPA software (http://www.ingenuity.com/) for canonical pathway and network analysis. We inputted all the significant genes in the VEGAS output (FDR < 2%) for IPA analysis. In the IPA core analysis, we selected the Ingenuity Knowledge Base (Genes Only) as the reference set, including both direct and indirect relationships. We used the filter setting of relationships in human and experimentally observed only. Information regarding canonical pathways was obtained from IPA output.

GSEA[115,116]: We conducted gene set enrichment analysis with the software GSEA (http://www.broadinstitute.org/gsea) using as input the pre-ranked gene list generated on the basis of the –log(P value) from VEGAS using all genes. We selected the following settings for our analysis: number of permutations, 5,000; enrichment statistic, weighted; maximum size of gene set, 500; minimum size of gene set, 15; and with normalization.

Interdisease genetic sharing analysis. To examine the degree of overlap in genetic risk susceptibility between any two autoimmune diseases, we developed and/or implemented the following statistical measures to quantify interdisease genetic sharing:

1. LPS test, optimized to evaluate whether two pAIDs share more loci in common than would be expected to occur by chance; the score 'penalizes' disease pairs if many of the loci are disease specific. The test is helpful if only data on whether diseases share specific candidate genes or association loci in common are known.
2. GPS test, optimized to assess the correlation between the set of association test statistics observed genome-wide across any two pAIDs. This test is valuable because it is independent of the gene sets chosen and thus does not require the use of any arbitrary method to define a significance 'threshold' of input data.

LPS analysis. To quantify the similarity between any two diseases D_1 and D_2 on the basis of the degree to which D_1 and D_2 share independent genetic risk associations (i.e., loci, SNPs or candidate genes), we considered the following model.

We began with a list of candidate genes, association loci or LD-independent SNPs n_r, identified as having reached a predefined GWAS significance threshold (e.g., GWS or GWM) across one or more SNPs from n_r for a set of diseases with expected or hypothesized sharing (i.e., all autoimmune diseases in this study and those reported on by the Immunochip studies catalogued by ImmunoBase[83]).

For any two diseases D_1 and D_2, a given candidate gene or SNP x_i could be uniquely classified in one of four ways: associated with D_1 and D_2 (n_{11}), associated only with D_1 (n_{12}) or D_2 (n_{21}), or associated with neither D_1 nor D_2 (n_{22}). For any given list of TOP associations (i.e., n_r), the distribution across the four possible categories can be tabulated as follows:

Locus x_i	D_2 (yes)	D_2 (no)
D_1 (yes)	n_{11}	n_{12}
D_1 (no)	n_{21}	n_{22}

where $n_{11} + n_{12} + n_{21} + n_{22} = n_r$ and D_1 (yes) or (no) means the SNP x_i is or is not associated with that marker, respectively.

© 2015 Nature America, Inc. All rights reserved.

The probability P_x that an SNP x_i from the list n_r is associated with either D_1 or D_2 can be expressed as

$$P_1 = \frac{n_{11} + n_{12}}{n_r} \quad (\text{for } D_1)$$

$$P_2 = \frac{n_{12} + n_{21}}{n_r} \quad (\text{for } D_2)$$

for any two pAIDs D_1 and D_2.

Thus, the frequency at which x_i should truly be associated with two distinct disease subtypes is given by $n_r(P_1P_2)$, and the observed number of overlapping associations is represented by n_{11}. Therefore, under the null hypothesis H_0, for a given pair of diseases D_1 and D_2, the variance of the difference between the numbers of expected and observed associations of all those tested (n_T) shared by both D_1 and D_2 should follow a normal distribution.

$$Z = \frac{n_{11} - n_r(P_1P_2)}{\sqrt{n_r(P_1P_2)(1-P_1P_2)}} \sim N(0,1)$$

We used the one-sided Z-test to examine whether the degree of overlap was significantly greater than expected, assuming a normal distribution under the null hypothesis that D_1 and D_2 do not share more associations than they would by chance. We used a Bonferroni adjustment to correct for 45 pairwise disease-combination tests.

GPS analysis. The GPS test determines whether two pAIDs are genetically related. For the ith SNP, let $X_i = 1$ if the SNP is truly associated with one disease, and let $X_i = 0$ otherwise. Similarly, define Y_i as the indicator of whether the SNP is associated with the other disease in the pair. We can therefore consider the diseases to be genetically related if there are more SNPs with $(X_i,Y_i) = (1,1)$ than would be expected to occur by chance. This amounts to testing the independence of X_i and Y_i.

However, we do not directly observe X_i and Y_i and instead observe P values U_i and V_i, which come from the two GWAS studies for the two diseases. When $X_i = 1$, the P value U_i will tend to be small, and otherwise U_i will be uniformly distributed; the same is true of Y_i and V_i. If U_i and V_i are independent, then X_i and Y_i must be as well. We can therefore test for genetic relatedness by testing whether the P values are dependent.

Most existing methods may not take advantage of the availability of the full genome data set for testing genetic sharing using U_i and V_i. To address this limitation, we developed a novel, threshold-free method to detect genetic relatedness. Our test statistic is defined by

$$D = \sup_{u,v} \sqrt{\frac{n}{\ln n}} \frac{\left|F_{uv}(u,v) - F_u(u)F_v(v)\right|}{\sqrt{F_u(u)F_v(v) - F_u(u)^2F_v(v)^2}}$$

where n is the total number of SNPs, $F_{uv}(u,v)$ is the empirical bivariate distribution function of (U_i,V_i), and $F_u(u)$ and $F_v(v)$ are the empirical univariate distribution functions of U_i and V_i, respectively. Intuitively, the numerator of D is motivated by the fact that if U_i and V_i are truly independent, their bivariate distribution is equal to the product of their univariate distributions. The denominator of D makes the test capable of detecting even very weak correlations. Under the null hypothesis of no genetic sharing, it can be shown that D is approximately distributed like the inverse square root of a standard exponential random variable. This gives us an analytic expression for calculating P values. Note that no significance threshold is required.

The asymptotic null distribution of D is derived under the assumption that the genetic markers examined across the genome are statistically independent. We therefore pruned the SNPs for each pair of diseases before applying our test. We conducted inverse χ^2 meta-analyses separately for each pair of diseases and pruned the resulting P values using a threshold of $r^2 < 0.5$ within a 500-kB up- and downstream region. This left about 800,000 SNPs for each disease pair analyzed. The use of more stringent r^2 thresholds (for example, $r^2 < 0.3$ or 0.2) gave comparable results.

Undirected weighted cyclic network visualization of results from the locus-specific sharing test. In graphic representations, pairwise relationships between autoimmune diseases (nodes) are represented by edges, whose weights are determined by the magnitude of the LPS test statistic (R statistical software package q-graph). Specifically, the width and density of the edges are the standardized transformations of the test statistic, and the colors denote whether the direction of the test statistic is positive (blue, meaning more sharing than expected) or negative (red, meaning less sharing than expected). Although graphs are constructed from all 45 pairwise interactions, for simplicity and improved visualization, we showed only those edges that represented a pairwise interaction that reached a Bonferroni-adjusted or nominal (**Supplementary Fig. 4c**) significance threshold ($P < 0.05$). The nodes are positioned on the basis of a force-directed layout based on the Fruchterman-Reingold algorithm.

In silico replication of novel pAID-association loci using previously published autoimmune disease cohort data sets. Replication set I: The following data sets were used in the first replication set: CASP[117], CIDR Celiac Disease[118], NIDDK Crohn's Disease[119], Wellcome Trust Case Control Consortium (WT) Crohn's Disease and Type 1 Diabetes[120], WT Ulcerative Colitis[121] and WT Ankylosing Spondylitis[122]. These data sets were obtained via dbGaP or the Wellcome Trust Case Control Consortium. In order to maximize the power, we sought replication for each of the 12 significant SNPs in all of the seven available data sets. Full results are summarized in **Supplementary Table 2e**.

Each data set was subjected to strict QC filtering as follows: we removed individuals that were inferred to be related on the basis of genetic data, individuals with >10% missing data, individuals with a reported sex that did not match the observed heterozygosity rates on chromosome X, and individuals not of European ancestry. We further removed variants with >10% missingness, variants not in HWE, variants with missingness significantly correlated to phenotype, and variants with MAF < 0.005. Variants to be replicated that were not observed in the original data set were imputed using IMPUTE2 (ref. 123) and the 1KGP-RP haplotype data[124]. Markers across the X chromosome, which were previously considered by most of these studies, were reanalyzed using the XWAS toolset[125,126].

Replication-association analysis was carried out by logistic regression implemented in PLINK[127]. The first ten principal components calculated using EIGENSOFT[128] were added as covariates for all data sets except CASP, where no population stratification was observed.

Replication set II: The second replication set consisted of the following data sets: Rheumatoid Arthritis meta-analysis[129], IBDG Ulcerative Colitis meta-analysis[130], IBDG Crohn's Disease meta-analysis[131], Systemic Lupus Erythematosus GWAS[132], and SLEGEN[133]. Individuals from these data sets were of European ancestry. Summary statistics from the original studies were publicly available and were used for the replication analysis. Details regarding QC procedures and association analysis can be obtained from the original studies[129–133].

LD-based replication for replication sets I and II: We further assessed replication in SNPs that were in LD with the significant SNPs in the discovery set. For each associated SNP, a list of SNPs in LD ($r^2 > 0.5$) within 500 kb of the original SNP was obtained from SNAP[87] using the 1KGP-RP.

61. Denny, J.C. *et al.* PheWAS: demonstrating the feasibility of a phenome-wide scan to discover gene-disease associations. *Bioinformatics* **26**, 1205–1210 (2010).
62. Ritchie, M.D. *et al.* Robust replication of genotype-phenotype associations across multiple diseases in an electronic medical record. *Am. J. Hum. Genet.* **86**, 560–572 (2010).
63. Liao, K.P. *et al.* Associations of autoantibodies, autoimmune risk alleles, and clinical diagnoses from the electronic medical records in rheumatoid arthritis cases and non-rheumatoid arthritis controls. *Arthritis Rheum.* **65**, 571–581 (2013).
64. Hakonarson, H. *et al.* A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene. *Nature* **448**, 591–594 (2007).
65. Imielinski, M. *et al.* Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat. Genet.* **41**, 1335–1340 (2009).
66. Kugathasan, S. *et al.* Loci on 20q13 and 21q22 are associated with pediatric-onset inflammatory bowel disease. *Nat. Genet.* **40**, 1211–1215 (2008).
67. Orange, J.S. *et al.* Genome-wide association identifies diverse causes of common variable immunodeficiency. *J. Allergy Clin. Immunol.* **127**, 1360–1367.e6 (2011).
68. Behrens, E.M. *et al.* Association of the TRAF1-C5 locus on chromosome 9 with juvenile idiopathic arthritis. *Arthritis Rheum.* **58**, 2206–2207 (2008).
69. Grant, S.F. *et al.* Association of the *BANK 1* R61H variant with systemic lupus erythematosus in Americans of European and African ancestry. *Appl. Clin. Genet.* **2**, 1–5 (2009).

© 2015 Nature America, Inc. All rights reserved.

70. Liao, K.P. *et al.* Electronic medical records for discovery research in rheumatoid arthritis. *Arthritis Care Res. (Hoboken)* **62**, 1120–1127 (2010).
71. Petty, R.E. *et al.* International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J. Rheumatol.* **31**, 390–392 (2004).
72. Behrens, E.M. *et al.* Evaluation of the presentation of systemic onset juvenile rheumatoid arthritis: data from the Pennsylvania Systemic Onset Juvenile Arthritis Registry (PASOJAR). *J. Rheumatol.* **35**, 343–348 (2008).
73. Conley, M.E., Notarangelo, L.D. & Etzioni, A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin. Immunol.* **93**, 190–197 (1999).
74. Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904–909 (2006).
75. Delaneau, O., Coulonges, C. & Zagury, J.-F. Shape-IT: new rapid and accurate algorithm for haplotype inference. *BMC Bioinformatics* **9**, 540 (2008).
76. Howie, B.N., Donnelly, P. & Marchini, J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet.* **5**, e1000529 (2009).
77. Howie, B., Marchini, J. & Stephens, M. Genotype imputation with thousands of genomes. *G3 (Bethesda)* **1**, 457–470 (2011).
78. Stucky, B.J. SeqTrace: a graphical tool for rapidly processing DNA sequencing chromatograms. *J. Biomol. Tech.* **23**, 90–93 (2012).
79. Price, A.L. *et al.* Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* **38**, 904 (2006).
80. Zaykin, D.V. & Kozbur, D.O. *P*-value based analysis for shared controls design in genome-wide association studies. *Genet. Epidemiol.* **34**, 725–738 (2010).
81. De Bakker, P.I. *et al.* Practical aspects of imputation-driven meta-analysis of genome-wide association studies. *Hum. Mol. Genet.* **17**, R122–R128 (2008).
82. Yang, J., Lee, S.H., Goddard, M.E. & Visscher, P.M. GCTA: a tool for genome-wide complex trait analysis. *Am. J. Hum. Genet.* **88**, 76–82 (2011).
83. Institute for Systems Biology and Juvenile Diabetes Research Foundation–Wellcome Trust Diabetes and Inflammation Laboratory. *ImmunoBase* http://www.immunobase.org (2013).
84. NHGRI. Published GWAS through 08/01/2014. *NHGRI GWA Catalog* http://www.genome.gov/multimedia/illustrations/GWAS_2011_3.pdf (2014).
85. McLaren, W. *et al.* Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics* **26**, 2069–2070 (2010).
86. Rossin, E.J. *et al.* Proteins encoded in genomic regions associated with immune-mediated disease physically interact and suggest underlying biology. *PLoS Genet.* **7**, e1001273 (2011).
87. Johnson, A.D. *et al.* SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics* **24**, 2938 (2008).
88. Chelala, C., Khan, A. & Lemoine, N.R. SNPnexus: a web database for functional annotation of newly discovered and public domain single nucleotide polymorphisms. *Bioinformatics* **25**, 655–661 (2009).
89. Cunningham, F. *et al.* Ensembl 2015. *Nucleic Acids Res.* **43**, D662–D669 (2015).
90. Kent, W.J. *et al.* The human genome browser at UCSC. *Genome Res.* **12**, 996 (2002).
91. Boyle, A.P. *et al.* Annotation of functional variation in personal genomes using RegulomeDB. *Genome Res.* **22**, 1790–1797 (2012).
92. National Institutes of Health Genotype-Tissue Expression (GTEx) http://commonfund.nih.gov/GTEx/index (2015).
93. Liang, L. *et al.* A cross-platform analysis of 14,177 expression quantitative trait loci derived from lymphoblastoid cell lines. *Genome Res.* **23**, 716–726 (2013).
94. Kumar, P., Henikoff, S. & Ng, P.C. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat. Protoc.* **4**, 1073–1081 (2009).
95. Adzhubei, I., Jordan, D.M. & Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr. Protoc. Hum. Genet.* Chapter 7, Unit 7.20 (2013).
96. Liu, C. *et al.* MirSNP, a database of polymorphisms altering miRNA target sites, identifies miRNA-related SNPs in GWAS SNPs and eQTLs. *BMC Genomics* **13**, 661 (2012).
97. Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A. & Enright, A.J. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res.* **34**, D140–D144 (2006).
98. Davydov, E.V. *et al.* Identifying a high fraction of the human genome to be under selective constraint using GERP. *PLoS Comput. Biol.* **6**, e1001025 (2010).
99. Nguyen, D.-Q. *et al.* Reduced purifying selection prevails over positive selection in human copy number variant evolution. *Genome Res.* **18**, 1711–1723 (2008).
100. Bird, A.P. CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213 (1986).
101. Becker, K.G., Barnes, K.C., Bright, T.J. & Wang, S.A. The genetic association database. *Nat. Genet.* **36**, 431–432 (2004).
102. Heng, T.S.P. & Painter, M.W. The Immunological Genome Project: networks of gene expression in immune cells. *Nat. Immunol.* **9**, 1091–1094 (2008).
103. Mailman, M.D. *et al.* The NCBI dbGaP database of genotypes and phenotypes. *Nat. Genet.* **39**, 1181–1186 (2007).
104. Benita, Y. *et al.* Gene enrichment profiles reveal T-cell development, differentiation, and lineage-specific transcription factors including ZBTB25 as a novel NF-AT repressor. *Blood* **115**, 5376–5384 (2010).
105. Franceschini, A. *et al.* STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* **41**, D808–D815 (2013).
106. Ashburner, M. *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25–29 (2000).
107. Wang, J., Duncan, D., Shi, Z. & Zhang, B. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res.* **41**, W77–W83 (2013).
108. Kelder, T. *et al.* WikiPathways: building research communities on biological pathways. *Nucleic Acids Res.* **40**, D1301–D1307 (2012).
109. Ingenuity Systems Ingenuity Pathway Analysis http://www.ingenuity.com/products/ipa (2015).
110. Huang, D.W. *et al.* The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol.* **8**, R183 (2007).
111. Wang, K., Li, M. & Bucan, M. Pathway-based approaches for analysis of genomewide association studies. *Am. J. Hum. Genet.* **81**, 1278–1283 (2007).
112. Cerami, E.G. *et al.* Pathway Commons, a web resource for biological pathway data. *Nucleic Acids Res.* **39**, D685–D690 (2011).
113. Raychaudhuri, S. *et al.* Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. *PLoS Genet.* **5**, e1000534 (2009).
114. Liu, J.Z. *et al.* A versatile gene-based test for genome-wide association studies. *Am. J. Hum. Genet.* **87**, 139–145 (2010).
115. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* **102**, 15545–15550 (2005).
116. Mootha, V.K. *et al.* PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat. Genet.* **34**, 267–273 (2003).
117. Nair, R.P. *et al.* Genome-wide scan reveals association of psoriasis with IL-23 and NF-κB pathways. *Nat. Genet.* **41**, 199–204 (2009).
118. Ahn, R. *et al.* Association analysis of the extended MHC region in celiac disease implicates multiple independent susceptibility loci. *PLoS ONE* **7**, e36926 (2012).
119. Duerr, R.H. *et al.* A genome-wide association study identifies *IL23R* as an inflammatory bowel disease gene. *Science* **314**, 1461–1463 (2006).
120. Wellcome Trust Case Control Consortium. *et al.* Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661–678 (2007).
121. Barrett, J.C. *et al.* Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. *Nat. Genet.* **41**, 1330–1334 (2009).
122. Evans, D.M. *et al.* Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility. *Nat. Genet.* **43**, 761–767 (2011).
123. Marchini, J. *et al.* A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat. Genet.* **39**, 906–913 (2007).
124. Abecasis, G.R. *et al.* An integrated map of genetic variation from 1,092 human genomes. *Nature* **491**, 56–65 (2012).
125. Gao, F. *et al.* XWAS: a software toolset for genetic data analysis and association studies of the X chromosome. *bioRxiv* doi:10.1101/009795.
126. Chang, D. *et al.* Accounting for eXentricities: analysis of the X chromosome in GWAS reveals X-linked genes implicated in autoimmune diseases. *PLoS One* **9**, e113684 (2014).
127. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
128. Patterson, N., Price, A.L. & Reich, D. Population structure and eigenanalysis. *PLoS Genet.* **2**, e190 (2006).
129. Stahl, E.A. *et al.* Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. *Nat. Genet.* **42**, 508–514 (2010).
130. Anderson, C.A. *et al.* Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nat. Genet.* **43**, 246–252 (2011).
131. Franke, A. *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42**, 1118–1125 (2010).
132. Hom, G. *et al.* Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N. Engl. J. Med.* **358**, 900 (2008).
133. Harley, J.B. *et al.* Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in *ITGAM*, *PXK*, *KIAA1542* and other loci. *Nat. Genet.* **40**, 204–210 (2008).

© 2015 Nature America, Inc. All rights reserved.

3.5 References

1. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schäffer AA, Noyan F, Perro M, Diestelhorst J, Allroth A, Murugan D, Hätscher N, Pfeifer D, Sykora KW, Sauer M, Kreipe H, Lacher M, Nustede R, Woellner C, Baumann U, Salzer U, Koletzko S, Shah N, Segal AW, Sauerbrey A, Buderus S, Snapper SB, Grimbacher B, Klein C. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009; 361: 2033-2045 [PMID: 19890111 DOI: 10.1056/NEJMoa0907206]
2. Kotenko SV, Krause CD, Izotova LS, Pollack BP, Wu W, Pestka S. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *EMBO J* 1997; 16: 5894-5903 [PMID: 9312047 DOI: 10.1093/emboj/ 16.19.5894]
3. Moran CJ, Walters TD, Guo CH, Kugathasan S, Klein C, Turner D, Wolters VM, Bandsma RH, Mouzaki M, Zachos M, Langer JC, Cutz E, Benseler SM, Roifman CM, Silverberg MS, Griffiths AM, Snapper SB, Muise AM. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* 2013; 19: 115-123 [PMID: 22550014 DOI:10.1002/ibd.22974]
4. Duraturo F, Liccardo R, Cavallo A, De Rosa M, Grosso M, Izzo P. Association of low-risk MSH3 and MSH2 variant alleles with Lynch syndrome: probability of synergistic effects. *Int J Cancer* 2011; 129: 1643-1650 [PMID: 21128252 DOI: 10.1002/ijc.25824]
5. Chang F, Lacey MR, Bouljihad M, Höner Zu Bentrup K, Fortgang IS. Tumor necrosis factor receptor 1 functions as a tumor suppressor. *Am J Physiol Gastrointest Liver Physiol* 2012; 302: G195-G206 [PMID: 22052015 DOI: 10.1152/ajpgi. 00209.2011]
6. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med* 2009; 361: 2066-2078 [PMID: 19923578 DOI: 10.1056/ NEJMra0804647]
7. De Rosa M, Galatola M, Borriello S, Duraturo F, Masone S, Izzo P. Implication of adenomatous polyposis coli and MUTYH mutations in familial colorectal polyposis. *Dis Colon Rectum* 2009; 52: 268-274 [PMID: 19279422 DOI: 10.1007/DCR.0b013e318197d15c]
8. De Rosa M, Morelli G, Cesaro E, Duraturo F, Turano M,Rossi GB, Delrio P, Izzo P. Alternative splicing and nonsensemediatedmRNA decay in the regulation of a new adenomatouspolyposis coli transcript. *Gene* 2007; 395: 8-14 [PMID:17360132 DOI: 10.1016/j.gene.2006.10.027]
9. Lee G, Goretsky T, Managlia E, Dirisina R, Singh AP, Brown JB, May R, Yang GY, Ragheb JW, Evers BM, Weber CR, Turner JR, He XC, Katzman RB, Li L, Barrett TA.

Phosphoinositide 3-kinase signaling mediates beta-catenin activation in intestinal epithelial stem and progenitor cells in colitis. *Gastroenterology* 2010; 139: 869-881, 881.e1-9 [PMID: 20580720 DOI:10.1053/j.gastro.2010.05.037]

10. Glocker EO, Kotlarz D, Klein C, Shah N, Grimbacher B. IL-10 and IL-10 receptor defects in humans. *Ann N Y Acad Sci* 2011; 1246: 102-107 [PMID: 22236434 DOI: 10.1111/j.1749-6632.2011.06339.x]
11. Naftali T, Mechulam R, Lev LB, Konikoff FM. Cannabis for inflammatory bowel disease. Dig Dis. 2014;32(4):468-74. doi: 10.1159/000358155.
12. Alhouayek M, Muccioli GG. The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. Trends Mol Med. 2012 Oct;18(10):615-25. doi: 10.1016/j.molmed.2012.07.009.
13. Maccarrone M, Bab I, Bíró T, Cabral GA, Dey SK, Di Marzo V, et al. Endocannabinoid signaling at the periphery: 50 years after THC. Trends Pharmacol Sci. 2015;36: 277–296. doi: 10.1016/j.tips.2015.02.008
14. Sipe JC, Arbour N, Gerber A, Beutler E. Reduced endocannabinoid immunemodulation by a common cannabinoid 2 (CB2) receptor gene polymorphism: possible risk for autoimmune disorders. J Leukoc Biol. 2005;78: 231–238.
15. Bellini G, Olivieri AN, Grandone A, Alessio M, Gicchino MF, Nobili B, et al. Association between cannabinoid receptor type 2 Q63R variant and oligo/polyarticular juvenile idiopathic arthritis. Scand J Rheumatol. 2015;14:1–4.
16. Rossi F, Mancusi S, Bellini G, Roberti D, Punzo F, Vetrella S, et al. CNR2 functional variant (Q63R) influences childhood immune thrombocytopenic purpura. Haematologica. 2011;96: 1883–1885. doi: 10.3324/haematol.2011.045732
17. Rossi F, Bellini G, Tolone C, Luongo L, Mancusi S, Papparella A, et al. The cannabinoid receptor type 2 Q63R variant increases the risk of celiac disease: implication for a novel molecular biomarker and future therapeutic intervention. Pharmacol Res. 2012;66: 88–94. doi: 10.1016/j.phrs.2012.03.011 Epub 2012 Mar 24. PubMed PMID: 22465144.).
18. Borrelli, F., & Izzo, A. A. (2009). Role of acylethanolamides in the gastrointestinal tract with special reference to food intake and energy balance. Best Pract Res Clin Endocrinol Metab 23, 33−49.)
19. Ligresti, A., Bisogno, T., Matias, I., De Petrocellis, L., Cascio, M. G., Cosenza, V., et al. (2003). Possibile endocannabinoid control of colorectal cancer growth. Gastroenterology 125,−687.

20. Ihenetu, K., Molleman, A., Parsons, M. E., & Whelan, C. J. (2003). Inhibition of interleukin-8 release in the human colonic epithelial cell line HT-29 by cannabinoids. Eur J Pharmacol 458, 207−215.
21. Lu T, Newton C, Perkins I, Friedman H, Klein TW Role of cannabinoid receptors in Delta-9-tetrahydrocannabinol suppression of IL-12p40 in mouse bone marrow-derived dendritic cells infected with Legionella pneumophila.Eur J Pharmacol. 2006 Feb 17;532(1-2):170-7).
22. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 in the gut of patients with Crohn's disease. Am J Clin Pathol. 1997; 150(3):823–832.
23. Niessner M, Volk BA. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). Clin Exp Immunol. 1995; 101:428–435. [PubMed: 7664489]
24. Karttunnen R, Breese EJ, Walker-Smith JA, et al. Decreased mucosal interleukin-4 (IL-4) production in gut inflammation. J Clin Pathol. 1994; 47:1015–1018. [PubMed: 7829675]
25. Chang, Y. H., Lee, S. T., & Lin, W. W. (2001). Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrophages: involvement of eicosanoids. J Cell Biochem 81, 715−723.

Chapter 4

4. Peculiar clinical features in inflammatory bowel disease

4.1 Natural history of pancreatic involvement in paediatric inflammatory bowel disease.

Inflammatory bowel disease (IBD), characterized by chronic, relapsing immune-mediated inflammation of the gastrointestinal tract is often associated with extra-intestinal manifestations (EIMs) affecting multiple organs. EIM are reported to occur in 18–47% of paediatric and adult patients with IBD. [1-5]Acute and chronic pancreatitis as well as pancreatic insufficiency have been reportedas one of EIMs in IBD.[6]There are only limited published dataon the incidence of acute pancreatitis in paediatric patients with IBD. [7,8]

Despite scattered case reports, the relationship between pancreatic involvement and IBD has not been further investigated. The primary aim of our study was to investigate prevalence and disease course of paediatric IBD patients presenting with pancreatitis; secondary aim was to evaluate the clinical significance of exclusive hyperamylasemia and hyperlipasemia in children with IBD.

To the best of our knowledge our paper represents the first paediatric multicentre IBD registry-based study, characterizing the natural history of pancreatic involvement. Consistent with the published literature in the present cohort, the prevalence of pancreatic involvement was 4.1% and acute pancreatitis was diagnosed in 1.6% of cases.

The question of whether pancreatitis is an EIM of IBD remains unclear.[9]The aetiology and pathogenesis of AP (acute pancreatitis) are elusive and seem to be multifactorial in themajority of IBD patients. It is possible that epithelial cells of the gastro-intestinal tract and pancreatic tissue, may share similar target molecular or cellular structures vulnerable to injury. To support this hypothesis in our cohort ofpatients acute pancreatitis was present at IBDonset in 18%, suggesting that in somecases pancreatic dysfunction is part of a common immune disorder.

As previously described in paediatric literature, in our study AP incidence was not different according to the IBD type, either CD or UC.[7] Moreover according to Heikius et al. who demonstrated a correlation between the lipase increase and the histological activity of the disease, 91% of our patients with pancreatic involvement presented active disease.[6] Furthermore, patients with recurrent episodes of AP and hyperamylasemia/hyperlipasemia showed higher PCDAI/PUCAI scores at 6 and 12 months. These data may suggest that pancreatic involvement could be strictly related to the activity of disease at least in a 320 subset of patients.

Interestingly, the majority of our patients with CD and AP showed a colonic involvement, as previously described.[7] Nevertheless, It is well known that paediatric patients with CD present with more colonic involvement.[10] The association between colonic disease and AP remains obscure. One possible explanation may be that the colon is considered a major source of the bacteria causing pancreatic necrosis in AP. Supporting this hypothesis subtotal colectomy before AP in rats was found to reduce mortality.

Finally female gender resulted to be significantly associated with the onset of pancreatitis in our patients, also when comparing with the total IBD registry population. Female predominance in the majority of autoimmune disorders may be one of the possible explanations.

Our study has some limitations besides the retrospective nature. Firstly, we did not have a control group of patients with AP without IBD; second, we did not evaluate serological markers of autoimmune pancreatitis. One could argue that the number of IBDpatients with pancreatic involvement is too small to draw definitive conclusions; furthermore, in this subset of patients it is difficult to clearly define the aetiology of AP, as IBD itself is a predisposing factor, and development of pancreatitis is certainly multifactorial.

Nevertheless our data, based on a large paediatric IBD population, highlight once again that pancreatic involvement has a relatively low prevalence. However, a specific attention has to be paid to the monitoring of pancreatic function IBD children, considering that in a proportion of patients the pancreatic involvement tends to persist and in some cases pancreatic damage may evolve. Future studies on the pathogenesis of pancreatitis and its relationship to the long-term outcome in IBD are required.

AUTHOR QUERY FORM

ELSEVIER	**Journal:** YDLD **Article Number:** 2820	**Please e-mail or fax your responses and any corrections to:** **E-mail:** corrections.esnl@elsevier.thomsondigital.com **Fax:** +353 6170 9272

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. Note: if you opt to annotate the file with software other than Adobe Reader then please also highlight the appropriate place in the PDF file. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please confirm that given names and surnames have been identified correctly.
Q2	"Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact f.kop@elsevier.com immediately prior to returning your corrections.".
Q3	Please note that Refs. [5,15] were identical, and Ref. [15] has been deleted. The subsequent references have been renumbered.
	Please check this box or indicate your approval if you have no corrections to make to the PDF file ☐

Thank you for your assistance.

