"FEDERICO II"
UNIVERSITY OF NAPLES

PhD Program
"Human Reproduction, Development and Growth"

Director
Prof. Claudio Pignata

PhD Thesis

Genetical, Immunological and Clinical Aspects of Gastrointestinal Inflammatory Disorders in Childhood

TUTOR
Prof. Riccardo Troncone

STUDENT
Dr. Lucia Quaglietta

CONTENTS

Miele E, Pascarella F, **Quaglietta L**, Giannetti E, Greco L, Troncone R, Staiano A. Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis; *J Pediatr Gastroenterol Nutr* 2007;44: e256. 40th ESPGHAN Annual meeting, May 9-12 2007 Barcelona-Spain

Miele E, Pascarella F, **Quaglietta L**, Giannetti E, Greco L, Troncone R, Staiano A. Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis. *Aliment Pharmacol Ther* 2007, 15;25 (8):933-9

3.2 Immunohistochemical markers of small bowel inflammation in children with ulcerative colitis

Quaglietta L, Giannetti E, Aquino C, Paparo F, miele E, Friano C, Troncone R, Staiano A. Immunohistochemical markers of small bowel inflammation in children with ulcerative colitis. *42nd Annual meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), Budapest June 3-6 2009.*

Quaglietta L, Giannetti E, Aquino C, Paparo F, Miele E, Friano C, Troncone R, Staiano A. Immunohistochemical markers of small bowel inflammation in children with ulcerative colitis. Accettato come comunicazione orale: *International Symposium on Paediatric Inflammatory Bowel Disease, Parigi 9-12 Settembre 2009*

CHAPTER 4:
CLINICAL AND THERAPEUTICAL ASPECTS OF INFLAMMATORY BOWEL DISEASE (IBD)

4.1 Effect of a probiotic (VSL#3) on Induction and Manteinance of Remission in Children with Ulcerative colitis.

E. Miele, F. Pascarella, E. Giannetti, **L. Quaglietta**, R.N. Baldassano, A. Staiano. Effect of a probiotic (VSL#3) on Induction and Manteinance of Remission in Children with Ulcerative colitis. *Am J Gastroenterol* 2009 Feb;104(2):437-43

CHAPTER 5
A NEWEST ESOPHAGEAL INFLAMMATORY DISEASE: EOSINOPHILIC ESOPHAGITIS.

5.2 Eosinophilic Esophagitis and Celiac Disease: Is There an Association?

L. Quaglietta, P. Coccorullo, E. Miele, F. Pascarella, R. Troncone, A. Staiano. Eosinophilic Esophagitis and Celiac Disease: Is There an Association? *Dig Liv Dis* 2007; 39 (10): PP30

L. Quaglietta, P. Coccorullo, E. Miele, F. Pascarella, R. Troncone, A. Staiano. Eosinophilic Esophagitis and Celiac Disease: Is There an Association? *Aliment Pharmacol Ther* 2007; 26 (3):487-93.

CHAPTER 1

INTRODUCTION

CD and UC represent the two major subtypes within idiopathic IBD and share clinical, epidemiologic, and genetic features. Both are chronic, often relapsing inflammatory disorders of the gastrointestinal tract. Patients typically suffer from diarrhea, abdominal pain, rectal bleeding, malnutrition, and diminished healthrelated quality of life. The incidence of IBD is highest in the second to fourth decades of life (1). Patients with IBD are more likely to also have other chronic inflammatory diseases, including primary sclerosing cholangitis, ankylosing spondylitis, psoriasis, and multiple sclerosis (2). CD may be distinguished from UC by the distribution of intestinal inflammation. In CD, the inflammation is often transmural, focal, discontinuous, and asymmetric in distribution. The distribution of inflammation in UC is typically continuous, symmetric, and confined to the superficial mucosa and submucosa. CD most commonly involves the ileum and colon but can affect any region of the gut. UC always involves the rectum, and inflammation may extend as far as the cecum in a contiguous pattern (3). CD is associated with granulomas in 30% of cases and the deeper, transmural involvement of inflammation observed can be associated with the development of stricturing and fistulous complications, whereas UC is typically not associated with these features.

Medical therapies for both CD and UC include the use of aminosalicylates, antibiotics, immunosuppressives (azathioprine, methotrexate), corticosteroids, and monoclonal antibodies directed toward blockade of pathways contributing to the inflammatory process, with anti-TNF antibodies dramatically improving medical therapies for both CD and UC. One third of patients with UC eventually require total proctocolectomy (removal of the colon and rectum to treat UC or its complications), and up to 57% of patients with CD required one or more surgeries to remove

portions of their small or large intestines in the period prior to anti-TNF therapy (4). For these reasons, there is a significant need to more efficiently prioritize, test, and apply improved medical therapies for these disorders.

The importance of genetic factors in disease pathogenesis was first suggested through the observation that cases of IBD tended to cluster within families. The relative risk to siblings compared to general population risk ranges from 30 to 40 for CD and from 10 to 20 for UC (5). Furthermore, the relative risk for UC to siblings of a CD proband was observed to be 3.9, and the converse risk given a UC proband was observed to be 1.8. Taken together, these epidemiologic data suggest a genetic model of IBD in which some risk alleles will be unique to either CD or UC, and other risk alleles will be common to both. That genetic factors contribute to disease pathogenesis is definitively established through twin studies, which demonstrate a significantly higher rate of disease concordance in monozygotic twins than dizygotic twins, and higher concordance rates for CD (20%–50% monozygotic concordance, 0%–7% dizygotic twin concordance) than UC (14%–19% monozygotic concordance, 0%–5% dizygotic concordance) (6-9). That monozygotic twin concordance is significantly less than 100% reflects the roles of developmental and environmental factors in disease expression. By definition, expression of complex genetic disorders does not follow a simple Mendelian pattern of inheritance (e.g., autosomal recessive, autosomal dominant), and no single genetic variant alone drives disease expression. Instead, multiple genetic loci of varying statistical significance and functional effects have been associated with both CD and UC. The engine for these discoveries has been the application of the genome-wide association (GWA) study to large case-control cohorts, which has recently resulted in the identification of >30 distinct genetic loci associated with CD (10). The magnitude of the association effects, as well as the certainty with which given genes and specific causal alleles can be assigned in these regions, varies

broadly. However, genes of both the innate and adaptive immune system have been implicated in IBD.

Genes of the innate immune system associated with CD and not UC include *NOD2* and the autophagy genes, *ATG16L1* (autophagy) (11, 12) and *IRGM* (immunity related GTPaseMprotein) (13, 14). On the other hand, multiple genes involved in IL- 23 signaling as well as Th17 cell subsets have been identified in both CD and UC, notably the IL23R, interleukin 12A (IL-12A, p40), and STAT3 (signal transducer and activator of transcription) (10). The aim of this thesis is to define the genetical, immunological and clinical aspects of inflammatory gastrointestinal disorders in pediatrics, with paricolar attention to Inflammatory Bowel disease (Crohn's disease and Ulcerative Colitis) and to Eosinophilic Esophagitis.

REFERENCES

1) Loftus EV Jr, Sandborn WJ. 2002. Epidemiology of inflammatory bowel disease. *Gastroenterol. Clin. North Am.* 31:1–20
2) Urlep D, Mamula P, Baldassano R. 2005. Extraintestinal manifestations of inflammatory bowel disease. *Minerva Gastroenterol. Dietol.* 51:147–63
3) Podolsky DK. 2002. Inflammatory bowel disease. *N. Engl. J. Med.* 347:417–29
4) Loftus EV Jr, Schoenfeld P, Sandborn WJ. 2002. The epidemiology and natural history of Crohn's disease in population-based patient cohorts from North America: a systematic review. *Aliment. Pharmacol. Ther.* 16:51–60
5) Satsangi J, Jewell DP, Bell JI. 1997. The genetics of inflammatory bowel disease. *Gut* 40:572–74
6) Halfvarson J, Bodin L, Tysk C, et al. 2003. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. *Gastroenterology* 124:1767–73
7) Orholm M, Binder V, Sorensen TI, et al. 2000. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand. J. Gastroenterol.* 35:1075–81
8) Thompson NP, Driscoll R, Pounder RE, et al. 1996. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ* 312:95–96
9) Tysk C, Lindberg E, Jarnerot G, et al. 1988. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 29:990–96
10) Barrett JC, Hansoul S, Nicolae DL, et al. 2008. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* 40:955–62

CHAPTER 2

GENETICAL ASPECTS OF INFLAMMATORY BOWEL DISEASE (IBD)

2.1 Functional Consequences of NOD 2 Mutations in Crohn Disease

Abstract

Crohn's disease (CD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract. Its aetiology remained obscure until recent years, when through an overwhelming body of research, the main theme of its origin became clear. Crohn´s disease develops in those individuals who carry risk alleles for the disease that can result in loss of physiologic tolerance to commensal bacteria. As a consequence, immune responses develop that activate a whole range of immunocompetent cells, resulting in the secretion of proinflammatory mediators that ultimately cause mucosal breaks and the formation of ulceration, edema and loss of proper function.

Clinical phenotype

The disease usually develops in the young adult, with the majority of cases diagnosed between 15-35 years. However, CD can affect people at any age and approximately 10 percent of cases are under age 18 upon presentation. CD predominantly affects Caucasians; with a prevalence rate of 150 per 100.000. IBD is largely a disease of the industrialized world, especially the United States and Europe, and is more common in urban areas and northern climates. No clear-cut Mendelian pattern of inheritance in CD has been established but CD has a known genetic

component, with 25 percent of Crohn's patients having a family member with some form of IBD (1). Signs and symptoms of CD include frequent (bloody) diarrhoea, abdominal pain, fatigue, loss of appetite, weight loss, fever, stomatitis, and perianal fistula or fissures. A proportion of patients present with extraintestinal manifestations such as arthritis, erythema nodosum or pyoderma gangrenosum. In paediatric cases of CD, growth failure is observed in 75 percent of patients (1).

Clear evidence exists for the activation of the immune response in CD. The lamina propria is infiltrated with lymphocytes, macrophages, and other cells of the immune system. In any immune response, a specific antigen serves as a trigger for the response and as a target for the effector arm of the response. Over the past 35 years, an intensive search has been conducted for the antigens that trigger the immune response in CD. Immune activation in CD is largely confined to the gastrointestinal tract; therefore, the search for the antigenic trigger has focused on the intestinal lumen. Most of the foreign antigens in the intestinal lumen are of either microbial or dietary origin (2). Three major hypotheses as to the antigenic trigger in CD have been postulated. One hypothesis is that the antigenic triggers are microbial pathogens that have not yet been identified because of fastidious culture requirements. According to this hypothesis, the immune response in CD is an appropriate but ineffective response to these pathogens. Various viruses and bacteria have been proposed as candidate organisms, but little evidence has been found to support any of these organisms as having a causative role in CD. The second hypothesis is that the antigenic trigger in CD is some common dietary antigen, or usually nonpathogenic microbial agent, against which the patient mounts an abnormal immune response. In healthy persons, a finely tuned, low-grade chronic inflammation is present in the intestinal lamina propria. Presumably, this chronic inflammation is a product of chronic exposure of the lamina propria to luminal antigens. Failure to suppress this inflammatory response could result in the uncontrolled immune activation seen in CD. As a result

of failure of normal suppressor mechanisms, immune activation in CD may be an inappropriate vigorous and prolonged response to some normal luminal antigens. The third hypothesis for CD is that the antigenic trigger is one expressed on the patient's own cells, particularly intestinal epithelial cells. This is an autoimmune theory for the pathogenesis of CD. In this theory, the patient mounts an appropriate immune response against some luminal antigen, either dietary or microbial; however, because of similarities between proteins on epithelial cells and the luminal antigen, the patient's immune system also attacks the epithelial cells (3).

CD immune response

Crohn's disease is a consequence of a disturbance in the normal immunological unresponsiveness of the mucosal immune system to components of the mucosal microflora. The hyper-responsiveness to these components that ensues gives rise to the T helper type 1 (Th1)-cell mediated inflammation that underlies all forms of the disease. Activated Th1 cells secrete cytokines, such as TNF-α, IL-2 and IFN-γ. IFN-γ, in turn, activates macrophages causing them to secrete excess proinflammatory cytokines (IL-1β, TNF-α). Activated macrophages contribute to epithelial injury by secreting TNF-α and reactive oxygen species and by recruiting neutrophils, which also produce free radicals. Neutrophils are also recruited by IL-8 secreted by epithelial cells following activation or injury by bacteria. Neutrophils release reactive oxygen species, and oxygen species injury the epithelial cells. Together, the macrophages and neutrophils produce prostaglandin E2 and leukotriene B4 that contribute to the vasodilatation and enhanced vascular permeability characteristic of CD (4). CD bears the immunological stigmata of an exaggerated CD4+ T helper cell type I response. Thus, intestinal CD4+ T cells isolated from Crohn's patients produce large amounts of the Th1 signature cytokine IFN-γ21-22. Mucosal macrophages from CD patients also produce large amounts of the Th1 inducing cytokines IL-12, IL-18 and TNF-α. Th1 cell resistance

to apoptosis and increased cell cycling in CD inflammation appear to be sustained by these cytokines(5). Blocking the pathways that confer resistance of Th1 cells to apoptotic stimuli and the use of drugs that enhance mucosal T cell death, such as the immunosuppressive agent azathioprine or the antibody to TNF-α, infliximab, are effective in down-modulating intestinal inflammation (6). Identifying the particular antigen(s) that drive the Th1 inflammatory response in the face of the large amount of potential antigens in the gut, has proven difficult. Nevertheless, likelihood is that bacterial antigens are involved, because stimulation of mucosal CD4 cells from CD patients with extracts of their own commensal flora can induce IFN-γ production (7). Clinical observations also support a role for antigens derived from the commensal flora. Thus, for example, the antibiotic metronidazole is of therapeutic benefit in CD of the distal colon, eliminating the commensal flora, resulting in decreased inflammation (8).

Conclusion and rationale

The cellular and molecular mechanisms of interaction between intestinal mucosal cells and the resident luminal bacteria in healthy individuals and patients with CD, is not yet fully understood, but is currently an area of active investigation.

Recently mutations in NOD2/CARD15, encoding an intracellular bacteria-sensing protein, expressed mainly by macrophages and dendritic cells, have been associated with CD.

Different theories exist for NF-κB activation in NOD2 variants. Mutation(s) in NOD2 causing a loss-offunction of NOD2, resulting in a decreased suppression of the TLR2-driven Th1 response, via NF-κB. On the other hand, it has been hypothesized that mutated NOD2 enhances (gain-of-function) the sensitivity of macrophages to MDP potentiating NF-κB activity. This review focuses on the functional consequences of NOD2/CARD15 mutations in the pathogenesis of CD.

Pattern recognition molecules (PRRs)

The host mucosa is exposed to vast numbers of metabolically active microbial cells and cell wall components, such as lipopolysaccharide (LPS) a component of the outer membrane of Gram negative bacteria, and peptidoglycan (PGN), a complex amino sugar present in Gram negative as well in Gram positive bacteria. A unique feature of host-microbial interactions in the intestine is the lack of proinflammatory responses in the mucosa exposed to the resident luminal microflora, whilst retaining the capability to respond to luminal pathogenic bacteria via the recruitment of acute inflammatory cells from the systemic circulation. The gut epithelium itself can also directly sense commensal bacteria and pathogens; integral to this are the mammalian pattern recognition receptors (PRRs), which recognize conserved structures of bacteria and viruses and generally activate pro-inflammatory pathways alerting the host to infection. Two different classes of PRRs are involved (9,10). The Toll-like receptors (TLRs) are usually associated with cell membranes and have an external leucine-rich repeat (LRR) recognition domain and an intracellular interleukin-1 receptor (IL-1R)- like signaling domain that activates intracellular signaling pathways (11). To date, 11 members of the TLR family have been identified in mammals. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast TLR3, TLR7 and TLR9, all of which are involved in the recognition of nucleic-acid like structures, are not expressed on the cell surface. For example, TLR9 has recently been shown to be expressed in the endoplasmic reticulum (11). After ligand binding, TLRs/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signaling molecules (10). These include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), which activates the serine/threonine kinases of the IL-1R-associated kinase family (IRAK), and the tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), subsequently leading to the degradation of the inhibitor of NF-κB activity protein IκB.

This results in the activation of NF-κB and its translocation to the nucleus (12). In addition to TLRs, microbial products can be recognized by members of the nucleotide-binding oligomerization domain protein (NOD) family.

Nucleotide-binding oligomerization domain (NOD)

Introduction

Nods are cytosolic proteins that contain a nucleotide-binding oligomerization domain (NOD). These proteins include key regulators of apoptosis and pathogen resistance in mammals and plants. A large number of Nods contain leucine-rich repeats (LRRs), hence referred to as NOD-LRR proteins. Genetic variation in several NOD-LRR proteins, including human Nod2, Cryopyrin, and CIITA, as well as mouse Naip5, is associated with inflammatory disease or increased susceptibility to microbial infections. Nod1, Nod2, Cryopyrin, and Ipaf have been implicated in protective immune responses against pathogens. Together with Toll-like receptors, Nod1 and Nod2 appear to play important roles in innate and acquired immunity as sensors of bacterial components. Specifically, Nod1 and Nod2 participate in the signaling events triggered by host recognition of specific motifs in bacterial peptidoglycan and, upon activation, induce the production of proinflammatory mediators. Naip5 is involved in host resistance to *Legionella pneumophila* through cell autonomous mechanisms, whereas CIITA plays a critical role in antigen presentation and development of antigen-specific T lymphocytes. Thus, NOD-LRR proteins appear to be involved in a diverse array of processes required for host immune reactions against pathogens.

Family of NOD-LRR proteins

The NOD-LRR proteins are also referred to as the CATERPILLER (CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats) family (13). NOD-LRR proteins are expressed primarily in immune cells, although the expression of certain proteins, such as Nod1, is

ubiquitous. The majority of animal and plant NOD-LRR proteins are comprised of three distinct functional domains: an amino-terminal effector domain involved in signaling, a centrally located regulatory NOD-domain, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (Figure 2).

The effector domains of mammalian NOD-LRR proteins are structurally variable, linking these proteins to multiple signaling pathways and biological functions. These effector domains are involved in homophilic and heterophilic interactions with downstream signaling partners. The diversity of the effector domains allows NOD-LRR proteins to interact with a wide array of binding partners and to activate multiple signaling pathways. Effector domains involved in homophilic association include the caspase-recruitment domain (CARD) (14) and the pyrin domain (PYD, also called DAPIN and PAAD) (15). Both the PYD and the CARD belong to the death domain-fold family characterized by six α-helices that are tightly packed and include the death domain (DD) and death effector domain (DED) (16). Only 3 human NOD-LRR proteins possess aminoterminal CARDs, whereas NOD-LRR proteins possessing a PYD are, by far, the most numerous and include 14 proteins designated NALPs.

Several NOD-LRR proteins contain amino-terminal sequences that are not involved in homophilic protein interactions, including neuronal apoptosis inhibitor proteins (NAIPs) and CIITA (17,18). Instead of a CARD or PYD, the amino termini of NAIPs are composed of amino-terminal baculovirus-inhibitor-of-apoptosis repeats (BIRs) (19). CIITA is a transcriptional co-activator involved in the regulation of major histocompatibility complex class (MHC) genes, especially class II (MHC-II) (17, 19). CIITA contains an amino-terminal transcriptional activation domain that is essential for MHC gene trans-activation through its interaction with multiple nuclear factors, including CBP/p300, RFX5, NF-Y, and CREB (19).