G Model
YDLD 2820 1–6
ARTICLE IN PRESS

Digestive and Liver Disease xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary Tract

Natural history of pancreatic involvement in paediatric inflammatory bowel disease

Massimo Martinelli[a], Caterina Strisciuglio[a], Maria Teresa Illiceto[b], Sabrina Cardile[c], Graziella Guariso[d], Silvia Vignola[e], Marina Aloi[f], Mario Rocco D'Altilia[g], Patrizia Alvisi[h], Silvia Salvatore[i], Annamaria Staiano[a], Salvatore Cucchiara[f], Erasmo Miele[a,*], on behalf of IMIBD Group of the Italian Society for Paediatric Gastroenterology, Hepatology and Nutrition

[a] Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Naples, Italy
[b] Pediatric Gastroenterology Unit, Spirito Santo Hospital, Pescara, Italy
[c] Endoscopy and Gastroenterology Unit, Department of Pediatrics, University of Messina, Italy
[d] Department of Pediatrics, Unit of Gastroenterology, University Hospital of Padova, Italy
[e] Gastroenterology and Endoscopy Unit, G. Gaslini Institute for Children, Genoa, Italy
[f] Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, Italy
[g] Division of Pediatrics, General Hospital I.R.C.C.S., S. G. Rotondo (Fg), Italy
[h] Pediatric Unit, Maggiore Hospital, Bologna, Italy
[i] Department of Pediatrics, University of Insubria, Varese, Italy

ARTICLE INFO

Article history:
Received 17 September 2014
Accepted 27 January 2015
Available online xxx

Keywords:
Amylase
Crohn's disease
Lipase
Pancreas
Pancreatitis
Ulcerative colitis

ABSTRACT

Background: Few case reports describe the clinical features of pancreatic involvement in inflammatory bowel disease.
Aim: To investigate prevalence and disease course of inflammatory bowel disease children with pancreatitis and with exclusive hyperamylasemia and hyperlipasemia.
Methods: We used a web-registry to retrospectively identify paediatric inflammatory bowel disease patients with hyperamylasemia and hyperlipasemia. Participants were re-evaluated at 6 months and 1 year.
Results: From a total of 649 paediatric patients, we found 27 with hyperamylasemia and hyperlipasemia (4.1%). Eleven patients (1.6%) fulfilled diagnostic criteria for acute pancreatitis. Female gender was significantly associated with acute pancreatitis ($p = 0.04$). Twenty-five children (92.5%) had colonic disease. At 6 months 1/11 children with acute pancreatitis (9%) showed acute recurrent pancreatitis, while 1 patient (9%) had persistent hyperamylasemia and hyperlipasemia. At 12 months, 1 patient showed chronic pancreatitis (9.1%). Of the 16 children with exclusive hyperamylasemia and hyperlipasemia, 4 developed acute pancreatitis (25%), while 1 patient (6.2%) still presented exclusive hyperamylasemia and hyperlipasemia at 6 months. At 12 months, 11/16 patients (68.7%) reached a remission of pancreatic involvement, whereas 5 remaining patients (32.3%) had persistent hyperamylasemia and hyperlipasemia.
Conclusions: In inflammatory bowel disease children, acute pancreatitis is more common in colonic disease and in female gender. Pancreatic function should be monitored, considering that pancreatic damage may evolve.

© 2015 Published by Elsevier Ltd on behalf of Editrice Gastroenterologica Italiana S.r.l.

1. Introduction

Inflammatory bowel disease (IBD), characterized by chronic, relapsing immune-mediated inflammation of the gastrointestinal tract is often associated with extra-intestinal manifestations (EIMs) affecting multiple organs. EIM are reported to occur in 18–47% of paediatric and adult patients with IBD [1–5]. Acute and chronic

* Corresponding author at: Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5, 80131 Naples, Italy. Tel.: +39 081 7464565; fax: +39 081 7464565.
E-mail address: erasmo.miele@unina.it (E. Miele).

http://dx.doi.org/10.1016/j.dld.2015.01.155
1590-8658/© 2015 Published by Elsevier Ltd on behalf of Editrice Gastroenterologica Italiana S.r.l.

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

UNCORRECTED PROOF

pancreatitis as well as pancreatic insufficiency have been reported as one of EIMs in IBD [6].

Acute pancreatitis (AP) in children is a costly and increasingly recognized disease. Several studies have documented an increase during the past 10–15 years [7]. Estimated incidences range from 3.6 to 13.2 cases per 100,000 children per year [8,9]. The reasons for the increase are not entirely clear and may be multifactorial. An Australian study suggests that the increasing number is mainly due to the complications of systemic illness [9]. Patients with IBD are at increased risk of developing both acute and chronic pancreatitis. Clinical symptoms of IBD-associated pancreatitis are found in about 2% of patients but the actual frequency of the disease could be much higher. According to several studies, hyperamylasemia and exocrine pancreatic insufficiency are found in 6–16 and 21–80% of adult patients, respectively, whereas histological changes are observed in 38–53% of postmortem pathological examinations [6,10–12]. There are only limited published data on the incidence of acute pancreatitis in paediatric patients with IBD [13,14]. Although pancreatitis can be seen in association to drugs assumption, biliary lithiasis, Crohn's disease (CD) duodenal involvement or sclerosing cholangitis, the contribution of these etiological factors to histopathology-proved pancreatitis appears to be low and IBD itself seems to contribute to the pathogenesis [5]. In addition, a previous study also indicates that the rarer variant, autoimmune pancreatitis occurs more often among IBD patients [15]. Regards to drugs, several case reports about drug-induced pancreatitis have been published [16]. Nevertheless, it is always difficult to establish a causal role for medications in the pathogenesis of pancreatitis, but a few medications are clearly associated with a high risk for drug-induced pancreatitis. This is true with regard to some medications used in IBD management. Of the medications, the possible agents inducing pancreatitis include sulfasalazine, 5-aminosalicylic acid (ASA) compounds, azathioprine (AZA), metronidazole and steroids [17,18]. Complicating the scenario it appears that IBD-associated pancreatic involvement may be often a silent disease in children. Various possible explanations for asymptomatic hyperamylasemia and hyperlipasemia in IBD patients have been proposed. The pancreatic enzyme elevation observed in more extensive or active disease can represent the abnormal passage of pancreatic amylase from the gut lumen to the blood due to increased permeability of the inflamed mucosa [19]. In addition, there are several potential mechanisms for the suggested enzyme leakage from the pancreas. First, the pancreas might be affected in some way directly by the extent of IBD. Another explanation could be an enzyme increase related to the pancreatic effects of inflammatory mediators and cytokines released from the inflamed gut. A third mechanism might be associated with inflammation of pancreatic ducts [6].

Despite scattered case reports, the relationship between pancreatic involvement and IBD has not been further investigated. The primary aim of the present study was to investigate prevalence and disease course of paediatric IBD patients presenting with pancreatitis; secondary aim was to evaluate the clinical significance of exclusive hyperamylasemia and hyperlipasemia in children with IBD.

2. Subjects and methods

We retrospectively reviewed data collected in the IBD web-registry of the Italian Society for Paediatric Gastroenterology, Hepatology and Nutrition (SIGENP). Paediatric gastroenterologists from all the Italian paediatric IBD centers belonging to the SIGENP, established in 2008 a prospective registry to collect demographic, clinical, and epidemiologic data from paediatric patients with IBD. The registry started the 1st January 2009 and included patients less than 18 years with a new diagnosis of IBD. Data of all paediatric patients enrolled and stored in the registry from January 1, 2009 to November 30, 2012 (data retrieval date) were used for this study. Nine sites participated to this study; trained investigators at each centre obtained information from the medical records (electronic and paper charts) and standardized information was entered into the registry. Eligible subjects included all patients with any form of IBD [ulcerative colitis (UC), CD and inflammatory bowel disease unclassified (IBD-U)]. Diagnosis of IBD was based on clinical history, physical examination, endoscopic appearance, histologic findings, and radiologic studies, according to Porto criteria [20]. All patients presenting with serum amylase ≥100 IU/L (normal range: 28–100 IU/L) and serum lipase ≥60 IU/L (normal range: 13–60 IU/L) were included in the study. Participants were additionally evaluated within 6 months, and 1 year from enrolment. AP was defined as the presence of 2 of the following criteria: (a) abdominal pain compatible with AP, (b) serum amylase and/or lipase values ≥3 times upper limits of normal, (c) imaging findings of AP [21]. Acute recurrent pancreatitis (ARP) was defined as: ≥2 distinct episodes of AP with intervening return to baseline. The severity of AP episodes was assessed with the Paediatric Acute Pancreatitis Score (PAPS), developed by DeBanto and colleagues [22]. The system has eight parameters, scored at admission and at 48 h. The admission criteria include: age <7 years, weight <23 kg, white blood cell count ≥18,500/mm^3, and LDH > 2000 U/L. The 48-h criteria are trough calcium < 8.3 mg/dl, trough albumin < 2.6 mg/dl, fluid sequestration > 75 ml/kg/48 h, and a rise in BUN > 5 mg/dl. One point is assigned for each criterion met; a score of ≥3 is predictable of a severe course of disease [22]. Chronic pancreatitis (CP) was diagnosed if one of the following criteria was present: (a) typical abdominal pain plus characteristic imaging findings; (b) exocrine insufficiency plus imaging findings; (c) endocrine insufficiency plus imaging findings. Exclusive hyperamylasemia and hyperlipasemia was used to describe those patients who did not meet diagnostic criteria for pancreatitis [20].

The information retrieved for the purpose of this study included demographic features (age, gender), IBD type (CD, UC, IBD-U), median lag time period between the diagnosis of IBD and pancreatic involvement episodes, and disease location. The disease location at the diagnosis and at follow-up was established by endoscopic and imaging evaluations in all patients according to the availability of individual methods for each centre and reported in the registry. For the purpose of this manuscript, disease location was described according to Paris classification [23]. Disease activity at the diagnosis was scored by the Paediatric Crohn's Disease Activity Index (PCDAI) [24] or the Paediatric Ulcerative Colitis Activity Index (PUCAI) [25] for CD and UC, respectively. Laboratory tests included full blood count, C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR), nutritional, renal, and liver function parameters. In addition, pancreatic laboratory studies including serum amylase and lipase, were collected. Data on imaging methods used for the diagnosis of pancreatic involvement including transabdominal ultrasound (US), magnetic resonance cholangiopancreatography (MRCP), abdominal computed tomography scan (CT) or endoscopic retrograde cholangiopancreatography (ERCP), were evaluated. In patients with CP if available, details on genetic testing (CFTR, SPINK1, PRSS1) or on exocrine pancreatic function assessed with the faecal elastase, were recorded. In addition, pancreatic involvement episode characteristics, including drug exposure, severity, complications, in-hospital stay, actions taken post-pancreatic involvement were reported.

Institutional review board approval for the registry protocol and the informed consent and assent forms were obtained at each site before subject enrolment and data collection. Signed parental and patient informed consent and signed youth assent when appropriate were required from all patients enrolled.

UNCORRECTED PROOF

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

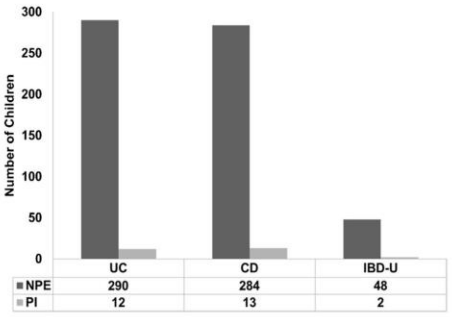


Fig. 1. Prevalence of pancreatic involvement in different inflammatory bowel disease type. CD: Crohn's disease; UC: ulcerative colitis; IBD-U: unclassified-IBD; NPE: normal pancreatic enzymes; PI: pancreatic involvement.

2.1. Statistical analysis

Statistical analysis was performed using SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). Means and medians were calculated for dimensional variables after controlling for normality of distribution. Categorical data were expressed as frequencies and percentages. The Student's *t*-test and the Mann–Whitney test for continuous variables and the χ^2 and Fisher's exact tests for categorical variables were used where appropriate. A *p* value of 0.05 or less was considered significant.

3. Results

3.1. Patients characteristics

From 2009 to 2012 we identified 649 paediatric patients with a diagnosis of IBD, of whom 27 met the inclusion criteria and were enrolled in the study (4.1%, Fig. 1). Demographic and clinical characteristics for the study group are reported in Table 1. Median lag time between the diagnosis of IBD and pancreatic involvement was 7 months (range 0–65 months). In 5/27 children (18.5%), pancreatic involvement was present at time of IBD diagnosis (Table 2). Median serum amylase level was 196 (range 61–413 IU/L), and median serum lipase level was 299 (range 37–2565 IU/L). Eleven patients (40.7%) fulfilled diagnostic criteria for AP (6 CD, 4 UC, 1 IBD-U). On the basis of PAPS all 11 patients presented with an episode of mild AP (Table 3). The remaining 16 patients (60.3%) presented with exclusive hyperamylasemia and hyperlipasemia. Median serum amylase level and median serum lipase level were significantly higher in AP patients compared with patients with exclusive hyperamylasemia and/or hyperlipasemia ($p = 0.009$ and $p = 0.001$ respectively; Table 2). Comparing the total IBD registry population with patients with pancreatic involvement, female gender resulted to be significantly associated with AP ($p = 0.04$; OR: 4.8; 95% confidence interval 1–22), whereas no significant difference in gender was observed in patients with exclusive hyperamylasemia and/or hyperlipasemia ($p = 0.7$). IBD type, ongoing treatments, and extension of disease were not significant risk factors when comparing patients with hyperamylasemia/hyperlipasemia and children with AP (Table 2). Twenty-three patients (85.1%) with pancreatic involvement presented with active disease, but no significant difference was found among children with exclusive hyperamylasemia and/or hyperlipasemia and subjects with AP ($p = 0.6$; Table 2). Twenty-five patients (92.5%) with pancreatic involvement had colonic disease [11 CD, 12 UC and 2 IBDU]. Eight of the 12 children with UC and pancreatic involvement (66.6%) were affected by pancolitis (Table 2). Regarding symptoms associated with pancreatic involvement, 21 patients (77.8%) reported epigastric pain as the dominant clinical characteristic, followed by nausea and/or vomiting (22.2%), and fever (22.2%). No patient experienced jaundice. No statistical significant difference was observed when comparing symptoms in patients with exclusive increase of pancreatic enzymes and AP (Table 2).

Table 1
Baseline characteristics of 27 inflammatory bowel disease children with pancreatic involvement.

Characteristics	
Patients (*N*)	27
Median age (years; range)	12.3 (5.4–15.9)
PI onset (years; range)	12.2 (0–65)
Gender (*N*, %)	
Males	13/27 (48.1)
IBD type (*N*, %)	
CD	13/27 (48.2)
UC	12/27 (44.4)
IBD-U	2/27 (7.4)
Paris classification	
CD (*N*, %)	
Ileum only (L1)	2/13 (15.3)
Colon only (L2)	0/13 (0)
Ileum and colon (L3)	11/13 (84.6)
Upper gastrointestinal tract (L4a)	5/13 (38.4)
UC (*N*, %)	
Proctosigmoiditis (E1)	2/12 (16.6)
Left-sided colitis (E2)	2/12 (16.6)
Extensive colitis (E3)	2/12 (16.6)
Pancolitis (E4)	6/12 (50)
IBD therapy (*N*, %)	
5-ASA	16/27 (59.3)
CCS	3/27 (11.1)
AZT	12/27 (44.4)
MTX	1/27 (3.7)
Biologic therapy	2/27 (7.4)

PI: pancreatic involvement; CD: Crohn's disease; UC: ulcerative colitis; IBD-U: unclassified-IBD; 5-ASA: 5-aminosalicylic acid; CCS: corticosteroids; AZT: azathioprine; MTX: methotrexate.

3.2. Imaging

All 11 patients with AP underwent abdominal US imaging, 6 also underwent MRCP (54.5%) and 2 a CT scan (18.1%). Pancreatic pathological findings were found in all subjects with AP. Main pancreatic findings were: enlargement of the head and the body ($n = 2$); tail and head enlargement ($n = 2$); tail enlargement ($n = 3$); diffuse oedema ($n = 3$) and peripancreatic/pancreatic fluid collections ($n = 1$). Primary sclerosing cholangitis (PSC) was diagnosed in 1 (9%) out of 11 patients. None of the patients with exclusive hyperamylasemia and/or hyperlipasemia showed pathological findings at imaging studies.

3.3. Treatment

Nine patients (33.3%) were receiving mesalamine (5-ASA), 14 (51.8%) were receiving immunomodulatory therapy [1 steroids, 6 AZA, 5 AZA + 5-ASA, 1 methotrexate, 1 infliximab (IFX), and 1 IFX + ASA + AZA], and 4 patients (14.8%) were not treated. Nine of 11 (81.8%) patients with AP needed therapeutic measures compared to only 5/16 patients with serum hyperamylasemia and/or hyperlipasemia (31.2%; $p = 0.04$). Therapeutic measures in children with AP and hyperamylasemia/hyperlipasemia are reported in Table 4. None of the patients with AP developed early complications.

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

Table 2
Clinical differences between patients with exclusive hyperamylasemia/hyperlipasemia and acute pancreatitis.

Characteristics	Exclusive HA/HL $n=16$	Pancreatitis $n=11$	p
Median age (years; range)	14 (6–17)	14.4 (10–16.7)	0.07
Gender			0.01
Males	11 (68.8)	2 (18.2)	
Females	5 (31.2)	9 (81.8)	
PI at IBD disease onset	2 (18.2)	3 (18.8)	1
Symptoms			
Epigastric pain	11 (68.8)	10 (90.9)	0.3
Fever	2 (12.5)	4 (36.4)	0.2
Nausea/vomiting	3 (18.3)	3 (27.3)	0.6
Amylase (median, range)	160 (61–320)	248 (150–413)	0.009
Lipase (median, range)	140.5 (37–508)	817 (512–2565)	0.001
IBD type			0.7
CD	7 (43.7)	6 (54.5)	
UC	8 (50)	4 (36.3)	
IBD-U	1 (6.2)	1 (9)	
Median disease duration (months, range)	9 (0–33)	7 (0–65)	0.4
Active disease	13 (81.2)	10 (90.9)	0.6
PUCAI (median, range)	20 (0–45)	22.5 (15–35)	1
PCDAI (median, range)	30 (18–54)	28.7 (10–70)	1
Colonic involvement	16 (100%)	9 (81.8)	0.1
Disease location			
Ileum only (L1)	0 (0)	1 (9)	0.4
Colon only (L2)	0 (0)	0 (0)	1
Ileum and colon (L3)	7 (43.7)	4 (36.3)	1
Upper GI tract (L4a)	1 (6.2)	3 (27.3)	0.1
Upper GI tract (L4b)	0 (0)	1 (9)	0.4
Proctosigmoiditis (E1)	1 (6.2)	1 (9)	1
Left-sided colitis (E2)	1 (6.2)	1 (9)	1
Extensive colitis (E3)	(6.2)	1 (9)	1
Pancolitis (E4)	5 (31.2)	1 (9)	0.3
IBD therapy			
5-ASA	10 (62.5)	6 (54.5)	0.7
CCS	3 (18.3)	1 (9)	0.6
AZT	5 (31.2)	6 (54.5)	0.3
MTX	0 (0)	1 (9)	0.4
BIO	2 (12.5)	0 (0)	0.5
Imaging findings			NA
Enlargement of the head and the body	–	2 (18.1)	
Tail and head enlargement	–	2 (18.1)	
Diffuse oedema	–	3 (25)	
Peripancreatic/pancreatic fluid collections	–	1 (9)	

5-ASA: 5-aminosalicylic acid; AP: acute pancreatitis; AZT: azathioprine; BIO: biologic therapy; CCS: corticosteroids; CD: Crohn's disease; HA/HL: hyperamylasemia/hyperlipasemia; IBD: inflammatory bowel disease; IBD-U: unclassified-IBD; MTX: methotrexate; PI: pancreatic involvement; PUCAI: Paediatric Ulcerative Colitis Activity Index; PCDAI: Paediatric Crohn's Disease Activity Index; UC: ulcerative colitis.

Table 3
Paediatric acute pancreatitis score criteria.

	Patients with AP
Admission criteria	
Median age (years; range)	14.5 (10–17)
Median weight (kg, range)	43 (26–63)
White blood cell count, $\times 10^3$/mm (median, range)	6.9 (4.1–14.8)
LDH, U/L (median, range)	377.5 (206–639)
48-h criteria	
Calcium, mg/dl (median, range)	9.4 (8.5–10)
Albumin, mg/dl (median, range)	4.4 (2.5–4.8)
Fluid sequestration, ml/kg/48 h (median, range)	18.4 (6–54)
Rise in BUN, mg/dl (median, range)	3.2 (1.2–6)
Score (N, %)	
0–2 points (mild)	11 (100%)
3–8 points (severe)	0

BUN: blood urea nitrogen; LDH: lactic dehydrogenase.

3.4. Natural history

At 6 and 12 months follow-up evaluations, median serum amylase level was 56 (range 8–240 IU/L) and 66.5 (range, 23–480 IU/L), respectively; median serum lipase level was 28 (range 8–633 IU/L) and 51.1 (range, 12–206 IU/L), respectively. Natural history of pancreatic involvement is reported in Fig. 2.

Table 4
Therapeutic measures in patients with acute pancreatitis and hyperamylasemia/hyperlipasemia.

Therapeutic measures	AP (11)	HA/HL (16)
Fasting with rehydration (n, %)	6 (54.5)	0 (0)
Antibiotic therapy (n, %)	3 (27.2)	0 (0)
PPIs (n, %)	1 (9)	0 (0)
Octreotide (n, %)	1 (9)	0 (0)
AZT suspension	5 (45.5)	4 (25)
5-ASA suspension	2 (18.1)	1 (6.3)
None	2 (18.1)	11 (68.7)

AP: acute pancreatitis; HA/HL: exclusive hyperamylasemia/hyperlipasemia; TPN: total parenteral nutrition; PPIs: proton pump inhibitors; AZT: azathioprine; 5-ASA: 5-aminosalicylic acid.

3.4.1. Acute pancreatitis

At 6 months of follow-up 1/11 children with AP (9%) was diagnosed with ARP, while 1 patient presented with hyperamylasemia/hyperlipasemia. At 6 months, patients presenting with recurrent pancreatic involvement showed higher values of PUCAI/PCDAI scores compared with the remaining patients with a trend towards statistical significance (median: 17.5 vs 5; range: 0–47.5; $p=0.07$). At 12 months follow-up 10 patients (90.9%) had a remission from AP, while 1 patient (9.1%) showed laboratory

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

UNCORRECTED PROOF

and radiological signs of CP with reduced faecal elastase. The same patient resulted to carry a F1052V CFTR mutation in heterozygosis.

3.4.2. Hyperamylasemia/hyperlipasemia

At 6 months follow-up 4/16 children with exclusive increase of pancreatic enzymes developed AP (25%), while 1 (6.2%) still presented hyperamylasemia/hyperlipasemia. At 12 months follow-up, 11/16 patients (68.7%) reached a complete remission of pancreatic involvement, whereas in the 5 remaining patients (32.3%) exclusive hyperamylasemia/hyperlipasemia persisted. At 12 months, patients with recurrent pancreatic involvement had higher PUCAI/PCDAI scores compared to children who did not, without reaching statistical significance (median: 13 vs 5; range: 0–30; $p = 0.08$).

4. Discussion

IBD patients are affected by an increased incidence of pancreatic involvement, including AP and exclusive hyperamylasemia/hyperlipasemia, when compared with the general population [26]. The incidence of pancreatic involvement varies widely in IBD adult patients, ranging from 5% to 21% [6,11]. A possible explanation for this wide range is that diagnosis of mild disease may be easily missed. There are only limited and not recent published data on the incidence of pancreatic involvement in paediatric patients with IBD. To the best of our knowledge our paper represents the first paediatric multicentre IBD registry-based study, characterizing the natural history of pancreatic involvement. Consistent with the published literature in the present cohort, the prevalence of pancreatic involvement was 4.1% and AP was diagnosed in 1.6% of cases.

The question of whether pancreatitis is an EIM of IBD remains unclear [26]. The aetiology and pathogenesis of AP are elusive and seem to be multifactorial in the majority of IBD patients. It is possible that epithelial cells of the gastrointestinal tract and pancreatic tissue may share similar target molecular or cellular structures vulnerable to injury. To support this hypothesis in our cohort of patients AP was present at IBD onset in 18%, suggesting that in some cases pancreatic dysfunction is part of a common immune disorder. The mouse model of trinitrobenzene sulfonic acid-induced colitis was shown to have concurrent pancreatic lesions [27]. Nausea and vomiting as associated features often suggest a diagnosis of acute or chronic pancreatitis. More often in CD than in UC, recurrent abdominal pain is the presenting feature [18]. Unfortunately, an elevation of serum amylase and lipase without symptoms or signs of pancreatitis is more frequent in IBD patients than in controls [6,11]. Various hypotheses for exclusive hyperamylasemia and hyperlipasemia in IBD patients have been proposed and are still matter of discussion [19]. Complicating the picture, symptoms of pancreatitis often overlap with IBD flares. Indeed abdominal pain in an episode of pancreatitis may be falsely attributed to active IBD and serum levels of amylase or lipase never tested resulting in an underestimation of AP incidence [18]. According to this finding, in our cohort of patients symptoms were not able to discriminate patients with exclusive increase of pancreatic enzymes from patients with AP, suggesting that pancreatic imaging should be routinely evaluated in IBD patients.

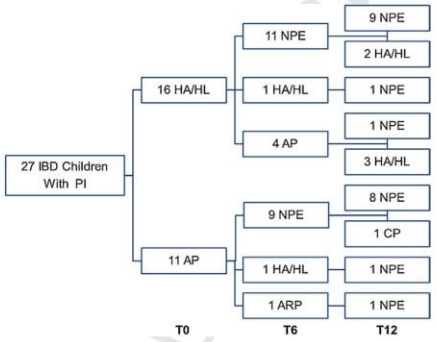


Fig. 2. Natural history of 27 children with inflammatory bowel disease and pancreatic involvement at one-year follow-up. AP: acute pancreatitis; ARP: acute recurrent pancreatitis; CP: chronic pancreatitis; HA/HL: hyperamylasemia/hyperlipasemia; IBD: inflammatory bowel disease; NPE: normal pancreatic enzymes; PI: pancreatic involvement; T0: baseline; T6: 6 months; T12: 12 months.

AP in adults has been reported much more commonly in CD than in UC [18]. As previously described in paediatric literature, in our study AP incidence was not different according to the IBD type, either CD or UC [13]. The relationships between pancreatitis and the extent and severity of the disease led to controversial conclusions in the recent literature [10]. According to Heikius et al. who demonstrated a correlation between the lipase increase and the histological activity of the disease, 91% of our patients with pancreatic involvement presented active disease [6]. Furthermore, patients with recurrent episodes of AP and hyperamylasemia/hyperlipasemia showed higher PCDAI/PUCAI scores at 6 and 12 months. These data may suggest that pancreatic involvement could be strictly related to the activity of disease at least in a subset of patients.

Interestingly, the majority of our patients with CD and AP showed a colonic involvement, as previously described [13]. Nevertheless, It is well known that paediatric patients with CD present with more colonic involvement [28]. The association between colonic disease and AP remains obscure. One possible explanation may be that the colon is considered a major source of the bacteria causing pancreatic necrosis in AP. Supporting this hypothesis subtotal colectomy before AP in rats was found to reduce mortality [29].

Female gender resulted to be significantly associated with the onset of pancreatitis in our patients, also when comparing with the total IBD registry population. Bermejo et al. reported that female gender was a risk factor for AZA/Mercaptopurine-associated acute pancreatitis [30]. Female predominance in the majority of autoimmune disorders may be one of the possible explanations.

Among IBD children with pancreatic involvement needing therapeutic measures, 85% of subjects withdrew AZA and/or suspended ASA. Indeed, AP is a well-recognized adverse effect occurring in 2–4% of IBD patients receiving thiopurines and it is usually considered as an absolute contraindication to reintroduction of a thiopurine [30–32]. However, the actual impact of thiopurines in AP episodes of IBD children is still questioned. In fact, pancreatic involvement is much rarer or not associated with AZA therapy in many other conditions, including rheumatoid arthritis, systemic lupus erythematosus, or in post-transplant patients [33]. This finding may suggest that AP could be more likely IBD-related rather than drug induced. Concerning mesalazine, its association with AP remains controversial. Munk et al. found no increased risk for mesalazine using data from a Danish hospital discharge registry [34]. In contrast, the UK study on AP mentioned above showed a nine-fold increased risk in patients receiving mesalazine up to 3 months before the onset of the disease [35].

No paediatric data are reported about the clinical course of IBD patients with AP. Although in all our cases the severity of AP episodes was mild and self-limiting, some of them tend to recur during the follow up. Furthermore, in our cohort, at one-year follow-up, ~~and~~ one showed a rapid evolution towards CP and

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

consequent pancreatic insufficiency, suggesting that, in a subgroup of IBD patients, pancreatic dysfunction has an unfavourable course. However, the severe evolution of the patient developing CP may be partially explained by the concomitant CFTR mutation in heterozygosis, not causing cystic fibrosis, but associated with pancreatic disorders in a subgroup of patients [36]. This finding highlights the need for checking other causes of pancreatic involvement in IBD children. Although the clinical significance of exclusive hyperamylasemia/hyperlipasemia has been questioned, the natural history of our patients seems not to be always benign. In our cohort, at 6 months follow-up, 25% of children with exclusive increase of pancreatic enzymes at the enrolment developed AP and at 12 months follow-up, 32.3% had persistent hyperamylasemia/hyperlipasemia.

Our study has some limitations besides the retrospective nature. Firstly, we did not have a control group of patients with AP without IBD; second, we did not evaluate serological markers of autoimmune pancreatitis. One could argue that the number of IBD patients with pancreatic involvement is too small to draw definitive conclusions; furthermore, in this subset of patients it is difficult to clearly define the aetiology of AP, as IBD itself is a predisposing factor, and development of pancreatitis is certainly multifactorial. Nevertheless our data, based on a large paediatric IBD population, highlight once again that pancreatic involvement has a relatively low prevalence.

In conclusion, this multicentre, retrospective registry-based study suggests that prevalence of AP in children is similar to that reported in adults. AP is more common in colonic disease and female gender seems to be significantly associated with the development of AP in IBD children. This study underlines that specific attention has to be paid to the monitoring of pancreatic function in IBD children, considering that in a proportion of patients the pancreatic involvement tends to persist and in some cases pancreatic damage may evolve. Future studies on the pathogenesis of pancreatitis and its relationship to the long-term outcome in IBD are required.

Conflict of interest

None declared.