Detection of microbial products by NOD-LRR proteins

Whereas initial studies identified lipopolysaccharide (LPS) as a NOD2 ligand (20), it is now well established that the NOD1 and NOD2 ligands are the peptidoglycan (PGN)-derived peptides γ-d-glutamyl*meso*- diaminopimelic acid (iE-DAP) (21,22) and muramyl dipeptide (MDP) respectively (23, 24). Because PGN from both Gram-positive and Gram-negative bacteria contains MDP, NOD2 functions as a general sensor of most, if not all, bacteria. By contrast, because PGNs from Gram-positive bacteria do not contain iE-DAP (except for PGNs derived from specific Gram-positive bacteria such as *Listeria* and *Bacillus* spp. and from many Gram-positive bacteria in the soil)(25) NOD1 mainly senses products from Gram-negative bacteria. Confirmation of these specificities has come from the finding that macrophages isolated from NOD1- or NOD2-deficient mice are completely unresponsive to their respective ligands (21, 26). The NODprotein ligands need to reach the LRR domains of the respective NOD protein for activation of this protein to be initiated. However, information on how this is accomplished is sparse at present, especially in the case of APCs. One possibility that relates to phagocytic cells such as macrophages or DCs is that these cells generate the peptide ligands by ingesting whole bacteria and then digesting them in phagolysosomes (27, 28). In epithelial cells, a slightly different process might occur in that the apical peptide transporter PEPT1 seems to have a role in the delivery of MDP. This is indicated by the finding that MDP that is taken up by PEPT1 into colonic epithelial cells subsequently mediates the activation of NF-κB (29). In addition, it has recently been shown that *H. pylori* can 'inject' PGN into cells through a type IV secretion system, which is encoded by a pathogenicity island (30). This discovery indicates that PGN can enter cells by various mechanisms that involve bacteria–host interactions. After small peptides derived from PGN have been released into the cytosol, they are thought to interact with NOD1 or NOD2 through the LRR domains of these molecules. However, it should be noted that, as is the case for activation of most TLRs by their respective ligands (31), there is, as yet, no direct evidence for the binding of the NOD1 and NOD2 ligands to these

domains. The postulated interaction is then proposed to initiate the activation of NOD1 and NOD2 through the induction of a complex conformational change (32,33). Our understanding of this change comes, in part, from studies of activation of apoptotic-protease-activating factor 1 (APAF1), an NLR-family (*NACHT-LRR family-domain present in NAIP, CIITA, HET-E and TP1-LRR family*) member that is involved in caspase activation and apoptosis (34,35). Activation of APAF1 is initiated by the interaction of its WD40 domain with its ligand (cytochrome *c*), as well as by the binding of dATP or ATP to an ATP-binding cassette (ABC) or oligomerization cassette in the NOD. The molecule then undergoes self-oligomerization, which enables it to bind its downstream effector molecule, caspase-9, through a CARD–CARD interaction. The large molecular complex that is formed in this way, which is known as the apoptosome, then facilitates activation of the bound caspase-9, possibly by bringing caspase molecules into juxtaposition (34,35). That this activation model applies to NLRs in general (and to NOD1 and NOD2 in particular) is indicated by the presence of structural similarities between NOD proteins and APAF1: the N-terminal region of the central NOD in NLRs contains both an ABC and an oligomerization module. At least in the case of NOD2, the introduction of mutations into the ABC region abolishes NOD2 signalling (33). In addition, it has been shown that both NOD1 and NOD2 undergo self-oligomerization following the binding of PGN-derived ligand (32, 33). In one model of NOD-protein activation based on the APAF1–caspase-9 pathway, Inohara *et al.*(25) proposed that oligomerization of NOD1 or NOD2 also allows binding to a downstream effector molecule through a CARD–CARD interaction, in this case involving the serine/threonine kinase RICK (receptor-interacting serine/threonine kinase; also known as RIP2 or CARDIAK), and this, in turn, leads to crossactivation of RICK. However, further work is necessary to establish this possibility.

Signal transduction pathways of NOD-LRR

One of the main outcomes of NOD1 and NOD2 activation by their respective ligands is the activation of NF-κB. Whereas such activation was clearly evident in iE-DAP- or MDP-stimulated epithelial cells that had been transfected with constructs encoding wild-type NOD1 or NOD2, it was reduced in cells that were transfected with constructs encoding a mutated NOD2 that had alterations in the LRR domain (23).

Consistent with this, after stimulation with MDP, translocation of NF-κB subunits to the nucleus is observed in human and mouse APCs that have intact NOD2 but not in APCs that are deficient in NOD2 or have a mutation in NOD2 (23, 34). The activation of NF-κB by NOD1 and NOD2 occurs exclusively through the downstream effector molecule RICK. This is shown by the finding that transfection of RICK-deficient fibroblasts with constructs encoding NOD1 or NOD2 results in severely defective NF-κB activation (35). It should be noted, however, that RICK-deficient macrophages also have reduced cytokine responses following stimulation with LPS, lipoteichoic acid and PGN, indicating that TLR2 and TLR4 might also use RICK as a downstream effector molecule, although the existence of a TLR4–RICK pathway is controversial (35). RICK is a CARD-containing serine/threonine kinase that physically associates with the CARD(s) of NOD1 and NOD2 through CARD–CARD interactions (36, 37). As shown recently by Abbott *et al.* (38), following its activation by NOD2, RICK mediates K63-linked polyubiquitylation of inhibitor of NF-κB (IB)-kinase-γ (IKKγ; also known as NEMO; the key member of the IKK complex) at a unique ubiquitylation site (the lysine residue at position 285). As shown previously, K63-linked polyubiquitylation is associated with activation of the NF-κB pathway (39), and in the case of the RICK–IKKγ interaction, it is, indeed, followed by phosphorylation of IKKγ and downstream activation of NF-κB, leading to the translocation of transcriptional components of NF-κB to the nucleus. So, in activating the IKK complex, RICK either activates an E3 ubiquitin ligase that promotes K63-linked polyubiquitylation or inhibits an enzyme (such as cylindromatosis protein,

CYLD) that de-ubiquitylates proteins that are modified with K63-linked polyubiquitin, so RICK does not require its own kinase activity for this function. Recently, it has been shown that activated NOD2 (but not NOD1) also interacts with the intracellular molecule GRIM19 (gene associated with retinoid-IFN-induced mortality 19) and that such an interaction might be required for optimal NF-Kb activation. However, neither the structural basis of this interaction nor the mechanism of its relation to NF-B activation is known (40). Another outcome of NOD1 and NOD2 activation is the activation of the mitogenactivated protein kinase (MAPK) pathway. So, stimulation of wild-type macrophages, but not NOD2- deficient macrophages, with MDP leads to activation of p38MAPK and extracellularsignal- regulated kinase (ERK) (41 ,42). In addition, activation of NOD1 by its ligand leads to the activation of JUN N-terminal kinase (JNK) (43). The mechanism of such NF-κB-independent signalling is unknown at present. Finally, it has been shown in transfection and immunoprecipitation studies that NOD2 binds procaspase-1, and when cells are transfected with constructs encoding NOD2 and procaspase-1, NOD2 induces interleukin-1β (IL- 1β) secretion (44). Because caspase-1 is required for processing pro-IL-1β (45) into mature IL-1β45, NOD2 might bind procaspase-1 through a CARD–CARD interaction in the same way that it binds RICK and, in doing so, convert the procaspase into a caspase. However, whether NOD2 has this function under physiological conditions remains to be seen.

Genetic NOD2 polymorphisms

Introduction (IBD loci)

A role for genetic factors in CD was first suggested by epidemiologic studies showing familial aggregation of disease and by twin studies that reported greater concordance for disease in monozygotic twins compared with dizygotic twins (46). Over the past 8 years, this evidence has been supplemented by molecular data from genome-wide linkage studies of multiple affected IBD

families. These studies have been remarkably successful in identifying a number of susceptibility loci, with convincing replication shown for at least 7 loci (IBD1-7). Some loci have been shown to be specific to either ulcerative colitis (e.g., IBD2) or Crohn's disease (IBD1), whereas others confer common susceptibility to IBD. Collectively, these genome scans reaffirm the concept that IBDs are complex genetic disorders with several predisposing genes (47).

In 2001, 3 independent groups reported the identification of the first Crohn's disease susceptibility gene, NOD2 (renamed CARD15 by the international nomenclature committee), on chromosome 16q12 (IBD1 (47). This major breakthrough firmly established a role for genetics in determining susceptibility to CD and has provided a proof of principle that model-free linkage analyses may be used successfully to identify disease susceptibility gene loci. Recent studies have highlighted a number of associations between genotype and phenotype. These suggest that genetics also may influence the clinical manifestations of CD including disease location, behavior, natural history and response, and side affects of drug therapy (46). These discoveries may in the future allow more accurate prediction of disease, permitting the implementation of highly specific therapy tailored to an individual's genotype.

NOD2 polymorphisms

NOD2/CARD15 has gained recent prominence through its association with increased susceptibility to CD. Thirty non-conservative mutations, also called variants or polymorphisms, associated with CD have been identified within the NOD2/CARD15 gene, but only three are common. *Lesage et al* (48) showed that the three common mutations- Arg702Trp, Gly908Arg and Leu1007fsinsC- account for 82% of the mutated alleles. The relative risk to develop the disease when carrying one mutation is 2-3, but increases dramatically to 20-40 in case of two mutations (49). Around the 40-50% patients carry at least one mutation in the NOD2/CARD15 gene but heterogeneity has been reported and in at least three populations (Japanese, Korean, African-American) the gene is not

implicated in CD (50-54). The prevalence of CD in Western Europe is 1-2/1000, so it is possible to deduce from these relative risks that probability of developing the disease is 4-8% in the group with two mutations. However, the penetrance is modest: less than 10% of all persons carrying two CARD15 risk alleles will develop CD: it means that other genes and environmental stimuli are needed for disease expression (55).

Functional consequences

Introduction

The mechanism by which CARD15 mutations cause susceptibility to CD is poorly understood: three main views are being considered at present. Because signaling via mutated NOD2 proteins leads to defective activation of the transcription factor NF-κB, one proposal is that mutations cause deficient NF-κB-dependant Th-1 responses and increased susceptibility to infection.

Two recent studies used genetically altered mouse models to address this hypothesis and have come to surprisingly different conclusions.

In the first study *Meada et al.* (56) introduced into mice a mutation of CARD15 homologous to the major mutation in human Crohn's disease (Leu007fsinsCys), which resulted in a truncated protein, lacking the last 33 amino acids. These model theoretically mimics the genetic defect in Crohn's disease. They examined the effect of the NOD2 mutation on NF-κB activation in bone marrow derived macrophages after stimulation with various TRL ligands or TRL2 ligand (PGN) plus MDP. No differences between wild-type and mutated mice were seen after stimulation, except in the case of stimulation with MDP alone, in which the authors found that macrophages of these mutant mice had enhanced sensitivity to MDP relative to WT counterparts with increased production of IL-1β, a target of the pro-inflammatory mediator NF-κB. These data together with the finding that knock-in mice were more suscettible to dextran-sulphate induced colitis (DSS) and showed increased amount of IL-1β, IL-6 and cyclooxygenase-2 (Cox-2) protein in colons relative to WT counterparts,

indicated that the frameshift mutation associated with Crohn's disease is a gain of function, that results in disease associated with IL-1β (and perhaps IL-6) production. Thus, the authors argue that individuals with NOD2 mutations may have an enhanced responsiveness to bacterial PGN, resulting in high levels of production of pro-inflammatory cytokines by intestinal macrophages.

However, this model cannot explain *in vitro* data showing that epithelial cells, transfected with *CARD15* that contains Crohn's-disease-associated mutations, have defective NF-κB activation in response to stimulation with MDP and, more importantly, it also cannot explain why peripheral-blood mononuclear cells isolated from patients with Crohn's disease that have a frameshift mutation in *CARD15* show a marked defect in IL- 1β production, rather than increased IL-1β production. Finally, these knock-in mice did not have any abnormality in the production of TH1 cytokines, which is an almost universal finding in Crohn's disease (57). However in order to this "gain of function" hypothesis in human, *Zelinkova et al.*(58) used monocyte-derived dendritic cells from CD patients carrying double-dose NOD2 mutations and wild type controls. Mature DCs were stimulated with MDP and the production of the pro-inflammatory cytokines, TNF-α and IL-12 was measured. Mature DC from NOD2 mutants showed significantly increased IL-12p70 and TNF-α production upon stimulation with MDP compared with wild type controls. These first observations in humans support the hypothesis of NOD2 variants associates with CD acting as a gain-of-function allele supporting the regulatory role of NOD2 as the basic cause of disease.

On the other hand, *Kobayashi et al.*(42) proposed a loss-of-function mutation in NOD2, in vivo, which affects mainly epithelial cells rather than macrophages. They generated NOD2 -/- mice using a targeting construct to replace the NOD, which is essential for the activation of the protein. The mutant animals displayed no symptoms of intestinal inflammation when observed for up to 6 months, and there was no significantly enhanced susceptibility to colitis in the DSS model. Their mutant mice lack responsiveness to MDP in several assays. The mice also developed a more severe

infection with *Listeria monocytogenes* when given orally compared to systematically, indicating a loss of control over intestinal infection, but not an overall suppressed ability to defend against the pathogen in NOD2 deficient mice. To investigate potential genes that might be induced by NOD2 during intestinal infection, they isolated RNA samples from wild-type and NOD2 -/- terminal ileum Paneth cells before and after *Listeria* infection and screened them by microarray analysis. The most significant difference was in the expression of a subgroup of cryptdins (analogous to α-defensins in human). Cryptidins are antimicrobial peptides that are produced in intestinal Paneth cells of mice, and their antimicrobial activity is important in suppressing infection with pathogenic bacteria. Their results indicate that NOD2 is essential in the detection of bacterial MDP, and in the regulation of cryptdin (α-defensins in human) expression in Paneth cells. Paneth cells are located at the (terminal) ileum, the side where CD patients having NOD2 gene mutations, are mostly affected. These data supportive of the concept that NOD2 mutations produce a kind of immunodeficiency state that predispose to a type of bacterial. A third resolution emerged from a study by *Watanabe et al.* (34) In this study a second mouse strain was also made completely deficient in NOD2 (target delection of exon 1); these mice had a reduced responses to MDP, yet had enhanced responses to PGN inducing elevated levels of IL-12. To explain this finding, the authors invoked an additional signal delivered through toll-like receptor -2 (TLR2)- which was supported by

studies with a second TRL2 ligand and purified MDP. Thus, activation of normal NOD2 inhibited signals codelivered through TLR2. A loss of function- mutation of NOD2 together with TLR2 signals delivered by other bacterial products will result in enhanced cytokine responses by macrophages (or dendritic cells) to commensal bacteria and result in inflammation. The data by Watanabe and colleagues emphasize the importance of innate immunity in driving a chronic inflammatory disease. Targeting TLR2 signaling may therefore be a useful approach in the treatment of individuals with Crohn disease that carry the *NOD2* mutation. Another outcome of this

study is the possibility of using NOD2 ligands as anti-inflammatory agents. This may explain the anti-inflammatory effects of certain Gram-positive bacteria used in the treatment of inflammatory bowel disease In addition to the results in mice, *Wehkamp et al.*(59) suggest that the expression of α-defensins is diminished in human CD patients, particularly those who have NOD2 gene mutations. They compared mucosal levels of the human α-defensins: epithelial human defensin 5 (HD5) and epithelial human defensin 6 (HD6) in CD patients with respect to NOD2 genotypes and healthy controls. They found diminished HD5 and HD6 expression in the ileum of patients carrying a NOD2 mutation compared with either controls or those with non-ileal CD. In addition to the NOD2 -/- mouse model, they suggest that the defensin deficiency secondary to NOD2 mutations in human could lead to a breakdown of the mucosal barrier with a secondary inflammation, leading to the development of CD.

Recent reports have shown synergy between NOD2 activation and several TLR ligands in cellular responses. *van Heel et al.*(60) demonstrated, using primary human cells of differing NOD2 genotypes, that NOD2 stimulation normally synergistically enhances TRL9 responses (TNF-α and IL-8 secretion) and that synergy is lost in Crohn's disease associated NOD2 homozygotes, with implications for TRL- mediated intestinal homeostasis and inflammation. Peripheral blood mononuclear cells (PBMCs) were stimulated with CpG DNA (TRL9 ligand) and MDP. MDP stimulation of PBMCs from normal individuals results in 2-3-fold

enhancement of CpG DNA stimulation of PBMC production of TNF-a and IL-8, such enhancement is not seen in PBMCs from patients with Crohn's disease bearing NOD2 mutations. Concomitant studies of IL-12 secretion were not reported in this study or in a previous study of normal individuals and Crohn's disease patients with NOD2 mutations (61), perhaps because secretion of IL-12 by PBMCs is low and thus difficult to assess. It's not kown if the loss of the enhancing effect of NOD2 is counterbalanced by loss of an inhibitory effect and if the same or similar findings

would be obtained if the authors had studied intestinal cells that differ considerable from peripheral cells in response to various stimuli. In any case, based on these findings, van Heel and colleagues propose that synergistic cytokine response between TLR9 and NOD2 might be beneficial in maintaining intestinal homeostasis and the lack of such synergism is a cause of Crohn's disease.

Conclusion

A tightly regulated response allows the immune system to co-exist with the large amount of antigenic material present in the gastrointestinal tract in the form of commensal microorganisms and food antigens, while retaining the ability to respond to pathogens. In genetically susceptible individuals however, alterations in responses to the resident luminal bacteria may lead to the development of CD, a complex multifactorial disease whose pathogenesis is still not well understood. Errors in interpretation or regulation of immune perception and responsiveness disrupt mucosal homoeostasis, and predispose the individual to uncontrolled or pathological inflammation. Tissue damage in most patients with CD can be accounted for by the downstream effects of activated Th 1 cells. T helper type 1 cell differentiation takes place when T cells interact with APCs that produce pro-inflammatory cytokines in response to exposure to bacteria. In Crohn's disease the type 1 activation is exaggerated and results in the secretion of excess pro-inflammatory cytokines, such as IL-1β and TNF-α. At the same time, anti-inflammatory cytokines such as IL-10 and TGF-β, which are responsible for downregulating the Th 1 response, are not produced to counteract this misbalance in CD (61). Recent studies have begun to define the mechanisms through which crucial protein recognition receptors may regulate intestinal innate immunity. Cooperative as well as competitive interactions may occur between different bacterial and nonbacterial ligands via TLRs and NODs leading to differential pro- as well as anti-inflammatory immune responses in different cell types. Both, TLRs and NOD2, are significantly involved in host defense and tissue repair responses, thus crucially maintaining mucosal homeostasis. TLR signaling protects intestinal

epithelial barrier and maintains tolerance, while NOD2 signaling exerts antimicrobial activity and prevents bacterial invasion. Thus, both receptors collectively exhibit distinct features that ensure commensal as well as mucosal homeostasis. Imbalance of the complex interactions between commensals and PRRs may result in tissue injury and subsequent inflammation of the intestinal mucosa. Aberrant TLR and/or NOD signaling may stimulate diverse inflammatory responses leading to chronic intestinal inflammation, seen in CD.

The discovery of an association of NOD2 variants with CD in humans has led to intensive research in this field for the last few years. Unraveling the signaling transduction cascade of NOD2 has proven difficult, and the link to the development of CD has been demonstrated by epidemiologic and linkage studies, but the exact cellular mechanism responsible for the CD phenotype in NOD2 variants still needs further analysis. Wild type NOD2 signaling by its specific ligand MDP leads to NF-κB activation and the subsequent production of pro-inflammatory cytokines. However, a loss-of-function mutation of NOD2 variants would lead to deficient NF-κB activation and subsequent decrease of Th 1 responses. Kobayashi et al (42). and Wehkamp et al.(34) showed that normal NOD2 function is responsible for the production of antibacterial peptides (defensins) in Paneth cells. In NOD2 variants, deficient defensin production leads to diminished bacterial clearance at the epithelial surface, and enhanced activation of macrophages or dendritic cells within the intestine. Together these effects would heighten the cellular immune responses mediated by high levels of IL-12 and TNF-α. This theory is particularly interesting because Paneth cells are concentrated most highly in the terminal ileum which is the most common site of inflammation in CD. Furthermore, NOD2 mutations have been consistently associated with ileal involvement in CD. On the other hand, Meada et al.(56) and Zelinkova et al.(57) found that macrophages and dendritic cells, respectively, with NOD2 mutations, had enhanced responsiveness to MDP, releasing excess pro-inflammatory cytokines compared to WT cells. This surprising gain-of-function finding is at odds

with previously generated data. Despite some reservations, however, it remains possible that more complex interactions between mutated NOD2 and downstream signaling complexes result in enhanced NF-κB activation in macrophages and dendritic cells in response to MDP. Finally, Watanabe et al. (34) propose that intact NOD2 signaling prevents the development of CD by controlling PGN-mediated Th 1 responses via TLR2. A loss-of-function mutation of NOD2 together with TLR2 signals delivered by other bacterial products will result in enhanced cytokine responses by macrophages or dendritic cells to commensal bacteria and result in inflammation.

Inflammation not only results from an up-regulation of pro-inflammatory proteins by APCs or effector T cells, but is balanced by regulatory cells which secrete IL-10 or TGF-β which are cytokines known to inhibit T cell proliferation. Netea et al.(60) demonstrated that NOD2 mutations in mononuclear cells produce less IL-10 in response to various bacterial ligands compared to WT cells. The IL-10/TNF-α ratio was only half of patients bearing the WT allele, favoring mucosal inflammation. Finally, linkage studies show an association between the TLR4 polymorphism Asp299Gly and the development of CD. However other research groups weren't able to significantly reproduce these findings. Further studies of the physiologic and pathophysiologic mechanisms within this network of possible cell-cell, ligand-ligand and PRR-PRR signaling interactions that may favor or prevent CD could lead to promising, novel approaches that may differentially exploit the TLR/NOD pathways, and lead to novel therapeutic strategies. It is likely that differential therapeutic strategies will need to include agonists as well as antagonists of PRRs, taking into account differences of PRR pathophysiology at different stages of disease as well as phenotypic and genotypic heterogeneity between distinct subgroups of CD patients. Prophylactic application of selective TLR/NOD2 ligands could enhance desired commensal mediated tissue protective processes in order to prevent disease. Once an acute inflammatory episode has broken out, some of the untoward effects of intestinal inflammation could be stopped by blocking

uncontrolled signal transduction by specific TLR/NOD2 inhibitors, thus dampening the tissue destructive effects. One key element to this disease-modifying approach might be to stop, rather than entirely eliminate the dysregulated innate responses in CD. In this context, careful assessments of adverse effects will be critical when modulating such fundamental host defense pathways of innate immunity. Given the rapid and exciting advancements of research in this field over the last few years, it is reasonable to presume that more immunological evidence and concrete directions for the potential value of these PRRs as therapeutic targets in CD, will emerge in the near future.

REFERENCES

1. Head, K., Jurenka, J. Inflammatory bowel disease part II: Crohn's disease pathophysiology and conventional and alternative treatment options. Alternative Medicine Review 2004; 9: 360-401.
2. Philpott, D.J., Viala, J. Towards an understanding of the role of NOD2/CARD15 in the pathogenesis of Crohn's disease. Clinical Gastroenterology 2004; 18: 555-568.
3. Yamada, T., Alpers, D.H., Laine, L. et al. Textbook of Gastroenterology Volume 1, Third edition (1999)
4. Ohkusa, T., Nomura, T., Sato, N. The role of bacterial infection in the pathogenesis of inflammatory bowel disease. Internal Medicine 2004; 43: 534-539.
5. MacDonald, T.T., Monteleone, G. IL-12 and Th1 immune responses in human Peyer's pathes. Trends Immunol 2001; 22: 244-247.
6. Dhillon, S., Loftus, E.V. Medical therapy of Crohn's disease. Curr. Treat Options Gastroenterol 2005; 8: 19-30.
7. Duchmann, R., Kaiser, I., Hermann, E. et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995; 102: 448-455.
8. Greenberg, G.R. Antibiotics should be used as first-line therapy for Crohn's disease. Inflamm Bowel Dis 2004; 10: 318-320.
9. Akira, S., Takeda, K. Toll-like receptor signaling. Nature Immunol 2004; 4: 499-511
10. Russell, R.K., Wilson, D.C., Satsangi, J. Unraveling the complex genetics of inflammatory bowel disease. Arch Dis Child 2004; 89: 598-603.
11. Cario, E. Bacterial interactions with cells of the intestinal mucosa: toll-like receptors and NOD2. Gut 2005; 54: 1182-1193.
12. Dunne, A., O'Neill, L.A. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE. 2003 Feb 25;2003(171):re3
13. Harton JA, Linhoff MW, Zhang J, et al. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. J Immunol 2002;169:4088–93.
14. Hofmann K, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. Trends Biochem. Sci. 1997; 22:155 56
15. Bertin J, DiStefano PS. The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. Cell Death Differ. 2000 Dec;7(12):1273–1274
16. Liepinsh, E., Barbals, R., Dahl, E., Sharipo, A., Staub, E. and Otting, G. (2003) The death-domain fold of the ASC PYRIN domain, presenting a basis for PYRIN/PYRIN recognition. J Mol Biol, 2003; 332, 1155-63.
17. Steimle V, Otten LA, Zufferey M, et al. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell. 1993;75:135–146

18. Roy N, Mahadevan MS, McLean M, *et al.* The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 1995;80:167-178
19. Reith W, Mach B. The bare lymphocyte syndrome and the regulation of MHC expression. Annu Rev Immunol 2001;19: 331–73.
20. 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.
21. Chamaillard M; Hashimoto M; Horie Y *et al.* An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nature Immunol 2003; . 4, 702–707.
22. Girardin S, Boneca I, Carneiro LAM et al. Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Science 2003; 300, 1584–1587.
23. Inohara, N, Ogura Y, Fontalba A, *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J Biol Chem 2003; 278, 5509–5512.
24. Girardin, S. E, Boneca I, Viala J, *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003; 278, 8869–8872.
25. Inohara, N., Chamaillard, M., McDonald, et al. NOD-LRR proteins: role in host–microbial interactions and inflammatory disease. Annu Rev Biochem 2004; 74, 355–383.
26. Pauleau, A. L., Murray, P. J. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. Mol Cell Biol 2003; 23, 7531–7539.
27. Gupta, D. K., Theisen, N., von Figura, K. et al. Comparison of biosynthesis and subcellular distribution of lysozyme and lysosomal enzymes in U937 monocytes. Biochim Biophys Acta 1985; 847, 217–222.
28. Araki, Y., Nakatani, T., Makino, et al. Isolation of glucosaminyl-â (1–4)-muramic acid and phosphoric acid ester of this disaccharide from acid hydrolysates of peptidoglycan of *Bacillus cereus* AHU 1356 cell walls. Biochem Biophys Res Commun 1971; 42, 684–690.
29. Vavricka, S. R. Musch MW, Chang JE, et al. hPepT1 transports muramyl dipeptide, activating NF-κB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. Gastroenterology 2004; 127, 1401–1409.
30. Viala, J. Chaput C, Boneca IG et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. Nature Immunol 2004; 5, 1166–1174.
31. Bell, J. K. Mullen G.E., Leifer C.A *et al.* Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol 2003; 24, 528–533.
32. Inohara N, Koseki T, Lin J, *et al.* An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signalling pathways. J Biol Chem 2000; 275, 27823–27831.
33. Tanabe, T, Chamaillard M, Ogura Y et al. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. EMBO J 2004; 23, 1587–1597.
34. Watanabe, T., Kitani, A., Murray, et al, W. NOD2 is a negative regulator of Toll-like receptor 2- mediated T helper type 1 responses. Nature Immunol 2004; 5, 800–808.
35. Kobayashi, K., N. Inohara, L. D. Hernandez, J. E. *et al.* RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 2002; 416, 194–199.
36. Ogura Y, Inohara N, Benito A et al., Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem. 2001 Feb 16;276(7):4812-8.
37. Inohara N, Koseki T, del Peso L, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. J Biol Chem 1999; 274: 14560–14567.
38. Abbott, D. W., Wilkins, A., Asara, J. M. et al. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr. Biol.* 2004; 14, 2217–2227.
39. Zhou, H. Wertz I, O'Rourke K, et al. Bcl10 activates the NF-κB pathway through ubiquitination of NEMO. *Nature* 2004; 427, 167–171.

40. Barnich, N, Hisamatsu T, Aguirre JE et al. GRIM-19 interacts with nucleotide oligomerization domain 2 and serves as downstream effector of anti-bacterial function in intestinal epithelial cells. J. Biol. Chem.2005; 280, 19021–19026.
41. Pauleau, A. L., Murray, P. J. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. Mol. Cell. Biol. 2003; 23, 7531–7539.
42. Kobayashi KS, Chamaillard M, Ogura Y, *et al.* Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science *2005;* 307, 731–734.
43. Girardin, S. E., Tournebize R, Mavris M, et al. CARD4/Nod1 mediates NF-κB and JNK activation by invasive Shigella flexneri. EMBO Rep. 2001; 2, 736–742.
44. Damiano, J. S., Oliveira, V., Welsh, K. et al. Heterotypic interactions among NACHT domains:implications for regulation of innate immune responses. Biochem. J. *2004;* 381, 213–219.
45. Martinon, F., Tschopp, J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell 2004*;* 117, 561–574 .
46. Hugot, J.P., Chamaillard, M., Zouali, H., et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001; 411: 599-603.
47. Ahmad, T., Tamboli, C.P, Jewel, D. et al. Clinical relevance of advances in genetics and pharmacogenetics of IBD. Gastroenterology 2004; 126: 1533-1549.
48. Lesage S, Zouali H, Cezard JP et al. NOD2/CARD15 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. Am J Hum Genet 2002; 70: 845-857
49. Economou M, Trikalinos TA, Loizou KT, et al. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations : a metaanalysis. Am J Gastroenterol, 2001; 99: 2393-2404.
50. SugimuraM, Kinouchi Y, takahashi S et al. NOD2/CARD15 mutational analysis inJapanese patients with Crohn's disease. Clinical genetics 2003; 63: 160-162.
51. Inoue N, Tamura K, Kinoudhi Y et al. lack of common NOD2 variants in Japanese patients with Crohn's disease. Gastroenterol 2003; 123: 86-91.
52. Leong RWL, Armuzzi a, Ahmad T et al. NOD2/CARD15 gene polymorphism and Crohn's disease in the Chinese population. Alim Pharmacol Ther 2003;17: 1465-1470.
53. Bonen DK, Niclolae DL, Moran T et al. Racial differences in NOD2 variation: characterization of NOD2 in African-Americans with Crohn's disease. Gastroenterol 122: (suppl): A-29
54. Croucher PJ, Mascheretti S, Hampe J et al. Haplotype structure and association to Crohn's disease of CARD15 mutations in two ethnically divergent population. European J Hum Genet 2003; 11: 6-16.
55. Vermeire S. NOD2/CARD15: relevance in clinical practice. Best Pract Research Clin Gastroenterol 2004; 18: 569-575.
56. Maeda, S., Hsu, L., Liu, H. et al. Nod 2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing. Science 2005; 307: 734-738.
57. Strober W, Murray PJ, Kitani A et al. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2005; 1-12
58. Zelinkova, Z, de Kort F, Pronk I, *et al.* Functional consequences of NOD2 deficiency in Crohn's disease patients peripheral blood monocytes derived dendritic cells. *Gastroenterol 2005;* 128 (4), A510.
59. Wehkamp, J., Harder, J., Weichenthal, M. et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal0020-defensin expression. Gut 2004; 53: 1658-1664.
60. van Heel D. A., Ghosh S, Hunt KA, et al. Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. Gut 2005; 54, 1553–1557.

61. Netea, M. G. Ferwerda G, de Jong DJ, et al. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. J Immunol 2005; 174, 6518–6523.
62. Netea, M.G., Kullberg, B.J., de Jong, D.J. et al. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. Eur J Imuunol 2004; 34: 2052-2059.

FIGURES LEGEND

Figure 1: IBD linkage areas.

Figure 2: Domain organization of the CATERPILLER proteins.

MHC class II transactivator (CIITA), cryopyrin, nucleotide-oligomerization domain 2 (NOD2), and NAIP (neuronal apoptosis inhibitor protein) represent the four main groups of CATERPILLER proteins, which are distinguished by their distinct amino (N)-terminal domains. The four possible N-terminal domains are the acidic, pyrin, caspase-recruitment domain (CARD) and baculoviral inhibitory repeat (BIR) domains. All have the nucleotide-binding domain (NBD) leucine-rich repeat (LRR) (NBD-LRR) configuration, with A and B depicting the Walker A and B motifs of the NBD. CIITA is the only family member with a documented role as a transcription factor.

Figure 3: Structure of the CARD15 gene and location of the CD-associated variants. The numbers represent the amino acid position. NBN, nucleotide binding domain; LRR, leucine rich repeat.

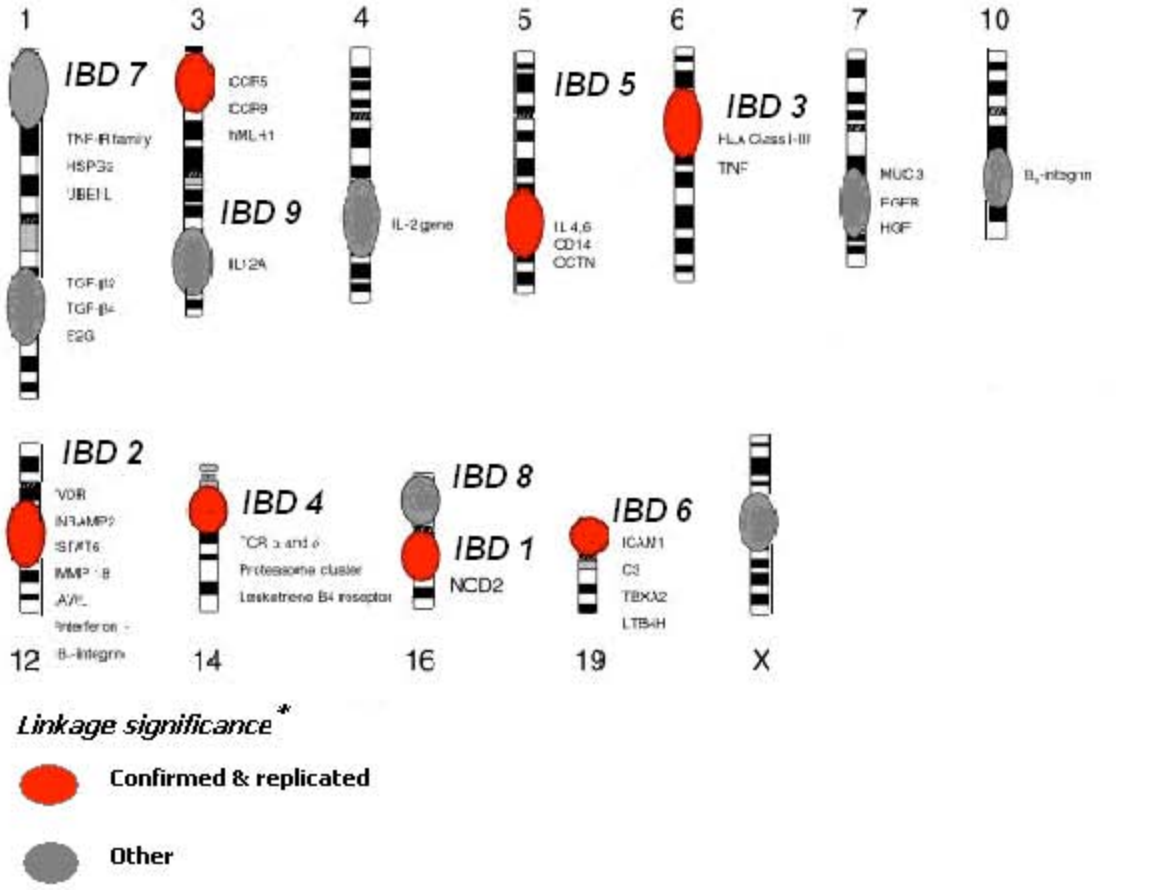
Figure 4: Possible mechanisms of Crohn's disease caused by NOD 2 mutations.

A) defective function of macrophages leads to persistent intracellular infection of macrophages and chronic stimulation of T cells by macrophage-infecting organism

B) defective epithelial-cell responses lead to loss of barrier function and increased exposure to the mucosal microflora

C) Defective "conditioning" of antigen –presenting cells (APCs) leads to inappropriate activation of APCs and disruption of the homeostatic balances of affector and regulatory cells.

Figure 1



*Significance levels defined by Lander and Kruglyak

Figure 2

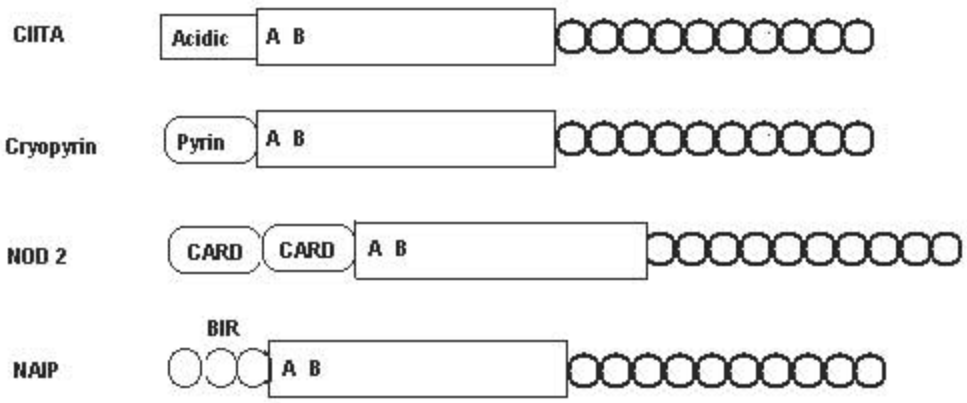

CIITA
Acidic
A B
Cryopyrin
Pyrin
A B
NOD 2
CARD
CARD
A B
BIR
NAIP
A B

Figure 3

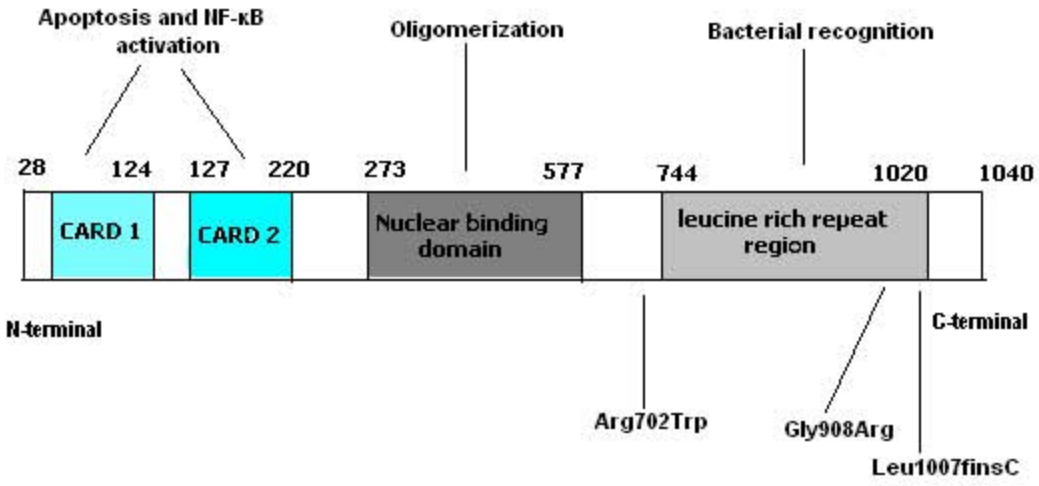
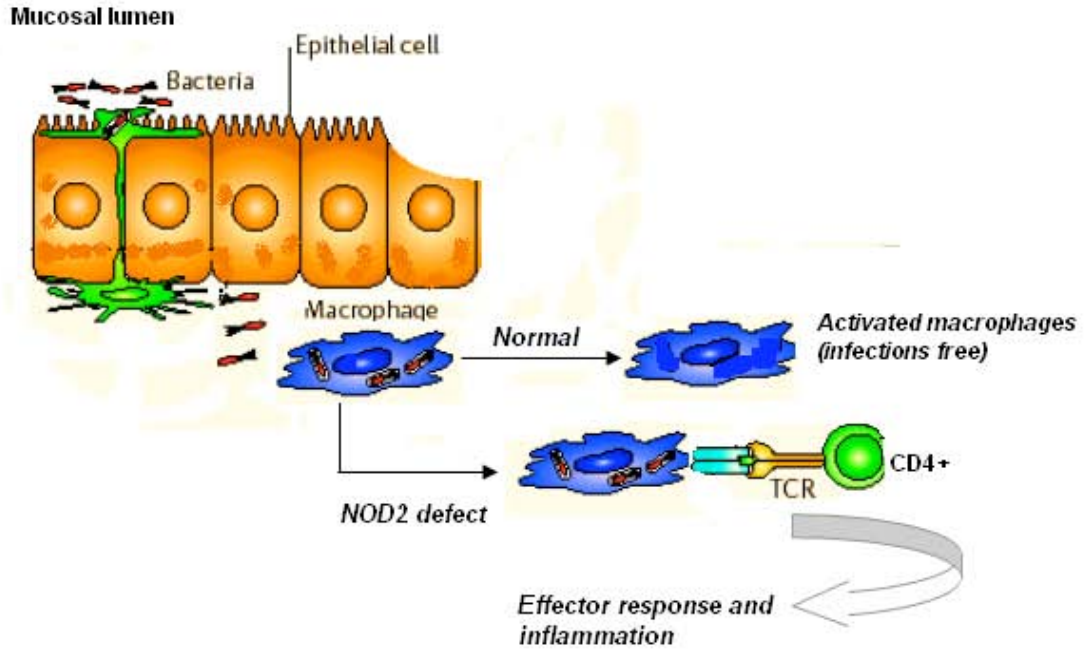
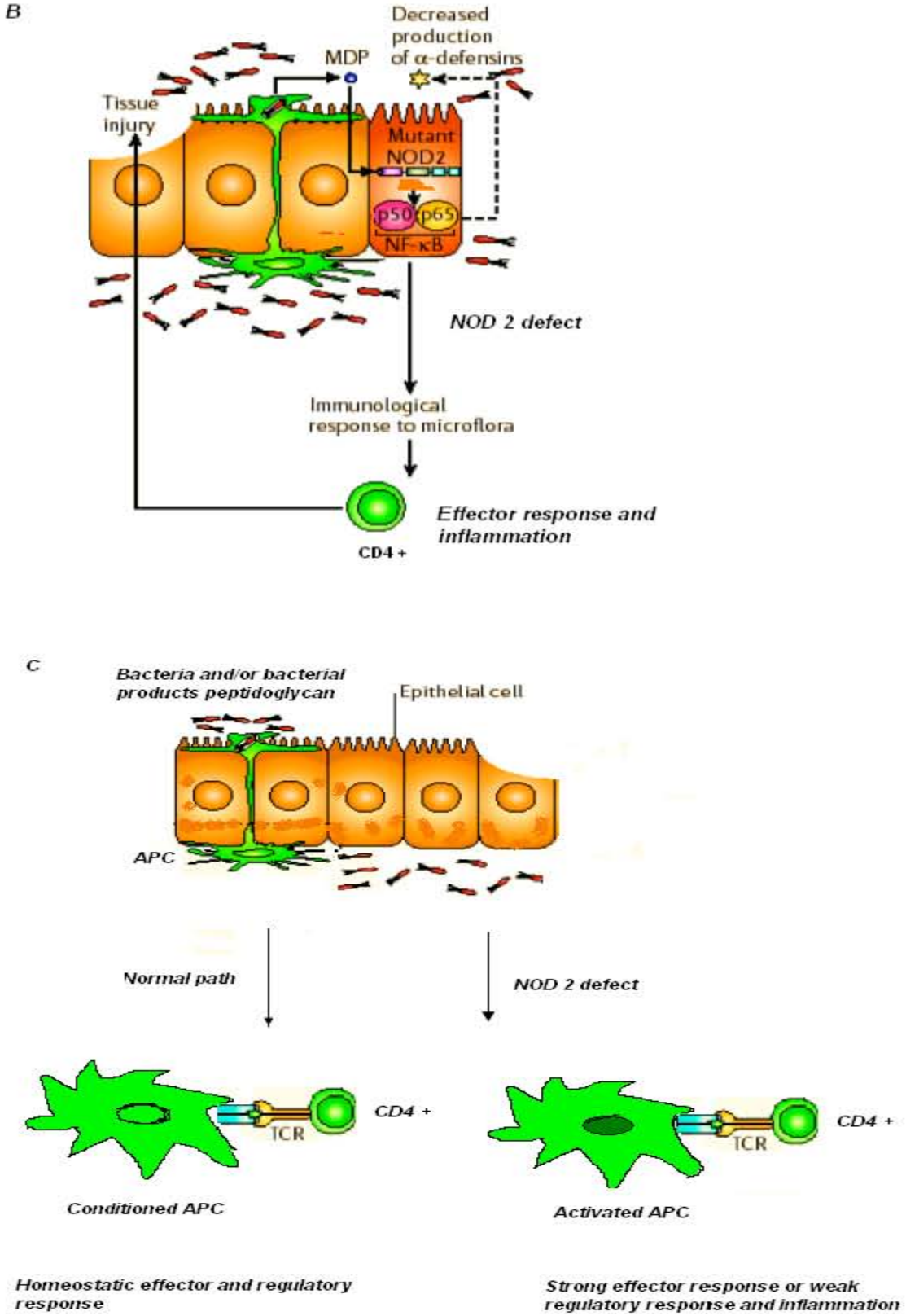