References

[1] Bernstein CN, Blanchard JF, Rawsthorne P, et al. The prevalence of extraintestinal diseases in inflammatory bowel disease: a population-based study. American Journal of Gastroenterology 2001;96:1116–22.
[2] Timmer A, Behrens R, Buderus S, et al., CEDATA-GPGE Study Group. Childhood onset inflammatory bowel disease: predictors of delayed diagnosis from the CEDATA German-language paediatric inflammatory bowel disease registry. Journal of Pediatrics 2011;158:467–73, e2.
[3] Ricart E, Panaccione R, Loftus Jr EV, et al. Autoimmune disorders and extraintestinal manifestations in first-degree familial and sporadic inflammatory bowel disease: a case–control study. Inflammatory Bowel Diseases 2004;10:207–14.
[4] Bernstein CN, Wajda A, Blanchard JF. The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study. Gastroenterology 2005;129:827–36.
[5] Navaneethan U, Shen B. Hepatopancreatobiliary manifestations and complications associated with inflammatory bowel disease. Inflammatory Bowel Diseases 2010;16:1598–619.
[6] Heikius B, Niemelä S, Lehtola J, et al. Elevated pancreatic enzymes in inflammatory bowel disease are associated with extensive disease. American Journal of Gastroenterology 1999;94:1062–9.
[7] Bai HX, Lowe ME, Husain SZ. What have we learned about acute pancreatitis in children? Journal of Pediatric Gastroenterology and Nutrition 2011;52:262–70.
[8] Morinville VD, Barmada MM, Lowe ME. Increasing incidence of acute pancreatitis at an American paediatric tertiary care center: is greater awareness among physicians responsible? Pancreas 2010;39:5–8.
[9] Nydegger A, Heine RG, Ranuh R, et al. Changing incidence of acute pancreatitis: 10-year experience at the Royal Children's Hospital, Melbourne. Journal of Gastroenterology and Hepatology 2007;22:1313–6.
[10] Barthet M, Lesavre N, Desplats S, et al. Frequency and characteristics of pancreatitis in patients with inflammatory bowel disease. Pancreatology 2006;6:464–71.
[11] Katz S, Bank S, Greenberg R, et al. Hyperamylasemia in inflammatory bowel disease. Journal of Clinical Gastroenterology 1988;6:627–30.
[12] Angelini G, Cavallini G, Bovo P, et al. Pancreatic function in chronic inflammatory bowel disease. International Journal of Pancreatology 1988;3:185–93.
[13] Broide E, Dotan I, Weiss B, et al. Idiopathic pancreatitis preceding the diagnosis of inflammatory bowel disease is more frequent in paediatric patients. Journal of Pediatric Gastroenterology and Nutrition 2011;52:714–7.
[14] Stawarski A, Iwanczak F. Incidence of acute pancreatitis in children with inflammatory bowel disease. Polski Merkuriusz Lekarski 2004;17:33–6.
[15] Ravi K, Chari ST, Vege SS, et al. Inflammatory bowel disease in the setting of autoimmune pancreatitis. Inflammatory Bowel Diseases 2009;15:1326–30.
[16] Lankisch PG, Droge M, Gottesleben F. Drug induced acute pancreatitis: incidence and severity. Gut 1995;37:565–7.
[17] Stein RB, Hanauer SB. Comparative tolerability of treatments for inflammatory bowel disease. Drug Safety 2000;23:429–48.
[18] Pitchumoni CS, Rubin A, Das K. Pancreatitis in inflammatory bowel diseases. Journal of Clinical Gastroenterology 2010;44:246–53.
[19] Pieper-Bigelow C, Strocchi A, Levitt MD. Where does serum amylase come from and where does it go? Gastroenterology Clinics of North America 1990;19:793–810.
[20] IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis–the Porto criteria. Journal of Pediatric Gastroenterology and Nutrition 2005;41:1–7.
[21] Morinville VD, Husain SZ, Bai H, et al., INSPPIRE Group. Definitions of pediatric pancreatitis and survey of present clinical practices. Journal of Pediatric Gastroenterology and Nutrition 2012;55:261–5.
[22] DeBanto JR, Goday PS, Pedroso MR, et al., Midwest Multicenter Pancreatic Study Group. Acute pancreatitis in children. American Journal of Gastroenterology 2002;97:1726–31.
[23] Levine A, Griffiths A, Markowitz J, et al. Paediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. Inflammatory Bowel Diseases 2011;17:1314–21.
[24] Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a paediatric Crohn's disease activity index. Journal of Pediatric Gastroenterology and Nutrition 1991;12:439–47.
[25] Turner D, Otley AR, Mack D, et al. Development, validation, and evaluation of a paediatric ulcerative colitis activity index: a prospective multicenter study. Gastroenterology 2007;133:423–32.
[26] Herrlinger KR, Stange EF. The pancreas and inflammatory bowel diseases. International Journal of Pancreatology 2000;27:171–9.
[27] Barthet M, Dubucquoy L, Garcia S, et al. Pancreatic changes in TNBS-induced colitis in mice. Gastroenterologie Clinique et Biologique 2003;27:895–900.
[28] de Bie CI, Paerregaard A, Kolacek S, et al., EUROKIDS Porto IBD Working Group of ESPGHAN. Disease phenotype at diagnosis in paediatric Crohn's disease: 5-year analyses of the EUROKIDS Registry. Inflammatory Bowel Diseases 2013;19:378–85.
[29] Van Minnen LP, Nieumwenhuijs VB, de Bruijn MT, et al. Effects of subtotal colectomy on bacterial translocation during experimental acute pancreatitis. Pancreas 2006;32:110–4.
[30] Bermejo F, Lopez-Sanroman A, Taxonera C, et al. Acute pancreatitis in inflammatory bowel disease, with special reference to azathioprine-induced pancreatitis. Alimentary Pharmacology and Therapeutics 2008;28:623–8.
[31] Chaparro M, Ordàs I, Cabré E, et al. Safety of thiopurine therapy in inflammatory bowel disease: long-term follow-up study of 3931 patients. Inflammatory Bowel Diseases 2013;19:1404–10.
[32] Lopez-Martìn C, Chaparro M, Espinosa L, et al. Adverse events of thiopurine immunomodulators in patients with inflammatory bowel disease. Gastroenterologia y Hepatologia 2011;34:385–92.
[33] Weersma RK, Peters FT, Oostenbrug LE, et al. Increased incidence of azathioprine-induced pancreatitis in Crohn's disease compared with other diseases. Alimentary Pharmacology and Therapeutics 2004;20:843–50.
[34] Munk EM, Pedersen L, Floyd A, et al. Inflammatory bowel diseases, 5-aminosalicylic acid and sulfasalazine treatment and risk of acute pancreatitis: a population-based case–control study. American Journal of Gastroenterology 2004;99:884–8.
[35] Lancashire RJ, Cheng K, Langman MJ. Discrepancies between population-based data and adverse reaction reports in assessing drugs as causes of acute pancreatitis. Alimentary Pharmacology and Therapeutics 2003;17:887–93.
[36] Bombieri C, Claustres M, De Boeck K, et al. Recommendations for the classification of diseases as CFTR-related disorders. Journal of Cystic Fibrosis 2011;10(Suppl. 2):S86–102.

UNCORRECTED PROOF

Please cite this article in press as: Martinelli M, et al. Natural history of pancreatic involvement in paediatric inflammatory bowel disease. Dig Liver Dis (2015), http://dx.doi.org/10.1016/j.dld.2015.01.155

4.2 Clostridium difficile and Pediatric Inflammatory Bowel Disease: A Prospective, Comparative, Multicenter, ESPGHAN.

The prevalence of C. difficile infection in pediatric patients with IBD is reported over a broad range from 3.5% to 69%.[11-16] Indeed, despite considerable advances in understanding the epidemiology, immunology, and pathogenesis, Clostridium difficile (C. difficile) infection is still the most frequent cause of nosocomial bacterial infectious diarrhea in developed countries. [17]

Most of our current knowledge stemsfrom studies in adults with numerous confounding factors.

Despite the association with pediatric IBD, data regarding the health care burden related to C. difficile infection in children withIBD are limited to few studies. The primary aim of our study was to investigate the occurrence of C. difficile infection in pediatric patients with IBD and to compare with a group of children affected by celiac disease; secondary aim of this study was to evaluate natural history and disease course of C. difficile infection in pediatric patients with IBD.

To the best of our knowledge, this is the first prospective, multicenter pediatric study characterizing the occurrence and natural history of C. difficile infection/colonization in patients withIBD. We found that although infection is common in certain countries and rarer in others, it seems to be associated with approximately 15% of relapses. Clostridium difficile infection was significantly higherin patients with IBD than patients with celiac disease. We decided to test patients with celiac as control group, to verify whether another gastrointestinal pathology with described microbiota alterations, such as celiac disease, may confer a higher susceptibility to C. difficile infection. Our data confirm that C. difficile increased risk is related to IBD itself.

In addition we found that there was no specific IBD type predisposing to C. difficile colonization. This is consistent with published pediatric literature.[18,19] Our data seem to indicate that colonic involvement rather than disease type is the explanation for the association with UC. Most patients with IBD with C. difficile in our cohort (85.7%) showed colonic involvement, independent of the type of disease, as previouslyreported.[20] In agreement with previous articles, C. difficile was mainly community-acquired.[11-16] This is a further warning that C. difficile epidemiology is changing, and classical risk factors are often not involved.

Aside from disease location, other traditional risk factors seem to be less important or have no association with pediatricIBD. We could not demonstrate that medications such as PPIs or immune modulators were associated, and hospitalization did notincrease the risk for acquiring C. difficile.

In conclusion, this prospective, multicenter study confirms that pediatric IBD is associated with increased C. difficile detection.Clostridium difficile is associated with a severe disease course, as

demonstrated by the escalation of immunosuppressive therapy, the higher frequency of active disease, the colectomyrate, and the higher number of hospitalizations at 6 months.

A consistent number of patients show an asymptomatic carriage, which should be carefully evaluated, considering the possibility ofa quick worsening of disease. Future studies will clarify whether C. difficile has a causative role on IBD course exacerbation or if may simply colonize those patients with a more severe phenotype.

In addition, the relationship between C. difficile and IBD pathogenesis should be further investigated.

Clostridium difficile and Pediatric Inflammatory Bowel Disease: A Prospective, Comparative, Multicenter, ESPGHAN Study

Massimo Martinelli, MD, Caterina Strisciuglio, MD,* Gabor Veres, MD,† Anders Paerregaard, MD,‡ Ana M. Pavic, MD,§ Marina Aloi, MD, PhD,‖ Javier Martín-de-Carpi, MD,¶ Arie Levine, MD,** Dan Turner, MD,†† Mariassunta Del Pezzo, MD,‡‡ Annamaria Staiano, MD,* and Erasmo Miele, MD, PhD* on behalf of Porto IBD Working Group of European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)*

Background: *Clostridium difficile* (*C. difficile*) infection is associated with pediatric inflammatory bowel disease (IBD) in several ways. We sought to investigate *C. difficile* infection in pediatric patients with IBD in comparison with a group of children with celiac disease and to evaluate IBD disease course of *C. difficile* infected patients.

Methods: In this prospective, comparative, multicenter study, 211 pediatric patients with IBD were enrolled from October 2010 to October 2011 and tested for the presence of *C. difficile* toxins A and B in their stools at 0, 6, and 12 months. During the same study period, stool specimens for *C. difficile* toxins analysis were collected from 112 children with celiac disease as controls.

Results: *Clostridium difficile* occurrence was significantly higher in patients with IBD compared with patients with celiac disease (7.5% versus 0.8%; $P = 0.008$). *Clostridium difficile* was associated with active disease in 71.4% of patients with IBD ($P = 0.01$). Colonic involvement was found in 85.7% of patients with *C. difficile*. Antibiotics, proton pump inhibitors, hospitalization, and IBD therapies were not associated with increased *C. difficile* detection. At 12 months, a higher number of *C. difficile*–positive patients at the enrollment started immunosuppressant/biological therapy compared with patients without *C. difficile* ($P = 0.01$). At 6 and 12 months, patients with *C. difficile* were more frequently in active disease than patients without *C. difficile* ($P = 0.04$; $P = 0.08$, respectively). Hospitalizations were higher at 6 months in *C. difficile* group ($P = 0.05$).

Conclusions: In conclusion, this study demonstrates that pediatric IBD is associated with increased *C. difficile* detection. Patients with *C. difficile* tend to have active colonic disease and a more severe disease course.

(Inflamm Bowel Dis 2014;0:1–7)

Key Words: *Clostridium difficile*, Crohn's disease, ulcerative colitis

Despite considerable advances in understanding the epidemiology, immunology, and pathogenesis, *Clostridium difficile* (*C. difficile*) infection is still the most frequent cause of nosocomial bacterial infectious diarrhea in developed countries, and it has grown in frequency and severity over the 3 decades that has passed since the identification of the organism as a pathogen.[1] Recent changes in the epidemiology of *C. difficile* infection include the identification of adult and pediatric patients with inflammatory bowel disease (IBD) as a group at risk in comparison with the general population.[2–5] The prevalence of *C. difficile* infection in pediatric patients with IBD is reported over a broad range from 3.5% to 69%.[6–11] It also seems that antibiotic exposure, a uniformly identified risk factor in the general population, is frequently absent in patients with IBD infected with *C. difficile*. The increased risk of infection is believed to be due not only to the frequent use of immunosuppressive agents and hospital based-services but also

Received for publication July 24, 2014; Accepted August 7, 2014.

From the *Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Naples, Italy; †First Department of Pediatrics, Semmelweis University, Budapest, Europe; ‡Department of Pediatrics, Hvidovre University Hospital, Hvidovre, Denmark; §Referral Center for Pediatric Gastroenterology and Nutrition, Children's Hospital Zagreb, Zagreb, Croatia; ‖Department of Pediatrics, University of Rome "La Sapienza," Rome, Italy; ¶Pediatric IBD Unit, Department of Pediatric Gastroenterology, Hepatology and Nutrition, Hospital Sant Joan de Déu, Barcelona, Spain; **Pediatric Gastroenterology and Nutrition Unit, Wolfson Medical Center, Tel Aviv University, Tel Aviv, Israel; ††Pediatric Gastroenterology Unit, Shaare Zedek Medical Center, The Hebrew University of Jerusalem, Jerusalem, Israel; and ‡‡Department of Cellular and Molecular Biology and Pathology "Luigi Califano", University of Naples "Federico II," Naples, Italy.

The authors declare no conflict of interest to disclose regards to this paper. A.L. was a consultant for Abbvie, a speaker for Abvie, MSD and Nestle and received travel expenses for meetings from MSD, Abvie, Jannsen, Ferring and Nestle; A.S. is a consultant of DMG Italy.

Reprints: Annamaria Staiano, MD, Department of Translational Medical Sciences, Section of Pediatrics, University of Naples "Federico II," Via S. Pansini, 5, 80131 Naples, Italy (e-mail: staiano@unina.it).

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1097/MIB.0000000000000219

Published online.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

to IBD itself. However, whether the underlying inflammatory disease process per se confers an additional risk of infection in patients with IBD is still unclear.[12] An additional concern is that *C. difficile* infection in patients with IBD seems to be mostly community-acquired.[6–9] *Clostridium difficile* may associate with the course of IBD in several ways, including triggering disease flares, sustaining activity, and in some cases, acting as an "innocent" bystander.[13] Therefore, when a flaring patient with IBD is found to be infected with *C. difficile*, the clinician usually faces a therapeutic dilemma whether to withhold immunosuppression or alternatively to administer antibiotics along with intensified immunosuppressant therapy to treat a possible concurrent IBD exacerbation.[14] The complexity of this situation has increased with recent reports showing that IBD may deteriorate after *C. difficile* infection.[8,10,15,16] Most of our current knowledge stems from studies in adults with numerous confounding factors. Despite the association with pediatric IBD, data regarding the health care burden related to *C. difficile* infection in children with IBD are limited to few studies. The primary aim of this study was to investigate the occurrence of *C. difficile* infection in pediatric patients with IBD and to compare with a group of children affected by celiac disease; secondary aim of this study was to evaluate natural history and disease course of *C. difficile* infection in pediatric patients with IBD.

METHODS

Patient Population

We conducted a prospective, comparative, multicenter study in pediatric inpatients and outpatients with a diagnosis of IBD. Patients affected by Crohn's disease (CD) or ulcerative colitis (UC) were consecutively enrolled between October 2010 and October 2011 and tested for the presence of *C. difficile* toxins A and B in their stools. During the same study period, stool samples for testing *C. difficile* toxins A and B were also obtained from a control group of children, admitted to the outpatient clinic of the enrolling centers, with a diagnosis of celiac disease. Patients with IBD were followed as part of normal follow-up necessary to control the underlying disease and stool samples for detection of *C. difficile* toxins A and B were further examined after 6 and 12 months. The control patients enrolled delivered stool samples for detection of toxins A and B to *C. difficile* only at the time of the enrollment and if negative no more. Eight tertiary care sites from 5 countries participated in this study: the Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Italy; Pediatric Gastroenterology and Liver Unit, Sapienza University of Rome, Rome, Italy; Department of Pediatric Gastroenterology, Hepatology and Nutrition, Hospital San Joan De Deu, Barcelona, Spain; Pediatric Gastroenterology and Nutrition Unit, Wolfson Medical Center, Tel Aviv University, Tel Aviv, Israel; Shaare Zedek Medical Center, The Hebrew University of Jerusalem, Jerusalem, Israel; Children's Hospital, Zagreb, Croatia; Department of Pediatrics, Hvidovre University Hospital, Hvidovre, Denmark; and Semmelweis University, Budapest, Hungary. For all children data on age and sex, duration of bowel disorder, consistency of feces, presence of abdominal pain or fever (>38°C), and antibiotic treatments 30 days before the stool collection were recorded. Data were also obtained about use of proton pump inhibitors (PPIs) and hospitalization within the preceding 2 months before *C. difficile* toxin collection. For the IBD group, type of IBD, anatomical distribution of disease, symptoms, disease activity, and treatments, including surgery, were recorded at each time point. The diagnosis of IBD was established on the basis of clinical, endoscopic, radiological, and histological criteria according to the Porto criteria.[17] For the purpose of this article, disease location was described according to Paris classification.[18] Disease activity was scored by the Pediatric Crohn's Disease Activity Index or the Pediatric Ulcerative Colitis Activity Index for CD and UC, respectively.[19,20] Exclusion criteria from the study were age: ≤2 years or >18 years; patients with inflammatory bowel disease unclassified; inability or unwillingness to give informed consent. Children with *C. difficile* toxins in the stools and symptoms suggestive of *C. difficile* infection were treated according to the physicians' discretion. The treatment for each patient was recorded. Asymptomatic patients with a positive test were not treated. *Clostridium difficile* recurrence was defined as a stool study positive within 60 days of the previous infection, with at least 1 stool test negative for *C. difficile* between the 2 infections. All parents or guardians signed a consent form indicating their awareness of the investigational nature and possible risks of this study. Where appropriate, we also obtained children's assent. The study was approved by the institutional review board of each involved center.

Microbiological Methods

Each laboratory of the units involved in the study, performed the immunoenzymatic test, routinely used. *Clostridium difficile* toxins A and B enzyme immunocard (Meridian Bioscience, Cincinnati, OH) was used in Hvidovre, Naples, Rome, and Zagreb. This qualitative, horizontal-flow enzyme immunoassay has a sensitivity of 83 ± 6.7% and a specificity of 95 ± 1.6%. *C. diff* Quik Chek Complete (TechLab, Blacksburg, VA) was performed in Barcelona, Budapest, Jerusalem, and Tel Aviv. This assay, comprising a dual rapid membrane enzyme immunoassay for toxins A and B and for glutamate dehydrogenase antigen, has a sensitivity of 87.8% and a specificity of 99.4%. The assays were performed according to the manufacturer's instructions.

Statistical Analysis

Variables were screened for their distribution, and appropriate parametric or nonparametric tests were adopted as necessary. The Student's t test and the Mann–Whitney test for continuous variables and the χ^2 and Fisher's exact tests for categorical variables were used where appropriate. Statistical significance was predetermined as $P < 0.05$. Percentages were rounded to the nearest whole numbers. To evaluate the association between *C. difficile* detection and the primary and secondary factors, we conducted 2 tests for matched-pair data along with multivariate conditional

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

TABLE 1. Number of Enrolled Children for Participating Centers

Centers	Patients with IBD (n = 211)	Patients with Celiac Disease (n = 112)
Barcelona	11	8
Budapest	29	26
Hvidovre	28	17
Jerusalem	10	1
Naples	76	41
Rome	24	—
Tel Aviv	14	—
Zagreb	19	19

logistic regression analysis. SPSS version 15 was used for all statistical analyses. The sample size of 100 children in each group was estimated with a 90% power to detect a difference of at least 20%, between the 2 groups with an alpha of 0.05.

RESULTS

Three hundred twenty-three patients met the inclusion criteria and were enrolled between October 2010 and October 2011, of whom 211 were affected by IBD (UC: 93; CD: 118; median age: 13.1 yr; range, 2–18 yr; M/F: 121/90) and 112 by celiac disease (median age: 11.1 yr; range, 2–18 yr; M/F: 53/59). Number of enrolled patients for participating center and baseline characteristics are shown in Table 1 and Table 2, respectively. At the enrollment, *C. difficile* was detected significantly more often in patients affected by IBD compared with patients with celiac disease (16/211 [7.5%] versus 1/112 [0.8%]; $P = 0.008$; odds ratio = 9; 95% confidence interval, 1.8–68). One hundred ninety-six patients completed follow-up at 6 months. Six of 196 patients with IBD (3%) were positive to *C. difficile* toxins test at 6 months of follow-up. One hundred seventy-three patients completed the follow-up at 12 months. Two patients (1.1%) were positive at 12 months. Twenty of 21 *C. difficile*–positive patients (95.2%) completed the study. Considering patients completing the follow-up at 12 months, the overall 1-year occurrence was 11.5% (20/173 total patients). *Clostridium difficile* occurrence varied among different enrolling centers. Geographical distribution of *C. difficile* infection is shown in Figure 1. Recurrence rate among *C. difficile*–positive patients completing the follow-up was 10% (2/20 patients). One patient had 2 recurrences. Twelve of 224 patients (5.3%) tested with Meridian Immunocard resulted to be *C. difficile* positive compared with 10 of 99 patients (10.1%) undergoing *C. diff* Quik Chek Complete, without any statistical difference ($P = 0.1$).

Clinical Associations with *Clostridium difficile* Detection in Patients with IBD

We did not identify a specific type of IBD predisposing to *C. difficile* infection ($P = 0.3$). *Clostridium difficile* infection was not associated with age of patients at the time of infection and duration of IBD ($P = 0.9$ and $P = 0.8$, respectively). Three of 21 patients with *C. difficile* (14.2%) detection had new-onset IBD compared with 24 of 195 patients without *C. difficile* detection (12.3%) ($P = 0.4$). The presence of *C. difficile* toxins was associated with active disease in 15 of 21 patients with IBD (71.4%) ($P = 0.01$). In particular, *C. difficile* infection was found in 15.4% of all patients with IBD in relapse (15/82). Mean Pediatric Ulcerative Colitis Activity Index score was significantly increased in *C. difficile* infected patients with UC compared with noninfected patients, whereas mean Pediatric Crohn's Disease Activity Index score was higher in *C. difficile*–positive patients with CD, but statistical significance was not reached ($P = 0.01$ and $P = 0.5$, respectively) (Table 3). Six patients with IBD (28.5%) with positive *C. difficile* toxins were totally asymptomatic. Eighteen of 21 patients with IBD with *C. difficile* infection (85.7%) showed a colonic disease. However, disease location did not result to be significant different between *C. difficile*–positive and negative

TABLE 2. Baseline Characteristics

Characteristics (n, %)	Patients with IBD	Patients with Celiac Disease	*P*
Median age (range), yr	13.1 (2–18)	11.1 (2–18)	0.01
Gender			0.1
Male	121 (57.3)	53 (47.3)	
Female	90 (42.7)	59 (52.7)	
C. difficile detection	16 (7.5)	1 (0.8)	0.008
IBD characteristics			
Type			NA
CD	118 (55.9)		
UC	93 (44.1)		
Disease location			NA
CD			
Ileum only (L1)	27 (22.8)		
Colon only (L2)	30 (25.4)		
Ileum and colon (L3)	59 (50)		
Upper gastrointestinal tract (L4a)	10 (8.5)		
Upper gastrointestinal tract (L4b)	14 (11.8)		
UC			
Proctosigmoiditis (E1)	19 (20.4)		
Left-sided colitis (E2)	22 (23.6)		
Extensive colitis (E3)	12 (12.9)		
Pancolitis (E4)	40 (43)		
Treatment			NA
Steroids	34 (16.1)		
Mesalazine	103 (48.8)		
Immunosuppressants	85 (40.2)		
Enteral nutrition	4 (1.8)		
Biologics	29 (13.7)		

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

TABLE 3. Clinical Associations with *Clostridium difficile* Detection in Patients with IBD

Characteristics (n, %)	*C. difficile* Positive (n = 21)	*C. difficile* Negative (n = 190)	*P*
Gender			
Male	15 (71.4)	106 (55.8)	0.2
Female	6 (28.6)	84 (44.2)	
Mean age (range), yr	13.4 (2–18)	12.8 (2–18)	0.9
Mean disease duration (range), mo	28.2 (0–118)	30.3 (0–132)	0.8
Disease onset	3 (14.2)	24 (12.3)	0.4
IBD type			0.3
CD	14 (66.7)	104 (54.7)	
UC	7 (33.3)	86 (45.3)	
Active disease	15 (71.4)	82 (43.1)	0.01
Mean PCDAI (range)	22.7 (5–35)	20.4 (0–45)	0.3
Mean PUCAI (range)	26.2 (0–35)	20 (0–50)	0.01
Disease location			
Ileum only (L1)	3 (14.2)	24 (12.6)	0.5
Colon only (L2)	4 (19)	26 (13.7)	0.7
Ileum and colon (L3)	7 (33.3)	52 (26.8)	0.2
Upper gastrointestinal tract (L4a)	2 (9.5)	8 (4.2)	0.3
Upper gastrointestinal tract (L4b)	2 (9.5)	12 (6.3)	0.6
Proctosigmoiditis (E1)	0 (0)	19 (10)	0.2
Left-sided colitis (E2)	2 (9.5)	20 (10.5)	0.2
Extensive colitis (E3)	1 (4.7)	11 (5.7)	1
Pancolitis (E4)	4 (19)	36 (18.9)	1
Colonic involvement	18 (85.7)	166 (87.4)	0.7
Treatment			
Antibiotics	3 (14.2)	21 (11)	0.1
PPIs	3 (14.2)	23 (12.1)	1
Immunosuppressants	12 (57.1)	88 (46.3)	0.3
Recent Hospitalization	2 (9.5)	39 (20.1)	0.05

PCDAI, Pediatric Crohn's Disease Activity Index; PUCAI, Pediatric Ulcerative Colitis Activity Index.

groups (Table 3). Antibiotics, PPIs, and immunosuppressive therapies did not predispose patients to *C. difficile* infection ($P = 0.1$, $P = 1$, and $P = 0.3$, respectively). Previous hospitalizations were registered significantly more frequently in patients with IBD without *C. difficile* infection than in patients with IBD with *C. difficile* infection ($P = 0.05$). Multivariate logistic regression analysis with the risk of *C. difficile* detection as the dependent variable, confirmed that having IBD was the only factor that significantly contributed to the development of the infection (odds ratio = 2.6; 95% confidence interval, 1.8–7.3; $P = 0.03$). None of the other factors resulted to be independently associated to the infection.

Natural History of *Clostridium difficile* Infected Patients

To evaluate the effect of *C. difficile* detection on IBD course, we examined the escalation of immunosuppressant and/or biological therapy, disease activity, rate of surgery, and number of hospitalizations during the study follow-up in *C. difficile*–positive patients at the enrollment. Fifteen of 16 patients with IBD (93%) *C. difficile* positive at the enrollment completed the follow-up at 6 and 12 months. Natural history of *C. difficile*–positive patients at the enrollment performing all the follow-up is shown in Table 4. Within 12 months, there was a higher number of *C. difficile*–positive patients who started immunosuppressant or biological therapy compared with patients without *C. difficile* (7/15 [46.6%] versus 30/158 [18.9%]; $P = 0.01$; odds ratio = 3.7; 95% confidence interval, 1.2–11). In details, among *C. difficile*–positive patients, 5 switched from mesalamine to an immunomodulator, and 2 patients, already under immunosuppressants, started biological therapy. At 6 and 12 months, patients with *C. difficile*–positive stools at the enrollment were significantly more often in active disease compared with patients without *C. difficile* (7/15 [46.6%] versus 38/181 [20.9%], $P = 0.04$; 6/15 [40%] versus 28/158 [17.7%], $P = 0.08$, respectively). One of 15 patients with *C. difficile* detection (6.6%) underwent total colectomy within 12 months compared with 1 of 158 (0.6%), but the difference was not statistically significant ($P = 0.1$). Number of hospitalizations was significantly higher at 6 months in *C. difficile*–positive group (4/15 [25%] versus 19/196 [9.6%], $P < 0.05$) but not different at 12 months ($P = 0.4$).

Treatment of *Clostridium difficile* Cases

Fifteen of 21 total patients with IBD with *C. difficile*–positive stools (71.4%) showed signs and symptoms compatible with the infection. Eight of them (53.3%) were treated with oral vancomycin, whereas 7 patients (46.6%) were treated with oral metronidazole for 14 days. None of the patients withdrew immunosuppressive therapy. In 2 of 15 patients (13.3%), both treated with oral vancomycin, *C. difficile* was not eradicated, and another cycle of oral vancomycin was needed. The only patient with positive celiac was totally asymptomatic and therefore was not treated.

DISCUSSION

To the best of our knowledge, this is the first prospective, multicenter pediatric study characterizing the occurrence and natural history of *C. difficile* infection/colonization in patients with IBD. We found that although infection is common in certain countries and rarer in others, it seems to be associated with approximately 15% of relapses. A number of adult studies[2–5,12,21] have examined the incidence of *C. difficile* infection, whereas only few

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

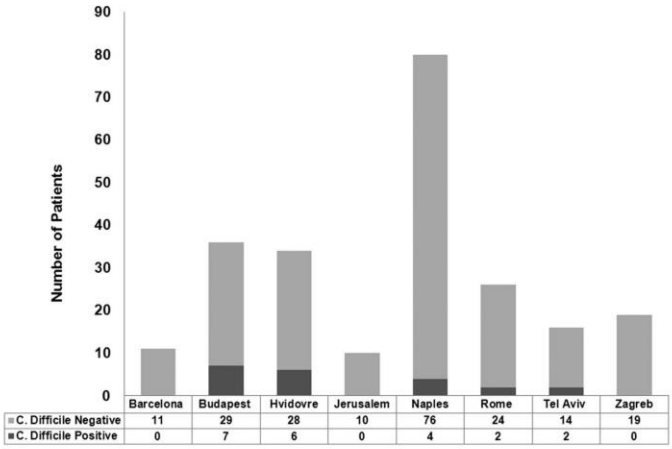


FIGURE 1. *Clostridium difficile* occurrence among different enrolling centers.

retrospective pediatric studies[6–11] tried to assess the prevalence, with different values. Pascarella et al[6] first reported a prevalence of 24.7% in pediatric IBD population, whereas more recently, Pant et al[11] assessed a rate of 3.5%. In our study population, *C. difficile* detection varied hugely among the enrolling centers, ranging from 0% to 24.1%. *Clostridium difficile* infection was significantly higher in patients with IBD than patients with celiac disease. We decided to test patients with celiac as control group, to verify whether another gastrointestinal pathology with described microbiota alterations,[22] such as celiac disease, may confer a higher

TABLE 4. Natural History of *Clostridium difficile* Infected Patients at the Enrollment Completing the Follow-up

Patients	Diagnosis	Baseline		T6		T12	
		C. difficile	Act	*C. difficile*	Act	*C. difficile*	Act
1	CD	Positive	Moderate	Negative	Moderate	Negative	Remission
2	CD	Positive	Severe	Negative	Moderate	Negative	Remission
3	CD	Positive	Moderate	Negative	Remission	Negative	Remission
4	CD	Positive	Moderate	Negative	Severe	Negative	Moderate
5	UC	Positive	Moderate	Negative	Remission	Negative	Remission
6	UC	Positive	Severe	Negative	Remission	Negative	Remission
7	CD	Positive	Mild	Positive	Remission	Negative	Remission
8	CD	Positive	Moderate	Negative	Remission	Negative	Moderate
9	CD	Positive	Remission	Negative	Moderate	Negative	Remission
10	UC	Positive	Severe	Positive	Moderate	Positive	Severe (colectomy)
11	CD	Positive	Remission	Negative	Remission	Negative	Remission
12	UC	Positive	Remission	Negative	Remission	Negative	Remission
13	CD	Positive	Mild	Negative	Moderate	Negative	Moderate
14	UC	Positive	Remission	Negative	Remission	Negative	Moderate
15	CD	Positive	Mild	Negative	Mild	Negative	Severe

Act, disease activity; T6, 6 months; T12, 12 months.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

susceptibility to *C. difficile* infection. Our data confirm that *C. difficile* increased risk is related to IBD itself. Although adult studies[3,4,12] documented an increased incidence of *C. difficile* in patients with UC, we could not confirm a similar association, and there was no specific IBD type predisposing to *C. difficile* colonization. This is consistent with published pediatric literature.[8,9] Our data seem to indicate that colonic involvement rather than disease type is the explanation for the association with UC. Most patients with IBD with *C. difficile* in our cohort (85.7%) showed colonic involvement, independent of the type of disease, as previously reported.[23] In agreement with previous articles, *C. difficile* was mainly community-acquired.[6–11] This is a further warning that *C. difficile* epidemiology is changing, and classical risk factors are often not involved.