Apoptosis and NF-κB activation
Oligomerization
Bacterial recognition
28
124
127
220
273
577
744
1020
1040
CARD 1
CARD 2
Nuclear binding domain
leucine rich repeat region
N-terminal
C-terminal
Arg702Trp
Gly908Arg
Leu1007finsC

Figure 4

A


Mucosal lumen
Epithelial cell
Bacteria
Macrophage
Normal
Activated macrophages
(infections free)
CD4+
TCR
NOD2 defect
Effector response and
inflammation


B
Decreased production of α-defensins
MDP
Tissue injury
Mutant NOD2
p50
p65
NF-κB
NOD 2 defect
Immunological response to microflora
Effector response and inflammation
CD4 +
C
Bacteria and/or bacterial products peptidoglycan
Epithelial cell
APC
Normal path
NOD 2 defect
CD4 +
TCR
Conditioned APC
CD4 +
TCR
Activated APC
Homeostatic effector and regulatory response
Strong effector response or weak regulatory response and inflammation

2.2 Characterization of mice with gut specific expression of Interleukin-12 (IL-12) family genes.

ABSTRACT

Inflammatory bowel disease (Crohn's disease and ulcerative colitis) are chronic inflammatory diseases leading to destruction of gastrointestinal tissue. In Crohn's disease, an increased expression of T-helper 1 cytokines is observed in which interleukine -12 (IL-12) has a pivotal role. Recently a novel heterodimeric cytokine, interleukine-23 (IL-23) also drives humen Th1 T-cell development, but compared to IL-12, Il-23 preferentially stimulates proliferation and cytokine production by memory T-cells. General overexpression of p19 by the β-actin promoter induced a lethal inflammatory phenotype in transgenic mice. IL-23 production was found in the terminal ileum of normal mice and it was found upregulated in the inflamed intestinal mucosa of patients with IBD. To investigate the function of IL-12, IL-23 and p40 in the gut in vivo and their role in experimental colitis, we generate transgenic mice with IL_12, Il-23 and p40 subunit expressed under a gut specific promoter, the fatty acid binding protein promoter (pFABP). A 0.6 kb fragment consisting of the short part L-FABP was excised from pELFABP plasmid promoter and cloned into BgI II/ PstI expression vector pCI-CMV (Promega), replacing the CMV promoter with the short L-FABP promoter. PCR fragments for p40, Il-12, IL-23 (subunit of IL-12 and IL-23 are linked together) were cloned into the pCI-FABP vector. The transgenic mice are characterized using southern blot, PCR, ELISA, immunohistochemical and western blot techniques. The cells population in the gut –associated lymphoid tissue and their susceptibility ton experimental colitis was analysed. Transgenic mice develop normally and do not display any abnormalities. Macroscopical and histopathological examination of the organs showed no apparent abnormalities or signs of inflammation in LFABPp40, LFABP/IL-12, LFABP/IL-23 transgenic mice up to the age

of one year. However the LFABP/IL-23 transgenic mice have reduced offspring compared to the normal FVB mce. In addition, LFABP/IL-23 transgenic mice showed an increased production of IL-17 in isolated splenocytes, evaluated by intracellular flow cytometry, when compared to LFABP/WT, LFABP/IL-12, LFABPp40 transgenic mice. Preliminary analysis of the susceptibility to experimental colitis after oral administration of TNBS or DSS demonstrated no major differences between the transgenic mice. These preliminary characterizations of transgenic mice with gut specific expression indicate that they are viable and demonstrate a specific phenotype.

INTRODUCTION

Inflammatory bowel diseases (IBD; Crohn's disease (CD) and ulcerative colitis) are chronic inflammatory diseases leading to destruction of gastrointestinal tissue. In CD, an increased expression of T-helper-1 (Th1) cytokines is observed in which interleukin-12 (IL-12) has a pivotal role.

IL-12 is a heterodimeric cytokine composed of two disulphide-linked subunits p35 and p40. We previously studied the role of IL-12 using IL-12p35-/- and IL-12p40-/- mice in experimental colitis. From these studies we conclude that IL-12p70, in contrast to IL-12p40, induces TNBS-induced colitis and IL-18 expression independent of IFN-γ (1). Anti-IL-12 strategies therefore seem to play a promising role because of the central position of this Th1-inducing cytokine in the inflammatory cascade. In the meantime a novel p40 linked subunit was described: p19 that forms a new cytokine IL-23 (2). IL-23 shares many, but not all of its biological activities with IL-12. IL-23 also drives human Th1 T cell development, but compared to IL-12, IL-23 preferentially stimulates proliferation and cytokine production by *memory* T cells. IL-23 deficient mice display compromised humoral and delayed-type hypersensitivity responses and a reduced production of IL-17 (3). The receptor for IL-12 is composed of two subunits: β1 (binding component) and β2 (signaling unit). Binding of IL-12 to this receptor results in rapid activation of Jak2 and Tyk2 and dimerization of STAT-4 and STAT-3 transcription factors (4). IL-23 also binds IL-12β1 but activates through a recently discovered new receptor (5). Based on the finding that anti IL-12 p40 antibodies abrogated experimental TNBS colitis (6) clinical trials with anti IL-12 antibodies have been conducted in patients with Crohn's disease. Since most of the antibodies under clinical development are anti-p40 (7) a detailed knowledge of the role of IL-12 and IL-23, that both share the p40 subunit, in the pathogenesis of IBD is mandatory. Recent studies collectively point to a pivotal and central role for IL-23, rather than IL-12, in mediating chronic, sustained autoimmune responses (8, 9, 10). Recent

data indicate that IL-23 is an essential promoter of end stage joint autoimmune inflammation, whereas IL-12 paradoxically mediates protection from autoimmune inflammation (11). Concerning the expression of IL-23 in the gut, Becker et al (13) demonstrated IL-23 production in the terminal ileum in normal mice and in the inflamed intestinal mucosa of patients with IBD the IL-23 expression was found to be upregulated (14). General over-expression of p19 by the β-actin promoter induced a lethal inflammatory phenotype in transgenic mice (8). We expect that local tissue expression of IL-12, IL-23 and p40 using a gut-specific promoter will allow us to generate viable animals that will help us to understand the role of IL-12 and IL-23 in the gut in vivo.

Interference with the biological activity of IL-12 or IL-23 more directly interferes with the induction of mucosal inflammation than the anti-TNF or anti-alpha$_4$ strategies that are currently used (15, 16).

Rationale and relevance to IBD

IL-12 and IL-23 are ideal targets for pharmacological intervention in the therapy of IBD. The production of IL-12 can be suppressed with several pharmacological approaches such as glucocorticoids. More specifically IL-12 and potentially also IL-23 can be inhibited using antibodies, soluble receptors or small interfering RNA (siRNA). However, IL-12 and possible also IL-23 are cytokines that are indispensable in the defense against certain pathogens, like intracellular pathogens and viruses where Th1-type responses are obligatory. We must therefore precisely unravel the exact role of IL-12 and IL-23 in intestinal inflammation.

This project will study the differential role of IL-12 and IL-23 in experimental colitis and provide insights in the biology of IL-12 and IL-23 and their possible therapeutic use.

METHODS

Transgene construction and microinjection

Cloning of the L-FABP fragment into pCI

A 6.0 Kb fragment consisting of the short L-FABP was excised from pELFABP plasmid promoter (Sweetser et al, 1988) and ligated in the EcoRI/PstI site of Puc2.1. the fragment was then excised from this plasmid with Bg1 II/PstI and inserted in the Bg1 II/PstI site of the expression vector pCI-CMV (promega), replacing the CMV promoter with the short L-FABP promoter.

Cloning of the p40 IL-12L and IL-23 L fragments

A RT-PCR reaction was performed for the mouse subunit p40 with primers containing enzymatic sites, the PCR fragments were cloned in PCRscript. After digestion the p40 subunit was cloned in the MCS of the FABP-pCI vector.

RT-PCR reactions were performed for p40, p35 and p19 with specific primers for the different genes. The products were cloned into a PCR-topo plasmid and screened by sequences analysis.

PCR's for the three different genes were performed with specific primers, the p40 rv primers contained a linker and the p19 and p35 fw primers contained a linker. The p40 and p35 bands and the p40 and p19 bands were mixwd and PCRs were performed with the p40 fw primer and the p35 or p19 rv primer generating PCR fragments for IL-12 and IL-23 (the subunits are linked together). The subunits were cloned into the pCI FABP p40 vector replacing the p40 subunit.

Generation and identification of transgenic mice

Transgenic mice (Tg mice) were generated by injecting DNA encoding for murine p40, IL-12, IL-23 under the L-FABP promoter into mouse oocytes and one mouse carrying p40, three mice carrying IL-12 and two mice carrying IL-23 were generated. Founder Tg mice (G0) were crossbred with wild type FVB mice. Heterozygous female and male off spring was crossed and off spring was checked for homozygosity.

Mice and induction of colitis

Experiments were approved by the Animal Studies Ethics Committee of the University of Amsterdam, the Netherlands. Mice were housed under standard conditions, and supplied with drinking water and food (AM-II 10 mm; Hope Farms, Woerden, the Netherlands). Experiments were conducted in FVB transgenic mice (age: between 8 and 10 weeks). TNBS colitis was induced as described previously (17). Dextran sulphate (DSS) colitis was induced by adding 3-5% (w/v) DSS (mol wt 40kD; TdB Consultancy, Uppsala, Sweden) to the drinking water for 6-7 days. The mice were sacrificed on day 6-7. Control mice received normal tap water for 6-7 days

TNBS colitis

Hapten-induced colitis was induced by rectal administration of two doses (separated by a seven day interval) of 1.5 mg of 2,4,6-trinitrobenzene sulphonic acid (TNBS; Sigma Chemical Co, St Louis, Missouri, USA) in 40% ethanol (Merck, Darmstadt, Germany) using a vinyl catheter positioned 3 cm from the anus (9 mice per group). Control mice (5 mice per group) underwent identical procedures but were given physiological salt. During administration of TNBS, mice were anaesthetised using isoflurane (1-chloro-2, 2,2,-trifluoroethyl-isoflurane-difluoromethyl-ether; Abbott Laboratories Ltd, Queenborough, Kent, UK), and after administration were kept vertical for

60 seconds. All mice were sacrificed nine days following the first TNBS administration (that is, 48 hours following the second TNBS challenge).

DSS colitis

Dextran sulphate (DSS) colitis was induced by adding 3 % (w/v) DSS (mol wt 40kD; TdB Consultancy, Uppsala, Sweden) to the drinking water for 7 days. The mice were sacrificed on day 7. Control mice received normal tap water for 7 days.

Assessment of inflammation

Body weight was recorded daily. Caudal lymph nodes and colons were harvested upon sacrifice. The colons were removed through a midline incision and opened longitudinally. For TNBS colitis the wet weight of the distal 6cm was recorded and used as an index of disease-related intestinal wall thickening. For DSS colitis the total length of the colon was measured as a parameter for disease severity. Subsequently, the colons were longitudinally divided in two parts, one of which was used for histological assessment.

The longitudinally divided colons were rolled up, fixed in 4% formaline and embedded in paraffin for routine histology. Histological scoring was performed as described previously (17). This score ranges from 0 to a maximum of 26 points.

Histological analysis

The longitudinally divided colons were rolled up, fixed in 4% formalin, and embedded in paraffin for routine histology. A pathologist scored the following parameters: (1) percentage of area involved, (2) number of follicle aggregates, (3) oedema, (4) erosion/ulceration, (5) crypt loss, and (6) infiltration of mono- and polymorphonuclear cells. The percentage of area involved and crypt loss was scored on a scale ranging from 0 to 4 as follows: 0, normal; 1, <10%; 2, 10%; 3, 10–50%;

and 4, >50%. Erosions were defined as 0 if the epithelium was intact, 1 for involvement of the lamina propria, 2 for ulcerations involving the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale from 0 to 3 as follows: 0, absent; 1, weak; 2, moderate; and 3, severe. Hence the score ranged from 0 to a maximum of 26 points.

Cytokine analysis

For cytokine determination in the supernatants, the Cytometric Bead Array kit (BD Biosciences, PharMingen) was used, according to the manufacturer's instructions.

Flow cytometry

Mouse spleen cells were incubated with or without PMA/ionomycin for 6 hours. After 2 hours brefeldin A was added. The cells were harvested, fixed, permeabilized, and intracellularly stained with Anti-IL-17-PE. Cell surface staining was performed with CD4-APC. Cells were analyzed by flow cytometry. Analysis was done on a FACSCalibur in conjunction with the Cellquest Pro (BD Biosciences, Pharmingen, San Diego, CA) software, and 5000 cells were counted.

RESULTS

FABPp40 Transgenic Mice (Tg)

Generation of the FABP/p40 transgenic mice

Transgenic mice (Tg mice) were generated by injecting DNA encoding for murine p40 under the L-FABP promoter into mouse oocytes and one mouse carrying p40were generated. These were mated with FVB mice and one line for FABP/p40 transmitted the transgene to the offspring. After several generations homozygotic transgenic mice were identified and these were mated to generate homozygotic offspring. To examine the tissue specificity of the p40 transgene expression, were performed ELISA for IL-12p40 subunit. In accordance with previouse report / expression of the murine p40 was detected in the liver.

Development of L-FABP p40 transgenic mice

LFABP/p40 Tg mice were of normal size and weight and both sexes were fully fertile. Upon macroscopical examination in LFABPp40 Tg mice up to the age of one year no apparent abnormalities were detected in liver, kidney, heart, spleen, lung and intestine and genital organs. Upon histopathological examination in LFABPp40 Tg mice no signs of inflammation were detected in intestine (small/large).

Colon weight, colon length, number of cells in the caudal lymph nodes in healthy control FABP/p40 mice

The colon length of FABP/p40 Tg mice was significant ($p < 0.05$) increased when compared to Wt mice. Upon the number of cells in caudal lymph nodes, the FABP/p40 Tg mice showed a significant ($p < 0.05$) increased number of CLN cells compared to FABP/IL12L Tg mice.

TNBS colitis results in FABP/p40 Tg mice

Hapten-mediated colitis was induced by administration of TNBS on day 0 and day 7 and mice were sacrificed on day 9. Induction of colitis was observed in both WT and Tg mice. About the body weight data are available from only one experiment.

The increase in colon weight of WT, FABP/p40 and FABP/IL-12L Tg mice after administration of TNBS was comparable (210.3 ± 11.95 in WT, 210.1 ± 10.66 in FABP/p40 Tg mice and 229.8±11.82 in FABP/IL-12L Tg mice). In FABP/p40 TNBS Tg mice the colon length shorted significantly ($p < 0.05$) when compared to the control FABP/p40 Tg mice group.

After administration of TNBS the number of CLN cells increased significantly ($p < 0.05$) in FABP/p40 mice compared to the control group and to TNBS Wt mice.

The small intestine weight of TNBS Wt mice was significantly ($p < 0.05$) increased compared to FABP/p40 TNBS Tg mice.

Histo-pathological analysis of the colon showed an equal total colitis score WT, FABP/p40 and FABP/IL-12L TNBS Tg mice and all the different components of which the score is composed were comparable. Granulocytes infiltration was significantly ($p < 0.05$) increased in Wt TNBS mice (N=9) compared to FABP/p40 Tg mice (N=9) (2.722±0.1884 vs 1.886±0.3093).

Cytokine production in FABP/p40 TNBS Tg mice

The IFN-γ, TNF-α, IL-4, IL-2 and IL-5 production of caudal lymph nodes was measured after stimulation with CD3/CD28 antibodies for 48 h.

IFN-γ and TNF-α production was enhanced in FAPB/p40 Tg mice trated compared to FABP/IL-12L Tg mice. FABP/p40 Tg mice ($p < 0.001$) had a significantly increased production of IL-4 compared to FABP/IL-12L Tg mice. FABP/p40 Tg mice ($p < 0.05$) had a significantly increased production of IL-2 compared to FABP/IL-12L Tg mice.

Upon the production of IL-5 no difference were been detected between the groups.

IL-12 Transgenic Mice (Tg)

Generation of the FABP/IL-12L transgenic mice

Transgenic mice (Tg mice) were generated by injecting DNA encoding for murine IL12 under the L-FABP promoter into mouse oocytes and three mice carrying Il 12 were generated. These were mated with FVB mice and three lines for FABP/IL12L transmitted the transgene to the offspring. After several generations homozygotic transgenic mice were identified and these were mated to generate homozygotic offspring. To examine the tissue specificity of the IL 12 L transgene expression, were performed ELISA (p40). In accordance with previouse report / expression of the murine p40 was detected in the liver.

Development of FABP/IL-12 L transgenic mice

FABP/IL-12L Tg mice were of normal size and weight and both sexes were fully fertile. Upon macroscopical examination in LFABP/IL-12L Tg mice up to the age of one year no apparent abnormalities were detected in liver, kidney, heart, spleen, lung and intestine and genital organs. Upon histopathological examination in LFABP/IL-12L Tg mice no signs of inflammation were detected in intestine (small/large).

Colon weight, colon length, number of cells in the caudal lymph nodes in healthy control FABP/IL-12L Tg mice

No differences were detected in colon weight between the control groups (Wt, FABP/p40, FABP/IL-12L Tg mice). Upon the number of cells in caudal lymph nodes, the FABP/p40 Tg mice showed a significant ($p < 0.05$) increased number of CLN cells compared to FABP/IL12L Tg mice.

TNBS colitis results in FABP/IL-12L Tg mice

Hapten-mediated colitis was induced by administration of TNBS on day 0 and day 7 and mice were sacrificed on day 9. Induction of colitis was observed in both WT and Tg mice. No data are available about the body weight.

No differences in colon weight were detected between FABP/IL-12L Tg compared to FABP/p40 Tg mice and WT. The colon weight of FABP/IL-23 Tg mice after administration of TNBS was significantly increase compared to FABP/IL-12 Tg mice (229.8± 11.82 [N=9] and 329.2± 19.12 [N= 10]).

After administration of TNBS the number of CLN cells increased significantly ($p< 0.05$) in FABP/IL-12L Tg mice compared to WT mice ($p< 0.01$) and FABP/p40 ($p< 0.05$).

Hysopathological analysis of the colon showed an equal total colitis score in WT, FABP/p40, FABP/IL-12L and FABP/IL-23L Tg mice. The area involved was significantly ($p< 0.001$) increased in FABP/Il-12L Tg mice (N=19) compared to FABP/IL-23 Tg mice (N= 10) (3.444±0.1757 vs 2.050± 0.2167). The granulocytes were significantly ($p< 0.01$) increased in FABP/IL-12 Tg mice (N=19) compared to FABP/IL-23 Tg mice (N = 10) (1.763±0.2767 vs 1.700 ± 0.2261) anf FABP / p40 Tg mice ($p< 0.05$; 1.763 ± 0.2727 vs 1.889± 0.1788).

The small intestine weight is significantly ($p< 0.05$) increased in FAPB/IL-12L Tg mice compared to FABP/p40 Tg mice.

Cytokine production in FABP/IL-12L TNBS Tg mice

The IFN-γ, TNF-α, IL-4, IL-2 and IL-5 production of caudal lymph nodes was measured after stimulation with CD3/CD28 antibodies for 48 h.

INF- γ production is significant ($p < 0.001$) increased in Wt TNBS mice compared to FABP/IL-12L TNBS Tg mice.

TNF-α production was enhanced in Wt and FAPB/p40 Tg mice trated with TNBS compared to FABP/IL-12L TNBS Tg mice.

Wt TNBS mice ($p < 0.05$) and FABP/p40 TNBS Tg mice ($p< 0.05$) had a significantly increased production of IL-4 compared to FABP/IL-12L TNBS Tg mice.

Wt TNBS mice ($p < 0.05$) and FABP/p40 TNBS Tg mice ($p< 0.05$) had a significantly increased production of IL-2 compared to FABP/IL-12L TNBS Tg mice.

Upon the production of IL-5 no difference were been detected between the groups.

FABP / IL-23 Transgenic mice

Generation of the, FABP/IL-23 transgenic mice

Transgenic mice (Tg mice) were generated by injecting DNA encoding for murine IL 23 under the L-FABP promoter into mouse oocytes and two mice carrying Il 23 were generated. These were mated with FVB mice and two lines for FABP/IL-23L transmitted the transgene to the offspring. After several generations homozygotic transgenic mice were identified and these were mated to generate homozygotic offspring.

Development of FABP/IL-23 L transgenic mice

Both lines of FABP/IL-23L mice presented infertility and exhibited breeding problems. Upon macroscopical examination in LFABP/IL-23L Tg mice up to the age of one year no apparent abnormalities were detected in liver, kidney, heart, spleen, lung and intestine and genital organs. Upon histopathological examination in LFABP/IL-23L Tg mice no signs of inflammation were detected in intestine (small/large).

Colon weight, colon length, number of cells in the caudal lymph nodes in healthy control FABP/IL-23L Tg mice:

Upon the number of cells in caudal lymph nodes, the FABP/IL-23 L tg mice showed a significant ($p< 0.05$) increased number of CLN cells compared to FABP/ p40 Tg mice.

No data are available regarding colon weight and length.

TNBS colitis results in FABP/Il-23 Tg mice

Hapten-mediated colitis was induced by administration of TNBS on day 0 and day 7 and mice were sacrificed on day 9. Induction of colitis was observed in both WT and Tg mice. About the body weight data are representative of only one experiment.

No differences in colon weight were detected between FABP/IL-23, WT and FABP/p40 Tg mice. The colon weight of FABP/IL-23 Tg mice after administration was significantly increased in compared to FABP/IL-12L Tg mice (392.2 ± 19.12 (N = 10) in FABP/IL23 Tg mice and 229.8 ± 11.82 (N = 9) in FABP/IL-12L Tg mice). After administration of TNBS no differences were detected in the number of CLN cells of IL-23 Tg mice compared to WT, p40 and IL-12 Tg mice.

Histo-pathological analysis of the colon showed an equal total colitis score WT, FABP/p40, FABP/IL-12L and FABP/IL-23L Tg mice. The mononuclear cells were significantly ($p< 0.001$) increased in FABP/IL-23 Tg mice (N=19) compared to WT, FABP/p40 Tg mice and FABP/IL-12 Tg mice. Granulocytes infiltration was significantly ($p < 0.05$) increased in FABP/IL-12 Tg mice (N=19) compared to FABP/IL-23 Tg mice (N=10) (1.763± 0.2767 vs 1.700±0.2261) and FABP/p40 Tg mice ($p < 0.05$; 1.763±0.2767 vs 1.889±0.1788).

Cytokine production in FABP/IL-23L TNBS Tg mice

IFN-γ, TNF-α, IL-6, MCP-1, IL-12p70 production of CLN and spleen cells was measured after stimulation with CD3/CD28 antibodies for 24 h.

TNF-α production of CLN was significantly enhanced in FABP/IL-23 Tg mice when compared to WT ($p< 0.01$), p40 Tg mice ($p< 0.01$) and FABP IL-12 Tg mice ($p< 0.001$).

IFN-γ production of CLN was significantly enhanced in FABP/IL-23 Tg mice when compared to WT ($p< 0.05$) and FABP IL-12 Tg mice ($p< 0.01$).

Upon the production of IL-5, Il-4, Il-2 no differences were detected between FABP/IL-23 Tg mice and the other groups.

Experimental Colitis: DSS colitis results

DSS colitis results in FAPB /p40 Tg mice

DSS colitis was induced by adding 3% dextran sulphate to the drinking water for 7 days. All the mice developed colitis, which was accompanied by minor weight loss and general symptoms of illness. No differences were detected in the colon weight ond CLN cells numbers. The colon length was evaluated upon sacrifice 7 days after the start of the experiment and was found to be significantly decreased in FAPB /p40 Tg mice compared to and FABp/IL-12LTg mice ($p< 0.01$). Histo-pathological analysis of the colon showed a significantly ($p< 0.05$) increased of colitis score in FABP/p40 Tg mice (N=10) compared to WT (N=11). A significantly increased score for Erosions/ulcerations ($p> 0.01$), edema ($p< 0.01$), crypt loss ($p< 0.01$), granulocytes ($p< 0.05$) and mononuclear cells ($p< 0.05$) was detected in FABPp40 Tg mice when compared to the WT grop. An increased value of the score for cript loss ($p< 0.01$), granulocytes ($p<0.05$) were detected in FABP/p40 Tg mice when compared to the FABP /Il-23 Tg mice.

Cytokine production in FABP/p40 DSS Tg mice

IL-12p70, IFN-γ, TNF-α, MCP-1, Il-6, Il-10, production in the sera, CLN and spleen cells was measured. The CLN and spleen cells were stimulated with CD3/CD28 antibodies for 24 h before evaluation.

TNF-α, IFN-γ, IL-6 production of CLN was significantly enhanced in FABP/p40 Tg mice when compared to FABPIL-23 Tg mice ($p<0.05$). No differences were detected upon the other cytokines and in the splenocytes and serum.

DSS colitis results in FAPB /IL-12L Tg mice

DSS colitis was induced by adding 3% or 5% dextran sulphate to the drinking water for 7 days. All the mice developed colitis, which was accompanied by minor weight loss and general symptoms of illness.

No differences were detected in the colon weight and CLN cells numbers. The colon length of was measured upon sacrifice, 7 days after the start of the experiment and was found to be significantly decreased FAPB /p40 compared to and FABp/IL-12LTg mice ($p<0.01$).

Histo-pathological analysis of the colon showed a significantly ($p< 0.05$) increased of the colitis score in FABP/IL-12L TNBS Tg mice (N=8) compared to WT (N=11) and FABP/IL-23 Tg mice (N=5). A significantly increased score for erosion/ulcerations ($p< 0.05$), crypt loss ($p< 0.05$), was detected in FABP/Il-12 Tg mice when compared to the WT mice. An increased value of the score for crypt loss ($p< 0.05$), detected in FABP/IL-12 Tg mice when compared to the FABP/IL-23 Tg mice.

Cytokines production in FAPB /IL-12L Tg mice

Il-12p70, IFN-γ, TNF-α, MCP-1, IL-6, IL-10 production in the sera and in the CLN and spleen cells was measured. The CLN and spleen cells were stimulated with CD3/Cd28 antibodies for 24 h before the evaluation.

TNF-α ($p< 0.05$), IFN-γ ($p< 0.05$), IL-10 ($p< 0.01$) and MCP-1 ($p< 0.05$) production of CLN was significantly enhanced in FABP/IL-12 Tg mice when compared to WT. An increased production of TNF-α ($p< 0.05$), IFN-γ ($p< 0.05$), IL-10 ($p< 0.01$) was found when compared to FABP/IL-23 Tg mice. An increase production of IL-10 ($p< 0.05$) was found when compared to FABP/p40 Tg mice. No differences were detected upon the other cytokines and in the splenocytes and serum.

DSS colitis results in FAPB /IL-23 Tg mice

DSS colitis was induced by adding 3% dextran sulphate to the drinking water for 7 days. All the mice developed colitis, which was accompanied by minor weight loss and general symptoms of illness. No differences were detected in the colon weight and CLN cells numbers and colon length. Hystopathological analysis of the colon didn't show a significant increased value of the total colitis score in FABP/Il-23 Tg mice (N=10) compared to WT (N=11), p40 and IL-12 Tg mice. A significantly increased score for follicles ($p< 0.001$) was detected in the FABP/IL-23 TG mice when compared to the WT mice.

Cytokines production in FAPB /IL-23 Tg mice

IL-12p70, IFN-γ, TNF-α, MCP-1, IL-6, IL-10 production in the sera and in the CLN and spleen cells was measured. The CLN and spleen cells were stimulated with CD3/CD28 antibodies for 24 h before the evaluation.

Il-6 evaluation in the serum was significantly enhanced in FABP/IL-23 Tg mice when compared to WT mice ($p < 0.05$). No differences were detected upon the other cytokines and the splenocytes and CLN.

DISCUSSION

The key role of the IL-23 pathway in mediating inflammation has been established through in vivo models targeting the IL-23p19 subunit by either increasing or blocking expression. IL-23p19 transgenic mice demonstrate severe systemic inflammation, including in the small and large intestine (3). In contrast, while IL-23p19-deficient mice demonstrate no overt abnormalities, they have defects in T cell-dependent antibody production, memory T cell function, and decreased IL-17 production (8). Importantly, murine models in which IL-23p19 expression is experimentally regulated have contributed to defining the role of this pathway in intestinal defense and inflammation.

Mucosal sites, such as the intestinal lamina propria, demonstrate the constitutive presence of IL-23 and of IL-17- producing cells (18,19,20). In addition to the IL-17 isoforms (e.g., IL-17A, IL-17F) that characterize Th17 cells, Th17 cells also produce IL-6, IL-21, IL-22, IL-26, and various chemokines, such as CCL20 (21, 22-26). IL-17 ultimately drives microbial defenses, as well as chronic inflammation during autoimmunity, through various mechanisms (9,27). For example, IL-17 can contribute to neutrophil migration, expansion, and function.56 Moreover, IL-17 enhances dendritic cell maturation, T cell priming, and cellular (e.g., fibroblasts, endothelial cells, macrophages, epithelial cells) production of inflammatory mediators (e.g., IL-1, IL-6, IL-8, TNF, GM-CSF, NOS-2, prostaglandin nE2, metalloproteases, and chemokines),28 although IL-17 can also contribute to certain downregulatory outcomes (29). In addition to proinflammatory cytokines, Th17 cells produce cytokines that can downregulate inflammation (e.g., IL-10 and IL-22).

The role of IL-23 in Th17 cell function has been an area of active research and is not yet fully defined. The role of IL-23 in Th17 differentiation specifically has been controversial; however, there is evidence for IL-23 contributions to Th17 cell expansion, stabilization, and/or conditioning

for a fully inflammatory cell phenotype (9, 30,31-42). For some of the implicated IL-23 contributions, in vitro and in vivo studies do not fully corroborate these IL-23 roles and additional studies will be needed to resolve these differences. In contrast to CD4+ and CD8+ naıve T cells, NKT cells constitutively express IL23R and RORγ (43). Both murine and human NKT cells can rapidly produce IL-17 on IL-23 stimulation, in particular in cooperation with T cell receptor stimulation, thereby implicating a role for the innate immune system in early production of IL-17 (43).

IL-23, a member of the IL-12 family of cytokines, was first described in 2000 as a heterodimer composed of a p19 subunit and the p40 subunit shared with IL-12 (44). The receptor for IL-23 was described as being expressed on activated/memoryTcell populations (45). First clue concerning the role of IL-23 in shaping the T cell immune response came from the analysis of IL-23p19-deficient mice. In 2003, Cua et al. (9) discovered that p19-deficient mice, in contrast to p35-deficient mice, were resistant to the development of EAE and had very few cells capable of secreting IL-17 in the CNS (8, 47). A stronger connection between IL- 23 and Th17 cells was established when investigators showed that IL-23 promotes the production of IL-17 by activated T cells (46) and that IL-23-expanded T cells are able to transfer EAE and CIA (47, 48). IL-23R is clearly not expressed on naiveTcells, and after the identification of the factors (IL-6, IL-21, and TGF-β) required for the differentiation of Th17 cells, it became clear that IL-23 was not involved in the initial differentiation of Th17 cells. Yet IL- 23 appears to be essential for the full and sustained differentiation of Th17 cells given that IL-23p19-deficient mice have limited numbers of Th17 cells and that prolonged culture of Th17 cells in vitro requires the addition of IL- 23. Similarly to IL-12 forTh1cells, IL-23 could serve to expand and stabilize Th17 responses.

Alternatively, IL-23 may induce proinflammatory effector cytokines and suppress anti-inflammatory cytokines like IL-10 in Th17 cells (49). But the precise function of IL-23 for Th17

cells remains elusive, in part because the timing and consistency of IL-23R expression on T cells have been difficult to investigate. Initial studies proposed that IL-23R could be induced by TGF-β (50). More recently, IL-6 and IL-21 were shown to induce IL-23R in a STAT3- dependent manner (21). But IL-23R appears also to be dependent on RORγt, as RORγtdeficient mice have reduced expression of IL- 23R (51). Thus, combined signals of RORγt and STAT3, induced by IL-6/IL-21 together with low amounts of TGF-β, might be required to promote IL-23R expression and confer IL-23 responsiveness.

In addition to its role for Th17 cells, IL- 23 also has an important role in the regulation of the innate immune response. The development of gut inflammation in T and B cell–deficient mice depends on IL-23 in that the loss of IL-23 but not IL-12 is associated with a decrease in gut inflammation induced by anti-CD40 antibody-activated cells of the innate immune system (52). IL-23 appears to induce IL-17, IL-1, TNF, and IL-6 from cells of the innate immune system (52, 53). Whether IL-23-mediated gut inflammation is entirely dependent on IL-17 produced by cells of the innate immune system has not been addressed. Consistent with the importance of IL-23 in preclinical models of inflammatory bowel disease (52,54-56), a genome-wide screen revealed that a particular coding variant of the *IL23R* gene (rs11209026, c.1142G>A, p.R381Q) conferred strong protection from Crohn's disease, whereas several variants in the noncoding region of this gene were associated with increased susceptibility to Crohn's disease (57). Similarly, other genome-wide association studies (58,59) revealed associations of *IL23R* gene SNPs [R381Q: rs11209026 (same as in Crohn's disease) and L310P: rs7530511] with psoriasis, further strengthening the idea that IL-23R may be involved in inducing human autoimmune diseases.

FIGURES AND TABLES

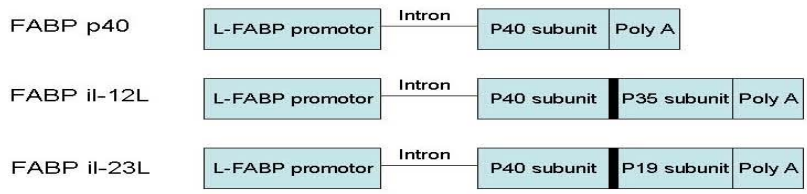


Figure 1: Transgene construction

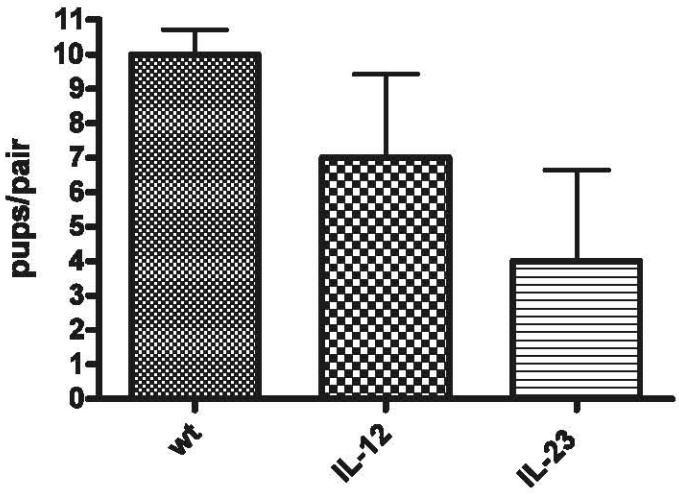
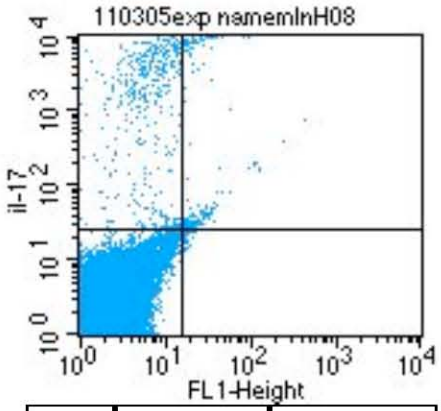
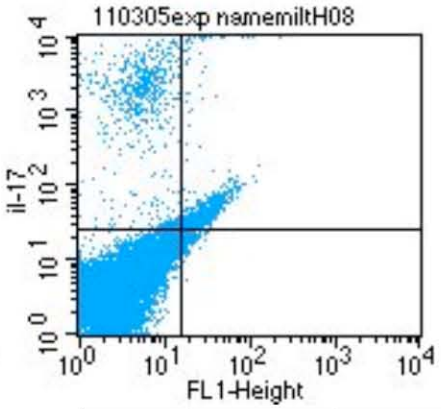


Figure 2: Transgene overexpression and fecundity



Mice	%Gated	%Total
wt	**0.67**	**0.26**
IL-12	**0.49**	**0.21**
IL-23	**2.64**	**0.96**



Mice	%Gated	%Total
wt	**0.88**	**0.19**
IL-12	**1.05**	**0.17**
IL-23	**6.54**	**1.05**

Figure 3: Intracellular IL-17 production by CD4+ from FABPIL-23 transgenic mice. The intracellular staining samples were stimulated with PMA/ionomycine before analysis. The cells are gated on live CD4+ cells and are rapresentative of four independent experiments

Table 1. Colon weight, colon length, number of cells in the caudal lymph nodes in healthy control mice (wild type and transgenic mice) and after induction of TNBS colitis

	Colon weight	**Colon length**	**Number of cells in CLN ($x10^3$)**	**Small intestine weight**
Nacl WT (N=5)	136.8± 4.821 (N=5)	8.800±0.2550	0.6430±0.1746	85.60 ± 4.600
TNBS WT (N=9)	210.3±11.95***	8.722±0.2778	1.255±0.1949	106.9±3.043**
Nacl FABP/p40 (N=5)	146.7± 10.07	9.917± 0.3516	1.138 ± 0.1645	86.00 ± 9.295
TNBS FABP/p40 (N=9)	210.1± 10.66**	8.778±0.344*	2.173±0.2850*	95.67±3.215 *
NaCl FABP/IL-12L (N=5)	151.0± 11.92	8.900± 0.2915	0.6100±0.09743	104.0± 8.495
TNBS FABP/IL-12L (N=9)	229.8± 11.82 **	8.444±0.2819	3.038±0.7051*	106.0±2.995

Number of mice: Nacl WT (n=5); TNBS WT (n=9); Nacl LFABP/p40 Tg (n=6); TNBS LFABP/p40 Tg (n=9); Nacl FABP/IL-12L Tg (n=5); TNBS FABP/IL-12L (n=9). Data are presented as mean and SEM.