Aside from disease location, other traditional risk factors seem to be less important or have no association with pediatric IBD. We could not demonstrate that medications such as PPIs or immunomodulators were associated, and hospitalization did not increase the risk for acquiring *C. difficile*. Freedberg et al[24] have recently questioned the role of PPIs. As described by Kelsen et al,[9] we found a high percentage of patients with *C. difficile* at onset of disease. Although not significant, this result underlines the possibility of a diagnostic delay and once more raises the question of a possible role of *C. difficile* in IBD pathogenesis. Furthermore, the presence of *C. difficile* toxins in the stools significantly correlated with an active disease, confirming the relevance of testing *C. difficile* during IBD flares.[25] As recently described, we found a considerable number of patients with IBD showing an asymptomatic carriage of *C. difficile*. Clayton et al[13] in a prospective evaluation reported that *C. difficile* was detected in stool cultures from 8% of patients with IBD in remission compared with 1% of healthy controls. More recently, Hourigan et al[26] found that asymptomatic *C. difficile* carriage was significantly more frequent in IBD (17%) versus controls (3%). Although the role of *C. difficile* carriage in subsequent *C. difficile*–associated disease or in IBD relapse needs to be further elucidated, our study provides important insight. Indeed, *C. difficile*–positive patients at the enrollment, including those with an asymptomatic carriage, seem to show a substantial risk for subsequent IBD exacerbation. This hypothesis is supported by the need for immunosuppressant escalation, the higher frequency of active disease, the increased colectomy rate, and the higher number of hospitalization at 6 months. Kelsen et al[9] in a retrospective analysis demonstrated that *C. difficile* infection worsened IBD severity, in terms of longer hospital admission, escalation of therapy, and colectomy rate. More recently, Pant et al[11] reported that *C. difficile* infected patients with IBD tended to have lengthier hospital stays, higher charges, and greater need for parenteral nutrition and blood transfusions. Differently from previous studies,[8] we found a high success rate of *C. difficile* eradication (86.6%) after treatment. However, this finding should be cautiously interpreted. The low treatment failure may partly reflect that European *C. difficile* strains are different from North-American B1/NAP1/027[27,28]; nevertheless, in most of the cases, eradication of the infection did not prevent a subsequent complicated IBD course, suggesting that *C. difficile* may act more as a relapse trigger rather than a "leading actor" of the symptoms. Another possibility is that *C. difficile* detection may be a expression of significant dysbiosis,[29] and therefore, it should be considered as an indicator of severe disease course. This study has some limitations. First, we limited our diagnosis to the use of immunoenzymatic assays. However in a recent report by Wang et al,[30] the outcomes of *C. difficile* diagnosed by polymerase chain reaction or enzyme-linked immunosorbent assay, seemed comparable despite the greater percentage of patients tested positive by polymerase chain reaction compared with enzyme-linked immunosorbent assay. There seemed to be a selection bias such that over 80% of patients had colonic involvement, which might reflect differences in phenotypes of disease, or enrollment of more severe colonic disease because of hospitalizations. It is well-known that Northern latitudes, such as Scandinavian and Scottish patients with IBD, as well as younger pediatric patients present with more colonic involvement in CD.[18] In addition, use of PPIs in our cohort was very limited, and we may have been underpowered to detect a difference. Furthermore, since the use of PPIs in children is low, this is less likely a priori to be a major risk factor in children. Finally, we did not perform *C. difficile* strains characterization, which could differ along the different countries.

CONCLUSION

In conclusion, this prospective, multicenter study confirms that pediatric IBD is associated with increased *C. difficile* detection. *Clostridium difficile* is associated with a severe disease course, as demonstrated by the escalation of immunosuppressive therapy, the higher frequency of active disease, the colectomy rate, and the higher number of hospitalizations at 6 months. A consistent number of patients show an asymptomatic carriage, which should be carefully evaluated, considering the possibility of a quick worsening of disease. Future studies will clarify whether *C. difficile* has a causative role on IBD course exacerbation or if may simply colonize those patients with a more severe phenotype. In addition, the relationship between *C. difficile* and IBD pathogenesis should be further investigated.

REFERENCES

1. Khanna S, Baddour LM, Huskins WC, et al. The epidemiology of *Clostridium difficile* infection in children: a population-based study. *Clin Infect Dis.* 2013;56:1401–1406.
2. Issa M, Vijayapal A, Graham MB, et al. Impact of *Clostridium difficile* on inflammatory bowel disease. *Clin Gastroenterol Hepatol.* 2007;5:345–351.
3. Rodemann JF, Dubberke ER, Reske KA, et al. Incidence of *Clostridium difficile* infection in inflammatory bowel disease. *Clin Gastroenterol Hepatol.* 2007;5:339–344.
4. Nguyen GC, Kaplan GG, Harris ML, et al. A national survey of the prevalence and impact of *Clostridium difficile* infection among hospitalized inflammatory bowel disease patients. *Am J Gastroenterol.* 2008;103:1443–1450.
5. Ananthakrishnan AN, McGinley EL, Binion DG. Excess hospitalisation burden associated with *Clostridium difficile* in patients with inflammatory bowel disease. *Gut.* 2008;57:205–210.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

6. Pascarella F, Martinelli M, Miele E, et al. Impact of *Clostridium difficile* infection on pediatric inflammatory bowel disease. *J Pediatr.* 2009;154: 854–858.
7. Wultanska D, Banaszkiewicz A, Radzikowski A, et al. *Clostridium difficile* infection in Polish pediatric outpatients with inflammatory bowel disease. *Eur J Clin Microbiol Infect Dis.* 2010;29:1265–1270.
8. Mezoff E, Mann EA, Hart KW, et al. *Clostridium difficile* infection and treatment in the pediatric inflammatory bowel disease population. *J Pediatr Gastroenterol Nutr.* 2011;52:437–441.
9. Kelsen JR, Kim J, Latta D, et al. Recurrence rate of *Clostridium difficile* infection in hospitalized pediatric patients with inflammatory bowel disease. *Inflamm Bowel Dis.* 2011;17:50–55.
10. Banaszkiewicz A, Kowalska-Duplaga K, Pytrus T, et al. *Clostridium difficile* infection in newly diagnosed pediatric patients with inflammatory bowel disease: prevalence and risk factors. *Inflamm Bowel Dis.* 2012;18: 844–848.
11. Pant C, Anderson MP, Deshpande A, et al. Health care burden of *Clostridium difficile* infection in hospitalized children with inflammatory bowel disease. *Inflamm Bowel Dis.* 2013;19:1080–1085.
12. Navaneethan U, Venkatesh PG, Shen B. *Clostridium difficile* infection and inflammatory bowel disease: understanding the evolving relationship. *World J Gastroenterol.* 2010;16:4892–4904.
13. Clayton EM, Rea MC, Shanahan F, et al. The vexed relationship between *Clostridium difficile* and inflammatory bowel disease: an assessment of carriage in an outpatient setting among patients in remission. *Am J Gastroenterol.* 2009;104:1162–1169.
14. Yanai H, Nguyen GC, Yun L, et al. Practice of gastroenterologists in treating flaring inflammatory bowel disease patients with *Clostridium difficile*: antibiotics alone or combined antibiotics/immunomodulators? *Inflamm Bowel Dis.* 2011;17:1540–1546.
15. Ananthakrishnan AN, McGinley EL, Saeian K, et al. Temporal trends in disease outcomes related to *Clostridium difficile* infection in patients with inflammatory bowel disease. *Inflamm Bowel Dis.* 2011;17:976–983.
16. Navaneethan U, Mukewar S, Venkatesh PG, et al. *Clostridium difficile* infection is associated with worse long term outcome in patients with ulcerative colitis. *J Crohns Colitis.* 2012;6:330–336.
17. IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis—the Porto criteria. *J Pediatr Gastroenterol Nutr.* 2005;41:1–7.
18. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis.* 2011;17:1314–1321.
19. Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr.* 1991;12:439–447.
20. Turner D, Otley AR, Mack D, et al. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology.* 2007;133:423–432.
21. Jen MH, Saxena S, Bottle A, et al. Increased health burden associated with *Clostridium difficile* diarrhoea in patients with inflammatory bowel disease. *Aliment Pharmacol Ther.* 2011;33:1322–1331.
22. Nistal E, Caminero A, Herrán AR, et al. Differences of small intestinal bacteria populations in adults and children with/without celiac disease: effect of age gluten diet, and disease. *Inflamm Bowel Dis.* 2012;18: 649–656.
23. Ananthakrishnan AN. Detecting and treating *Clostridium difficile* infections in patients with inflammatory bowel disease. *Gastroenterol Clin North Am.* 2012;41:339–353.
24. Freedberg DE, Salmasian H, Friedman C, et al. Proton pump inhibitors and risk for recurrent *Clostridium difficile* infection among inpatients. *Am J Gastroenterol.* 2013;108:1794–1801.
25. Mylonaki M, Langmead L, Pantes A, et al. Enteric infection in relapse of inflammatory bowel disease: importance of microbiological examination of stool. *Eur J Gastroenterol Hepatol.* 2004;16:775–778.
26. Hourigan SK, Chirumamilla SR, Ross T, et al. *Clostridium difficile* carriage and rerum antitoxin responses in children with inflammatory bowel disease. *Inflamm Bowel Dis.* 2013;19:2744–2752.
27. McDonald LC, Killgore GE, Thompson A, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl J Med.* 2005;353: 2433–2441.
28. Loo VG, Poirier L, Miller MA, et al. A predominantly clonal multiinstitutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Engl J Med.* 2005;353:2442–2449.
29. Bien J, Palagani V, Bozko P. The intestinal microbiota dysbiosis and *Clostridium difficile* infection: is there a relationship with inflammatory bowel disease? *Therap Adv Gastroenterol.* 2013;6:53–68.
30. Wang Y, Atreja A, Wu X, et al. Similar outcomes of IBD inpatients with *Clostridium difficile* infection detected by ELISA or PCR assay. *Dig Dis Sci.* 2013;58:2308–2313.

Copyright © 2014 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

4.3 Periappendiceal Inflammation in Pediatric Ulcerative Colitis

It is classically accepted that inflammation in ulcerative colitis (UC) originates in the rectum and spreads in continuity to the proximal portions of the colon.[21]

However, the proximal colon, especially at the base of the appendiceal orifice, is fre- quently involved by inflammatory infiltrates in patients with UC.[22]

Several prospective and retroprospective studies have confirmed that periappendiceal inflammation (PAI), both endoscopically and histologically, is common in UC.[22,23,24]

Only one pediatric study[25] looked at PAI involvement in children who required colonic resection. The purpose of our prospective study was, therefore, to evaluate, by endoscopy and histology, the prevalence of PAI in children affected by UC.

In this study, we found that PAI was endoscopically present in 32% of pediatric patients affected by UC not extending beyond the hepatic flexure. PAI was more frequent in children with new diagnosis of UC than in patients with pre-existing UC. In our study PAI seems to be related to the extent of diseaseWe can speculate that in pediatric patients, the disease is more extensive as reported by Van Limbergen et al,[26]who defined the phenotypic characteristics of pediatric UC. They found that children were more often affected by pancolitis and suggested a more severe phenotype in children than in adults. In our population, even if we ruled out children with pancolitis, we found that immunosuppressive use was significantly more common in children with PAI. This, in agreement with Van Limbergen's finding, suggests a more severe pediatric UC clinical picture, irrespective of the presence of pancolitis.[27]

We also found that clinical activity and the use of medical therapy were not involved in PAI.

However, PAI, in our patients, was significantly more frequent in newly diagnosed children. Whether this finding is related to the efficacy of the medical treatment or to the natural history of pediatric UC needs to be further evaluated.

At index colonoscopy, we found that the histologic grade of inflammation at the ascending colon was statistically higher in children with PAI. This higher grade of inflammation suggests that PAI may be representative of histologically active disease in the endoscopically unaffected proximal colon, although this involvement does not seem to contribute to clinical activity. Evidently, colonoscopy alone is unreliable for determining the extent of UC.[28]

The higher histologic grade in the group A in the ascending colon suggests that the PAI may be a hallmark for subclinical active disease, identified by histology. The histologic involvement at the ascending colon, even in the presence of macroscopically normal mucosa, suggests that the number of pancolitis in children is higher than observed only by endoscopy.

This study has some limitations. We did not include the findings on colonoscopy re-evaluation at follow-up because currently we do not have enough data. It is known that there is a little chance in the pediatric studies to scope all children as planned, in particular when they are on remission. In addition, we do not provide details on follow-up because its duration in most of the patient is too limited.

The importance of this study is that it is the first pediatric prospective study having the aim to define the features of UC with PAI that can be confused with Crohn's disease. In fact, the occasional patchy areas of mucosal inflammation often left to a change of diagnosis to Crohn's disease. Such a change in diagnosis from UC to Crohn's disease can affect management decision, especially in regard to surgery.[29]

In conclusion, to the best of our knowledge, this is the first pediatric study reporting the presence of PAI in children affected by UC not extending beyond the hepatic flexure. PAI seems to be more frequent in newly diagnosed children than in those with pre- existing diagnosis of UC, related to the extension of the disease and to an higher grade of histologic inflammation at level of the ascending colon.

ORIGINAL ARTICLE

Periappendiceal Inflammation in Pediatric Ulcerative Colitis

Caterina Strisciuglio, MD, Eleonora Giannetti, MD,* Francesca Paola Giugliano, MD,* Luigi Greco, MD, MSc (MCH), DCH,* Severo Campione, MD,† Mariarosaria D' Armiento, MD,† Annamaria Staiano, MD,* and Erasmo Miele, MD, PhD**

Background: An involvement of the appendiceal orifice as a distintive skip lesion in adults with left side ulcerative colitis (UC) has been reported. The aim of our prospective study was to evaluate, by endoscopy and histology, the prevalence of periappendiceal inflammation (PAI) in children affected by UC.

Methods: Fifty of 77 consecutive children undergoing total colonoscopy, who had a diagnosis of UC not extended beyond the hepatic flexure were enrolled.

Results: PAI was endoscopically present in 16 of 50 patients (32%) with UC. Patients were divided in 2 groups: group A included the 16 patients with PAI, whereas group B included 34 patients without PAI. We found that among the 2 groups, PAI was more frequent in patients with new diagnosis than in those with pre-existing UC ($P = 0.016$). At index colonoscopy, the patients of group A had a significant major extent of disease ($P = 0.013$). Moreover, the histologic grade of inflammation at the ascending colon was significantly higher in group A than in group B ($P = 0.014$). Clinical activity, measured by pediatric ulcerative colitis activity index, and use of medication did not show significant differences among groups ($P = 0.464$ and $P = 0.723$, respectively). The use of immunosuppressant was significantly higher in group A than in group B.

Conclusions: PAI is a frequent skip lesion in children with UC. It seems more frequent in patients with new diagnosis, and it is associated with a major extent of the disease and with a higher grade of histologic inflammation at the ascending colon.

(Inflamm Bowel Dis 2013;19:1617–1621)

Key Words: ulcerative colitis, periappendiceal inflammation

It is classically accepted that inflammation in ulcerative colitis (UC) originates in the rectum and spreads in continuity to the proximal portions of the colon.[1] It is a no specific inflammation with unknown etiology that affects the mucosa and extends proximally from the rectum, forming a continuous and diffuse lesion involving the whole colorectal region.[2] However, the proximal colon, especially at the base of the appendiceal orifice, is frequently involved by inflammatory infiltrates in patients with UC.[3] Although human appendix is considered as a vestigial remnant, recent observations have focused on the role of the appendix in the pathogenesis of UC.[4] Many case–control studies suggest that there is a negative association between previous appendectomy and patients with UC,[5-7] raising the possibility that appendectomy protects against the subsequent development of UC.[8-11] Patients with previous appendicectomy also have a delayed onset of UC,[8,9] a reduced need for immunosuppression therapy and proctocolectomy,[8,10] and a reduced relapse rate and extent of disease.[11] In contrast to Crohn's disease, in which location tends to be stable over time,[12,13] up to 35% of patients with ulcerative proctitis or distal UC present a proximal progression of their disease extent.[14,15] Patchy areas of inflammation have been observed in a certain proportion of patients with UC[16,17] and involvement at the appendiceal orifice has been demonstrated.[18,19] Several prospective and retroprospective studies have confirmed that periappendiceal inflammation (PAI), both endoscopically and histologically, is common in UC.[20-22] Only one pediatric study[23] looked at PAI involvement in children who required colonic resection. The purpose of our prospective study was, therefore, to evaluate, by endoscopy and histology, the prevalence of PAI in children affected by UC.

PATIENTS AND METHODS

The study population included a total of 50 of 77 consecutive children who underwent total colonoscopy from August 2011 to May 2012 and who had a diagnosis of UC not extended beyond the hepatic flexure. Twenty-two of 77 patients with more proximal involvement of the colon and 5 patients in whom the proximal extent of UC had not been determined by full colonoscopy were excluded from the study. In all patients, the diagnosis of UC was made on the basis of clinical symptoms and endoscopic and histologic features.

In each patient, clinical activity of the disease was evaluated at the time of endoscopy by using the Pediatric Ulcerative Colitis Activity Index.[24] Individual scores for each section of the test,

Received for publication November 8, 2012; Accepted November 19, 2012.

From the *Department of Pediatrics, University of Naples "Federico II", Italy; and †Department of Biomorphological and Functional Sciences, Section of Pathology, University of Naples "Federico II", Naples, Italy.

The authors have no conflicts of interest to disclose.

Reprints: Annamaria Staiano, MD, Department of Pediatrics, University "Federico II," Via S. Pansini, 5, 80131 Naples, Italy (e-mail: staiano@unina.it).

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1097/MIB.0b013e318281f469

Published online 2 April 2013.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

including symptoms, characteristics of stool, and physical examination, were computed. Additional information collected during the visits included demographic data, family history, and symptom onset. Physical examination was performed by a pediatrician and included an abdominal examination and evaluation for extraintestinal manifestations of UC. All children underwent ileocolonoscopy with mucosal biopsies by a videoendoscope after preparation by ingesting at least 2 L of an oral electrolyte lavage solution. Colonoscopy report consisted of segmental descriptions (rectum, sigmoid, descending, splenic flexure, hepatic flexure, transverse and ascending colon, and cecum), and each segment was independently assessed with regard to endoscopic and histologic grades of UC. From the information provided on the colonoscopy report, the extent of disease was described by the Paris classification: E0 for absence of disease, E1 for sigmoid colon/rectum involvement (proctitis), E2 for descending colon distal to splenic flexure involvement, E3 for extensive disease distally to hepatic flexure, and E4 for pancolitis.[25] All colonoscopies were performed by a single colonoscopist (E.M.). The base of the appendiceal orifice, the focus of the study, was observed and categorized as either positive (group A) or negative (group B) according to the presence or absence, respectively, of macroscopic involvement by UC. Appendiceal involvement was characterized colonoscopically by reddish and friable mucosa with mucinose exudates (Fig. 1). Colonoscopic grade of inflammation was determined by use of Mayo endoscopic score: 0 for normal or inactive disease, 1 for mild disease (erythema, decreased vascular pattern, mild friability), 2 for moderate disease (marked erythema, absent vascular pattern, friability erosions), and 3 for severe disease (spontaneous bleeding, ulceration).[26] Colonoscopy report system required segmental descriptions (rectum, sigmoid, descending, transverse and ascending colon, and cecum). From the information provided on the colonoscopy report, for each segment of the colon was designated a score, and a mean score for that colonoscopy was derived.

At least 2 biopsy specimens were obtained from each segment with an endoscopic forceps regardless of the endoscopic findings and processed for histologic assessment, stained with hematoxylin and eosin. Histologic grade of inflammation was determined by histopathologic assessment of the hematoxylin and eosin–stained biopsy sections (Fig. 1). Each specimen was graded by an expert pathologist (M.D.) who was masked to clinical features and endoscopic findings. The degree of inflammatory infiltrate and tissue destruction was graded according to Zhong's score as follows: 0, no neutrophilic leukocyte infiltration in lamina propria; 1, a small number of neutrophilic leukocytes (<10/high power field) in lamina propria with minimal infiltration of crypts; 2, prominent neutrophilic leukocytes (10–50/high power field) in lamina propria with infiltration of more than 50% of crypts; 3, a large number of neutrophilic leukocytes (>50/high power field) in lamina propria with crypt abscesses; and 4, significant acute inflammation with ulcerations in lamina propria.[27] Backwash ileitis was not observed in any of the specimens of children with UC.

All patients underwent abdominal ultrasound, 40 of 50 patients (80%) underwent upper gastrointestinal (GI) endoscopy, and 21 of 50 patients (42%) underwent either an abdominal magnetic resonance imaging or a small bowel follow-through. Moreover, for each patient, we considered the therapy at the time of index colonoscopy.

Statistical analysis was performed using SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). The Student's t test was used for normally distributed variables, and the Mann–Whitney U test and the χ^2 and Fisher's exact tests for categorical variables were used where appropriate. Written informed consent was obtained from participants' parents, and assent was obtained for all patients older than 10 years. The study was approved by the Institutional Review Board of the University of Naples "Federico II."

RESULTS

An involvement at the appendiceal orifice was found in 16 of 50 patients (32%, group A), whereas it was not present in the remaining 34 patients (68%, group B). Clinical characteristics of our patients and clinical activity, according to the pediatric ulcerative colitis activity index score, are described in Table 1.

We found that among the 2 groups, PAI was more frequent in patients with new diagnosis than in those with pre-existing UC ($P =$

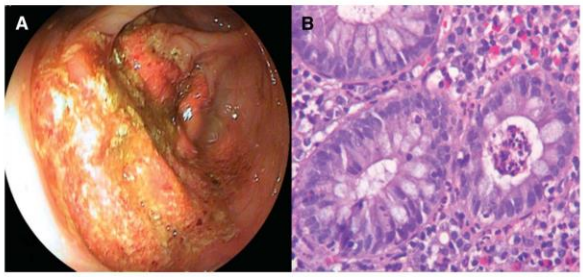


FIGURE 1. Typical PAI endoscopically (A) and histologically (B) found in one patient. A, Reddish and friable mucosa with mucinose exudate. B, Colonic mucosa with active inflammatory infiltrate in the lamina propria, cryptitis, and crypt microabscess.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

TABLE 1. Demographic and Clinic Characteristics in 50 Children with UC

Characteristics	Group A	Group B	*P*
Patients (%)	16 (32)	34 (68)	
Sex, n (%)			
Male	6 (37)	16 (47)	
Female	10 (62)	18 (53)	0.5[a]
Mean age (range), yr	13.1 (6–18)	14.3 (6–20)	0.2[b]
Duration of disease (mean ±SD, yr)	2.9 ± 2.9	4.2 ± 2.9	0.1[b]
Medical therapy, n (%)	12 (75)	31 (91)	0.7[a]
Aminosalycilates	11/12 (91)	27/31 (87)	0.5[c]
Immunosuppressant	12/12 (100)	16/31 (52)	0.001[c]
PUCAI, n (%)			
Remission	8 (50)	20 (59)	0.3[d]
Mild	2 (12)	4 (11)	
Moderate	6 (37)	6 (17)	
Severe	0 (0)	4 (11)	

[a]Calculated by chi-square test.
[b]Calculated by *T*-test.
[c]Calculated by Fisher's exact test.
[d]Calculated by Mann–Whitney *U* test.
PUCAI, pediatric ulcerative colitis activity index.

0.016), with an incidence of new diagnosis of 5 of 16 (31%) in group A and 3 of 34 (9%) in group B. The mean duration of UC from onset of symptoms until the index colonoscopy was not significantly ($P = 0.12$) different between the 2 groups as reported in Table 1.

At index colonoscopy, according to the Paris classification and the Mayo endoscopic score, an extensive disease and an higher level of inflammation were significantly more frequent in group A than in group B, as reported in Table 2 ($P = 0.013$; $P = 0.03$, respectively).

TABLE 2. Endoscopic Characteristics in 50 Children with UC

Characteristic	Group A	Group B	*P*
Paris classification			
E0	3 (19)	13 (38)	0.01[a]
E1	2 (12)	14 (41)	
E2	5 (31)	1 (3)	
E3	6 (37)	6 (18)	
Mayo endoscopic score			
0	3 (19)	13 (38)	0.03[a]
1	4 (25)	14 (41)	
2	8 (50)	5 (15)	
3	1 (6)	2 (6)	

Values are represented as n (%).
[a]Calculated by Mann–Whitney *U* test.

According to the Zhong's score, the histologic grade of inflammation at the ascending colon was significantly ($P = 0.014$) higher in group A than in group B (mean score: 0.75 versus 0.14). However, there was no statistically significant difference concerning the histologic grade of inflammation for the remaining colonic segments, as shown in Figure 2.

Forty of our 50 patients underwent upper GI endoscopy to exclude upper GI involvement. In these patients, there were no macroscopic and microscopic abnormalities at the level of both esophagus and duodenum. Five of 40 patients (12%) had a macroscopically abnormal-appearing stomach, and 9 of 40 patients (22%) had histologically gastric abnormalities. Seven of these 9 patients showed a diffuse, no specific gastritis containing mixed inflammatory cells infiltrate without giant cells or granulomas, whereas the remaining 2 patients had a chronic active gastritis, positive for *Helicobacter pylori* infection. No one of our patients showed a focal active gastritis, and no patient showed small bowel alterations by ultrasound, magnetic resonance imaging, and/or small bowel follow-through.

Concerning medical treatment, 5 patients (31%) in group A did not receive any therapy being newly diagnosed, 10 patients (62.5%) received aminosalicylates associated with an immunosuppressant, and 1 patient (6.2%) received immunosuppressant alone. In group B, 3 of 34 patients (9%) were at new diagnosis, and among the remaining 31 patients, 27 (87%) were treated with aminosalicylates, 12 (35.3%) of which associated with immunosuppressant, and 4 of 31 patients (13%) with immunosuppressant only. The use of therapy did not show significant differences between groups A and B ($P = 0.723$). However, when we considered individual treatment, we found that in patients with PAI (group A), the use of immunosuppressant was significantly higher than in UC children without PAI (group B) (Table 1).

DISCUSSION

In this study, we found that PAI was endoscopically present in 32% of pediatric patients affected by UC not extending beyond the hepatic flexure. PAI was more frequent in children with new diagnosis of UC than in patients with pre-existing UC.

Only one pediatric study[23] looked at PAI involvement in children who required colonic resection. Forty-one children with inflammatory bowel disease (24 consistent with Crohn's disease and 17 with UC) underwent intestinal resection because of failure of medical therapy. Of 17 specimens of children affected by UC, all appendices were abnormal. However, this study was conducted using colectomy specimens. Therefore, the reported higher prevalence of the appendiceal involvement might reflect the severity and extent of the disease. In the colectomy specimen, it is more likely to have pancolitis than patients in this study, where it was limited to hepatic flexure.

We excluded from our study those children affected by involvement of the ascending colon and/or pancolitis because it would have been difficult to evaluate the PAI. It is well known that the proximal margin of inflammation in UC may not be sharply

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

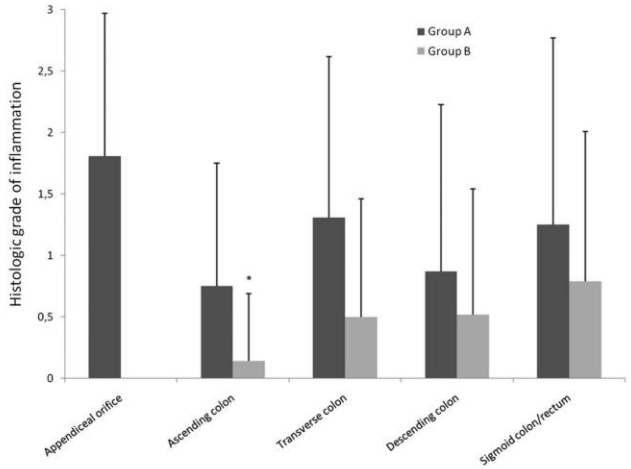


FIGURE 2. Comparison of histologic grade of inflammation by colonic site between Group A and Group B. There is a significant difference at the ascending colon (*$P = 0.014$).

demarcated, and this may contribute to falsely report the presence of PAI in children with involvement of the ascending colon.[18,28]

In our study PAI seems to be related to the extent of disease. Yang et al[21], in their study on adults patients affected by UC, reported that the involvement at the appendiceal orifice was not related to the duration of disease and it was more frequently observed in patients with less extensive disease. We can speculate that in pediatric patients, the disease is more extensive as reported by Van Limbergen et al,[29] who defined the phenotypic characteristics of pediatric UC. They found that children were more often affected by pancolitis and suggested a more severe phenotype in children than in adults. In our population, even if we ruled out children with pancolitis, we found that immunosuppressive use was significantly more common in children with PAI. This, in agreement with Van Limbergen's finding, suggests a more severe pediatric UC clinical picture, irrespective of the presence of pancolitis.[29] Time from diagnosis to the first surgery in UC is significantly shorter in children than in adults: "of the patients who underwent colectomy, surgery happened within 10 years in 40% of children and only in 20% of adult-onset UC patients."[29]

We also found that clinical activity and the use of medical therapy were not involved in PAI. Matsumoto et al[20] have shown that there is no difference between adult patients affected by UC with PAI and those without PAI, for that concerns clinical activity and duration of disease as well, as in our study. However, PAI, in our patients, was significantly more frequent in newly diagnosed children. Whether this finding is related to the efficacy of the medical treatment or to the natural history of pediatric UC needs to be further evaluated.

At index colonoscopy, we found that the histologic grade of inflammation at the ascending colon was statistically higher in children with PAI. This higher grade of inflammation suggests that PAI may be representative of histologically active disease in the endoscopically unaffected proximal colon, although this involvement does not seem to contribute to clinical activity. Evidently, colonoscopy alone is unreliable for determining the extent of UC.[30] The higher histologic grade in the group A in the ascending colon suggests that the PAI may be a hallmark for subclinical active disease, identified by histology. The histologic involvement at the ascending colon, even in the presence of macroscopically normal mucosa, suggests that the number of pancolitis in children is higher than observed only by endoscopy. Previous studies considered PAI only as an histologic feature because they were using colectomy specimens[16,23]; whereas more recently, with the implementation of GI endoscopy, PAI has been defined only endoscopically[20] or both endoscopically and histologically.[21] However, according to the unreliability of endoscopy for determining the extent of UC, we believe that it is more appropriate to assess PAI both histologically and endoscopically.

Crohn's disease was excluded on the basis of an absence of: classical macrosocpical lesions (e.g., skip lesions, snail track ulcers); no-caseating epithelioid and giant cell granulomas in any part of the GI tract; histologic findings of focal inflammation, submucosal or transmural inflammation, lymphocyte aggregates (without germinal centers), and mucous retention in the presence of more than minimal inflammation; fistulae and/or perianal abscesses; and typical stricturing small bowel disease on barium follow-through and/or magnetic resonance imaging.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

This study has some limitations. We did not include the findings on colonoscopy re-evaluation at follow-up because currently we do not have enough data. It is known that there is a little chance in the pediatric studies to scope all children as planned, in particular when they are on remission. In addition, we do not provide details on follow-up because its duration in most of the patient is too limited.

The importance of this study is that it is the first pediatric prospective study having the aim to define the features of UC with PAI that can be confused with Crohn's disease. In fact, the occasional patchy areas of mucosal inflammation often left to a change of diagnosis to Crohn's disease. Such a change in diagnosis from UC to Crohn's disease can affect management decision, especially in regard to surgery.[31]

In conclusion, to the best of our knowledge, this is the first pediatric study reporting the presence of PAI in children affected by UC not extending beyond the hepatic flexure. PAI seems to be more frequent in newly diagnosed children than in those with preexisting diagnosis of UC, related to the extension of the disease and to an higher grade of histologic inflammation at level of the ascending colon.

ACKNOWLEDGMENT

All authors contributed to write and revise the manuscript.

REFERENCES

1. Pera A, Bellando P, Caldera D, et al. Colonoscopy in inflammatory bowel disease: diagnostic accuracy and proposal of an endoscopic score. *Gastroenterology*. 1987;92:181–185.
2. Yamagishi N, Iizuka B, Nakamura T, et al. Clinical and colonoscopic investigation of skipped periappendiceal lesions in ulcerative colitis. *Scand J Gastroenterol.* 2002;37:177–182.
3. Lumb G, Protheroe RHB. Ulcerative colitis: a pathologic study of 152 surgical specimens. *Gastroenterology*. 1958;34:381–407.
4. Pannaccione R, Sandborn WJ. The appendix in ulcerative colitis: a not so innocent bystander. *Gastroenterology*. 1999;117:272–273.
5. Rutgeerts P, D' haens G, Hiele M, et al. Appendectomy protects against ulcerative colitis. *Gastroenterology*. 1994;106:1251–1253.
6. Russel MG, Dorant E, Brummer RJM, et al. Appendectomy and the risk of developing ulcerative colitis or Crohn' s disease: results of a large case-control study. *Gastroenterology*. 1997;113:377–382.
7. Andersson RE, Olaison G, Tysk C, et al. Appendectomy and protection against ulcerative colitis. *N Engl J Med.* 2001;344:808–814.
8. Radford-Smith GL, Edwards JE, Purdie DM, et al. Protective role of appendicectomy on onset and severity of ulcerative colitis and Crohn' s disease. *Gut*. 2002;51:808–813.
9. Selby WS, Griffin S, Abraham N, et al. Appendectomy protects against the development of ulcerative colitis but no affects its course. *Am J Gastroenterol.* 2002;97:2834–2838.
10. Cosnes J, Carbonnel F, Beaugerie L, et al. Effects of appendicectomy on the course of ulcerative colitis. *Gut*. 2002;51:803–807.
11. Naganuma M, Iizuka B, Torii A, et al. Appendectomy protects against the development of ulcerative colitis and reduces its recurrence: results of a multicenter case-controlled study in Japan. *Am J Gastroenterol.* 2001; 96:1123–1126.
12. Louis E, Collard A, Oger AF, et al. Behaviour of Crohn' s disease according to the Vienna classification: changing pattern over the course of disease. *Gut*. 2001;49:772–782.
13. Cosnes J, Cattan S, Blain A, et al. Long term evolution of disease behaviour of Crohn's disease. *Inflamm Bowel Dis.* 2002;8:244–2500.
14. Meucci G, Vecchi M, Astegiano M, et al. The natural history of ulcerative proctitis: a multicenter, retrospective study. *Am J Gastroenterol.* 2000;95: 469–473.
15. Etchevers MJ, Aceituno M, Garcia-Bosch O, et al. Risk factors and characteristics of extent progression in ulcerative colitis. *Inflamm Bowel Dis.* 2009;15:1320–1325.
16. Berstein CN, Shanahan F, Anton PA, et al. Patchiness of mucosal inflammation in treated ulcerative colitis: a prospective study. *Gastrointest Endosc.* 1995;42:232–237.
17. Markowitz J, Kahn E, Grancher K, et al. Atypical rectosigmoid histology in children with newly diagnosed ulcerative colitis. *Am J Gastroenterol.* 1993;88:2034–2037.
18. D' Haens G, Geboes K, Peeters M, et al. Patchy cecal inflammation associated with distal ulcerative colitis: a prospective endoscopic study. *Am J Gastroenterol.* 1997;92:1275–1279.
19. Takizawa H, Koike M, Hirose S, et al. Appendiceal involvement in patients with ulcerative colitis. *Digest Endosc.* 1997;9:217–221.
20. Matsumoto T, Nakamura S, Shimizu M, et al. Significance of appendiceal involvement in patients with ulcerative colitis. *Gastrointest Endosc.* 2002; 55:180–185.
21. Yang SK, Jung HY, Kang GH, et al. Appendiceal orifice inflammation as a skip lesion in ulcerative colitis: an analysis in relation to medical therapy and disease extent. *Gastrointest Endosc.* 1999;49:743–747.
22. Ladefiged K, Munck L.K, Jorgensen F, et al. Skip inflammation of the appediceal orifice: a prospective endoscopic study. *Scand J Gastroenterol.* 2005;40:1192–1196.
23. Kahn E, Markowitz J, Daum F. The appendix in inflammatory bowel disease in children. *Mod Pathol.* 1992;5:380–383.
24. Turner D, Otley AR, Mack D, et al. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology*. 2007;133:423–432.
25. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: The Paris Classification. *Inflamm Bowel Dis.* 2011;17:1314–1321.
26. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med.* 1987;317:1625–1629.
27. Zhong Y-Q, Huang H-R, Zhu Z-H, et al. Effects of sulfasalazine on biopsy mucosal pathologies and histological grading of patients with active ulcerative colitis. *World J Gastroenterol.* 2005;11:4435–4438.
28. Riley SA, Mani V, Goodman MJ, et al. Microscopic activity in ulcerative colitis: report of a case. *Am J Gastroenterol.* 1974;62:151–155.
29. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology*. 2008;135:1114–1122.
30. Moum B, Ekbom A, Vatn MH, et al. Changes in the extent of colonoscopic and histologic involvement in ulcerative colitis over time. *Am J Gastroenterol.* 1999;94:1564–1569.
31. Fozard BJ, Pemberton JH. Results of pouch surgery after ileoanal anostomosis: the implication of pouchitis. *World J Surg.* 1992;16: 880–884.

Copyright © 2013 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

4.4 References

1. Bernstein CN, Blanchard JF, Rawsthorne P, et al. The prevalence of extraintestinal diseases in inflammatory bowel disease: a population-based study. American Journal of Gastroenterology 2001;96:1116–22.
2. Timmer A, Behrens R, Buderus S, et al., CEDATA-GPGE Study Group. Childhood onset inflammatory bowel disease: predictors of delayed diagnosis from the CEDATA German-language paediatric inflammatory bowel disease registry. Journal of Pediatrics 2011;158:467–73.
3. Ricart E, Panaccione R, Loftus Jr EV, et al. Autoimmune disorders and extraintestinal manifestations in first-degree familial and sporadic inflammatory bowel disease: a case–control study. Inflammatory Bowel Diseases 2004;10:207–14.
4. Bernstein CN, Wajda A, Blanchard JF. The clustering of other chronic inflammatory diseases in inflammatory bowel disease: a population-based study. Gastroenterology 2005;129:827–36.
5. Navaneethan U, Shen B. Hepatopancreatobiliary manifestations and complications associated with inflammatory bowel disease. Inflammatory Bowel Diseases 2010;16:1598–619.
6. Heikius B, Niemelä S, Lehtola J, et al. Elevated pancreatic enzymes in inflammatory bowel disease are associated with extensive disease. American Journal of Gastroenterology 1999;94:1062–9.
7. Broide E, Dotan I, Weiss B, et al. Idiopathic pancreatitis preceding the diagnosis of inflammatory bowel disease is more frequent in paediatric patients. Journalof Pediatric Gastroenterology and Nutrition 2011;52:714–7.
8. Stawarski A, Iwanczak F. Incidence of acute pancreatitis in children with inflammatory bowel disease. Polski Merkuriusz Lekarski 2004;17:33–6.
9. Herrlinger KR, Stange EF. The pancreas and inflammatory bowel diseases. International Journal of Pancreatology 2000;27:171–9.
10. de Bie CI, Paerregaard A, Kolacek S, et al., EUROKIDS Porto IBD Working Group of ESPGHAN. Disease phenotype at diagnosis in paediatric Crohn's disease: 5-year analyses of the EUROKIDS Registry. Inflammatory Bowel Diseases 2013;19:378–85.
11. Pascarella F, Martinelli M, Miele E, et al. Impact of Clostridium difficileinfection on pediatric inflammatory bowel disease. J Pediatr. 2009;154:854–858.

12. Wultanska D, Banaszkiewicz A, Radzikowski A, et al. Clostridium difficile infection in Polish pediatric outpatients with inflammatory bowel disease. Eur J Clin Microbiol Infect Dis. 2010;29:1265–1270.
13. Mezoff E, Mann EA, Hart KW, et al. Clostridium difficile infection and treatment in the pediatric inflammatory bowel disease population. J Pediatr Gastroenterol Nutr. 2011;52:437–441.
14. Kelsen JR, Kim J, Latta D, et al. Recurrence rate of Clostridium difficileinfection in hospitalized pediatric patients with inflammatory bowel disease.Inflamm Bowel Dis. 2011;17:50–55.
15. Banaszkiewicz A, Kowalska-Duplaga K, Pytrus T, et al. Clostridiumdifficile infection in newly diagnosed pediatric patients with inflammatory bowel disease: prevalence and risk factors. Inflamm Bowel Dis. 2012;18:844–848.
16. Pant C, Anderson MP, Deshpande A, et al. Health care burden of Clostridiumdifficile infection in hospitalized children with inflammatory bowel disease. Inflamm Bowel Dis. 2013;19:1080–1085.
17. Khanna S, Baddour LM, Huskins WC, et al. The epidemiology of Clostridiumdifficile infection in children: a population-based study. Clin InfectDis. 2013;56:1401–1406.
18. Mezoff E, Mann EA, Hart KW, et al. Clostridium difficile infection and treatment in the pediatric inflammatory bowel disease population.J Pediatr Gastroenterol Nutr. 2011;52:437–441.
19. Kelsen JR, Kim J, Latta D, et al. Recurrence rate of Clostridium difficileinfection in hospitalized pediatric patients with inflammatory bowel disease. Inflamm Bowel Dis. 2011;17:50–55.
20. Ananthakrishnan AN. Detecting and treating Clostridium difficile infectionsin patients with inflammatory bowel disease. Gastroenterol ClinNorth Am. 2012;41:339–353.
21. Pera A, Bellando P, Caldera D, et al. Colonoscopy in inflammatory boweldisease: diagnostic accuracy and proposal of an endoscopic score. Gastroenterology.1987;92:181–185.
22. Lumb G, Protheroe RHB. Ulcerative colitis: a pathologic study of 152surgical specimens. Gastroenterology. 1958;34:381–407.
23. Matsumoto T, Nakamura S, Shimizu M, et al. Significance of appendiceal involvement in patients with ulcerative colitis. Gastrointest Endosc. 2002;55:180–185.Yang SK, Jung HY, Kang GH, et al. Appendiceal orifice inflammation asa skip lesion in ulcerative colitis: an

analysis in relation to medical therapyand disease extent. Gastrointest Endosc. 1999;49:743–747.

24. Ladefiged K, Munck L.K, Jorgensen F, et al. Skip inflammation of theappediceal orifice: a prospective endoscopic study. Scand J Gastroenterol.2005;40:1192–1196.
25. Kahn E, Markowitz J, Daum F. The appendix in inflammatory boweldisease in children. Mod Pathol. 1992;5:380–383.
26. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. Gastroenterology. 2008;135:1114–1122.
27. Yamagishi N, Iizuka B, Nakamura T, et al. Clinical and colonoscopic investigation of skipped periappendiceal lesions in ulcerative colitis.Scand J Gastroenterol. 2002;37:177–182.
28. Moum B, Ekbom A, Vatn MH, et al. Changes in the extent of colonoscopicand histologic involvement in ulcerative colitis over time. Am JGastroenterol. 1999;94:1564–1569.
29. Fozard BJ, Pemberton JH. Results of pouch surgery after ileoanalanostomosis: the implication of pouchitis. World J Surg. 1992;16:880–884.

Chapter 5

5. New therapeutic strategies in inflammatory bowel disease.

5.1 Bifidobacteria Enhance Antigen Sampling and Processing by Dendritic Cells in Pediatric Inflammatory Bowel Diseas

In the last years, probiotics have bee tested as treatment agents for induction of remission in IBD, however the outcome of their use in clinical practice has not always been so encouraging[1].

The Bifidobacteriahave been largely studied among the probiotic species for their specific immunoregulatory effect on epithelial cells, lymphocytes and, above all, on DCs[2].

A recent study conducted in patients with IBD indicated that oral administration of Bifidobacterium sub- species have the capability of reducing inflammation in UC, through the increase of regulatory T cells, and reduction in the serum level of inflammatory markers, such as tumor necrosis factor (TNF)-α, IL-6, and C-reactive protein.[3] However, no experimental studies assessing the role of Bifidobacterium strains on DC maturation and functionality are available in either adult or pediatric IBD patients. In the current study, we aimed to analyze the effects of a mixture of 3 Bifidobacterium strains (B. longum, B. breve, B. infantis), on the phenotype and on antigen processing of monocyte-derived DCs in a pediatric cohort with IBD.

In this study, we demonstrate that a commercial probiotic mixture (Tribif) composed by 3 Bifidobacteria species (B. longum, B. breve, and B. infantis) is able to ameliorate the impaired ability of antigen sampling and processing by DC from pediatric patients with CD. We found a marked reduction in the uptake of cellular fragments from the Enterobacteria E. coli by DC from children with CD, compared with both DC from either UC or non-IBD children. DCs from patients with CD were also impaired in processing soluble antigens, as shown by reduced intracellular degradation of the dietary protein ovalbumin. These results confirmed a defect of particulate antigen sampling in specialized antigen presenting cells from patients with CD, likely due to an impairment of autophagy, as we previously reported[4].

Interestingly, the probiotic mixture had no significant effect on the antigen uptake in DC from patients with UC, in which the autophagy functionality is not impaired as in CD. This difference in antigen handling is probably due to the different molecular pathway at the base of the pathogen-esis of these 2 intestinal diseases[5].

The beneficial effect on DC functionality reported in our study is a specific property of the probiotics, since the Enterobacterium Salmonella had an opposite effect, reducing significantly the

sampling efficiency of the DC. Moreover, we found that Tribif ameliorate the antigen phagocytosis and processing without enhancing the DC maturation or inducing a cytokine production. In particular, the proinflammatory TNF-α, which was significantly enhanced on the stimulation of particulate antigens from the Enterobacterium E. coli, was not induced in DC cell supernatant after the treatment with the BifidobacteriaTribif. This finding indicates that DC from children with CD are sensitized to produce TNF-α, an important player in CD pathogenesis, in response to specific bacteria strains[5].Most current probiotics are marketed as foods or supplements that improve gut health and were developed based on ease of production or stability, rather than based on specific mechanisms of disease prevention or treatment. Many commercial probiotics have limited purity and viability making difficult conclusions about efficacy in the treatment or prevention of disease[6]. "Second generation probiotics" may be a useful term to describe probiotics selected based on established mechanisms of prevention or treatment of a specific disease. Bifidobacteria would fit in this category, suggesting mechanistic advantages compared with other probiotics.[7]Studying Bifidobacteria and other probiotics for metabolites and ligands that modulate the host immune function will likely lead to a new class of immunotherapeutic agents for inflammatory states.

Bifidobacteria Enhance Antigen Sampling and Processing by Dendritic Cells in Pediatric Inflammatory Bowel Disease

Caterina Strisciuglio, MD, Erasmo Miele, MD, PhD,* Francesca P. Giugliano, MD,* Serena Vitale, Biol Sc,*† Marialuisa Andreozzi, MD,* Alessandra Vitale, Biol Sc,* Maria R. Catania, Biol Sc,‡ Annamaria Staiano, MD,* Riccardo Troncone, MD,* and Carmen Gianfrani, Biol Sc*†*

Abstract: Bifidobacteria have been reported to reduce inflammation and contribute to intestinal homeostasis. However, the interaction between these bacteria and the gut immune system remains largely unknown. Because of the central role played by dendritic cells (DCs) in immune responses, we examined in vitro the effects of a Bifidobacteria mixture (probiotic) on DC functionality from children with inflammatory bowel disease. DCs obtained from peripheral blood monocytes of patients with Crohn's disease (CD), ulcerative colitis, and noninflammatory bowel disease controls (HC) were incubated with fluorochrome-conjugated particles of *Escherichia coli* or DQ-Ovalbumin (DQ-OVA) after a pretreatment with the probiotic, to evaluate DC phenotype, antigen sampling and processing. Moreover, cell supernatants were collected to measure tumor necrosis factor alpha, interferon gamma, interleukin 17, and interleukin 10 production by enzyme-linked immunosorbent assay. DCs from CD children showed a higher bacteria particles uptake and DQ-OVA processing after incubation with the probiotic; in contrast, DC from both ulcerative colitis and HC showed no significant changes. Moreover, a marked tumor necrosis factor alpha release was observed in DC from CD after exposure to *E. coli* particles, whereas the probiotic did not affect the production of this proinflammatory cytokine. In conclusion, the Bifidobacteria significantly improved the antigen uptake and processing by DCs from patients with CD, which are known to present an impaired autophagic functionality, whereas, in DCs from ulcerative colitis and HC, no prominent effect of probiotic mixture was observed. This improvement of antigen sampling and processing could partially solve the impairment of intestinal innate immunity and reduce uncontrolled microorganism growth in the intestine of children with inflammatory bowel disease.

(Inflamm Bowel Dis 2015;21:1491–1498)

Key Words: dendritic cells, inflammatory bowel diseases, probiotics

The spectrum of inflammatory bowel disease (IBD) is represented by Crohn's disease (CD) and ulcerative colitis (UC), which are chronic and relapsing disorders. CD can affect, in a discontinuous way, any segment of alimentary canal, although involving mainly the distal ileum and colon. Instead, inflammation associated with UC is continuous and confined within the colon extending from the rectum proximally along the large bowel. Although the etiopathogenesis of IBD is still unknown, it has been suggested that the interaction of environmental factors and host immune response with genetic individual susceptibility play an important role.[1] Both human and animal studies have demonstrated the central role of intestinal microbiota in flaring up and perpetuating inflammation in patients with IBD.[1] Gut microbiota is considered a "metabolic organ" getting energy from nutrients escaped from the gastrointestinal (GI) absorption. Together with gut mucosal barrier, the microbiota contributes to intestinal immune homeostasis and protection against pathogen invasion.[2] IBD are associated with significant shifts in the composition of the normal enteric microbiota compared with control subjects, with an almost depletion of Lactobacillus and Bifidobacterium,[3] normally present in healthy intestinal conditions, and increase of harmful species, such as Proteobacteria and Actinobacteria.[4] This kind of dysbiosis might determine in the host organism the loss of tolerance toward microbes and induce an inappropriate inflammation. In the past years, probiotics have been successfully studied as prophylaxis therapy for postoperative infections[5] and as adjuvants during broad-spectrum antibiotic treatment.[6] However, when tested as treatment agents for induction of remission in IBD, the outcome of their use in clinical practice has not always been so encouraging.[7] There are few evidences of benefit by probiotics in CD either in adult or in pediatric populations, whereas in UC, only VSL#3, a high-concentrated probiotic mixture, has been effective as adjuvant therapy.[8]

Received for publication December 10, 2014; Accepted February 4, 2015.

From the *Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Naples, Italy; †Department of Biomedicine, Institute of Protein Biochemistry, CNR, Naples, Italy; and ‡Department of Molecular Medicine and Medical Biotechnology, University of Naples "Federico II," Naples, Italy.

Supported by Valeas S.P.A. (Milan, Italy).

A. Staiano is a speaker for Valeas S.P.A. (Milan, Italy), Angelini, Menarini, Milte S.P.A.; and consultant for DMG (Italy). The remaining authors have no conflicts of interest to disclose.

C. Strisciuglio, E. Miele, and F. P. Giugliano have contributed equally to this study.

Reprints: Annamaria Staiano, MD, Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II," Via S. Pansini, 5, 80131 Naples, Italy (e-mail: staiano@unina.it).

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1097/MIB.0000000000000389

Published online 17 April 2015.

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Dendritic cells (DCs) may play a determinant role in IBD pathogenesis, as they prime T-cell responses against bacteria, and induce proinflammatory cytokine release by intestinal mucosa T cells.[9] In the past years, the Bifidobacteria have been largely studied among the probiotic species for their specific immunoregulatory effect on epithelial cells, lymphocytes and, above all, on DCs.[10] Hoarau et al found that the supernatant of a *Bifidobacterium breve* strain (BbC50) culture can induce prolonged DC survival through an upregulated maturation and interleukin (IL)-10 production, suggesting a regulatory role of this probiotic on inflammation.[11] Moreover, Lopez et al[12] found differences in the immunomodulatory activity on T-helper-mediated response, which were strictly dependent on the specific probiotic strain. A recent study conducted in patients with IBD indicated that oral administration of Bifidobacterium subspecies have the capability of reducing inflammation in UC, through the increase of regulatory T cells, and reduction in the serum level of inflammatory markers, such as tumor necrosis factor (TNF)-α, IL-6, and C-reactive protein.[13] However, no experimental studies assessing the role of Bifidobacterium strains on DC maturation and functionality are available in either adult or pediatric IBD patients. In the current study, we aimed to analyze the effects of a mixture of 3 Bifidobacterium strains (*B. longum*, *B. breve*, *B. infantis*), on the phenotype and on antigen processing of monocyte-derived DCs in a pediatric cohort with IBD.

MATERIALS AND METHODS

Study Population

The study population included a total of 25 young subjects (mean age, 13 yr; range, 1–18 yr) stratified into 3 groups on the basis of the clinical diagnosis: 12 were affected by CD, 7 affected by UC, and 6 were non-IBD controls (HC). The 12 patients with CD had been genotyped for the ATG16L1 polymorphism in a previous study[14]; of these, 7 were GG homozygous (risk genotype), 4 were AG heterozygous, and 1 was AA homozygous (wild type). HC were subjects affected by functional GI disorders, who performed blood analysis to exclude any organic diseases and an inflammatory condition. The diagnosis of CD and UC was based on clinical, endoscopic, radiologic, and histopathologic criteria.[15] Demographic and clinical characteristics of enrolled subjects are described in Table 1. For the IBD diagnosis, all children underwent ileocolonoscopy, upper GI endoscopy, and imaging studies, including abdominal ultrasound and entero magnetic resonance imaging, or a small bowel follow-through. For each patient, clinical activity of disease was evaluated at the time of diagnosis using the Pediatric Crohn's Disease and the Pediatric Ulcerative Colitis Activity Index.[16,17] Written informed consents were obtained from participants' parents and from patients themselves if older than 10 years. The study was approved by the Institutional Review Board of the University of Naples "Federico II."

TABLE 1. Clinical Features of 25 Pediatric Patients Enrolled

Characteristics	CD	UC	HC
Patients, n	12	7	6
Gender, male/female	6/6	2/5	4/2
Mean age (range), yr	13.9 (10–18)	15 (11–18)	10.16 (8–17)
Mean age at diagnosis (range), yr	10.6 (3–18)	13.8 (3–13)	—
Mean disease duration (range), mo	45 (12–84)	90.8 (12–168)	—
Therapy, n			
Aminosalicylates	6	7	—
Immunosuppressants	4	0	—
Aminosalicylates and immunosuppressants	2	0	—

DC Generation and Stimulation

DCs were generated from peripheral blood mononuclear cells according to a well-established procedure as previously described.[18] Briefly, adherent monocytes were cultured at 2×10^6/mL in 24-well plate (Starstedt, Numbrecht, Germany) for 6 days in complete culture medium (RPMI supplied with penicillin/streptomycin, nonessential amino acids, and 10% fetal calf serum), in the presence of recombinant human GM-CSF and IL-4 (both at 100 ng/mL; R&D Systems, Inc., Minneapolis, MN).

Stimulation of Monocyte-derived DCs with Bifidobacteria Tribif

After 6 days of incubation, DCs were resuspended at 3 to 5×10^5/mL in 48-well flat bottom (Sarstedt) in complete culture medium. To examine the effects of Bifidobacterium on DC maturation, cell cultures were incubated in the presence of the probiotic mixture Tribif (Valeas s.p.a, Milan, Italy), composed of 3 different Bifidobacterium strains, *B. longum, B. breve,* and *B. infantis*. Live probiotics were reconstituted from lyophilized samples and used as stimuli at a bacteria:DC ratio of 1:50/100 for 24 hours of incubation. Subsequently, to study the uptake of particulate antigens, the DCs were treated with 20 μg/mL of Alexa-488-labeled *E. coli* bioparticles (hereafter indicated as bacteria particles; Invitrogen, Carlsbad, CA), or left untreated, as a negative control. After 2 hours of incubation, supernatants from these cultures were collected, clarified by centrifugation, and stored at −20°C for cytokine analysis, whereas DCs were harvested for phenotypic characterization. In the experiments evaluating antigen processing, DCs were incubated with DQ-Ovalbumin (DQ-OVA; Molecular Probes, Eugene, OR) at 90 μg/mL for 4 hours, as previously reported.[14] After antigen pulsing, cells were washed and analyzed by flow cytometry. In some experiments, DCs were preincubated for 24 hours with the pathogen bacteria Salmonella before adding the bacteria particles. Salmonella was cultured in XLD Agar medium (Xylose Lysine

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Desoxycholate Agar, Becton Dickinson) selective for gram-negative pathogen species and added at a final bacteria:DC ratio of 1:50/100 for 24 hours of incubation.

Cytokine Quantification in Culture Supernatants

TNF-α, interferon (IFN)-γ, and IL-17 were analyzed using commercially available sandwich enzyme-linked immunosorbent assay kits provided by Mabtech (Nacka Strand, Sweden) according to the manufacturer's instructions. For IL-10 detection, a sandwich enzyme-linked immunosorbent assay was performed using specific monoclonal Ab purchased from Mabtech. The sensitivity of each enzyme-linked immunosorbent assay was TNF-α: 12 pg/mL; IFN-γ: 2 pg/mL; IL-17: 2 pg/mL, and IL-10: 2 pg/mL.

Flow Cytometry and Confocal Microscopy

Fluorochrome-conjugated anti-CD86, anti-CD11c, anti-HLA-DR, and isotype control antibodies were obtained from BD Biosciences (San Jose, CA) or Miltenyi Biotec (Bologna, Italy). Cells were washed and stained in phosphate buffered saline per 1% bovine serum albumin and analyzed at FACSCalibur (Becton Dickenson, Franklin Lakes, NJ), using the CellQuest software. For confocal microscopy analysis of the bacteria particles uptake, DCs previously stained for flow cytometry analysis were collected. Cells were adhered to poly-L-lysine-coated coverslips (Sigma-Aldrich), fixed in 4% paraformaldehyde, and embedded in SlowFade Gold (Invitrogen). Images were obtained on an LSM Zeiss 510 confocal system equipped with 405 nm ultraviolet, 488 nm argon, and 543 HeNe lasers (ZEISS Germany) and processed using AIS Zeiss software.

Statistical Analysis

Statistical Analysis was performed using SPSS software package for Windows (version 13.0; SPSS, Chicago, IL). The χ^2 and Fisher's exact tests were used for categorical variables, the Student's *t* test, the Mann–Whitney U test, and the Wilcoxon test for normally distributed variables. Differences were considered to be statistically significant when the *P* value was <0.05.

RESULTS

Effect of Tribif on Bacterial Particle Uptake by DCs

We have previously shown a defective bacteria handling by DCs in pediatric CD with specific single nucleotide polymorphisms in autophagy-related genes. In the current study, we aimed to investigate whether the treatment with a commercial probiotic mixture (Tribif) can ameliorate sampling efficiency of the DCs obtained from pediatric IBD, either patients with CD or patients with UC. DCs were cultured from peripheral blood and defined as the HLA-DR and CD11c double-positive population. Bacterial particle uptake was evaluated as the mean fluorescence intensity measured in the gate of $HLA\text{-}DR^+CD11c^+$ cells obtained from each subject (Fig. 1A). Although we found that the great majority of DCs from all 3 children groups (CD, UC, and HC) were able to sample particulate antigens, the amount of fluorochrome-conjugated particles taken up was significantly lower in patients with CD compared with HC ($P = 0.04$) or UC (Fig. 1A, B). After the treatment with the probiotic mixture, the amount of particles taken up by DCs from CD group was significantly higher compared with basal level ($P = 0.01$). Interestingly, the 3 patients who showed the highest implementation of the bacteria sampling after the probiotic treatment carried the *ATG16L1* polymorphism T300A in homozygosis, found to be associated with a defective autophagy (Fig. 1B, C). By contrast, DCs from both UC and HC phagocyted bacteria particles more efficiently, even at basal level, compared with patients with CD and were not affected by probiotic mixture treatment. The effect of probiotic mixture on the uptake of bacteria particles was next evaluated at confocal microscopy. The images showed an increased intracellular bacteria particles staining in DCs from children with CD after probiotic mixture exposure, thus confirming the flow cytometry findings (Fig. 1C).

To evaluate whether the increased uptake observed in the DCs from CD was a specific effect of the probiotic mixture, we treated the DCs with the pathogen bacteria, Salmonella. Although, we found that the exposure to the Salmonella resulted in a marked reduction of DCs vitality, most likely due to a cell toxicity, the amount of particles taken up from the vital DCs was significantly lower compared with the level observed either at baseline, or after the probiotic mixture treatment (Fig. 1D).

Effect of Tribif on DC Maturation

To further examine whether the treatment with the probiotic mixture might affect the maturation of DCs, we determined the expression of activation markers HLA-DR and CD86 induced by bacterial particles, or by the probiotic mixture, used both as antigenic stimuli. The expression levels of HLA-DR in DCs did not substantially differ among the bacterial particles and probiotic mixture in any of the 3 groups. The CD86 expression increased in DCs from patients with both CD and UC after the bacterial particle stimulation, compared with unstimulated cells, although the increments did not reach a statistical significance. In contrast, no substantial variation of the membrane levels of CD86 was observed in IBD DCs after the probiotic treatment. The CD86 expression remained almost unchanged in DC from HC independently from the stimuli (Fig. 2A, B). Overall, these data indicate that the specific Bifidobacteria strains analyzed in this study had no substantial effect on DC maturation, at least at the experimental condition used in our study.

Effect of Tribif on DC Antigen Processing

The uptake of an antigen is the first step for its presentation to immune competent cells and is followed by processing that involves antigen degradation and peptide loading to HLA restriction molecules. To investigate whether the probiotic mixture might affect the processing of the antigens, as well as their uptake, we tested the ability of DCs to phagocytize and process DQ-ovalbumin, a dietary protein labeled with a quenched fluorochrome that becomes

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

Desoxycholate Agar, Becton Dickinson) selective for gram-negative pathogen species and added at a final bacteria:DC ratio of 1:50/100 for 24 hours of incubation.

Cytokine Quantification in Culture Supernatants

TNF-α, interferon (IFN)-γ, and IL-17 were analyzed using commercially available sandwich enzyme-linked immunosorbent assay kits provided by Mabtech (Nacka Strand, Sweden) according to the manufacturer's instructions. For IL-10 detection, a sandwich enzyme-linked immunosorbent assay was performed using specific monoclonal Ab purchased from Mabtech. The sensitivity of each enzyme-linked immunosorbent assay was TNF-α: 12 pg/mL; IFN-γ: 2 pg/mL; IL-17: 2 pg/mL, and IL-10: 2 pg/mL.

Flow Cytometry and Confocal Microscopy

Fluorochrome-conjugated anti-CD86, anti-CD11c, anti-HLA-DR, and isotype control antibodies were obtained from BD Biosciences (San Jose, CA) or Miltenyi Biotec (Bologna, Italy). Cells were washed and stained in phosphate buffered saline per 1% bovine serum albumin and analyzed at FACSCalibur (Becton Dickenson, Franklin Lakes, NJ), using the CellQuest software. For confocal microscopy analysis of the bacteria particles uptake, DCs previously stained for flow cytometry analysis were collected. Cells were adhered to poly-L-lysine-coated coverslips (Sigma-Aldrich), fixed in 4% paraformaldehyde, and embedded in SlowFade Gold (Invitrogen). Images were obtained on an LSM Zeiss 510 confocal system equipped with 405 nm ultraviolet, 488 nm argon, and 543 HeNe lasers (ZEISS Germany) and processed using AIS Zeiss software.

Statistical Analysis

Statistical Analysis was performed using SPSS software package for Windows (version 13.0; SPSS, Chicago, IL). The χ^2 and Fisher's exact tests were used for categorical variables, the Student's *t* test, the Mann–Whitney U test, and the Wilcoxon test for normally distributed variables. Differences were considered to be statistically significant when the *P* value was <0.05.

RESULTS

Effect of Tribif on Bacterial Particle Uptake by DCs

We have previously shown a defective bacteria handling by DCs in pediatric CD with specific single nucleotide polymorphisms in autophagy-related genes. In the current study, we aimed to investigate whether the treatment with a commercial probiotic mixture (Tribif) can ameliorate sampling efficiency of the DCs obtained from pediatric IBD, either patients with CD or patients with UC. DCs were cultured from peripheral blood and defined as the HLA-DR and CD11c double-positive population. Bacterial particle uptake was evaluated as the mean fluorescence intensity measured in the gate of $HLA\text{-}DR^+CD11c^+$ cells obtained from each subject (Fig. 1A). Although we found that the great majority of DCs from all 3 children groups (CD, UC, and HC) were able to sample particulate antigens, the amount of fluorochrome-conjugated particles taken up was significantly lower in patients with CD compared with HC ($P = 0.04$) or UC (Fig. 1A, B). After the treatment with the probiotic mixture, the amount of particles taken up by DCs from CD group was significantly higher compared with basal level ($P = 0.01$). Interestingly, the 3 patients who showed the highest implementation of the bacteria sampling after the probiotic treatment carried the *ATG16L1* polymorphism T300A in homozygosis, found to be associated with a defective autophagy (Fig. 1B, C). By contrast, DCs from both UC and HC phagocyted bacteria particles more efficiently, even at basal level, compared with patients with CD and were not affected by probiotic mixture treatment. The effect of probiotic mixture on the uptake of bacteria particles was next evaluated at confocal microscopy. The images showed an increased intracellular bacteria particles staining in DCs from children with CD after probiotic mixture exposure, thus confirming the flow cytometry findings (Fig. 1C).