* represent a significant difference ($p < 0.05$)

** represent a high significant difference ($p<0.001$)

*** represent a very high significant difference ($p<0.0001$)

Table 2. Colon weight, colon length, number of cells in the caudal lymph nodes, IFN-γ, IL-4 and IL-2 production of isolated CLN cells in TNBS colitis

	Colon weight	Colon length	Number of cells in CLN ($x10^3$)	Small intestine weight
WT (N=9)	210.3± 11.95	8.722± 0.2778	1.255 ± 0.1949	106.9 ± 3.043*
FABP/p40 (N=9)	210.1± 10.66	8.778± 0.3447	2.173 ± 0.2850*	95.67± 3.215
FABP/IL-12L (N=9)	229.8± 11.82	8.444± 0.2819	3.038 ± 0.7051*	106.0 ± 2.995*

Number of mice: Nacl WT (n=5); TNBS WT (n=9); Nacl LFABP/p40 Tg (n=6); TNBS LFABP/p40 Tg (n=9); Nacl FABP/IL-12L Tg (n=5); TNBS FABP/IL-12L (n=9). Data are presented as mean and SEM.

* represent a significant difference ($p < .05$)

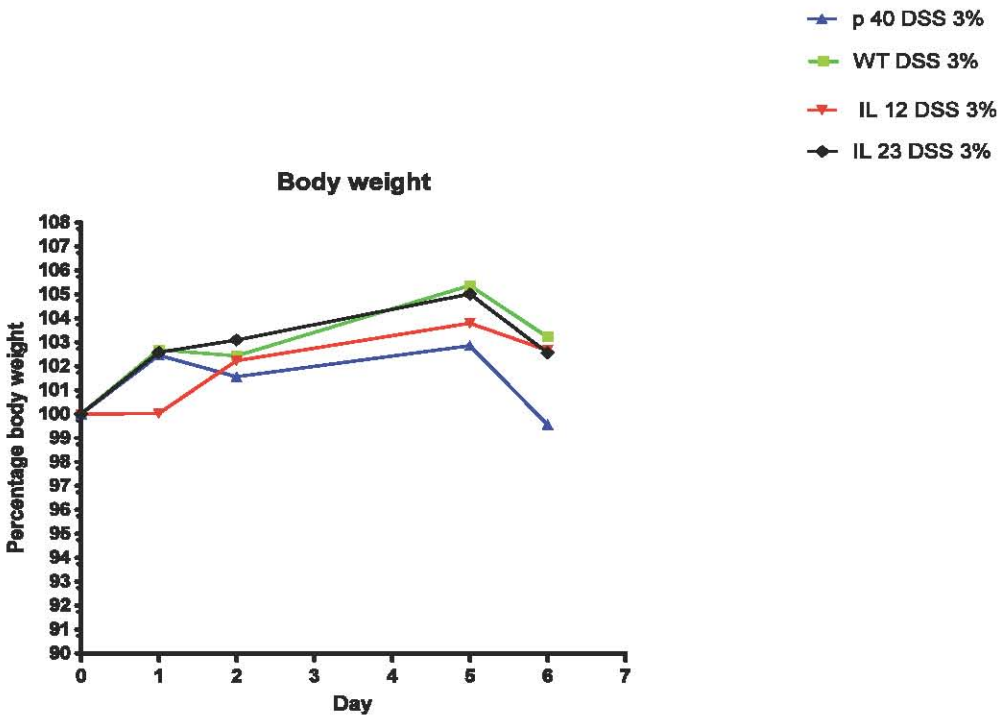
Table 3. IFN-γ, TNF-α, IL-4 and IL-2 production of isolated CLN cells in TNBS colitis

	INF-γ production (pg/ml)	TNF-α production (pg/ml	IL-4 production (pg/ml)	IL-2 production (pg/ml)	IL-5 production (pg/ml)
WT (N=9)	1604 ±354.0 ** N=9	232.4±86.69 * N=9	48.07±14.58 * N=9	95.17±33.98 * N=9	61.93±23.79 N=9
FABP/p40 (N=9)	1808 ±783.3 N=9	221.6±87.36** N=9	52.80±13.00 ** N=9	39.08±6.144 * N=9	117.6±53.12 N=9
FABP/IL-12L (N=9)	198.9±70.43** N=9	18.88±5.641* N=9	12.10±2.022 N=9	18.24 ± 3.670 *N=9	61.40±37.26 N=9

Data are presented as mean and SEM.

* represent a significant difference ($p < 0.05$)

** represent a high significant difference ($p < 0.001$)



b

Figure 4: The body weight of transgenic mice after induction of destran sodium solphate (DSS) experimental colitis

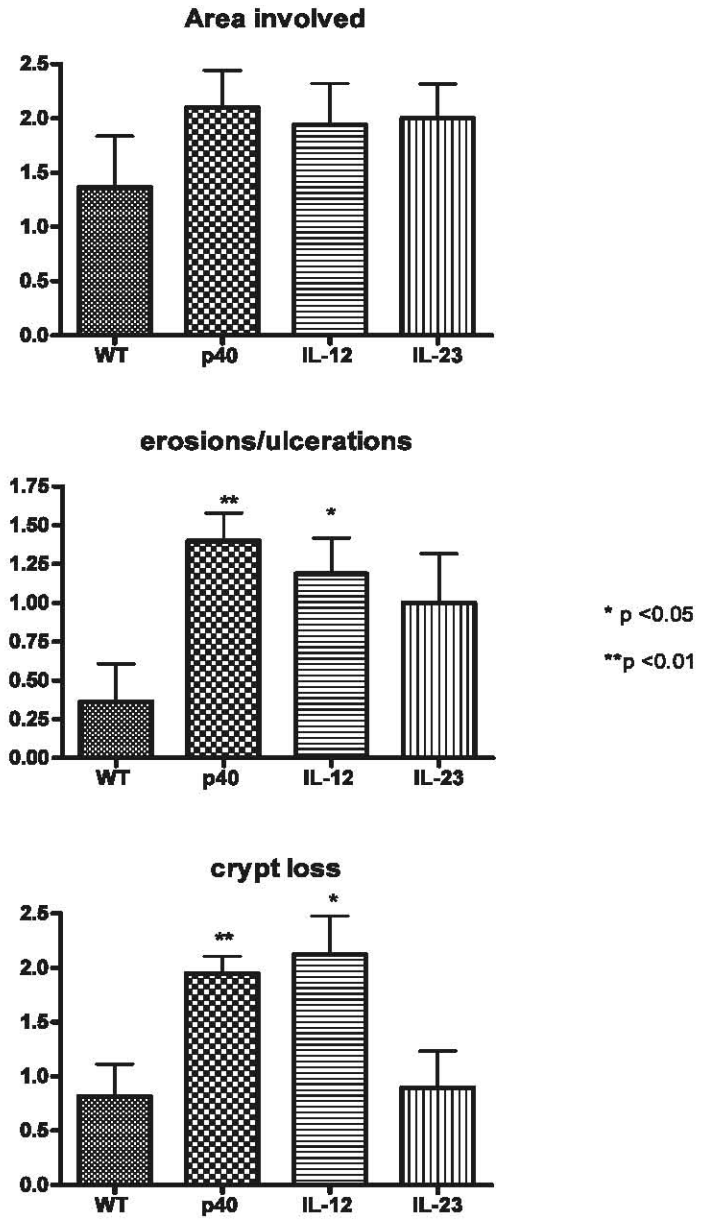
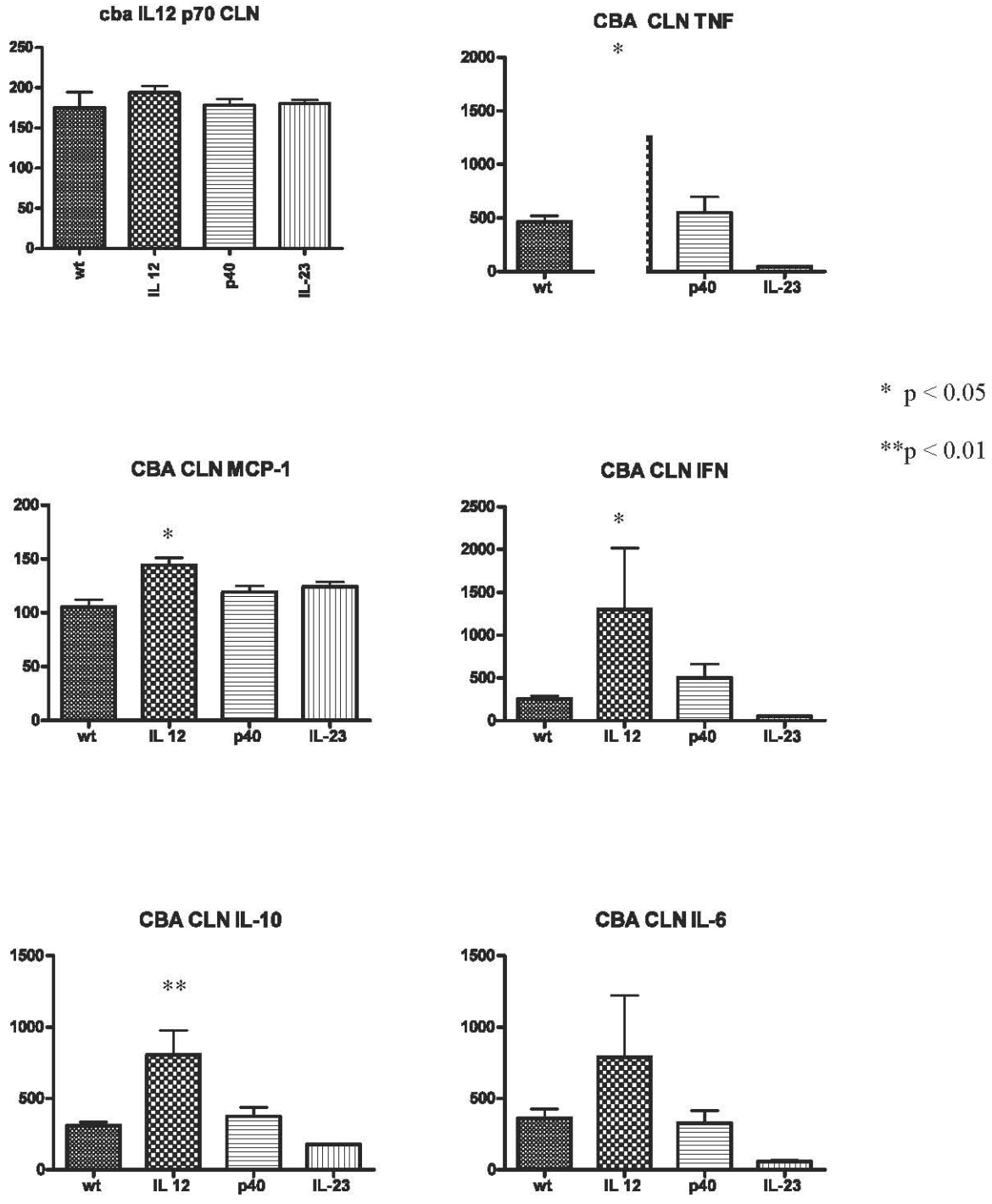


Figure 5: Colitis score in transgenic mice after induction of destran sodium solphate (DSS) experimental colitis



* $p < 0.05$

**$p < 0.01$

Figure 6: Cytokines production evacuate by Cytometric Bead Array in transgenic mice after induction of destran sodium solphate (DSS) experimental colitis

*

Table 4. Colon weight, colon length, number of cells in the caudal lymph nodes in healthy control mice (wild type and transgenic mice) and after induction of DSS colitis

	Colon weight	Colon length	Number of cells in CLN ($x10^3$)	Small intestine weight
WT (N=5)	136.8±4.821	8.800±0.2550 N=5	0.6430± 0.1746 N=5	85.60±4.600 N=5
FABP/p40 (N=6)	146.7±10.07	9.917±0.3516 N=6	1.138±0.1645 N=6	86.00 ±9.295 N=5
FABP/IL-12L (N=5)	151.0±11.92	8.900±0.2915 N=5	0.6100±0.09743 N=5	104.0 ±8.495 N=4
WT 3% (N=5)	274.4±13.61***	5.740±0.1965*** N=5	2.480 ± 0.4616 ** N=5	104.8 ±18.82 N=5
FABP/p40 3% (N=5)	293.6±15.82***	5.900±0.3578 *** N=5	1.125± 0.1954 N=5	102.2 ±7.324 N=5
FABP/IL-12L 3% (N=5)	291.5±28.12**	7.300±0.2145 ** N=5	1.304 ± 0.1638 ** N=5	104.0 ±11.00 N=5

Data are representative of 1 experiment and presented as mean and SEM.

* represent a significant difference ($p< 0.05$)

** represent a high significant difference ($p< 0.001$)

*** represent a very high significant difference ($p<0.0001$)

Table 5. Colon weight, colon length, number of cells in the caudal lymph nodes and small intestine weight in DSS colitis

	Colon weight	Colon length	Number of cells in CLN ($x10^3$)	Small intestine weight
WT 3% (N=5)	274.4±13.61	5.740±0.1965	2.480 ± 0.4616	104.8 ± 18.82
FABP/p40 3% (N=5)	293.6±15.82	5.900±0.3578	1.125 ± 0.1954*	102.2 ± 7.324
FABP/IL-12L 3% (N=5)	291.5±28.12	7.300±0.2145	1.304 ± 0.1638 *	104.0 ± 11.00

Data are representative of 1 experiment and presented as mean and SEM.

* represent a significant difference ($p < 0.05$)

REFERENCES

1. Camoglio et al (2002) Contrasting roles of IL-12p40 and IL-12p35 subunits in the development of experimental colitis. *Eur J Immunol* 32: 261-269.
2. Oppmann et al (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715-25.
3. Ghilardi et al (2004) Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. *J of Immunol* 172: 2827-2833.
4. Ferrante et al (1997) Characterization of IL-12 receptor beta1 chain (IL-12Rbeta1)-deficient mice: IL-12Rbeta1 is an essential component of the functional mouse IL-12 receptor. *J Immunol.* 15;159(4):1658-65
5. Parham et al (2002) A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rβ1 and a novel cytokine receptor subunit, IL-23R. *J. Immunol.* 168: 5699-5708.
6. Neurath et al (1995) Antibodies to IL-12 abrogate established experimental colitis in mice. *J. Exp. Med.* 182: 1281-1290.
7. Mannon et al (2004) Anti-Interleukin-12 treats active Crohn's disease. *Gastroenterology* 126: A84.
8. Wiekowski et al (2001) Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol.* 15;166(12):7563-70.
9. Cua et al. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421: 744-8.
10. Lee et al (2004). Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 199: 125-30.
11. Murphy et al (2003) Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* 198: 1951-7.
12. Becker et al (2003) Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. *J Clin Invest* 112: 693-706.
13. Guarino et al (2004) Interleukin-23, a novel IL-12-like cytokine with Th1 properties is upregulated in the inflamed intestinal mucosa of patients with IBD. *Gastroenterology* A564.
14. Van Deventer et al (1997 a) Tumour necrosis factor and Crohn's disease. *Gut* 40: 443-8.
15. Van Deventer S et al. (1997 b). Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. *Gastroenterology* 113: 383-9
16. Ghosh et al (2003) Natalizumab for active Crohn's disease. *New Eng J Med* 348:24-32.
17. Camoglio L, te Velde AA, de Boer A *et al.*. Hapten-induced colitis associated with maintained Th1 and inflammatory responses in IFN-gamma receptor-deficient mice. *Eur J Immunol* 2000;**30**:1486-95
18. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, et al. 2006. Transforming growth factor-β induces development of the TH17 lineage. *Nature* 441:231–34
19. Wurster AL, Rodgers VL, Satoskar AR, Whitters MJ, Young DA, et al. 2002. Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon γ-producing Th1 cells. *J. Exp. Med.* 196:969–77
20. Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, et al. 2008. Development and characterization of IL-21-producing CD4+ T cells. *J. Exp. Med.* 205:1369–79

21. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, et al. 2007. IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8:967–74
22. King C, Ilic A, Koelsch K, Sarvetnick N. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117:265–77
23. Jain R, Tartar DM, Gregg RK, Divekar RD, Bell JJ, et al. 2008. Innocuous IFNγ induced by adjuvantfree antigen restores normoglycemia inNODmice through inhibition of IL-17 production. *J. Exp. Med.* 205:207–18
24. Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, et al. 2008. Regulation of gut inflammation and Th17 cell response by interleukin-21. *Gastroenterology* 134:1038–48
25. Coquet JM, Chakravarti S, Smyth MJ, Godfrey DI. 2008. Cutting edge: IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyelitis. *J. Immunol.* 180:7097–101
26. Sonderegger I, Kisielow J, Meier R, King C, Kopf M. 2008. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. *Eur. J. Immunol.* 38:1833–38
27. Holmdahl R. 2008. IL-21 and autoimmune disease—hypothesis and reality? *Eur. J. Immunol.* 38:1800–2
28. He YW, Deftos ML, Ojala EW, Bevan MJ. 1998. RORγt, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* 9:797–806
29. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, et al. 2006. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. USA* 103:8137–42
30. Li MO,Wan YY, Sanjabi S, Robertson AK, Flavell RA. 2006. Transforming growth factor-β regulation of immune responses. *Annu. Rev. Immunol.* 24:99–146
31. Mathur AN, Chang HC, Zisoulis DG, Stritesky GL,Yu Q, et al. 2007. Stat3 and Stat4 direct developme of IL-17-secreting Th cells. *J. Immunol.* 178:4901–7
32. Kimura A, Naka T, Kishimoto T. 2007. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc. Natl. Acad. Sci. USA* 104:12099–104
33. Lohoff M, Mittrucker HW, Prechtl S, Bischof S, Sommer F, et al. 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc. Natl. Acad. Sci. USA* 99:11808–12
34. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. 2002. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J. Exp. Med.* 195:1003– 12
35. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, et al. 2007. The development of inflammatory TH-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8:958–66
36. Chen W, Jin W, Hardegen N, Lei KJ, Li L, et al. 2003. Conversion of peripheral CD4+CD25− naïve T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198:1875–86
37. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6:1219–27
38. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29:44–56

39. DuJ, Huang C, Zhou B, Ziegler SF. 2008. Isoform-specific inhibition ofRORα-mediated transcriptional activation by human FOXP3. *J. Immunol.* 180:4785–92
40. Zhang F, Meng G, Strober W. 2008. Interactions among the transcription factors Runx1, RORγt and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat. Immunol.* 9:1297–306
41. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, et al. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26:371–81
42. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, et al. 2007. Reciprocal Th17 and regulatory T cell differentiation mediated by retinoic acid. *Science* 317:256–60
43. Antov A, Yang L, Vig M, Baltimore D, Van Parijs L. 2003. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. *J. Immunol.* 171:3435–41
44. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:715–25
45. Kastelein RA, Hunter CA, Cua DJ. 2007. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. *Annu. Rev. Immunol.* 25:221–42
46. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. 2003. Interleukin-23 promotes a distinct CD4Tcell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278:1910–14
47. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, et al. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201:233–40
48. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, et al. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J. Exp. Med.* 198:1951–57
49. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, et al. 2007. TGF-β and IL-6 rive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nat. Immunol.* 8:1390–97
50. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, et al. 2006. Transforming growth factor-β induces development of the TH17 lineage. *Nature* 441:231–34
51. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, et al. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448:480–83
52. Uhlig HH, McKenzie BS, Hue S, Thompson C, Joyce-Shaikh B, et al. 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. *Immunity* 25:309–18
53. 88. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203:1685–91
54. Hue S, Ahern P, Buonocore S, Kullberg MC, Cua DJ, et al. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J. Exp. Med.* 203:2473–83
55. Izcue A, Hue S, Buonocore S, Arancibia-Carcamo CV, Ahern PP, et al. 2008. Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* 28:559–70
56. Kullberg MC, Jankovic D, Feng CG, Hue S, Gorelick PL, et al. 2006. IL-23 plays a key role in *Helicobacter hepaticus*-induced T cell-dependent colitis. *J. Exp. Med.* 203:2485–94
57. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, et al. 2006. A genome-wide association study identifies *IL23R* as an inflammatory bowel disease gene. *Science* 314:1461–63

58. Cargill M, Schrodi SJ, Chang M, Garcia VE, Brandon R, et al. 2007. A large-scale genetic associatio study confirms *IL12B* and leads to the identification of *IL23R* as psoriasis-risk genes. *Am. J. Hum. Genet.* 80:273–90
59. 94. Liu Y, Helms C, Liao W, Zaba LC, Duan S, et al. 2008. A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci. *PLoS Genet.* 4:e1000041

2.3 Role of Interleukin-23 receptor (IL-23R) in Pediatric-Onset IBD and Genotype-Phenotype Association

ABSTRACT

Aim. In the present study we aimed to establish the allele frequencies of IL23R and NOD2/CARD15 (R702W, G908R, and 3020insC) gene polymorphisms and to assess the contribution of variants of IL-23R and Nod2/CARD15 in the phenotype in our paediatric population affected by IBD. **Methods.** The DNA of the patients was collected and screened for the R702W, G908R, and 3020insC polymorphisms of the CARD15 gene and the rs11209026 Arg381Gln, rs7517847 intron and rs10889677 exon-3'UTR of IL23 gene. In addition a genotype/phenotype analysis was performer. **Results.** We investigated the frequencies of the three NOD2/CARD15 mutations (Arg702Trp, Gly908Arg, 3020insC) in 71 patients with inflammatory bowel disease and compared them with 919 controls. In total, 22,7% of CD patients carried at least one mutant allele within NOD2/CARD15 compared with 22,4% of patients with UC and to 2.6% of controls. The frequencies of the three IL-23 R gene variants (rs11209026 Arg381Gln; rs7517847 intron and rs10889677 exon-3'UTR) showed that all CD patients 100% carried at least one mutant allele within IL-23R gene, compared with 96% of patients with UC. Genotype frequency in UC and CD patients showed that: NOD2/CARD15 mutations seems to be more associated to ileal and ileocolonic involvement in CD affected children; whereas IL-23R mutations are associated to a reduced ileum involvement. IL-23R mutations in our children with UC seem to be associated with a more extensive disease (pancolitis is reported in 42.8% of patients carrying at least one mutation of IL-23R gene) and with a moderate activity disease. **Conclusion**. In conclusion, our study provides additional support for the association of the IL-23R gene in CD and UC pathogenesis. Future studies on larger cohorts of patients need to be undertaken to further explore the role of this gene in the determination of specific CD and UC phenotypes.