To evaluate whether the increased uptake observed in the DCs from CD was a specific effect of the probiotic mixture, we treated the DCs with the pathogen bacteria, Salmonella. Although, we found that the exposure to the Salmonella resulted in a marked reduction of DCs vitality, most likely due to a cell toxicity, the amount of particles taken up from the vital DCs was significantly lower compared with the level observed either at baseline, or after the probiotic mixture treatment (Fig. 1D).

Effect of Tribif on DC Maturation

To further examine whether the treatment with the probiotic mixture might affect the maturation of DCs, we determined the expression of activation markers HLA-DR and CD86 induced by bacterial particles, or by the probiotic mixture, used both as antigenic stimuli. The expression levels of HLA-DR in DCs did not substantially differ among the bacterial particles and probiotic mixture in any of the 3 groups. The CD86 expression increased in DCs from patients with both CD and UC after the bacterial particle stimulation, compared with unstimulated cells, although the increments did not reach a statistical significance. In contrast, no substantial variation of the membrane levels of CD86 was observed in IBD DCs after the probiotic treatment. The CD86 expression remained almost unchanged in DC from HC independently from the stimuli (Fig. 2A, B). Overall, these data indicate that the specific Bifidobacteria strains analyzed in this study had no substantial effect on DC maturation, at least at the experimental condition used in our study.

Effect of Tribif on DC Antigen Processing

The uptake of an antigen is the first step for its presentation to immune competent cells and is followed by processing that involves antigen degradation and peptide loading to HLA restriction molecules. To investigate whether the probiotic mixture might affect the processing of the antigens, as well as their uptake, we tested the ability of DCs to phagocytize and process DQ-ovalbumin, a dietary protein labeled with a quenched fluorochrome that becomes

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

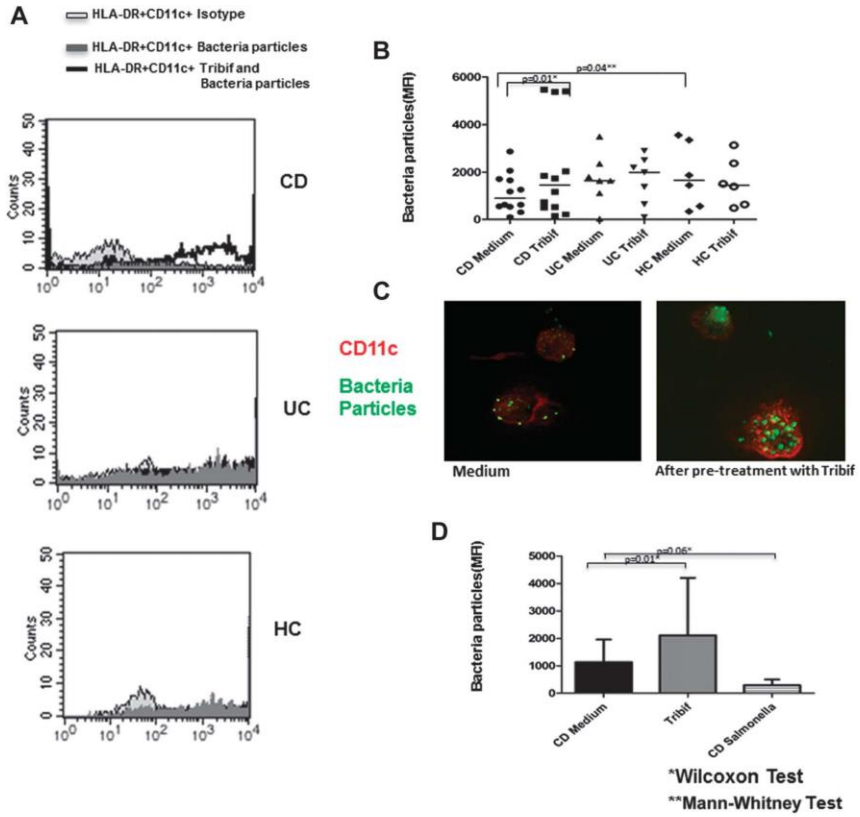


FIGURE 1. Bifidobacteria Tribif enhance uptake of *E. coli*-derived particles by DC from pediatric patients with CD; monocyte-derived dendritic cells were obtained from 12 pediatric patients affected by CD, 7 UC, and 6 non-IBD controls (HC). The DCs were incubated for 2 hours with Alexa-fluor 488-labeled *E. coli* particles after a pretreatment of 24 hours with live probiotics and soon after analyzed by flow cytometry or confocal microscopy. DCs are identified as HLA-DR$^+$CD11c$^+$ cells, and the analysis is performed in the double positive-gated cells. A, Mean fluorescence intensity (MFI) of bacteria particles staining in DCs of a representative patient from each group. B, Overall MFI results of bacteria particles staining in DCs from CD, UC, and HC children. Dashes indicate the median MFI value. C, Confocal images of DCs (CD11c$^+$ cells in red) after the uptake of Alexa-fluor 488-conjugated bacteria particles (green) from a representative patient with CD, before and after pretreatment with the probiotic mixture. D, Bacteria particles uptaken in DC from patients with CD after pretreatment with the probiotic Tribif or the pathogen Enterobacterium Salmonella. Data are shown as mean ± SD of MFI.

detectable only after proteolytic cutting in antigen presenting cells. In agreement to our previous observation, DCs from pediatric patients with CD showed an impairment of uptake and processing of soluble antigen, as DQ-OVA.[14] In fact, the intensity of DQ-OVA stained cells from children with CD resulted markedly reduced compared with both UC and HC groups ($P = 0.01$). Interestingly, a more efficient DQ-OVA processing was observed in DCs from children with CD after the treatment with the probiotic mixture ($P = 0.01$), whereas Tribif had no significant effect in DQ-OVA degradation in the other 2 groups of patients (Fig. 3A, B).

Effect of Tribif on DC Cytokine Production

To further characterize the impact of the probiotic treatment on DC cell function, we tested the profile of cytokine production on

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

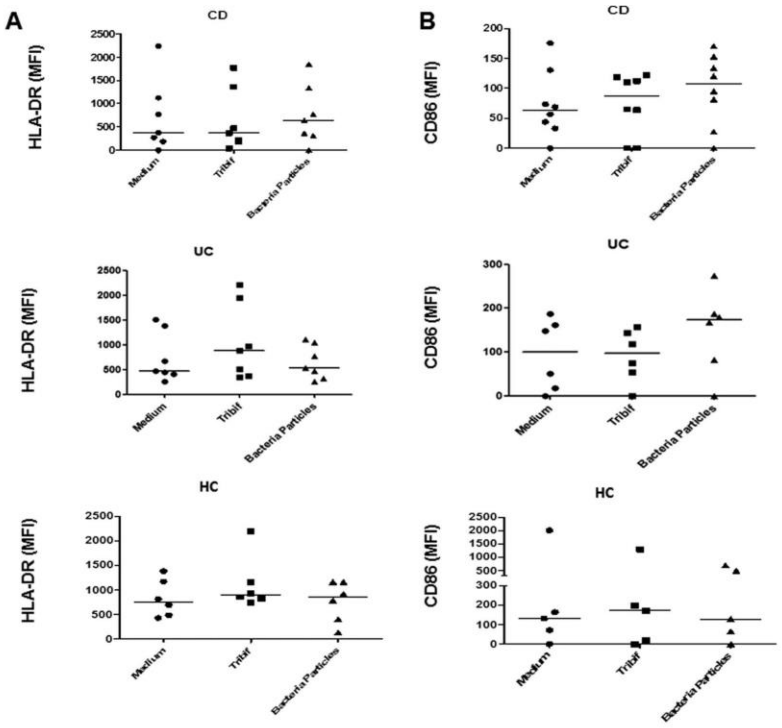


FIGURE 2. Bifidobacteria Tribif have no effect on DC maturation; The surface expression of HLA-DR and CD86 was analyzed in DCs after stimulation with *E. coli* particles or with the probiotic mixture, as indicated in Material and Methods. Data are shown as the mean fluorescence intensity (MFI) and are referred to 7 CD, 7 UC, and 6 HC patients for DR (A) and to 8 CD, 6 UC, and 5 HC patients for CD86 expression (B). Dashes indicate the median MFI value.

the exposure to alive Bifidobacteria or *E. coli* particles. Culture supernatants were analyzed for the content of proinflammatory cytokines, as TNF-α, INF-γ, IL-17, and regulatory cytokine IL-10. Interestingly, a significant production of TNF-α was detected in DC culture supernatant from patients with CD after exposure to bacteria particles compared with basal level ($P = 0.01$) or after the pretreatment with the probiotic mixture ($P = 0.03$) (Fig. 4A). Instead, if excepted few cases, either IFN-γ or IL-17 resulted almost undetectable in cellular medium of patients with CD, on bacteria particles or probiotic exposure (Fig. 4B, C). No significant proinflammatory cytokines production was detected in any of HC or UC patients, after incubation with the bacteria particles or probiotics mixture (data not shown). By contrast, high levels of IL-10 were found in DC supernatants in all groups, independently from the antigen stimulation (Fig. 4D, and data not shown).

DISCUSSION

Many experimental and clinical studies suggest that intestinal bacterial flora plays an important role in the pathogenesis of IBD, and manipulation of the luminal microorganism contents with antibiotics or probiotics represents a potential therapeutic option. The beneficial effect of probiotics was tested mainly in clinical study, where it was shown to contribute to the prevention and treatment of pouchitis, and in maintaining remission of mild-to-moderate UC. However, probiotics seemed to be less effective in patients with CD.[8] Furthermore, studies that investigated the effect of the probiotic Bifidobacteria in a pediatric cohort with IBD are very limited. In this study, we demonstrate that a commercial probiotic mixture (Tribif) composed by 3 Bifidobacteria species (*B. longum, B. breve,* and *B. infantis*) is able to ameliorate the impaired ability of antigen sampling and processing by DC from

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

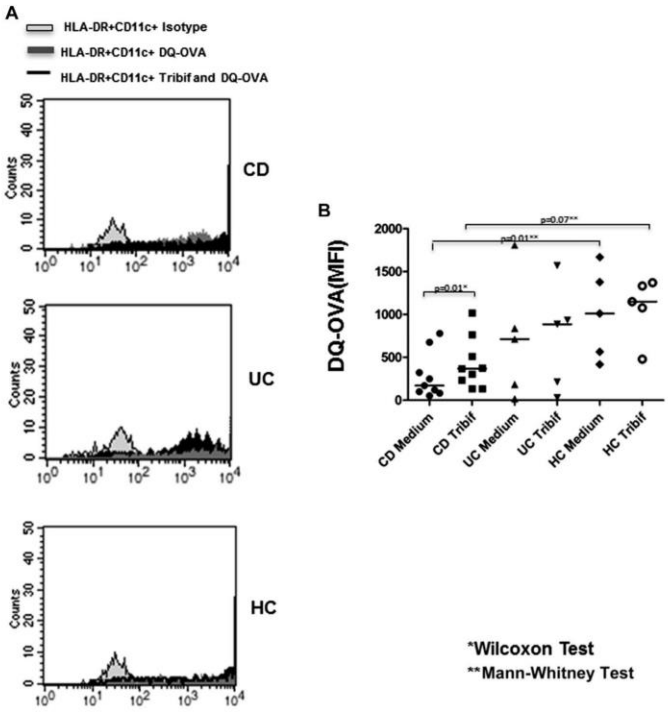


FIGURE 3. Effect of Bifidobacteria Tribif on DQ-OVA processing; DCs were incubated for 4 hours with a fluorochrome-labeled ovalbumin (DQ-OVA) after a 24 hours of pretreatment with the probiotic mixture, or medium alone, and soon after analyzed by flow cytometry. A, Histograms of mean fluorescence intensity (MFI) of DQ-OVA uptake and processing in a representative patient from each children group (CD, UC, and HC). B, Overall DQ-OVA stainings in DCs pretreated with medium alone or Tribif. Results are shown as MFI of DQ-OVA expression obtained in 9 CD, 6 UC, and 6 HC pediatric patients. Dashes indicate the median MFI value.

pediatric patients with CD. We found a marked reduction in the uptake of cellular fragments from the Enterobacteria *E. coli* by DC from children with CD, compared with both DC from either UC or non-IBD children. DCs from patients with CD were also impaired in processing soluble antigens, as shown by reduced intracellular degradation of the dietary protein ovalbumin. These results confirmed a defect of particulate antigen sampling in specialized antigen-presenting cells from patients with CD, likely due to an impairment of autophagy, as previously reported, either by us and other groups.[14,19,20] In particular, we have previously shown that DC from a subgroup of pediatric patients with CD carrying the ATG16L1 polymorphism T300A, have a reduced antigen sampling and processing.[14] Indeed, we found that after the probiotic treatment, the 3 patients who showed the highest improvement of bacteria particles uptake were homozygous fort the T300A variant of ATG16L1 gene. It would be interesting to asses, in a larger cohort of patients with CD, whether the ameliorative effect of the probiotic mixture on DC functionality prevalently occurs in association with the ATG16L1 polymorphism genotype. Interestingly, the probiotic mixture had no significant effect on the antigen uptake in DC from patients with UC, in which the autophagy functionality is not impaired as in CD. This difference in antigen handling is probably due to the different molecular pathway at the base of the pathogenesis of these 2 intestinal diseases.[21]

Since the specific composition of intestinal microorganisms has been suggested to be one of the causes responsible of the bowel inflammation,[1] it is likely that an alteration of phagocytosis might lead to an uncontrolled microorganism growth in the intestine of patients with CD. Probiotics, such as Bifidobacteria, are reported to stimulate the DC and to improve their ability to

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

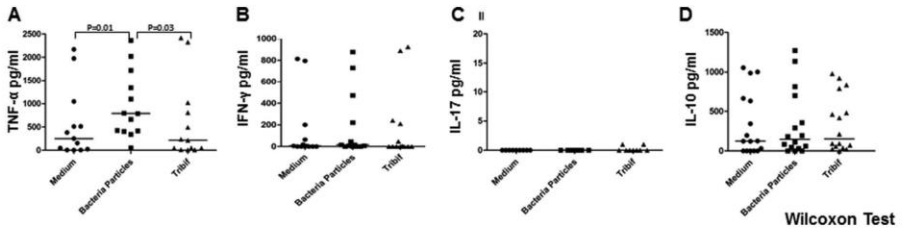


FIGURE 4. Effect of Bifidobacteria Tribif on cytokine production; The level of TNF-α (A), IFN-γ (B), IL-10 (C), and IL-17 (D) was measured by enzyme-linked immunosorbent assay in cell culture supernatant of DCs from children with CD. DCs (3×10^5/mL) were unstimulated or stimulated for 2 hours with *E. coli* particles (20 μg/mL), or for 24 hours with live probiotics (bacteria/DC ratio 1:50). Dashes indicate the median cytokine concentration.

phagocytize and to process particulate antigens of microbial origin.[11,22] In this contest, it has been shown that Bifidobacteria induce a DC-mediated polarization of regulatory T cells[23,24] through the interactions of lipoprotein with TLR2 in complex with TLR1 or TLR6. This ligation has been shown to stimulate an inhibitory signal preventing the peptidoglycan-mediated activation of NOD2, which is one of the key regulators of autophagy machinery.[25] It was recently shown that another probiotic, the Lactobacillus *PGN,* has anti-inflammatory capacities mediated by the release of a specific muropeptide sensed by NOD2.[26]

The beneficial effect on DC functionality reported in our study is a specific property of the probiotics, since the Enterobacterium Salmonella had an opposite effect, reducing significantly the sampling efficiency of the DC. Moreover, we found that Tribif ameliorate the antigen phagocytosis and processing without enhancing the DC maturation or inducing a cytokine production. In particular, the proinflammatory TNF-α, which was significantly enhanced on the stimulation of particulate antigens from the Enterobacterium *E. coli*, was not induced in DC cell supernatant after the treatment with the Bifidobacteria Tribif. This finding indicates that DC from children with CD are sensitized to produce TNF-α, an important player in CD pathogenesis, in response to specific bacteria strains.[21] Surprisingly, high levels of IL-10 were found in DC supernatants from all patient groups, independently from antigen stimulation, most likely due to the in vitro culturing conditions. Notwithstanding, we cannot exclude that IL-10 might be the mediator of the beneficial effect on DC functionality by the probiotic mixture, herein observed.

Our results differ from previous studies that have specifically investigated the maturation and activation of DC from healthy donors[11,12,27] after stimulation with Bifidobacteria because no effect of the probiotic mixture was found on the DC expression of maturation markers HLA-DR and CD86, either in controls or in patients with IBD. One possible explanation of the differences could be due to the fact that in the above studies,[11,12,27] a single strain, and not a mixture, of Bifidobacterium was used to stimulate DC. Furthermore, it is well known that the type of modulation of DC functionality (inflammatory toward regulatory phenotype, or vice versa) might depend on the specific probiotic strains.[28] In fact, Lopez et al[12] found significant differences on the expression of DC maturation markers depending on the specific Bifidobacteria strains. Furthermore, DCs were obtained from young children with IBD, or affected by other functional GI disorders, instead of adult subjects, as in previous reports.[11,12] However, the marked rising in the incidence of IBD in childhood,[27] renders of particular interest such studies, evaluating the beneficial effect of probiotics in the functionality of innate immune cells in young patients with IBD.

Most current probiotics are marketed as foods or supplements that improve gut health and were developed based on ease of production or stability, rather than based on specific mechanisms of disease prevention or treatment. Many commercial probiotics have limited purity and viability making difficult conclusions about efficacy in the treatment or prevention of disease.[29] "Second generation probiotics" may be a useful term to describe probiotics selected based on established mechanisms of prevention or treatment of a specific disease. Bifidobacteria would fit in this category, suggesting mechanistic advantages compared with other probiotics.[30] Studying Bifidobacteria and other probiotics for metabolites and ligands that modulate the host immune function will likely lead to a new class of immunotherapeutic agents for inflammatory states.

ACKNOWLEDGMENTS

Authors contributions: C. Strisciuglio and F. P. Giugliano performed the flow cytometry experiments, data analysis and manuscript drafting; E. Miele did patients' recruitment and gastrointestinal endoscopy and contributed to the study design and critical revision and manuscript drafting; S. Vitale, M. Andreozzi, and A. Vitale did all the DC generation, cellular experiments, and provided assistance in flow cytometry and data analysis; A. Staiano and R. Troncone contributed to the study design and critical revision of manuscript; C. Gianfrani conceived the study design, the critical revision of experiments and the manuscript drafting.

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

REFERENCES

1. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterology*. 2008;134:577–594.
2. Danese S, Fava F. Intestinal microbiota in inflammatory bowel disease: friend of foe? *World J Gastroenterol.* 2011;17:557–566.
3. Macfarlane S, Furrie E, Cummings JH, et al. Chemotaxonomic analysis of bacterial populations colonizing the rectal mucosa in patients with ulcerative colitis. *Clin Infect Dis.* 2004;38:1690–1699.
4. Frank DN, St Amand AL, Feldmn RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel disease. *Proc Natl Acad Sci U S A.* 2007;104:13780–13785.
5. Jeppsson B, Mangell P, Thorlacius H. Use of probiotics as prophylaxis for postoperative infections. *Nutrients*. 2011;3:604–612.
6. Shornikova AV, Casas IA, Isolauri E, et al. Lactobacillus reuteri as a therapeutic agent in acute diarrhea in young children. *J Pediatr Gastroenterol Nutr*. 1997;24:399–404.
7. Meijer BJ, Dieleman LA. Probiotics in the treatment of human inflammatory bowel diseases: update 2011. *J Clin Gastroenterol.* 2011;45(suppl): S139–S144.
8. Guendalini S. Update on the role of probiotics in the therapy of pediatric inflammatory bowel disease. *Expert Rev Clin Immunol.* 2010;6:47–54. Review.
9. Rescigno M, Di Sabatino A. Dendritic cells in intestinal homeostasis and disease. *J Clin Invest.* 2009;119:2441–2450.
10. Menard S, Candalh C, Bambou JC, et al. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. *Gut*. 2004;53:821–828.
11. Hoarau C, Lagaraine C, Martin L, et al. Supernatant of Bifidobacterium breve induces dendritic cell maturation, activation, and survival through a Toll-like receptor 2 pathway. *J Allergy Clin Immunol.* 2006;117:696–702.
12. Lopez P, Gueimonde M, Margolles A, et al. Distinct Bifidobacterium strains drive different immune response in vitro. *Int J Food Microbiol.* 2010;130:157–165.
13. Groeger D, O'Mahony L, Murphy EF, et al. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. *Gut Microbes*. 2013;4:325–339.
14. Strisciuglio C, Miele E, Wildenberg ME, et al. T300A variant of autophagy ATG16L1 gene is associated with decreased antigen sampling and processing by dendritic cells in pediatric Crohn's disease. *Inflamm Bowel Dis.* 2013;19:2339–2348.
15. Levine A, Griffiths A, Markowitz J, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis.* 2011;17:1314–1321.
16. Hyams JS, Ferry GD, Mandel FS, et al. Development and validation of a pediatric Crohn's disease activity index. *J Pediatr Gastroenterol Nutr.* 1991;12:439–447.
17. Turner D, Otley AR, Mack D, et al. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology*. 2007;133:423–432.
18. Sallusto L, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 1994;179:1109–1118.
19. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med.* 2010;16:90–97.
20. Brain O, Cooney R, Simmons A, et al. Functional consequences of mutations in the autophagy genes in the pathogenesis of Crohn's disease. *Inflamm Bowel Dis.* 2012;18:778–781.
21. Blumberg R, Cho J, Lewis J, et al. Inflammatory bowel disease: an update on the fundamental biology and clinical management. *Gastroenterology*. 2011;140:1701–1703.
22. Hart AL, Lammers K, Brigidi P, et al. Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut*. 2004;53:1602–1609.
23. Konieczna P, Groeger D, Ziegler M, et al. Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. *Gut*. 2012;61:354–366.
24. Baba N, Samson S, Bourdet-Sicard R, et al. Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. *J Leukoc Biol.* 2008;84:468–476.
25. Singh SB, Davis AS, Taylor GA, et al. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science*. 2006; 313:1438–1441.
26. Fernandez EM, Pot B, Grangette C. Beneficial effect of probiotics in IBD. Are peptidogycan and NOD2 the molecular key effectors?. *Gut Microbes.* 2011;2:280–286.
27. Birmingham CL, Smith AC, Bakowski MA, et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem.* 2006;281:11374–11383.
28. Grangette C. Bifidobacteria and subsets of dendritic cells: friendly players in immune regulation! *J Leuk Biol.* 2008;84:468–476.
29. Marcobal A, Underwood MA, Mills DA, et al. Rapid determination of the bacterial composition of commercial probiotic products by terminal restriction fragment length polymorphism analysis. *J Pediatr Gastroenterol Nutr.* 2008;46:608–611.
30. Underwood MA. Intestinal dysbiosis: novel mechanisms by which gut microbes trigger and prevent disease. *Prev Med.* 2014;65:133–137.

Copyright © 2015 Crohn's & Colitis Foundation of America, Inc. Unauthorized reproduction of this article is prohibited.

5.2 Does cow's milk protein elimination diet have a role on induction and maintenance of remission in children with ulcerative colitis?

Food sensitivity/intolerance has been suggested to play a role in the aetiology of IBD, and the culpable foods vary on an individual basis. The most common behaviour resulted the avoidance of milk and dairy products; this dietary change resulted in reduced calcium intakes but had no apparent effect on the rate of relapse [8]. On the other hand, malnutrition is very common in children with IBD.[9]The origins of malnutrition in IBD are multifactorial, but dietaryrestrictions (due to intolerance of diet or therapeutic fasting) are considered to be relevant.[10]Without further evidences, and considering the nutritional issues, it remains unclear whether dietary manipulation will continue to have a role solely in symptom control, or whether completeremission may be possible using these methods in combination with pharmacological agents.

The purposes of this study were to evaluate the efficacy of a cow's milk protein (CMP) elimination diet on induction and maintenance of remission and to define the association

with atopy in children with ulcerative colitis (UC). This was a prospective, single-centre, randomized, controlled, 1-year study of children with newly diagnosed UC.

In this study, we did not demonstrate a significant efficacy of CMP elimination diet in the induction and maintenance of remission in paediatric active UC. Patients receiving CMP elimination diet and concomitant conventional therapy did not have a significantly higher rate of remission compared to children on free diet with not significantly lower incidence of relapse within 1 year of follow-up.

The belief that dairy products can exacerbate the disease and that patients restrict their consumption has already been documented.[11]However, several reports have demonstrated that inadequate caloric intake is the primary cause of growth retardation in IBD.[12,13]All clinicians should be aware that iatrogenic dietary restrictions, which are often imposed without a sound scientific or clinical basis, further reduce the amount of caloriesprovided, contributing to the malnutrition and various micronutrient deficiencies in paediatric IBD patiens.[14]Analysing the different therapeutic subgroups (steroids and mesalazine vs. mesalazine alone) within 1 year of follow-up, we found a higher incidence of relapses inpatients under steroid therapy of both CMP and free diet groups compared with patients treated with mesalazine alone. This finding clearly highlights that neither steroids nor CMP elimination diet were able to modify the natural history of the disease. Patients eligible for steroid treatment, and therefore with the worst onset of disease, continue to act with the same severe phenotype despite a more aggressive therapy .

An important limitation of our study is the small number of patients. Because we had negative results (i.e., we did not find a statistical difference), and the sample size of thecohorts is somewhat

small, it is likely that the lack of significant difference occurred by chance. However, because there are no trends towards significance, we doubt that there is the risk of a type II statistical error in our study.

Therefore, we believe that CMP elimination diet does not appear to be useful in the therapy of UC of children.

In conclusion, data of this paediatric, randomized trial suggest that CMP elimination has no role in the management of UC in non-sensitized children. Further larger studies are needed to evaluate the efficacy of the CMP exclusion diet on the induction and maintenance of remission
in UC atopic children.

ACTA PÆDIATRICA
NURTURING THE CHILD

Acta Pædiatrica ISSN 0803-5253

REGULAR ARTICLE

Does cow's milk protein elimination diet have a role on induction and maintenance of remission in children with ulcerative colitis?

Caterina Strisciuglio, Eleonora Giannetti, Massimo Martinelli, Elisa Sciorio, Annamaria Staiano (staiano@unina.it), Erasmo Miele

Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Naples, Italy

Keywords
Cow's milk protein, Diet, Inflammatory bowel disease, Pediatrics, Ulcerative colitis

Correspondence
Annamaria Staiano, Department of Translational Medical Science, Section of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5, 80131 Naples, Italy
Tel: +39.081.7462679 |
Fax: +39.081.5469811 |
Email: staiano@unina.it

Received
12 December 2012; revised 11 February 2013; accepted 20 February 2013.

DOI:10.1111/apa.12215

ABSTRACT

Aim: Aims of this study were to evaluate the efficacy of a cow's milk protein (CMP) elimination diet on induction and maintenance of remission and to define association with atopy in children with ulcerative colitis (UC).

Methods: Twenty-nine consecutive patients (mean age: 11.2 years; range: 4.6–17 years; F/M: 15/14) with newly diagnosed UC were randomized either to receive a CMP elimination diet (n = 14) or to continue a free diet (n = 15) associated with concomitant steroid induction and mesalazine maintenance treatment. Children were prospectively evaluated at four time points: within 1 month, 6 months and 1 year after diagnosis or at the time of relapse.

Results: Twenty-five of the 29 enrolled patients responded to the UC induction therapy with a complete remission (86.2%), 13 belonging to CMP elimination diet group and 12 to free diet group (p = 0.59). Overall, our data showed that 7 of 13 (53.8%) patients treated with CMP elimination diet and 8 of 15 (53.3%) patients on free diet and UC therapy relapsed within 1 year of follow-up (p = 1).

Conclusions: In conclusion, data of this paediatric, randomized trial suggest that CMP elimination has no role in the management of UC in non-sensitized children.

INTRODUCTION

Food sensitivity/intolerance has been suggested to play a role in the aetiology of IBD, and the culpable foods vary on an individual basis. The foods excluded have been predominantly cereals, dairy products, yeast and certain types of fat (1). In particular, children with IBD often avoid dairy products more than they would need to, based on the prevalence of lactose malabsorption and/or allergy to milk proteins because of arbitrary advice from physicians. In a study by Jowett et al., 68% of UC adult patients believed that diet influenced their disease and modified their diet accordingly. The most common behaviour resulted the avoidance of milk and dairy products; this dietary change resulted in reduced calcium intakes but had no apparent effect on the rate of relapse (2). On the other hand, malnutrition is very common in children with IBD (3). At the time of diagnosis, up to 85% of paediatric patients with CD and 65% of patients with UC have weight loss (4). Unnecessary dietary exclusions are a concern if initiated without appropriate nutritional or medical supervision, given the importance of adequate calcium and vitamin D intakes in the prevention of osteoporosis in IBD (5). The origins of malnutrition in IBD are multifactorial, but dietary restrictions (due to intolerance of diet or therapeutic fasting) are considered to be relevant (6). Without further evidences, and considering the nutritional issues, it remains unclear whether dietary manipulation will continue to have a role solely in symptom control, or whether complete remission may be possible using these methods in combination with pharmacological agents.

The purposes of this study were to evaluate the efficacy of a cow's milk protein (CMP) elimination diet on induction and maintenance of remission and to define the association with atopy in children with ulcerative colitis (UC).

METHODS

This was a prospective, single-centre, randomized, controlled, 1-year study of children with newly diagnosed UC consecutively enrolled at the Department of Translational Medical Sciences, Section of Pediatrics, University of Naples 'Federico II', Italy. Twenty-nine consecutive patients (mean age: 11.2 years; range: 4.6–17 years; F/M:

Key notes

- Allergy to milk protein has been reported to play a possible role in the etiopathogenesis of UC.
- Unnecessary dietary exclusions are a concern if initiated without appropriate nutritional or medical supervision.
- Data of this paediatric, randomized trial suggest that cow's milk protein elimination diet does not modify the time of induction and the risk of relapse.

15/14) with newly diagnosed UC, based on accepted historical, endoscopic, histological and/or radiological criteria (7), were randomized either to receive a CMP elimination diet (n = 14) or to continue a free diet (n = 15).

All patients with a Pediatric Ulcerative Colitis Activity Index (PUCAI) score $\geq$35 received concomitant steroid induction treatment (oral methylprednisolon: 1 mg/kg/day, maximum 40 mg/day per 4 weeks) and oral mesalazine induction and mesalazine maintenance treatment (50 mg/kg/day), while subjects with a PUCAI score <35 received exclusively oral mesalazine induction and mesalazine maintenance treatment (50 mg/kg/day). The paediatric gastroenterologists (AS; EM) made all decisions regarding induction therapeutic intervention being unaware of diet group allocation. A detailed list of food containing milk proteins was given to each patient to avoid them during the elimination diet period. During the study period, parents and/or patients were asked to record in a daily diary only the amount and type of not allowed food. At the end of the study, these diaries were analysed to evaluate adherence to the diet and the quantity of milk consumed. The parents were able to contact one of the investigators (MM) whenever necessary, and frequent telephone contacts helped to ensure adherence to the diet. All children received supplemental elemental calcium 1000 mg/day and vitamin D3 0.25 mcg/day for 1 year. Exclusion criteria were children who had received therapy-inducing remission of UC and/or children who required surgery for complications related to UC.

Assignment to CMP elimination diet or free diet was determined according to a computer-generated randomization scheme. Excellent compliance was defined as no violation of the protocol with respect to the intake of the diet.

After 4 weeks, patients who were in remission began tapering off corticosteroids on a weekly basis (25%/week) on the basis of the PUCAI score. Upon induction of remission patients continued to receive concomitant therapy (CMP elimination diet group: mesalazine and CMP elimination diet; free diet group: mesalazine and free diet) for 1 year or until relapse.

Additional information collected at the first visit included demographic data, family history and symptom onset. At diagnosis, physical examination was performed by a paediatrician and included an abdominal examination and evaluation of extraintestinal manifestations of UC. Age- and gender-specific z-scores (standard deviation scores) for height and weight were calculated using National Center for Health Statistics 2000 Center for Disease Control data.

The PUCAI and physician global assessment (PGA) were used to measure disease activity (8). The extension of disease involvement was evaluated using the Paris Classification (9). Clinical remission was defined on the basis of PUCAI < 10, while clinical response to the induction treatment was identified as a PUCAI score a change of at least 20 points from baseline. Clinical relapse was defined as the occurrence or worsening of symptoms accompanied by an increase of PUCAI >10 points, sufficient to require rescue treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery. When a relapse occurred, the study protocol was stopped and the patient was treated according to the physician's preference. Laboratory studies including complete blood count, albumin, ESR, CRP, calcium and phosphorus were performed at first visit.

Children were prospectively evaluated at four time points: at diagnosis, within 1 month, 6 months and 1 year after diagnosis or at the time of relapse. At diagnosis and at 12 months or at the time of relapse all patients were assessed endoscopically and histologically. Colonoscopic grade of inflammation was determined by use of Matts' colonoscopy grading score (10). Colonoscopy report system required segmental descriptions (rectum, sigmoid, descending, transverse and ascending colon, and caecum). From the information provided on the colonoscopy report, for each segment of the colon was designated a score, and a mean score for that colonoscopy was derived. All histologic specimens were reviewed under code by a single pathologist experienced in analysing paediatric intestinal biopsies, blinded to the patients' clinical details, who scored biopsies according to the Matts' histologic criteria (10).

At baseline, a detailed questionnaire was completed for each subject and control, including details of family, personal history of atopic disease and the presence of allergic symptoms. Family and personal history of atopic disease as well as the presence of allergic symptoms in children were established through the use of the validated ISAAC questionnaire (11). Skin prick tests (SPT) were performed according to a standard technique for common inhalational (*Dermatophagoides pteronyssinus* and *farinae, graminacea, parietaria* and cat dander) and dietary allergens (whole milk, alpha-lactoalbumin, beta-lactoglobulin, casein, albumin, egg yolk and fish) (Lofarma®, Milano, Italy). The tests were carried out on the volar side of the forearm with disposable prick lancettes by one physician who was not aware of the subject's clinical history (MM). Histamine chloride at 10-mg/mL and glycerosaline solution were used as positive and negative controls. After 15 min, weal diameters >3 mm, compared with positive and negative controls were considered positive. Specific IgE levels were considered positive if >0.70 kU/L, as the reported reference ranges for the commercial test used in our laboratory (Unicap-specific IgE allergen immuno cap, manufactured by Pharmacia). Subjects with specific IgE and/or prick tests positive for at least one allergen and a personal history of atopy (such as recurrent symptoms of rhinitis, dermatitis, bronchospasms) were considered atopic children.

Statistical analysis

Data were analysed for all patients who had completed at least two visits. Means and medians were calculated for dimensional variables after controlling for normality of distribution. Statistical analysis was carried out using SPSS statistical software package for Windows (13.0; SPSS Inc., Chicago, IL, USA). The Student's t-test and Mann–Whitney *U*-test for normally distributed variables and the chi-

squared and Fisher exact tests for categorical variables were used where appropriate. Survival analysis was used to analyse the data set with respect to relapse. The Kaplan–Meier method was used to estimate the survivor function, and comparison of cumulative relapse rates between treatment groups was tested by the log-rank test. The study was approved by the Institutional Review Board of the University of Naples 'Federico II'. Written, informed consent was obtained from participants' parents, and assent was obtained for all patients older than 10 years of age.

RESULTS

Thirty-two patients with newly diagnosed UC were screened. Three subjects were excluded, because the parents of two refused consent and one had received previous steroid therapy for the presence of concomitant disease (asthma). Twenty-nine children were eligible and participated to this study and were randomly assigned to receive a CMP elimination diet or to continue a free diet associated with concomitant steroid and/or mesalazine induction and mesalazine maintenance treatment. Demographic data for the study groups are listed in Table 1.

The mean PUCAI performed at diagnosis by the paediatric gastroenterologist of each patients did not show significant differences between CMP elimination diet and free diet patients (p = 0.5; OR 1.25; 95% CI 0.9–1.7) (Table 1).

On the basis of the PGA, the severity of the disease was mild in 14.3% of CMP elimination diet patients vs. 33.3% of free diet patients, moderate in 42.9% of CMP elimination diet subjects vs. 53.3% of free diet subjects and severe in 42.9% of CMP elimination diet children vs. 13.3% of free diet group (p = 0.3).

Twenty-five of the 29 enrolled patients responded to the IBD induction therapy with a complete remission (86.2%), 12 belonging to free diet group and 13 to CMP elimination diet group (p = 0.59). Three patients had a partial response, whereas 1 patient did not respond to induction therapy. Overall, our data showed that 7 of 13 (53.8%) patients treated with CMP elimination diet and 8 of 15 (53.3%) patients on free diet and IBD therapy relapsed within 1 year of follow-up (p = 1; OR = 0.8; CI = 0.2–3.7). Among them, 26.6% (4/15) and 38.4% (5/13) relapsed within the first 6 months (p = 0.45). Life-table analysis of the relapses in the 2 groups is showed in Figure 1.

Among the CMP elimination diet group, 6 (42.8%) patients were treated with steroids and mesalazine, while 8 (57.2%) patients received mesalazine alone. Ten (66.6%) patients of the free diet group were treated with steroid

Table 1 Baseline characteristics and at 1 year follow-up or at time of relapse in 29 children with newly diagnosed UC

	Cow's milk protein (CMP) elimination diet group	Free diet group	p	CMP elimination diet group	Free diet group	p
Characteristics	T0			T12 or at Time of Relapse		
Sex (n, %)						
Male	9 (64.3%)	5 (33.3%)	0.1			
Female	5 (35.7%)	10 (66.7%)				
Median Age (months; range)	134.5 (57–196)	138 (55–204)	0.8			
Male	145 (57–196)	157 (81–172)	0.5			
Female	124 (112–173)	133 (55–204)	0.8			
Mean Z-Score (range)	−0.18 (−3 to 2)	0.3 (−1.5 to 2)	0.4	−0.23 (−0.5 to 2.47)	0.6 (−1.1 to 2.2)	0.3
Duration of breast feeding (days, median, range)	97.5 (1–745)	90 (1–262)	0.3			
Introduction of cow's milk (days, median, range)	106 (1–185)	100 (1–170)	0.4			
PUCAI (median, range)	36.2 (15–45)	31 (15–55)	0.5	24 (0–85)	17.5 (0–65)	0.6
Paris classification (n, %)						
Negative (E0)			0.2	3 (21.4)	2 (13.3)	0.2
Proctosigmoiditis (E1)	4 (28.5)	3 (20)		2 (14.3)	2 (13.3)	
Left-sided colitis (E2)	1 (7.1)	2 (13.3)		2 (14.3)	4 (26.6)	
Extensive colitis (E3)	1 (7.1)	5 (33.3)		2 (14.3)	3 (20)	
Pancolitis (E4)	8 (57.1)	5 (33.3)		5 (35.7)	4 (26.6)	
Endoscopic Matt score (mean, range)	1.7	2.2	0.7	2.2	2.5	0.5
Histological Matt score	2.6	2.6	0.8	1.8	2.1	0.4
Steroid therapy	6 (80%)	10 (50%)	0.3			
Time to weaning						
Steroids (days, median, range)	55 (30–60)	60 (30–120)	0.7			
Total amount of Steroid						
Induction Therapy (mean mg/kg)	0.78	0.83	0.3			
Hemoglobin (median, range)	11.7 (5.5–14.1)	11.7 (7–14.3)	0.9	11.5 (7–14.2)	12.1 (9.4–13.7)	0.6
CRP (median, range)	0.3 (0.2–9.1)	0.7 (0.2–19.1)	0.1	0.6 (0.3–86.7)	0.3 (0.3–33.5)	0.6
ESR (median, range)	6.5 (3–25)	13 (3–46)	0.1	12 (1–60)	4 (2–20)	0.3
Calprotectin (median, range)	434.5 (60–500)	369 (15–500)	0.5	144.5 (15–500)	416.5 (53–500)	0.3

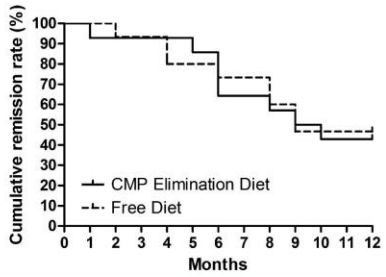


Figure 1 Kaplan–Meier estimates of relapse during treatment on cow's milk protein elimination diet or on free diet (log-rank test p = 0.87).

therapy and 5 (33.4%) with mesalazine alone. Overall, the number of relapses was significantly higher in patients treated with steroids and mesalazine versus patients receiving mesalazine alone (12 vs. 3; p = 0.009). No other significant differences were observed among the other therapeutic subgroups.

At time of enrolment, endoscopic and histological scores were not statistically different comparing the CMP elimination diet and free diet groups (p = 0.7 and p = 0.8) (Table 1). At baseline, laboratory parameters including haemoglobin, erythrocyte sedimentation rate and C-reactive protein were not significantly different between the two groups and were not predictive of response and/or relapse (Table 1). Hypereosinophilia was present in 2 patients (6.9%), one from the CMP elimination diet group and 1 from free diet group. At 12 months or at time of relapse, endoscopic and histological scores were not significantly different among the CMP elimination diet group and the free diet group. All patients were compliant to the dietary restrictions.

No side effects or significant changes from baseline values in any of the laboratory parameters examined attributable to CMP elimination diet was registered.

All 29 patients completed the questionnaire. Family history of atopy was present in 4 patients on CMP elimination diet (28.6%) and in five patients on free diet (33.3%) (p = 1). Based on the criteria listed under the Methods section, the prevalence of atopy in our study population was 27.5%. Four patients on CMP elimination diet (28.6%) and in five patients on free diet (33.3%) also satisfied the diagnostic criteria for atopy (p = 1). Positive laboratory results consistent with atopy for these patients are summarized in Table 2. None of the patients had symptoms of cow's milk allergy. On the basis of PUCAI, remission was achieved in four atopic patients (100%) treated with elimination diet and IBD therapy and in four atopic patients (80%) treated with free diet and IBD therapy (p = 1). One patient treated with free diet and IBD therapy had just a partial response. Two of four (50%) atopic patients treated with CMP elimination diet and IBD therapy and one of five (20%) atopic patients on free diet relapsed within 1 year of follow-up (p = 0.5).

Table 2 Clinical and laboratory results in UC atopic children who satisfy criteria for atopy

	Cow's milk protein elimination diet group n (%)	Free diet group n (%)
Family history of atopy	4 (28.6)	7 (46.6)
Associated symptoms	3 (21.4)	2 (13.3)
RAST	1 (7.1)	4 (26.6)
β Lactoglobulin	1 (7.1)	3 (20)
Dermatophagoides pteronyssinus	1 (7.1)	2 (13.3)
Grass pollens	1 (7.1)	0
Cat dander	1 (7.1)	0
Parietaria	0	1 (6.6)
PRICK	2 (14.3)	4 (26.6)
Dermatophagoides pteronyssinus	0	1 (6.6)
Parietaria	1 (7.1)	3 (20)
Grass pollens	2 (14.3)	1 (6.6)
Egg white	1 (7.1)	1 (6.6)
Cat dander	0	0
Casein	0	0
β Lactoglobulin	0	1 (6.6)

DISCUSSION

In this study, we did not demonstrate a significant efficacy of CMP elimination diet in the induction and maintenance of remission in paediatric active UC. Patients receiving CMP elimination diet and concomitant conventional therapy did not have a significantly higher rate of remission compared to children on free diet with not significantly lower incidence of relapse within 1 year of follow-up.

The aetiology of IBD is considered multifactorial. Genetic, infective and environmental theories exist, as well as those centred around host immunity, intraluminal gut flora, food allergies and hypersensitivity (12). Diet may influence gut inflammation through several biologically plausible mechanisms, including antigen presentation, change in prostaglandin balance, and alteration of the microflora (13). The belief that dairy products can exacerbate the disease and that patients restrict their consumption has already been documented (14). From a historical perspective, the concept of 'milk allergy' was introduced in the early part of this century when it was believed that foods and eating habits were involved in the pathogenesis of IBD (15,16). The concept of 'milk allergy' as initially proposed was not based on objective immunologic testing and gradually gave way to theories involving malabsorption of and 'intolerance' to lactose in the 1960s (17) and, more recently, intolerance to other components of dairy products (18). Allergy to milk proteins still remains a possible cause of 'dairy sensitivity' or milk intolerance in a small percentage of IBD patients (19). Even if, so far, the only

recognized evidence is that the lack of breast feeding and an increased prevalence of cow milk sensitivity may be risk factors for the later development of IBD, clinicians still focus on the foods that patients associate with their symptoms and therefore avoid (20,21). However, several reports have demonstrated that inadequate caloric intake is the primary cause of growth retardation in IBD (22,23). All clinicians should be aware that iatrogenic dietary restrictions, which are often imposed without a sound scientific or clinical basis, further reduce the amount of calories provided, contributing to the malnutrition and various micronutrient deficiencies in paediatric IBD patients (24).

Analysing the different therapeutic subgroups (steroids and mesalazine vs. mesalazine alone) within 1 year of follow-up, we found a higher incidence of relapses in patients under steroid therapy of both CMP and free diet groups compared with patients treated with mesalazine alone. This finding clearly highlights that neither steroids nor CMP elimination diet were able to modify the natural history of the disease. Patients eligible for steroid treatment, and therefore with the worst onset of disease, continue to act with the same severe phenotype despite a more aggressive therapy.

The prevalence of atopy in our study population was 27.5%. Our results showed no difference in the prevalence of atopy among UC children treated with elimination diet and those on free diet. Nevertheless, due to the small number of our patients, we cannot draw any conclusion in respect of the association between atopy and UC. Several studies tried to investigate the attractive hypothesis that IBD may be an allergic response to food (21,25) especially in individuals susceptible to various allergens. In particular, UC with Th2 cytokines pattern response has always been thought to be somehow related to allergic diseases. Most studies confirmed the observation that atopic features are more frequent in patients with IBD than in the general population (26,27). Only one study by Troncone et al. (28) showed that there was no correlation between atopy and IBD.

Our study has some limitations. First of all, we did not evaluate our children for lactase deficiency or lactose intolerance. Lactase deficiency is common in adults with chronic IBD (29). Complete avoidance of milk and milk products can obviate the symptoms of lactose malabsorption, although decreasing the milk intake may also prevent discomfort. As many as 80% of gastroenterologists surveyed indicated that they have recommended avoidance of milk and milk products to patients with UC (29). It has been reported that more than one-third of paediatric patients with IBD have lactase deficiency. Nevertheless, avoidance of a lactose-containing diet should not be routinely recommended to paediatric patients with IBD until evaluation for lactase deficiency or lactose intolerance is performed (30).

Another limitation of our study is the small number of patients. Because we had negative results (i.e., we did not find a statistical difference), and the sample size of the cohorts is somewhat small, it is likely that the lack of significant difference occurred by chance. However, because there are no trends towards significance, we doubt that there is the risk of a type II statistical error in our study. Therefore, we believe that CMP elimination diet does not appear to be useful in the therapy of UC of children.

In conclusion, data of this paediatric, randomized trial suggest that CMP elimination has no role in the management of UC in non-sensitized children. Further larger studies are needed to evaluate the efficacy of the CMP exclusion diet on the induction and maintenance of remission in UC atopic children.

References

1. Rajendran N, Kumar D. Role of diet in the management of inflammatory bowel disease. *World J Gastroenterol* 2010; 16: 1442–8.
2. Jowett SL, Seal CJ, Phillips E, Gregory W, Barton JR, Welfare MR. Dietary beliefs of people with ulcerative colitis and their effect on relapse and nutrient intake. *Clin Nutr* 2004; 23: 161–70.
3. Scott EM, Gaywood I, Scott BB. Guidelines for osteoporosis in coeliac disease and inflammatory bowel disease. British society of gastroenterology. *Gut* 2000; 46(Suppl. 1):i1–8.
4. Hartman C, Eliakim R, Shamir R. Nutritional status and nutritional therapy in inflammatory bowel diseases. *World J Gastroenterol* 2009; 15: 2570–8.
5. Shamir R, Phillip M, Levine A. Growth retardation in pediatric Crohn's disease: pathogenesis and interventions. *Inflamm Bowel Dis* 2007; 13: 620–8.
6. Kleinman RE, Baldassano RN, Caplan A, Griffiths AM, Heyman MB, Issenman RM et al. North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. Nutrition support for pediatric patients with inflammatory bowel disease: a clinical report of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2004; 39: 15–27.
7. IBD Working Group of the European Society for Paediatric Gastroenterology. Hepatology and Nutrition. Inflammatory bowel disease in children and adolescents: recommendations for diagnosis–the Porto criteria. *J Pediatr Gastroenterol Nutr* 2005; 41:1–7.
8. Turner D, Otley AR, Mack D, Hyams J, de Bruijne J, Uusoue K et al. Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study. *Gastroenterology* 2007; 133: 423–32.
9. Levine A, Griffiths A, Markowitz J, Wilson DC, Turner D, Russell RK, et al. Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflamm Bowel Dis* 2011; 17: 1314–21.
10. Matts SGF. The value of rectal biopsy in the diagnosis of ulcerative colitis. *Q J Med* 1961; 120:393–407.
11. Asher MI, Keil U, Anderson HR, Beasley R, Crane J, Martinez F, et al. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J* 1995; 8: 483–91.
12. Cashman KD, Shanahan F. Is nutrition an aetiological factor for inflammatory bowel disease? *Eur J Gastroenterol Hepatol* 2003; 15: 607–13.
13. Hou JK, Abraham B, El-Serag H. Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature. *Am J Gastroenterol* 2011; 106: 563–73.
14. Gerasimidis K, McGrogan P, Hassan K, Edwards CA. Dietary modifications, nutritional supplements and alternative medicine in paediatric patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2008; 27: 155–65.

15. Andresen AFR. Ulcerative colitis-an allergic phenomenon. *Am J Dig Dis* 1942; 9: 91–8.
16. Binder HJ, Gryboski JD, Thayer WR Jr, Spiro HM. Intolerance to milk in ulcerative colitis. A preliminary report. *Am J Dig Dis* 1966; 11: 858–64.
17. Pena AS, Truelove SC. Hypolactasia and ulcerative colitis. *Gastroenterology* 1973; 64:400–4.
18. Mishkin S. Controversies regarding the role of dairy products in inflammatory bowel disease. *Can J Gastroenterol* 1994; 8: 205–12.
19. Mishkin S. Dairy sensitivity, lactose malabsorption, and elimination diets in inflammatory bowel disease. *Am J Clin Nutr* 1997; 65: 564–7.
20. Koletzko S, Sherman P, Corey M, Griffiths A, Smith C. Role of infant feeding practices and ulcerative colitis in childhood. *Br Med J* 1991; 302: 1580–1.
21. Glassman MS, Newman U, Berezin S, Gryboski JD. Cow's milk protein sensitivity during infancy in patients with inflammatory bowel disease. *Am J Gastroenterol* 1990; 88: 227–32.
22. Seidman EG. Nutritional management of inflammatory bowel disease. *Gastroenterol Clin North Am* 1989; 18: 129–55.
23. Belli DC, Seidman E, Bouthillier L, Weber AM, Roy CC, Pletincx M, et al. Chronic intermittent elemental diet improves growth failure in children with Crohn's disease. *Gastroenterology* 1988; 94: 603–10.
24. Seidman E, LeLeiko N, Ament M, Berman W, Caplan D, Evans J, et al. Nutritional issues in pediatric inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 1991; 12: 424–38.
25. Smart HL, Mayberry JF. Atopy, food and ulcerative colitis. *Hepatogastroenterology* 1986; 33: 47–8.
26. Mee AS, Brown D, Jewell DP. Atopy in inflammatory bowel disease. *Scand J Gastroenterol* 1979; 14: 743–6.
27. Tzanakis NE, Tsiligianni IG, Siafakas NM. Pulmonary involvement and allergic disorders in inflammatory bowel disease. *World J Gastroenterol* 2010; 16:299–305.
28. Troncone R, Merrett TG, Ferguson A. Prevalence of atopy is unrelated to presence of inflammatory bowel disease. *Clin Allergy* 1988; 18: 111–7.
29. Bernstein CN, Ament M, Artinian L, Ridgeway J, Shanahan F. Milk tolerance in adults with ulcerative colitis. *Am J Gastroenterol* 1994; 89: 872–7.
30. Pfefferkorn MD, Fitzgerald JF, Croffie JM, Gupta SK, Corkins MR, Molleston JP. Lactase deficiency: not more common in pediatric patients with inflammatory bowel disease than in patients with chronic abdominal pain. *J Pediatr Gastroenterol Nutr* 2002; 35: 339–43.

 ©2013 Foundation Acta Pædiatrica. Published by Blackwell Publishing Ltd 2013

5.3 Use of Biosimilars in Paediatric Inflammatory Bowel Disease: A Position Statement of the ESPGHAN Paediatric IBD Porto Group

Biological medicines are complex protein-based compounds derived from a biological source as defined by the European Medicines Agency (EMA) .[15] These proteins have a much larger molecular structure than the standard pharmacological preparations. When such small molecule drugs' patents expire, generic products are introduced. Because of both their structure and biological activity, however, the counterpart of generics in terms of biologicals is called biosimilars.[15]The World Health Organization has defined these as ''a biotherapeutic product which is similar in terms of quality, safety and efficacy to an already licensed reference biotherapeutic product''.[16] Because of the structure of the biological molecules and trade secrets of the companies producing the original products, the new versions are very similar but not exactly identical to the originator drug.

Agencies including the US Food and Drug Administration (FDA) and EMA decided that for biosimilars, documentation of efficacy is not needed for all of the indications of the original molecule. Extrapolation may be acceptable provided that the pharmacokinetic (PK) and pharmacodynamic properties have been demonstrated in studies of all levels (in vitro, animal models, and clinical trials) in some of the indications.[17] Because biosimilars have now come to the market in some countries, an overview is given on behalf of the paediatric inflammatory bowel disease (IBD) Porto group on the use of biosimilars in paediatric IBD (PIBD). The PIBD Porto group is a group of PIBD experts from European Society for Paediatric Gastroenterology, Hepatology, and Nutrition whose goals are to generate collaborative international research and to provide a leadership role with regards to current diagnosis and management of IBD in children. Children with IBD on average have a more severe disease phenotype than in adult-onset IBD, potentially requiring antitumour necrosis factor (anti-TNF) treatment for even longer duration. Therefore, in addition to the European Crohn's and Colitis Organisation position statement on the use of biosimilars in the treatment of IBD, we hereby provide consensus-based recommendations specifically for paediatric gastroenterologists treating children with IBD.[18]

It appears that the experience so far with the introduction of biosimilar therapeutic mAb is encouraging with regards to drug safety and effectiveness in rheumatology. Even minor alterations, however, in the production process of biologics may lead to changes in cell behaviour and cause differences in structure, stability, or other quality aspects of the end product, commonly because of differences in glycosylation patterns. Any of these differences may affect the treatment's safety, efficacy, and, most importantly with biologics, the immunogenicity.[19] In children, the risk of developing immunogenicity to anti-TNF treatment is even more worrisome than in adult patients

because children both have more severe disease and potentially need anti-TNF treatment for a longer period.

Moreover immunogenicity is clinically a very relevant phenomenon with both IFX and adalimumab, and affects anti-TNF drug levels and clinical efficacy in both Crohn disease and ulcerative colitis.[20-27] Therefore, there is no guarantee that our understanding of immunogenicity of the originator biological will easily be extrapolated to the biosimilar that may be subtly different in molecular structure. New assays need to be developed and studies undertaken to explore and understand the immunogenicity of the biosimilars.

Concerns remain about the introduction of biosimilars, particularly in PIBD. These concerns should spark debates in the medical arena, and this information should be available to physicians who have to make the decisions about the welfare of their patients. In contrast, the introduction of biosimilars to the market will likely decrease the costs of anti-TNF drugs by 30%, thereby lowering the threshold of use of these highly effective but expensive drugs in IBD.

Altough the Porto IBD group conclude with the following statement:

The European Medicines Agency approved the use of biosimilars for infliximab for all indications, including adult and paediatric inflammatory bowel disease (IBD). The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition paediatric IBD Porto group advocates giving high priority to performing paediatric trials with long-term follow- up to support this decision

Treatment of a child with sustained remission on a specific medication should not be switched to a biosimilar until clinical trials in IBD are available to support the safety and efficacy of such a change.

Postmarketing surveillance programs for efficacy, safety, and immunogenicity in children with IBD should be a manda- tory requirement for the marketing of biologics and biosimilars with respective indications.

CME

Use of Biosimilars in Paediatric Inflammatory Bowel Disease: A Position Statement of the ESPGHAN Paediatric IBD Porto Group

*Lissy de Ridder, †Matti Waterman, ‡Dan Turner, §Jiri Bronsky, ||Almuthe C. Hauer, ¶Jorge A. Dias, #Caterina Strisciuglio, **Frank M. Ruemmele, ††Arie Levine, and ‡‡Paolo Lionetti, on Behalf of the ESPGHAN Paediatric IBD Porto Group

ABSTRACT

Because the patents for biopharmaceutical monoclonal antibodies have or soon will expire, biosimilars are coming to the market. This will most likely lead to decreased drug costs and so easier access to these expensive agents. Extrapolation, however, of the limited available clinical data from adults with rheumatologic diseases to children with inflammatory bowel disease (IBD) should be done with caution and needs some considerations. Post-marketing surveillance programs for efficacy, safety, and immunogenicity should become mandatory in children with IBD using biosimilars, as for all biological drugs.

Key Words: biosimilar, inflammatory bowel disease, infliximab, paediatric, position paper

(*JPGN* 2015;61: 503–508)

Received May 11, 2015; accepted July 2, 2015.

From the *Paediatric Gastroenterology, Erasmus Medical Center/Sophia Children's Hospital, Rotterdam, The Netherlands, the †Department of Gastroenterology, Inflammatory Bowel Diseases Service, Rambam Health Care Campus, Haifa, the ‡Institute of Paediatric Gastroenterology, Shaare Zedek Medical Center, The Hebrew University of Jerusalem, Israel, the §Department of Paediatrics, University Hospital Motol, Prague, Czech Republic, the ||Department of Paediatrics and Adolescence Medicine, GPGE Educational Center of Paediatric Gastroenterology, Medical University of Graz, Austria, the ¶Pediatric Gastroeneterology Department, Hospital Sao Joa, Porto, Portugal, the #Department of Woman, Child, and General and Specialized Surgery, Second University of Naples, Naples, Italy, the **Université Paris Descartes—Sorbonne Paris Cité, Assistance Publique—Hôpitaux de Paris, Hôpital Necker Enfants Malades, Service de Gastroentérologie Pédiatrique, INSERM U1163, Institute IMAGINE, Paris, France, the ††Paediatric Gastroenterology Unit, Wolfson Medical Center, Tel Aviv University, Israel, and the ‡‡Department of Neuroscience, Psychology, Pharmacology and Child's Health, University of Florence, Meyer Hospital, Florence, Italy.

Address correspondence and reprint requests to Lissy de Ridder, MD, PhD, Paediatric Gastroenterology, Erasmus Medical Center/Sophia Children's Hospital, Rotterdam, The Netherlands (e-mail: l.deridder@erasmusmc.nl).

This article has been developed as a Journal CME Activity by NASPGHAN. Visit *http://www.naspghan.org/content/59/en/Continuing-Medical-Education-CME* to view instructions, documentation, and the complete necessary steps to receive CME credit for reading this article.

All of the authors have contributed equally to this study.

In the last 3 years, consultation fees, research grants, or honoraria were received by L.R. from Shire, Merck, Janssen Biologics, AbbVie, and Hospira; by M.W. from Janssen Biologics, AbbVie, and Takeda; by D.T. from Merck Sharpe & Dohme (MSD), Janssen Biologics, Pfizer, The Hospital for Sick Children, Ferring, AbbVie, and Abbott; by J.B. from MSD, AbbVie, Abbott, Ferring, Nestlé, Nutricia, and Biocodex; by A.C.H. from MSD and Janssen; by F.M.R. from Janssen, AbbVie, Falk, MSD, Shire, Nestlé, Danone, and Celgene; and by A.L. from Janssen, AbbVie, Falk, and Nestlé. The other authors report no conflicts of interest.

Copyright © 2015 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

DOI: 10.1097/MPG.0000000000000903

Biological medicines are complex protein-based compounds derived from a biological source as defined by the European Medicines Agency (EMA) (1). These proteins have a much larger molecular structure than the standard pharmacological preparations. When such small molecule drugs' patents expire, generic products are introduced. Because of both their structure and biological activity, however, the counterpart of generics in terms of biologicals is called biosimilars (1). The World Health Organization has defined these as ''a biotherapeutic product which is similar in terms of quality, safety and efficacy to an already licensed reference biotherapeutic product'' (2). Because of the structure of the biological molecules and trade secrets of the companies producing the original products, the new versions are very similar but not exactly identical to the originator drug.

These products do not need to go through the same complex licensing process for approval as new small molecules, thereby bringing down their cost (Fig. 1) (3). Biosimilars may also be cheaper because it is possible to produce molecules which are almost identical to the original product through alternative methods. The pricing difference, however, may be less pronounced than when producing generics because of the complex manufacturing process which large-molecule biosimilars require (4,5). Overall, a lower price should result, allowing wider access to this expensive class of medications.

Agencies including the US Food and Drug Administration (FDA) and EMA decided that for biosimilars, documentation of efficacy is not needed for all of the indications of the original molecule. Extrapolation may be acceptable provided that the pharmacokinetic (PK) and pharmacodynamic properties have been demonstrated in studies of all levels (in vitro, animal models, and clinical trials) in some of the indications, (3).

Because biosimilars have now come to the market in some countries, an overview is given on behalf of the paediatric inflammatory bowel disease (IBD) Porto group on the use of biosimilars in paediatric IBD (PIBD). The PIBD Porto group is a group of PIBD experts from European Society for Paediatric Gastroenterology, Hepatology, and Nutrition whose goals are to generate collaborative

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

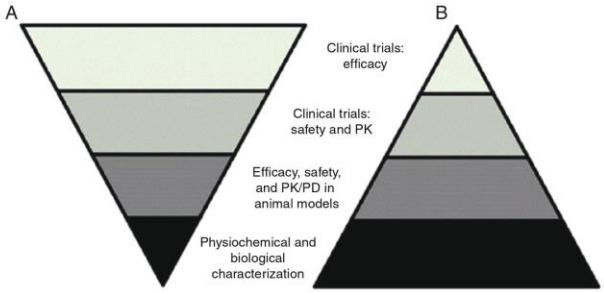


FIGURE 1. Biosimilar principle. A, Process development of new drug (original). B, Process development of biosimilar: reversed engineering and body of evidence. Although a new drug development starts with some physiochemical and biological characterization, the emphasis is on clinical trials. This is the other way around for the development of a biosimilar, in which the emphasis is on the proof of similarity (physiochemical and biological characterization). Revision of figure, derived from (3).

international research and to provide a leadership role with regards to current diagnosis and management of IBD in children. Children with IBD on average have a more severe disease phenotype than in adult-onset IBD, potentially requiring antitumour necrosis factor (anti-TNF) treatment for even longer duration. Therefore, in addition to the European Crohn's and Colitis Organisation position statement on the use of biosimilars in the treatment of IBD, we hereby provide consensus-based recommendations specifically for paediatric gastroenterologists treating children with IBD (6).

HOW BIOSIMILARS MAY DIFFER FROM CURRENT BIOLOGICS

Although the generic version of a small molecule drug is identical to the original product with respect to its structural and therapeutic identity, this cannot be said for biosimilars:

1. The reference biopharmaceuticals are characterised by marked molecular heterogeneity because of a variety of factors (ie, the interplay of primary, secondary, and higher-order protein structures, and intramolecular/intermolecular interactions and posttranslational modifications) leading to a magnitude of chemical forms.
2. Because biopharmaceuticals are made in living cell lines, they are sensitive to changes in the manufacturing process such as growth conditions, purification processes, formulation, or storage conditions (5).

The large and complex structure of monoclonal antibodies (mAbs) makes the synthesis of biosimilars more complicated than a biosimilar of small proteins. Moreover, even the original product drifts over time and is not fully identical to the drug that was licensed. Therefore, specific European guidelines published by the EMA do not refer to structural identity, rather they mandate that biosimilar mAb cannot have clinically meaningful differences from the reference product in terms of ''quality, safety, or efficacy'' (7,8). According to the FDA, there should not be any clinically meaningful differences in ''safety, purity, and potency'' (9).