INTRODUCTION

The currently accepted etiopathogenic hypothesis suggests that chronic intestinal inflammation and related systemic manifestations characteristic of inflammatory bowel disease (IBD) are due to an overly aggressive or pathologic immune response to resident luminal bacterial constituents. Predisposing factors are genetic dysregulation of mucosal immune responses and/ or barrier function, with onset triggered by environmental stimuli. The increased level of oncordance between identical twins, increased rates of IBD among the Ashkenazi Jewish population, and the familial risk of IBD provides strong evidence that genetic factors play an important role in the pathogenesis of IBD (1). Genetic advances have lead to the discovery that variants of CARD15 located at 16q12 and the 5q31 (IBD5) haplotype confer susceptibility to Crohn's disease (CD). However, the risks associated with these 2 genes account for only a portion of the overall genetic risk. This has led to additional gene findings efforts, the most recent being the recent genome-wide association study that identified the association of the interleukin -23 receptor gene (IL-23R) with small bowel CD. In that study the rare glutamine allele of Arg381Gln (R381Q single nucleotide polymorphism, SNP) conferred protection against CD (2). Distortion of allele transmission was also observed for non-Jewish ulcerative colitis (UC)-affected offspring. This study was conducted in an adult IBD cohort. Recently the gene has been also associated with pediatric-onset CD among Canadian children (3).

The IL-23R, consisting of an IL-12β1 and an IL-23R chain, is highly expressed on memory T cells (4). IL-23 is a novel cytokine formed via the binding of IL-12p40 to a p19 protein (5). After binding to the IL-23 receptor, IL-23 preferentially activates memory T cells. IL-23 does exhibit some similar biological activities to IL-12, however, IL-12 is more involved in the differentiation of naïve T cells into TH1 lymphocytes and subsequent IFNγ production. IL-23, on the other hand, mediates

proinflammatory activities in part by the production of IL-17 through activation of TH17 lymphocytes (6). Other research has shown that IL-23 increases IL-6 production that may account for the tissue injury characteristic of IBD (7). It has also been shown that IL-23, not IL-12, is an important promoter of chronic joint inflammation (6). Becker et al (8) suggested that there is a predisposition of IL-23-induced chronic inflammation in the terminal ileum, which strengthens the evidence for the recent association between IL-23R in small bowel CD patients (2). The elevation of IL-17 levels in the colonic mucosa of both CD and UC patients, combined with the recent association of IL-23R with UC,2 suggests that IL-23 may also play an important role in UC (9). Our aim was to establish the allele frequencies and to assess the contribution of variants (rs11209026 Arg381Gln; rs7517847 intron and rs10889677 exon-3'UTR) of IL23R in determining the phenotype in childhood onset IBD in our paediatric population. Additionally, we sought to investigate the to assess the contribution of the 3 common NOD2/CARD15 (R702W, G908R, and 3020insC) and the variants of IL-23R in the phenotype in our paediatric population affected by IBD.

MATERIALS AND METHODS

Study Patients

From November 1, 2006, to March, 2009, 72 patients with pediatric-onset IBD (younger than age 19) agreed to participate in this genetic study at the Department of Paediatrics, University of Naples "Federico II", (Italy). From January 1, 1997, to April 1, 2005, 919 patients with adult-onset IBD (age 19 or older) at the Academic Medical Centre agreed to participate in the study. IBD was diagnosed based on clinical, endoscopic, radiologic, and histologic criteria. Patients with indeterminate colitis were excluded. Written informed consent was obtained from parents, and the study protocol was approved by the institutional review board of the Department of Paediatrics, University of Naples "Federico II" (Italy).

Phenotype Analysis

Sex and age at diagnosis of all patients were ascertained. Phenotypic classification was based on disease localization and behavior. Localization was determined by endoscopic, histologic, and/or radiologic examination. Categories were made according to the Montreal Classification (10). Pediatric Crohn's disease activity index (PCDAI) (11) and Pediatric Ulcerative Colitis Activity Index (PUCAI) was used to measure disease activity (12).

Family history was defined as positive if at least 1 first- or second-degree relative was diagnosed with IBD. Information was obtained through endoscopic (including upper endoscopy, capsule endoscopy or colonoscopy), radiological [small bowel X-ray or computerized tomography (CT) enteroclysma] or histological examination. Stenotic CD was considered if persistent intestinal obstruction was found either in small bowel X-ray, CT enteroclysma, ultrasonography or colonoscopy. Perforating disease was recorded if patients had enterocutaneous, enteroenteric, enterovesical or enterovaginal fistula, intraabdominal abscess or small bowel perforation. Perianal disease was diagnosed if perianal fistula, ulcers or abscesses were present. Extraintestinal

manifestations were defined as follows: Typ 1 peripheral arthralgia (defined in Orchard et al [13]), presence of PSC diagnosed through endoscopic cholangiography, affections of skin (e.g. presence of erythema nodosum or pyoderma gangrenosum) or eye (e.g. presence of episcleritis or anterior uveitis).

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.

Genotype Analysis

Venous blood samples (10 mL from each pediatric patient) were collected. DNA was extracted following standard procedures and was stored at 5°C. Primers to amplify the polymorphic loci were selected using on-line Primer3 software. SNP genotyping was carried out using TaqMan PCR primer/probe sets designed through Applied Biosystems' Assay by Design service (Foster City, CA; http://myscience.appliedbiosystems. com/). SNP assay reactions were performed in 5-_L volumes and contained 25 ng of DNA, 1_ TaqMan Universal PCR Master Mix (Applied Biosystems), 100 nM of each primer, and 900 nM of each probe. Cycling conditions on the ABI prism 7900 HT (Applied Biosystems) were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 92°C and 1 minute at 60°C. End-point fluorescence was measured immediately after cycling. Alleles were assigned using SDS 2.0 software (Applied Biosystems). The DNA of the patients was screened for the R702W, G908R, and 3020insC polymorphisms of the CARD15 gene and the rs11209026 Arg381Gln, rs7517847 intron and rs10889677 exon-3'UTR of IL23 gene. The Table 1 shows the chromosomal location of the IL-23 SNPs and primers used for genotyping.

Statistical Analysis

Comparison of the frequency of the NOD2/CARD15 and IL-23R mutations between patients and controls and the association to the phenotype was performed by Chi-Square test or Fisher's

exact test where appropriate. The data were analysed using SPSS/PC+V10.01 software (SPSS, Chicago, IL, USA).

RESULTS

Seventy–two patients 72 (M/F: 42/30; mean age 10,8 years, range 4,1-15,1 years), with newly diagnosed IBD were enrolled in the study: 49 resulted affected by UC and 22 children by CD. One patient was excluded because affected by indeterminate colitis. Demographic data for the study group, including the PUCAI and PCDAI at diagnosis and the extension disease based on the Montreal guidelines for classification of UC/CD, are listed in Table 2.

We investigated the frequencies of the three NOD2/CARD15 mutations (Arg702Trp, Gly908Arg, 3020insC) in 71 patients with inflammatory bowel disease and compared them with 919 controls. In total, 22,7% of CD patients carried at least one mutant allele within NOD2/CARD15 compared with 22,4% of patients with UC and to 2.6% of controls.

The frequencies of the three IL-23 R gene variants (rs11209026 Arg381Gln; rs7517847 intron and rs10889677 exon-3'UTR) showed that all CD patients 100% carried at least one mutant allele within IL-23R gene, compared with 96% of patients with UC.

Genotype frequency in UC and CD patients is reported in Table 3.

In the next step a genotype/phenotype analysis was performed. The percentages of CD patients carrying at least one NOD2/CARD15 variant were correlated to demographic data, extension and disease activity and time of relapse as well as whose carrying at least one variants of IL-23R mutations. NOD2/CARD15 mutations seems to be associated to ileal and ileocolonic involvement. With respect to all other clinical and demographic data, including extraintestinal manifestations and fistulizing disease, no significant differences were found in respect to the NOD2/CARD15 genotype. The frequency of stenotic disease was higher in the patients carrying at least one NOD2/CARD15 mutation. No difference has been found regarding the time of relapse. Patients affected by CD carrying IL-23R mutation did not show a specific phenotype in our pediatric population except for a reduced percentage of ileal disease. IL-23R mutations in our children with

UC seem to be associated with a more extensive disease (pancolitis is reported in 42.8% of patients carrying at least one mutation of IL-23R gene) and with a moderate activity disease and not seem to predictive of a early relapse (within 6 months). Genotype –phenotype correlations of the pediatric-onset UC and CD patients are shown in Table 5 and Table 6.

DISCUSSION

In the present study we aimed to establish the allele frequencies of IL23R and NOD2/CARD15 (R702W, G908R, and 3020insC) gene polymorphisms and to assess the contribution of variants of IL-23R and NOD2/CARD15 in the phenotype in our paediatric population affected by IBD. We reported that our pediatric population affected by CD the NOD2/CARD15 mutations are responsible of a marked ileal and ileocolonic involvement with a higher frequency of stenotic disease in patients carrying at least one NOD2/CARD15 mutation. Patients affected by CD carrying IL-23R mutations did not show a specific phenotype except for a reduced percentage of ileal disease. Finally IL-23R mutations in our children with UC seem to be associated with a more extensive disease (pancolitis is reported in 42.8% of patients carrying at least one mutation of IL-23R gene) and with a moderate activity disease and not seem to be predictive of a early relapse (within 6 months). These data confirm that IL-23 pathway plays a pivotal role in the development of chronic mucosal inflammation seen in the inflammatory bowel diseases (14). Furthermore, the contribution of the interleukin 23 receptor gene (IL23R) to Crohn's Disease (CD) risk has now been established by a genome wide association study (GWAS), confirmed in a pediatric color, and confirmed the large multi-disease study from the UK (2007) (15). The association of IL23R variants with CD has also been observed in populations ascertained from Scotland (16), New Zealand (17), Continental Europe (18). North America (19, 20), Brazil (21), and Israel (22), but not Japan (23). In general, most of the confirmation studies have focused on the "protective" effect of the single-nucleotide polymorphism (SNP), rs11209026, a coding SNP that alters Arginine 381 to Glutamine (R381Q). At present, it can only be speculated how IL23R contributes to the pathophysiology of IBD. IL23 is produced by antigen-presenting cells and promotes the expansion of Th17 cells.[6] This pathway is suggested to be a novel non-Th1 pathway leading to the differentiation of distinct CD4+ Th17 inflammatory

effector cells and the IL23/Th17 axis is believed to be crucially involved in the pathogenesis of various inflammatory disorders. IL23 activity has been shown to be present in the terminal ileum[28] and colon. Furthermore, IL23 was reported to be essential for the development of colitis in IL10-deficient mice. In addition, IL23, but not IL12, was the key factor for the induction of chronic intestinal inflammation by both innate and adaptive immune mechanisms in two different mice models.

TABLES AND FIGURES

Table 1. Chromosomal Location of the IL-23 SNPs and Primers Used for Genotyping

SNP	*LOCATION*	*PRIMERS*
rs1 209026	67478546	GGAATGATCGTCTTTGCTGT (forward) (R381Q) TGTTCTTTTTATTTTCCTTTATCTGAA (reverse)
rs7517847	6739690	TTTCTGGATGCCCTTTCCT (forward) GAATTTGAGGGGCCTAGGAG (reverse)
rs10889677	67437141	ACGTTGGATGCACCTTCGGGACCTTAATTC (forward) ACGTTGGATGCATGTAAGAATTCCCGGGAG (reverse)

Characteristics	*Value*
Sex ✓ Male ✓ Female	 42 (44.5%) 30 (55.5%)
Mean age (years) ✓ Male ✓ Female	 11,3 (range 3,6- 15,11) 10,3 (range 4,6-15,10)
Montreal Classification (RCU=49 patients) ✓ Proctosigmoiditis (E1) ✓ Left-sided colitis (E2) ✓ Pancolitis (E3)	 20 (40,8%) 9 (18,36%) 20 (40,8%)
Pediatric ulcerative colitis Activity index (PUCAI) ✓ Inactive disease (≥10) ✓ Moderate disease activity (≥ 20) ✓ Severe disease activity (≥ 35)	 10 (20,4%) 21 (42,9%) 18 (36,7%)
Montreal Classification (CD=22 patients) ✓ Ileal ✓ Colonic ✓ Ileocolonic ✓ Isolated upper disease	 5 (22,7%) 8 (36,3%) 9 (41%) 0
Pediatric Crohn's disease activity index (PCDAI) ✓ Mild (>10) ✓ Moderate (>20) ✓ Severe (>35)	 4 (18,2%) 16 (72,7%) 2 (9 %)

Table 2. Baseline characteristics of patients affected by Inflammatory Bowel Disease (Crohn's disease patients- N= 22; Ulcerative colitis patients -N=49) at diagnosis.

MUTATION (NUMBER OF PATIENTS ANALYZED)	*GENOTYPE*	*TOTAL CROHN'S DISEASE PATIENTS (CD) N=22*	*TOTAL ULCERATIVE COLITIS PATIENTS (UC) N= 49*	*TOTAL CONTROL PATIENTS (CP) N= 919*
G908R (CD=22; UC=47; CP=906)	-/- +/- +/+	22 (100) 0 0	42 (89.3) 0 5 (10.6)	887 (97.2) 19 (2) 0
R702W (CD=8; UC=24; CP=843)	-/- +/- +/+	7 (87.5) 1(12.5) 0	21 (87.5) 3(12.5) 0	762 (90.3) 80 (9.5) 1 (0.1)
3020insC (CD=19; UC=43; CP=773)	-/- +/- +/+	16 (84.2) 2 (10.5) 1 (5.2)	40 (81.6) 3 (61.2) 0	749 (96.8) 24 (3.1) 0
rs11209026 Arg381Gln (CD=14; UC=39; CP=575)	-/- +/- +/+	12 (85.7) 2 (14.2) 0	36 (92.3) 3 (7.7) 0	212 (36.8) 280 (48.6) 83 (14.4)
rs7517847 intron (CD=8; UC=23; CP=919)	-/- +/- +/+	3 (37.5) 4 (50) 1	10 (43.4) 13 (56.5) 0	312 (33.9) 418 (45.4) 189 (20.5)
rs10889677 exon- 3'UTR (CD=20; UC=43; CP=908)	-/- +/- +/+	4 (20) 10 (50) 6 (30)	12 (27.9) 14 (32.5) 17 (39.5)	788 (86.7) 116 (12.7) 4 (0.4)

Table 3: Genotype of children affected by Crohn's disease (N=22), Ulcerative Colitis (N=49), and Controls (919). Wild-type is indicated by -/-, heterozygous is indicated by, +/-, homozygous is indicated by +/+. Data are expressed as number of patients and percentage

Chron's Disease Patients N=22				
	NOD+	**NOD-**	**IL-23R+**	**IL23R-**
Total number	4	18	19	3
Localization (%)				
Isolated Upper GI disease	-	-	-	-
Ileal	1 (25)	4 (22)	4 (21)	1 (33)
Ileo-colonic	3 (75)	6 (33)	8 (42)	1 (33)
Colonic	0	8 (44)	6 (31.5)	1 (33)
PCDAI at diagnosis (%)				
Mild	1 (25)	10 (55)	8 (42.1)	3 (100)
Moderate	3 (75)	7 (38.8)	10(52.6)	0
Severe	0	1 (5.5)	1 (5.3)	0
Behaviour (%)				
Non stricturing/penetrating	0	0	0	0
Stricturing	2 (50)	0	0	0
Penetrating	0	0	0	0
Timing of Relapse				
Within 6 months	2 (50)	7 (38.8)	7 (36.8)	1(33.3)
> 6 months	2 (50)	4 (22.2)	12(63.1)	2(66.6)

Table 5. Clinical Characteristics of children affected by Crohn's disease

Ulcerative Colitis Patients N=49				
	NOD+	**NOD-**	**IL-23R+**	**IL23R-**
Total number	11	38	35	14
Localization (%)				
Proctosigmoiditis	5(45.4)	15(39.5)	14(40.1)	5(35.7)
Left-sided colitis	1 (9)	8 (21)	6 (17.1)	5(35.7)
Pancolitis	5(45.4)	15(39.5)	15(42.8)	4(28.5)
PUCAI at diagnosis (%)				
Inactive disease	0	14(36.8)	8 (22.8)	9(64.2)
Moderate disease activity	7(63.6)	10(26.3)	14 (40)	1 (7.1)
Severe disease activity	4(36.3)	14(36.8)	13(37.1)	4(28.5)
Time of relapse (%)				
Within 6 months	7(63.6)	13(34.2)	16(45.7)	3 (21.4)
> 6 months	4(36.3)	25(65.7)	17(48.5)	11(78.5)

Table 6. Clinical Characteristics of children affected by Ulcerative Colitis

REFERENCES

1. Taylor, KD.; Yand, HY.; Rotter, JI. Inflammatory bowel disease. In: Rimoin, DL.; Connor, JM.; Pyeritz, RE.; Korf, B., editors. Emery and Rimoin's principles and practice of medical genetics. 5. London: Churchill Livingstone; 2007. p. 1549-1582.
2. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL-23R as an inflammatory bowel disease gene. Science 2006;314:1461–1463.
3. Amre DK, MackD, Israel D et al. Association Between Genetic Variants in the IL-23R Gene
and Early-Onset Crohn's Disease: Results From a Case-Control and Family-Based Study Among Canadian Children. Am J Gastroenterol 2008; 103:615-620
4. Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. J Immunol 2002;168:5699–5708.
5. Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23,with biological activities similar as well as distinct from IL-12. Immunity 2000;13:715–725.
6. Murphy CA, Langrish CL, Chen Y, et al. Divergent pro- and anti-inflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 2003;198:1951–1957.
7. Fuss IJ, Becker C, Yang Z, et al. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. Inflamm Bowel Dis 2006;12:9–15.
8. Becker C, Wirtz S, Blessing M, et al. Constitutive p40 promoter activation and IL-23 production in the terminal ileum mediated by dendritic cells. J Clin Invest 2003;112:693–706.
9. Fujino S, Andoh A, Bamba S, et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003;52:65–70.
10.
Satsangi J, Silverberg MS, Vermiere S et al. The Montreal Classification of Inflammatory bowel disease: controversies, consensus and implications. Gut 2006; 55: 749-753
11.
Turner, A. R. Otley, D. Mack, J Development, Validation, and Evaluation of a Pediatric Ulcerative Colitis Activity Index: A Prospective Multicenter Study Gastroenterology 2007;133:423–432
12. Hyams J, Markowitz J, Otley A et al Evaluation of the pediatric crohn disease activity index : A prospective multicenter experience. J pediatr Gastroenterol Nutr. 2005: 41: 416-421
13. Orchard TR, Wordsworth BP, Jewell DP. Peripheral arthropathies in inflammatory bowel disease: their articular distribution and natural history. Gut 1998; 42: 387-391.
14. McGovern D, Powrie F. The IL23 axis plays a key role in the pathogenesis of IBD. Gut. 2007 Oct;56(10):1333-6.
15. Duerr RH, Taylor KD, Brant SR, Rioux et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science. 2006 Dec 1;314(5804):1461-3