Proof of Biosimilarity

The primary goal of biosimilar development is to establish biosimilarity (10):

1. In vitro characterisation studies are required, in which the biosimilar and the reference product are compared in terms of binding and function. In vivo testing may be required if there are concerns identified in in vitro studies, such as alterations in receptor binding or stability, potentially resulting in altered safety or clinical efficacy. Clinical evaluations are required to evaluate PKs, pharmacodynamics, efficacy, and safety.
2. Analytical tools currently available remain limited in the ability to characterise all possible chemical variants of biologics. Therefore, the absence of detectable differences does not necessarily imply biosimilarity (11).
3. Because the manufacturing process of the originator product remains a trade secret even after patent expiration, there is no information on process steps (ie, vector, host cell expression system, cell expansion procedure, protein recovery mechanism, purification process, or formulation of the therapeutic protein into a drug) (12).
4. Changes in manufacturing over the years of production with subsequent incremental differences among multiple biological medicines, either original authorised products or biosimilars, have to be considered (13–15). Even after the demonstration of biosimilarity at the time of approval, a biosimilar and a reference medicine could then diverge over time. Therefore, demonstration of comparability between subsequent biosimilar products and the initial biosimilar is also necessary.

Because of the vast molecular structural heterogeneity and differences in manufacturing, biosimilars are unlikely to be identical with their reference products.

EXISTING BIOSIMILARS DESTINED FOR IBD AND CURRENT CLINICAL DATA ON EFFICACY

There are no randomised controlled trials (RCTs) published on the use of infliximab (IFX) or other anti-TNF biosimilars in IBD to date. The only data on the efficacy can be derived from 2 published RCTs in adult patients with rheumatoid arthritis (PLANETRA) and ankylosing spondylitis (PLANETAS) (16,17). Data from long-term extensions of both studies are available as abstracts (18,19).

Recently, a Hungarian IBD cohort treated with the biosimilar IFX is published as an abstract (20).

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

PLANETAS

PLANETAS was a randomised, double-blind, multicentre prospective study comparing the PKs, safety, and efficacy of biosimilar CT-P13 (an immunoglobulin G1 chimeric human-murine mAb IFX biosimilar) and IFX in patients with ankylosing spondylitis (16). The primary endpoint was PK equivalence at steady state and the observed maximal serum concentration assessed between weeks 22 and 30. Efficacy was a secondary endpoint.

Of the 250 randomised patients, 229 completed the 30-week study period. PK analyses included 223 patients. Efficacy and safety analyses were performed in all 250 patients. Patients were randomly assigned 1:1 to receive either 5 mg/kg of CT-P13 (Celltrion Inc, Incheon, Republic of Korea) or IFX (Janssen Biotech Inc, Horsham, PA), at weeks 0, 2, 6, and thereafter every 8 weeks until week 30. Steady-state PK was equivalent for CT-P13 and IFX in the overall PK population. Efficacy was highly similar between the 2 groups. No statistically significant difference in immunogenicity between the CT-P13 and IFX treatment groups was observed.

PLANETAS Extension

Of the 210 patients who completed PLANETAS, 174 patients entered the extension phase for an additional 48 weeks: 88 were continuously treated with CT-P13 (maintenance group) and 86 were switched from IFX to CT-P13 (switch group) (18). During the extension, disease activity scores were similar in the maintenance group and the switch group.

Antidrug antibody formation was comparable between the 2 groups, and positivity was maintained throughout the study. Patients without anti-drug antibody formation achieved better responses compared with patients with anti-drug antibody formation, whereas there were no differences between the maintenance and switch groups.

PLANETRA

PLANETRA was a randomised, double-blind, multicentre prospective study comparing CT-P13 and IFX, both coadministered with methotrexate in adult patients with active rheumatoid arthritis (17).

Patients with active rheumatoid arthritis were randomly assigned 1:1 to receive 3 mg/kg of CT-P13 or IFX at weeks 0, 2, and 6 and thereafter every 8 weeks until week 30. The primary endpoint was to demonstrate equivalent efficacy of CT-P13 to IFX at week 30, as determined by rheumatological disease activity scores. Of the 606 randomised patients, 494 completed the study without protocol violations. Discontinuation was primarily because of adverse events (8.9%) and patient withdrawal of consent (4.1%). Clinical responses at week 30 were equivalent (60.9% and 58.6%) between treatment groups according to the intention-to-treat analysis for CT-P13 and IFX, respectively, as were the PK profile and immunogenicity.

PLANETRA Extension

Out of the 455 patients who completed the PLANETRA study, 302 patients were entered into the open-label extension for an additional 48 weeks (19). Patients either continued CT-P13 (n = 158) or switched from IFX to CT-P13 (n = 144). Through week 102, clinical response rates were maintained and similar within each group. Antidrug antibody formation positivity was comparable between both groups and did not increase significantly during year 2 while on CT-P13.

Biosimilar IFX in an IBD Cohort

Gecse et al (20) describe the first prospective nationwide cohort examining the efficacy and safety of the biosimilar IFX in adult IBD patients. In total, 90 CD and 51 UC patients have been treated with the biosimilar IFX (total number of infusions not reported). A total of 26% of patients had previous exposure to anti-TNF treatment. Patients were followed for 52 weeks but only results until week 14 have been reported to date. Early clinical response and remission rates were comparable to previously reported original IFX trials.

These studies have shown that the efficacy between the biosimilar CT-P13 and IFX was comparable both in ankylosing spondylitis and rheumatoid arthritis patients until week 102. Switching from IFX to CT-P13 was efficacious during 2 years in both patient groups. Extrapolation of these data to children with IBD, however, should be done with caution and needs some considerations.

First, it needs to be taken into account that biological medicines such as IFX are used to treat IBD. IBD and rheumatoid arthritis are not identical in pathogenesis, and there are several examples of biologics which are effective in rheumatoid arthritis but ineffective (21) or even harmful in IBD (22). In vitro studies show substantial differences in the mechanism of action among the anti-TNF drugs currently used (23). Therefore, extrapolation of the biosimilar agent to be approved across the indications for which the reference agent is approved is not well established, and this should be weighed in the decision to support approval. Moreover, pharmacodynamic markers as surrogate endpoints for efficacy, such as the absolute neutrophil count for granulocyte-colony stimulating factor (G-CSF) therapy, are lacking for TNF antagonists.

The second consideration pertains to the differences in doses. The dose of IFX for IBD, 5 mg/kg, differs from that used for rheumatoid arthritis, 3 mg/kg. The third consideration is that children with IBD are often treated with monotherapy, or combination therapy with concomitant immunosuppressive agents for short periods of time, yet the authorisation studies were in those on combination therapy. The presence of drug antibodies to anti-TNFs correlates with shorter duration of response and higher incidence of infusion reactions (24,25): immunosuppressive medications are known to reduce the risk of the development of neutralising antibodies against anti-TNFs (26). Therefore, the concomitant use of methotrexate in the phase III study may compromise the validity of extrapolation of safety and efficacy data to children with IBD who are not receiving concomitant methotrexate.

EXISTING BIOSIMILARS DESTINED FOR IBD AND CURRENT CLINICAL DATA ON SAFETY

Data concerning the safety of biosimilars is available only from the PLANETAS and PLANETRA trials and their extensions described above (16,17). In the PLANETAS trials, infusion reactions occurred in 3.9% of patients receiving CT-P13 and in 4.9% of patients treated with IFX. Patients who developed antidrug antibodies were 27.4% and 22.5% in the CT-P13 and IFX group, respectively. In the PLANETRA study, drug-related adverse events were observed in 35.3% versus 35.9% whereas antidrug antibodies were developed in 48.4% versus 48.2%, respectively. In both trials, the majority of adverse events were mild to moderate in severity. In patients with rheumatoid arthritis, the most common reported treatment-related adverse events in CT-P13 and reference IFX were latent tuberculosis, raised alanine aminotransferase/aspartate aminotransferase, urinary tract infection, flare in rheumatoid arthritis

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

activity, nasopharyngitis, and headache. Infusion reactions occurred in 6.6% of patients in the CT-P13 group and in 8.3% of the patients in the reference IFX group. Serious adverse events occurred in 10.0% of the rheumatoid arthritis patients receiving CT-P13 and 7.0% receiving IFX. Three cases of active tuberculosis occurred in the CT-P13 group and none in the reference IFX group. Two patients in the IFX group withdrew from the trial because of malignancy. In patients with ankylosing spondylitis, serious treatment-related adverse events occurred in 4.7 % of patients receiving CT-P13 and 6.4 % of patients receiving reference IFX. In both trials, there were no deaths. Safety data of the Hungarian IBD cohort treated with the biosimilar IFX is limited to the report of allergic reactions which were found in 2.8% of cases (all previously anti-TNF treated patients) (20).

CT-P13 is now being produced in Korea, and commercialised under the name Remsima by Celltrion Healthcare and Inflectra by Hospira (Lake Forest, IL). In September 2013, EMA approved both Remsima and Inflectra for the treatment of rheumatoid arthritis, ankylosing spondylitis, and psoriatic arthritis, as well as for adult IBD and PIBD. Until now, no postmarketing data on the safety of these agents have been published (27).

CONCERNS REGARDING THE INTRODUCTION OF BIOSIMILARS

It appears that the experience so far with the introduction of biosimilar therapeutic mAb is encouraging with regards to drug safety and effectiveness in rheumatology. Even minor alterations, however, in the production process of biologics may lead to changes in cell behaviour and cause differences in structure, stability, or other quality aspects of the end product, commonly because of differences in glycosylation patterns. Any of these differences may affect the treatment's safety, efficacy, and, most importantly with biologics, the immunogenicity (28). In children, the risk of developing immunogenicity to anti-TNF treatment is even more worrisome than in adult patients because children both have more severe disease and potentially need anti-TNF treatment for a longer period.

Previous studies have shown that these differences in glycosylation and protein structure between the original drugs and biosimilar products do occur. For instance, in a study comparing 7 brands of recombinant human G-CSF, potency differences of 82% to 105% were noted, as well as significant differences in the level of purity among various brands of G-CSF and erythropoietin biosimilars (29). Following a change in the stabilizer used in subcutaneous erythropoietin (Eprex, Janssen Cilag, High Wycombe, UK) syringes (ie, the originator drug), an unprecedented rise in the incidence of pure red-cell aplasia was noted between 1998 and 2003, with ~200 cases reported in patients with chronic renal failure. An interaction of the stabilizer with the rubber cap acted as an adjuvant that induced an immune response to erythropoietin that in turn attacked erythroblasts and caused red-cell aplasia (30). A study comparing the structure of 2 original erythropoietins and 2 erythropoietin biosimilars found considerable differences in structure and potency (31). Concerns about these experiences led to the development of EMA guidelines on biosimilar development in general (32) and specifically on mAb biosimilars (7).

Despite the potential for altered efficacy, increased immunogenicity, and adverse effects, generally the introduction of most biosimilars has turned out to be safe. In the largest study to date, with a total of 904 patients using a biosimilar G-CSF (520 with Ratiograstim [Ratiopharm, Ulm, Germany]/Tevagrastim [Pharmachemie, Haarlem, the Netherlands], 384 with Zarzio [Sandoz, Holzkirchen, Germany]), the adverse effect profile was comparable to historic controls treated with the originator G-CSF (34). Erythropoietin biosimilars such as HX575 were generally safe in most studies (31). Immunogenicity was not more than expected in some studies (30), whereas other studies did show increased prevalence of neutralizing antibodies in individuals who experienced a loss of response (35).

Still, monoclonal anti-TNF-antibody biosimilars may pose more concerns for immunogenicity and safety because these are much larger than proteins such as erythropoietin (148,000 Da vs 18,464 Da, respectively). Gaining a clear understanding of the immunogenicity impact of nonanti-TNF agents has taken several years. As stated before, immunogenicity is clinically a very relevant phenomenon with both IFX and adalimumab, and affects anti-TNF drug levels and clinical efficacy in both Crohn disease and ulcerative colitis (36–43). Therefore, there is no guarantee that our understanding of immunogenicity of the originator biological will easily be extrapolated to the biosimilar that may be subtly different in molecular structure. New assays need to be developed and studies undertaken to explore and understand the immunogenicity of the biosimilars.

Emerging study results on Remicade (Merck Sharp & Dohme, Kenilworth, NJ) biosimilars have been reassuring. Cross-reactivity between antibodies to Remicade and the biosimilar Remsima was recently investigated by Ben-Horin et al (published as an abstract) (44). They describe a cross-immunogenicity study in IBD patients. In total, 124 sera of Remicade-treated IBD patients with measurable antibodies to Remicade were tested by anti-λ enzyme-linked immunosorbent assay (ELISA) for their cross-reactivity to 2 batches of Remsima. Sera negative for anti-Remicade antibodies were tested in parallel as controls. All 68 positive anti-Remicade IBD sera were cross-reactive with Remsima. In negative controls (16 healthy individuals, 40 IBD patients), there was a slightly higher background signal in the enzyme-linked immunosorbent assay assay for Remsima compared with Remicade. Anti-Remicade antibodies of IBD patients ($n = 10$) exerted a similar functional inhibition on Remsima and Remicade TNFα-binding capacity (P = not significant for all points on the inhibition curves). Antibodies to adalimumab in adalimumab-treated IBD patients ($n = 7$) did not cross-react with neither Remicade nor Remsima. Ben-Horin et al (44) concluded that antibodies to Remicade in Remicade-treated IBD patients recognize Remsima to a similar extent, suggesting shared immunodominant epitopes on these 2 IFX agents. These currently available studies have included only adult patients, whereas no data are available in children. So far, the results suggest a strong similarity between the originator and the biosimilar product. An important implication of these findings is that patients who received Remicade and developed antibodies to IFX would not be candidates for IFX biosimilar therapy.

RESEARCH GAPS IN BIOSIMILAR RESEARCH IN IBD

Clinical trials in the IBD population could help ease concerns. The technical aspects of designing these trials of biosimilars for the treatment of IBD need careful consideration. Noninferiority trials are probably the best design, even though they are not often feasible given the required large sample size. It has been estimated that 1500 patients would be required to conclude with 95% confidence that the biosimilar would not be >7.5% inferior than the originator (45). Therefore, it is possible that regulatory decisions may be made based on trials of smaller size, increasing the likelihood of failure to detect small but clinically significant difference in therapeutic effect. Another important issue is whether regulatory agencies will require both induction and maintenance data or only induction data. We have learnt from existing TNF antagonists that attenuation of response with maintenance therapy is a key issue, and

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

it will be important to know whether the biosimilars will have similar performance characteristics in both the induction and maintenance phases of treatment. On the contrary, a lengthy approval pathway including prolonged IBD maintenance studies may result in significant delay of the introduction of biosimilars into the market and thus will result in continued elevation of therapy-related health expenditure.

Postmarketing surveillance programs for efficacy, safety, and immunogenicity should become mandatory in children with IBD using biosimilars and when using all biological drugs. For this purpose, the PIBD community recently established an international platform (PIBD-net). PIBD-net is a nonprofit organization founded in September 2014. The aim is to advance the care of children with IBD globally through investigator and industry initiated research, the development of optimal treatment plans, and monitoring safety and effectiveness of current and emerging treatments (46).

CONCLUSIONS

Concerns remain about the introduction of biosimilars, particularly in PIBD. These concerns should spark debates in the medical arena, and this information should be available to physicians who have to make the decisions about the welfare of their patients. In contrast, the introduction of biosimilars to the market will likely decrease the costs of anti-TNF drugs by $\geq$30%, thereby lowering the threshold of use of these highly effective but expensive drugs in IBD. Because of the absence of published trials on the use of biosimilars in adult IBD and PIBD, the following statements cannot be used as recommendations for management. They reflect expert opinion designed to inform paediatric gastroenterologists and to promote consensus on the proper usage of these agents in children with IBD. The statements are accompanied by the percentage of voting members of the PIBD Porto group expressing agreement: 83% (30/36) of all members voted.

The European Medicines Agency approved the use of biosimilars for infliximab for all indications, including adult and paediatric inflammatory bowel disease (IBD). The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition paediatric IBD Porto group advocates giving high priority to performing paediatric trials with long-term follow-up to support this decision: 97% agreement

Treatment of a child with sustained remission on a specific medication should not be switched to a biosimilar until clinical trials in IBD are available to support the safety and efficacy of such a change: 94% agreement

Postmarketing surveillance programs for efficacy, safety, and immunogenicity in children with IBD should be a mandatory requirement for the marketing of biologics and biosimilars with respective indications: 100% agreement

REFERENCES

1. European Medicines Agency. Biosimilar medicines. *http://www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/document_listing/document_listing_000318.jsp*. Published 2012. Accessed September 9, 2015.
2. Weise M, Bielsky MC, De Smet K, et al. Biosimilars—why terminology matters. *Nat Biotechnol* 2011;29:690–3.
3. McCamish M, Woollett G. The state of the art in the development of biosimilars. *Clin Pharmacol Ther* 2012;91:405–17.
4. World Health Organization Expert Committee on Biological Standardization. *Guidelines on Evaluation of Similar Biotherapeutic Products*. Geneva: World Health Organization; 2009.
5. Declerck PJ. Biosimilar monoclonal antibodies: a science-based regulatory challenge. *Expert Opin Biol Ther* 2013;13:153–6.
6. Danese S1, Gomollon F. ECCO position statement: the use of biosimilar medicines in the treatment of inflammatory bowel disease (IBD). *J Crohns Colitis* 2013;7:586–9.
7. European Medicines Agency, Committee for Medicinal Products for Human Use. Guideline on Similar Biological Medicinal Products Containing Monoclonal Antibodies—Non-clinical and Clinical Issues. *http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500128686.pdf*. Published 2012. Accessed September 9, 2015.
8. Mellstedt H, Niederwieser D, Ludwig H. The challenge of biosimilars. *Ann Oncol* 2008;19:411–9.
9. US Food and Drug Administration. Guidance for Industry. Quality Considerations in Demonstrating Biosimilarity to a Reference Protein Product. Draft Guidance. Washington, DC: US Food and Drug Administration; 2012.
10. Schneider CK, Vleminckx C, Gravanis I, et al. Setting the stage for biosimilar monoclonal antibodies. *Nat Biotechnol* 2012;30:1179–85.
11. Crommelin DJA, Storm G, Verrijk R, et al. Shifting paradigms: biopharmaceuticals versus low molecular weight drugs. *Int J Pharm* 2003;266:3–16.
12. Misra M. Biosimilars: current perspectives and future implications. *Indian J Pharmacol* 2012;44:12–4.
13. Schiestl M, Stangler T, Torella C, et al. Acceptable changes in quality attributes of glycosylated biopharmaceuticals. *Nat Biotechnol* 2011; 29:310–2.
14. Dorner T, Strand V, Castaneda-Hernandez G, et al. The role of biosimilars in the treatment of rheumatic diseases. *Ann Rheum Dis* 2013;72:322–8.
15. Scheinberg MA, Kay J. The advent of biosimilar therapies in rheumatology—"O brave new world." *Nat Rev Rheumatol* 2012;8: 430–6.
16. Park W, Hrycaj P, Jeka S, et al. A randomised, double-blind, multicentre, parallel-group, prospective study comparing the pharmacokinetics, safety, and efficacy of CT-P13 and innovator infliximab in patients with ankylosing spondylitis: the PLANETAS study. *Ann Rheum Dis* 2013;72:1605–12.
17. Yoo DH, Hrycaj P, Miranda P, et al. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. *Ann Rheum Dis* 2013;72:1613–20.
18. Park W, Miranda P, Brzosko M et al. Efficacy and safety of CT-P13 (infliximab biosimilar) over two years in patients with ankylosing spondylitis: comparison between continuing with CT-P13 and switching from infliximab to CT-P13 [abstract]. Paper presented at the American College of Rheumatology annual meeting. San Diego, CA; October 25–30, 2013.
19. Yoo DH, Prodanovic N, Miranda P, et al. AT Efficacy and safety of CT-P13 (infliximab biosimilar) over two years in patients with rheumatoid arthritis: comparison between continued CT-P13 and switching from infliximab to CT-P13 [abstract]. Paper presented at the American College of Rheumatology annual meeting. San Diego, CA; October 25–30, 2013.
20. Gecse K, Farkas K, Lovasz B et, al. Biosimilar infliximab in inflammatory bowel diseases: first interim results from a prospective nationwide observational cohort. *https://www.ecco-ibd.eu/index.php/publications/congress-abstract-s/abstracts-2015/item/p314-biosimilar-infliximab-in-inflammatory-bowel-diseases-first-interim-results-from-a-prospective-nationwide-observational-cohort.html*. Accessed September 9, 2015.
21. Sandborn WJ, Hanauer SB, Katz S, et al. Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2001;121:1088–94.
22. Sandborn WJ, Colombel JF, Sands BE, et al. Abatacept for Crohn's disease and ulcerative colitis. *Gastroenterology* 2012;143:62–9.
23. Nesbitt A, Fossati G, Bergin M, et al. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. *Inflamm Bowel Dis* 2007;13:1323–32.
24. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 2003;348:601–8.
25. Karmiris K, Paintaud G, Noman M, et al. Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease. *Gastroenterology* 2009;137:1628–40.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

26. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; 362:1383–95.
27. *http://www.hc-sc.gc.ca/dhp-mps/prodpharma/sbd-smd/drug-med/sbd smd2014 inflectra 159493-eng.php*.
28. Mendes de Abreu M, et al. Putting the value into biosimilar decision making: the judgment value criteria. *Autoimmun Rev* 2014;13:678–84.
29. Mody R. *http://www.pharmafocusasia.com/research_development/blind-comparative-study.html*.
30. McKoy JM, Stonecash RE, Cournoyer D, et al. Epoetin-associated pure red cell aplasia: past, present, and future considerations. *Transfusion* 2008;48:1754–62.
31. Brinks V, Hawe A, Basmeleh AH, et al. Quality of original and biosimilar epoetin products. *Pharm Res* 2011;28:386–93.
32. European Medicines Agency. Guideline on similar biological medicinal products. *http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/10/WC500176768.pdf*. Published October 23, 2014. Accessed September 9, 2015.
33. Deleted in proof.
34. Schmitt M, Xu X, Hilgendorf I, et al. Mobilization of PBSC for allogeneic transplantation by the use of the G-CSF biosimilar XM02 in healthy donors. *Bone Marrow Transplant* 2013;48:922–5.
35. Praditpornsilpa K, Tiranathanagul K, Kupatawintu P, et al. Biosimilar recombinant human erythropoietin induces the production of neutralizing antibodies. *Kidney Int* 2011;80:88–92.
36. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; 362:1383–95.
37. Hanauer SB, Wagner CL, Bala M, et al. Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. *Clin Gastroenterol Hepatol* 2004;2:542–53.
38. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 2003;348:601–8.
39. Karmiris K, Paintaud G, Noman M, et al. Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease. *Gastroenterology* 2009;137:1628–40.
40. Vermeire S, Noman M, Van Assche G, et al. Effectiveness of concomitant immunosuppressive therapy in suppressing the formation of antibodies to infliximab in Crohn's disease. *Gut* 2007;56: 1226–31.
41. Maser EA, Villela R, Silverberg MS, et al. Association of trough serum infliximab to clinical outcome after scheduled maintenance treatment for Crohn's disease. *Clin Gastroenterol Hepatol* 2006; 4:1248–54.
42. Seow CH, Newman A, Irwin SP, et al. Trough serum infliximab: a predictive factor of clinical outcome for infliximab treatment in acute ulcerative colitis. *Gut* 2010;59:49–54.
43. Panaccione R, Ghosh S, Middleton S, et al. Infliximab, azathioprine or infliximab + azathioprine for treatment of moderate to severe ulcerative colitis: the UC Success trial [abstract]. *Gastroenterology* 2011;140:A-202.
44. Ben-Horin S, Yavzori M, Fudim E, et al. Cross-immunogenicity: antibodies to infliximab in Remicade-treated IBD patients similarly recognize the bio-similar Remsima. *Gut* 2015 April 20. [Epub ahead of print]
45. Kay J, Feagan BG, Guirguis MS, et al. Summit on regulatory and clinical topics related to subsequent entry biologics (biosimilars), Ottawa: May 14, 2012. *Biologicals* 2012;40:517–27.
46. Pediatric Inflammatory Bowel Diseases Network. Mission statement. *http://pibd-net.org/index.php/mission-statement*. Accessed September 9, 2015.

Publisher's Note

Diagnostic and Therapeutic Roles of Endoscopic Ultrasound in Pediatric Pancreaticobiliary Disorders

''Diagnostic and Therapeutic Roles of Endoscopic Ultrasound in Pediatric Pancreaticobiliary Disorders'' by Scheers et al, which published in the August 2015 issue of the *Journal of Pediatric Gastroenterology and Nutrition*, is now available for Continuing Medical Education (CME) credit. Please visit *http://www.naspghan.org/content/59/en/Continuing-Medical-Education-CME* to view instructions, documentation, and the complete necessary steps to receive CME credit for reading this article.

Copyright 2015 by ESPGHAN and NASPGHAN. Unauthorized reproduction of this article is prohibited.

5.4 References

1. Meijer BJ, Dieleman LA. Probiotics in the treatment of human inflammatory bowel diseases: update 2011. J Clin Gastroenterol. 2011;45(suppl):S139–S144.
2. Menard S, Candalh C, Bambou JC, et al. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport.Gut. 2004;53:821–828.
3. Groeger D, O'Mahony L, Murphy EF, et al. Bifidobacterium infantis 35624 modulates host inflammatory processes beyond the gut. GutMicrobes. 2013;4:325–339.
4. Strisciuglio C, Miele E, Wildenberg ME, et al. T300A variant of autophagyATG16L1 gene is associated with decreased antigen sampling and processing by dendritic cells in pediatric Crohn's disease. Inflamm BowelDis. 2013;19:2339–2348.
5. Blumberg R, Cho J, Lewis J, et al. Inflammatory bowel disease: an updateon the fundamental biology and clinical management. Gastroenterology.2011;140:1701–1703.
6. Marcobal A, Underwood MA, Mills DA, et al. Rapid determination of thebacterial composition of commercial probiotic products by terminal restriction fragment length polymorphism analysis. J Pediatr GastroenterolNutr. 2008;46:608–611.
7. Underwood MA. Intestinal dysbiosis: novel mechanisms by which gutmicrobes trigger and prevent disease. Prev Med. 2014;65:133–137.
8. Jowett SL, Seal CJ, Phillips E, Gregory W, Barton JR, Welfare MR. Dietary beliefs of people with ulcerative colitis and their effect on relapse and nutrient intake. Clin Nutr 2004; 23: 161– 70.
9. Scott EM, Gaywood I, Scott BB. Guidelines for osteoporosis in coeliac disease an inflammatory bowel disease. British society of gastroenterology. Gut 2000; 46(Suppl. 1):i1–8.
10. Kleinman RE, Baldassano RN, Caplan A, Griffiths AM, Heyman MB, Issenman RM et al. North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. Nutrition support for pediatric patients with inflammatory bowel disease: a clinical report of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr 2004; 39: 15–27.
11. Gerasimidis K, McGrogan P, Hassan K, Edwards CA. Dietary modifications, nutritional supplements and alternative medicine in paediatric patients with inflammatory bowel disease. Aliment Pharmacol Ther 2008; 27: 155–65.
12. Seidman EG. Nutritional management of inflammatory bowel disease. Gastroenterol Clin North Am 1989; 18: 129–55.

13. Belli DC, Seidman E, Bouthillier L, Weber AM, Roy CC, Pletincx M, et al. Chronic intermittent elemental diet improves growth failure in children with Crohn's disease. Gastroenterology 1988; 94: 603–10
14. Seidman E, LeLeiko N, Ament M, Berman W, Caplan D, Evans J, et al. Nutritional issues in pediatric inflammatory bowel disease. J Pediatr Gastroenterol Nutr 1991; 12: 424–38.
15. European Medicines Agency. Biosimilar medicines. http://www.ema. europa.eu/ema/index.jsp?curl=pages/special_topics/document_listing/ document_listing_000318.jsp. Published 2012. Accessed September 9, 2015.
16. Weise M, Bielsky MC, De Smet K, et al. Biosimilars—why terminology matters. Nat Biotechnol 2011;29:690–3.
17. McCamish M, Woollett G. The state of the art in the development of biosimilars. Clin Pharmacol Ther 2012;91:405–17
18. Danese S1, Gomollon F. ECCO position statement: the use of biosimilar medicines in the treatment of inflammatory bowel disease (IBD). J Crohns Colitis 2013;7:586–9.
19. Mendes de Abreu M, et al. Putting the value into biosimilar decision making: the judgment value criteria. Autoimmun Rev 2014;13:678–84.
20. Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010; 362:1383–95. 37.
21. Hanauer SB, Wagner CL, Bala M, et al. Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. Clin Gastroenterol Hepatol 2004;2:542–53. 38.
22. Baert F, Noman M, Vermeire S, et al. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med 2003;348:601–8. 39.
23. Karmiris K, Paintaud G, Noman M, et al. Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease. Gastroenterology 2009;137:1628–40. 40.
24. Vermeire S, Noman M, Van Assche G, et al. Effectiveness of concomitant immunosuppressive therapy in suppressing the formation of antibodies to infliximab in Crohn's disease. Gut 2007;56: 1226–31. 41.
25. Maser EA, Villela R, Silverberg MS, et al. Association of trough serum infliximab to clinical outcome after scheduled maintenance treatment for Crohn's disease. Clin Gastroenterol Hepatol 2006; 4:1248–54. 42.
26. Seow CH, Newman A, Irwin SP, et al. Trough serum infliximab: a predictive factor of clinical outcome for infliximab treatment in acute ulcerative colitis. Gut 2010;59:49–54. 43.

27. Panaccione R, Ghosh S, Middleton S, et al. Infliximab, azathioprine or infliximab or azathioprine for treatment of moderate to severe ulcerative colitis: the UC Success trial [abstract]. Gastroenterology 2011;140:A- 202.

6. Conclusion

There is no doubt that we have achieved a big progress in the understanding of IBD pathogenesis during the past few years. IBD arises from an extremely complex interaction among genetic and environmental elements, dysregulated immune responses and alterations of the microbiome, and none of these factors alone is likely to cause the disease. More detailed information on their composition, function, and interaction is becoming increasingly accessible through high genomic approaches, investigation of environmental changes, molecular analysis of gut bacteria flora. Future research needs to further clarify the mechanisms and pathways of how bacteria, viruses or even fungi can modulate innate and adaptive immune responses.

A true understanding of IBD pathogenesis is mandatory to improve current therapeutic approaches to IBD. There is no doubt that there has been an enormous improvement in the management of IBD, however results are far from ideal, particularly in regard to rather predictable recurrence of disease.

As suggested from Fiocchi et al.[1] probably one of the reason why previous drugs fail to work is because they essentially target exclusively the immunome, with disregard of the other components of IBD pathogenesis: the exposome, the genome, and the gut microbiome. Since IBD is the result of a complex integration of different components, an effective therapy can only be achieved if an "IBD integrome" approach is implemented. Individual approaches in IBD are impractical, but a comprehensive approach, like the one that systems biology can offer, seems reasonable. Biobanking is now a reality in all major medical centers, as well as in our center, and it allows the collection of large amounts of molecular data from genomic, proteomic, and microbiomic arrays. The combination of these molecular data generate models that can improve patient classifications, predict clinical course, select the most logical treatment forms, and anticipate outcome.

Other publications:

- Galatola M, Izzo V, Cielo D, Morelli M, Gambino G, Zanzi D, Strisciuglio C, Sperandeo MP, Greco L, Auricchio R. Gene expression profile of peripheral blood monocytes: a steptowards the molecular diagnosis of celiac disease? PLoS One. 2013 Sep 17;8(9):e74747.
- Miraglia Del Giudice M, Indolfi C, Allegorico A, Cuppari C, Campana G, Strisciuglio C, Grandone A. Probiotics and allergic respiratory disease.J Biol Regul Homeost Agents. 2015 Apr-Jun;29(2 Suppl 1):80-3

6.1 References

1. Fiocchi C. Inflammatory bowel disease pathogenesis: where are we? J Gastroenterol Hepatol. 2015 Mar;30 Suppl 1:12-8. doi: 10.1111/jgh.12751. Review. PubMed PMID: 25827798.