16. Dubinsky MC, Wang D, Picornell Y, et al IL-23 receptor (IL-23R) gene protects against pediatric Crohn's disease. Inflamm Bowel Dis. 2007 May;13(5):511-5.
17. Van Limbergen JE, Russell RK, Nimmo ER, et al. IL23R Arg381Gln is associated with childhood onset inflammatory bowel disease in Scotland. Gut 2007;56:1173–4.tibody. Inflamm Bowel Dis 2006;12:9–15. Am J Gastroenterol. 2007 Dec;102(12):2754-61. Epub 2007 Sep 25.
18. Roberts RL, Gearry RB, Hollis-Moffatt JE IL23R R381Q and ATG16L1 T300A are strongly associated with Crohn's disease in a study of New Zealand Caucasians with inflammatory bowel disease.
19. Büning C, Genschel J, Weltrich R The interleukin-25 gene located in the inflammatory bowel disease (IBD) 4 region: no association with inflammatory bowel disease. Eur J Immunogenet. 2003 Oct;30(5):329-33.
20. Roberts RL, Gearry RB, Holli s-Moffatt JE, Miller AL, Reid J, Abkevich V, Timms KM, Gutin A, Lanchbury JS, Merriman TR, Barclay ML, Kennedy MA. IL23R R381Q and ATG16L1 T300A are strongly associated with Crohn's disease in a study of New Zealand Caucasians with inflammatory bowel disease. Am J Gastroenterol. 2007;102:2754–2761.
21. Baldassano RN, Bradfield JP, Monos DS, Kim CE, Glessner JT, Casalunovo T, Frackelton EC, Otieno FG, Kanterakis S, Shaner JL, Smith RM, Eckert AW, Robinson LJ, Onyiah CC, Abrams DJ, Chiavacci RM, Skraban R, Devoto M, Grant SF, Hakonarson H. Association of variants of the interleukin-23 receptor gene with susceptibility to pediatric Crohn's disease. Clin Gastroenterol Hepatol. 2007;5:972–9
22. Leshinsky-Silver E, Karban A, Dalal I, Eliakim R, Shirin H, Tzofi T, Boaz M, Levine A. Evaluation of the interleukin-23 receptor gene coding variant R381Q in pediatric and adult Crohn disease. J Pediatr Gastroenterol Nutr. 2007;45:405–408.
23. Yamazaki K, Onouchi Y, Takazoe M, Kubo M, Nakamura Y, Hata A. Association analysis of genetic variants in IL23R, ATG16L1 and 5p13.1 loci with Crohn's disease in Japanese patients. J Hum Genet. 2007;52:575–583.

CHAPTER 3

IMMUNOLOGICAL FEATURES OF INFLAMMATORY BOWEL DISEASE (IBD): MARKERS OF INFLAMMATION

3.1 Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis

ABSTRACT

The aim of this study was to determine if small bowel involvement at diagnosis could predict early relapse in children with ulcerative colitis. Children with newly diagnosed ulcerative colitis were evaluated prospectively at three time points: within 1 month, 6 months and 1 year after diagnosis. Clinical activity indices were used to measure disease activity. Laboratory studies were performed at each visit and/ or at the time of relapse. At diagnosis, all patients underwent colonoscopy and a cellobiose / mannitol small intestinal permeability study. Some children were further investigated with an upper gastrointestinal endoscopy. Thirty-three patients completed the 1-year study. Overall, nine patients (27.3%) relapsed within 6 months of diagnosis, one patient (3%) within 1 year, whereas 23 patients (69.7%) did not relapse. The mean clinical activity indices, laboratory parameters, extent of colonic involvement,nupper and lower gastrointestinal histological features were not predictive of early relapse. Results of the cellobiose / mannitol small intestinal permeability study were significantly higher in children who relapsed within 6 months compared with children who did not relapse ($P < 0.013$). The cellobiose / mannitol small intestinal permeability study was abnormal in 77.8% of early relapsers compared with only 8.3% of non-relapsers. Abnormal small intestinal permeability in children with ulcerative colitis could predict a more relapsing disease.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission with episodes of clinical relapse, which is characterized by an increase in symptoms (1, 2). A number of investigators have recently reported that diffuse gastritis is a common finding in patients with UC(3–6). In contrast, focal gastritis and granulomas are indicative of Crohn's disease (CD), although focal gastritis occasionally has been observed in UC. (7, 8). In addition, other authors have found that the gastroduodenitis in CD and UC may be indistinguishable unless aphthous ulcers and granulomas are found, even if the histological findings in the upper gastrointestinal (UGI) tract appear to be less severe in UC patients (3, 4, 9). The cause of the inflammation in the UGI tract of UC patients is not clear. It could be due to the patient's overall poor health or could be a response to yet-unknown immunological factors associated with the disease(9). Many histological and laboratory parameters [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count, white cell count, interleukin 6, tumour necrosis factor a, interleukin 1b, interleukin 15 and antineutrophil cytoplasmic antibody (ANCA)] that reflect the systemic consequences of inflammation, and clinical disease activity indices have been proposed as predictors of clinical relapse of IBD in adults (10). Faecal calprotectin has been suggested as a predictor of clinical relapse of disease activity in patients with CD and UC, whereas small intestinal permeability as a useful predictor of relapse in patients with small intestinal CD (2). However, the predictive values of these different parameters in identifying patients at risk of relapse have generally been disappointing (2,10). Recently, it has been demonstrated that lower serum albumin levels and haematocrit and elevated ESR in children with CD at diagnosis may predict the need for immunomodulator therapy earlier in the disease course (11). The purpose of this study was to determine if UGI involvement, evaluated by small intestinal permeability test and / or histological

studies, as well as clinical and laboratory parameters at diagnosis, could predict early relapse in children with UC.

MATERIALS AND METHODS

The study was a prospective, single-centre, 1-yearstudy of children with newly diagnosed UC consecutively enrolled over a 24-month period at the Department of Pediatrics of the University of Naples ''Federico II'', Italy. Patients were recruited to participate in this study if they had a new diagnosis of UC established based on accepted historical, endoscopic, histological and / or radiological criteria.12 Participants were evaluated a three time points: within 1 month, 6 months and 1 year after commencement of treatment. At each visit data were collected including patient questionnaires regarding disease activity (stool frequency, stool consistency, haematochezia, abdominal pain, extra-intestinal manifestations of disease and overall patient functioning). Additional information collected at the first visit included demographic data, family history and symptom onset. Physical examination was performed at each visit by a paediatrician and included an abdominal examination and examination for 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. Individual patients' paediatric gastroenterologists exclusively made all decisions regarding therapeutic interventions. Patients who failed to respond to corticosteroids (CS) (not showing regression of clinical symptoms to oral methylprednisolone, 1 mg / kg / day, max 40 mg/ day or equivalent within 30 days, or intravenous treatment within 7–10 days), defined as CS resistant or CS refractory, and patients who initially responded to CS but then relapse with CS tapering or shortly after CS discontinuation, defined as CS dependent, were excluded from the study (13). Lichtiger colitis activity index (LCAI) (Table 1) and a physician's global assessment (PGA) were used to measure disease activity (14).

Individual scores for each section of the test including symptoms, characteristics of stool and physical examination were computed. A sustained drop in the LCAI to ≤ 2 after steroid therapy was considered remission. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in the LCAI to >2, sufficient to require treatment with CS, azathioprine /immunosuppressive agents or surgery (2, 15) An early relapser was defined as a patient relapsing within 6 months from diagnosis, and a late relapser as a patient relapsing between 6 months and 1 year after diagnosis. Laboratory studies including complete blood count, albumin, ESR and CRP were performed at each visit and / or at the time of relapse. Celiac and infectious diseases at diagnosis were ruled out.

At baseline, before commencement of any treatment, ANCA titres were evaluated and all patients were asked to provide a single stool sample for the determination of faecal calprotectin levels according to the methods previously reported (16). In addition, all patients underwent CMPS. Cellobiose and mannitol are oligosaccharides and small intestinal permeability probes. They are degraded by the bacterial flora of the large intestine and yield no information regarding colonic permeability characteristics (17). The CMPS was performed after an overnight fast, prior to commencement of any treatment and in close temporal proximity to the endoscopic procedures. Each subject drank a solution containing 2 g mannitol, 5 g cellobiose and water to make 100 mL (osmolarity 270 mmol / L). Urine was collected for the next 5 h and stored at)20 °C. The subjects went without food during the test but were allowed to drink water after the first hour. Mannitol in urine was measured by the method of Corcoran and Page whereby it is oxidized to formaldehyde by periodic acid (18). Urine cellobiose was measured using the method described by Strobel et al. in which the compound is digested by D-glucosidase to glucose (19). The final ratio of percentage recovery of cellobiose to percentage recovery of mannitol was calculated. A cellobiose:mannitol ratio >0.022 was considered abnormal, as the value exceeded 2 s.d. over the mean derived from

age- and sex-matched normal children (20). At diagnosis, all children underwent colonoscopy with mucosal biopsy; some children were further investigated with an UGI endoscopy. Biopsies were taken from the distal oesophagus, the gastric body and antrum, the duodenal bulb and / or third / fourth part of the duodenum. All children had a barium meal and small bowel follow-through. All histological 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 histological criteria of Chong et al (21). Helicobacter pylori was systematically sought in gastric antral biopsies using Giemsa staining and CLO colorimetric urease test. Crohn's disease was excluded on the basis of the absence of: classical macroscopical lesions (e.g. skip lesions, snail track ulcers); non-caseating epithelioid and giant cell granulomas in any part of the gastrointestinal tract; histological findings of focal inflammation, submucosal or transmural inflammation, lymphocyte aggregates (without germinal centres) and mucous retention in the presence of more than minimal inflammation; fistulae and / or perianal abscesses; typical structuring small bowel disease on barium follow-through (22). 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 for normally distributed variables and the Mann–Whitney U-test and the chi-squared and Fisher exact tests for categorical variables were used where appropriate. Sample size calculation parameters for CMPS were: relapse in 30% of cases; evaluation of minimal test group differences = 50%; type 1 error = 0.05; power = 90%. Ratio test group / control group 1:1, 10 subjects in each group were required (23). Written, informed consent was obtained from 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'

RESULTS

Thirty-eight patients with newly diagnosed UC were enrolled in the study. Five patients were excluded because two (5.3%) children were CS refractory and three (7.9%) were CS dependent. Thirty-three patients completed the 1-year study. Demographic data for the study group are listed in Table 2. In all patients, the diagnosis of UC was confirmed by a median follow-up of 29 months (range: 17–40 months). A t diagnosis, all patients received oral methylprednisolone (1 mg / kg / day, max 40 mg / day per 4 weeks). After 4 weeks, all patients were in remission and began tapering off CS on a weekly basis (25% / week) according to the prescribed schedule on the basis of the LCAI. All patients received mesalamine maintenance therapy. Oral mesalamine 50 mg / kg was used in 20 patients (60.6%), one 2 g mesalamine enema every one to three nights was used in three patients (9.1%) and oral and topical mesalamine was used in 10 patients (30.3%). Overall, nine patients (27.3%) relapsed within 6 months of diagnosis, one patient (3%) within 1 year and 23 patients (69.7%) did not relapse during the follow-up. No significant differences in formulation of mesalimine used among early relapsers and non-relapsers were found (v2: 0.14; P = 0.93). Gender and age were not associated with early relapse (P = 0.1). Linear growth and weight at diagnosis were normal in all children. No significant differences in weight or height at diagnosis were observed in early relapsers and non-relapsers. The mean LCAI performed at diagnosis by individual patients' paediatric gastroenterologist did not show differences among patients who had early relapse and those who did not (mean: 8.3 [range: 6–12] vs. 8.3 [range: 6–12], respectively; P = 0.724). On the basis of the PGA, the disease severity was classified as mild in 13% of non-relapsers vs. 22% of early relapsers, moderate in 49% of non-relapsers vs. 33% of early relapsers and severe in 38% of non-relapsers vs. 45% of early relapsers (P = 0.647). Laboratory parameters at diagnosis including haematocrit, albumin, ESR, CRP and faecal calprotectin were not predictive of early

relapse. Table 3 shows mean serum markers before a relapse (study entry) in patients who went on to relapse within 6 months, as well as at baseline and 6 months in those who did not relapse. Although 12-month measurements were available for the non-relapsers, they were not used in the analysis. At baseline, ANCA was evaluated in 23 children (69.7%). Nine (39.1%) of 23 patients were perinuclear pANCA positive. ANCA status (i.e. presence / absence) did not change during the follow-up. The pANCA presence was not associated with earlier clinical relapse (P = 0.666).

Results of the CMPS were significantly higher for children who relapsed within 6 months (mean: 0.036, range: 0.008–0.1; P < 0.013) compared with children who did not relapse (mean: 0.017, range: 0.003–0.17) (Figure 1). Using a test cut-off of 0.022, the CMPS was abnormal in 77.8% of early relapsers compared with only 8.3% of non-relapsers. A cellobiose:mannitol ratio >0.022 gave a sensitivity of 77.7%, a specificity of 91.6%, a positive predictive value of 77.7% and a negative predictive value of 91.6% in predicting early relapse in UC paediatric patients. Full ileo - colonoscopy was completed in 29 children (88%). All patients had rectal involvement with confluent inflammation. Lower endoscopic findings included mucosal granularity, friability, aphthoid ulcers, superficial and deep ulcers and inflammatory polyps. There was a trend for early relapsers to present more extensive disease, but this finding did not meet statistical significance (P = 0.145) (Figure 2). No mucosal histological colonic feature was identified as a marker of earlier time to relapse. UGI endoscopy was performed in 15 (45.4%) children and revealed abnormalities in 13 (86%). The observed mucosal abnormalities included oesophageal erythema and / or erosions (n = 7), gastric oedema and erythema (n = 7), antral mucosal nodularity (n = 2) and duodenal oedema and hyperaemia (n = 9). Two (15.4%) patients had H. pylori on Giemsa staining of gastric antral biopsies. Histological examination included oesophagitis (n = 7), chronic inflammation of the oesophagus (n = 1), acute (n = 2) and chronic (n = 7) gastritis, duodenitis (erosions) (n = 3) and

diffuse chronic duodenal inflammation with glandular distortion (n = 3). Histological UGI involvement was not significantly different in early relapsers vs. non-relapsers (P = 0.530, 0.311 and 0.5 for oesophagus, stomach and duodenum respectively). Barium meal and small bowel follow-through were normal in all patients.

DISCUSSION

Several clinical and laboratory markers have been studied in IBD as diagnostic aids, indicators of disease activity or severity, and to predict the risk of relapse in those patients in remission. In particular, the ability to reliably predict the risk of recurrence would help direct appropriate therapy to those who would most likely benefit from it and avoid the expense and potential toxicity of chronic maintenance therapy in those who have a low risk of recurrence. To our knowledge, this study represents the first prospective evaluation of indicators for early relapse among paediatric patients with UC. The natural history of our patients with UC was similar to that recently reported in paediatric patients (24). Statistical analysis did not identify any differences in patient characteristics between the early relapsing and non-relapsing groups for age and sex. The extent of disease was not predictive of early relapse. This finding suggests that clinical severity may depend more upon deep inflammation than on superficial lesions accessible to the endoscopist, as suggested for adults with CD (25). Direct determination of mediators released by gut-associated lymphoid tissue, for example, lymphokines and their soluble receptors, may provide insights into the process taking place in the gut wall (26). Previous studies in CD have shown that endoscopic findings have no predictive value for either the response to steroid treatment of an acute attack or the clinical course after steroid withdrawal (27, 28). The LCAI and the PGA at diagnosis were similar in early relapsers and non-relapsers. Both indices depend almost exclusively on clinical features that are often subjective, such as severity of abdominal pain, and may therefore not be truly representative of the degree of active inflammation and predictive of the risk of relapse (29, 30). In our study, laboratory parameters at diagnosis, including haematocrit, albumin, ESR and CRP, were not predictive of early relapse, as reported in adult patients with UC (10, 30). Our study showed that faecal calprotectin concentration during the acute phase of disease is not useful in identifing early relapsing subjects. (p)ANCA presence was not associated with earlier clinical relapse, as has been

reported in adults (10). Tests of intestinal permeability are sensitive for the detection of patients with active small intestinal CD, are useful for assessing treatment, and may have prognostic implications (31, 32). In our study, we demonstrated that an abnormal small intestinal permeability could be predictive of early relapse in paediatric patients affected by UC. In fact, abnormal permeability to cellobiose was found in 77.8% of early relapsed patients compared with only 8.3% of non-relapsing subjects, even though histopathological evaluation of small bowel biopsy wasb normal in two-thirds of our cases. This finding could be related, in part, to a patchy distribution of enteropathy. This observation is in agreement with previous studies which demonstrated that abnormal sugar permeability can occur in the presence of unequivocally normal small bowel histology (18). This would suggest that there is a subtle alteration in function, independent bof inflammation, which can manifest as in increase in paracellular permeability. An intriguing question is whether or not altered intestinal permeability plays a pathogenic role in UC as well as in CD. In CD, it has been hypothesized that increased permeability may lead to the absorption of endotoxin and lipopolysaccharides from the lumen. Both these substances are potent stimulators of acute-phase reactants and liberation of interleukin-6, which has been shown to be an important mediator of inflammation in CD. On the basis of our study, in a subgroup of paediatric patients with UC, a similar process may cause early relapse. The cause of the increase in permeability, however, is still unknown (31, 33). A recent study suggests that intrarectal administration of trinitro-benzene sulfonate (TNBS) to rats influences not only their colon and terminal ileum, but also the proximal ileum and jejunum. Involvement of the ileum and jejunum in TNBSinduced colitis may be related to the systemic reaction of the immune system and mucosa to colitis (34). Our study confirms an endoscopic and histological UGI involvement in UC, as previously described, but no significant differences were observed in early relapsers and non-relapsers (4, 5). In conclusion, this study strongly suggests that the gut inflammatory reaction in patients with UC is not restricted to the large

intestine. Our results show that clinical, endoscopic and biological findings have no predictive value for early relapse. On the other hand, in children with UC an abnormal small intestinal permeability could predict a more relapsing disease. If these data are confirmed on a larger scale, the permeability test in presentation of disease may represent a noninvasive test useful in identifying those patients who will require more targeted treatment including immunomodulators. In addition, further studies on intestinal permeability may lead to a better understanding of the pathogenesis of UC.

REFERENCES

1. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. Gastroenterol Clin North Am 1999; 28: 445–58.
2. Tibble JA, Sigthorsson G, Bridger B, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroenterology 2000; 119: 15–22.
3. Ruuska T, Vaajalahti P, Arajarvi P, et al. Prospective evaluation of upper gastrointestinal mucosal lesions in children with ulcerative colitis. J Pediatr Gastroenterol Nutr 1994; 19: 181–6.
4. Kaufman SS, Vanderhoof JA, Young R, et al. Gastroenteric inflammation in children with ulcerative colitis. Am J Gastroenterol 1997; 92: 1209–12.
5. Tobin JM, Sinha B, Ramani P, et al. Upper gastrointestinal mucosal disease in pediatric Crohn disease and ulcerative colitis: a blinded, controlled study. J Pediatr Gastroenterol Nutr 2001; 32: 443–8.
6. Kundhal PS, Stormon MO, Zachos M, et al. Gastric antral biopsy in the differentiation of pediatric colitides. Am J Gastroenterol 2003; 98: 557–61.
7. Parente F, Cucino C, Bollani S, et al. Focal gastric inflammatory infiltrates in inflammatory bowel diseases: prevalence, immunohistochemical characteristics, and diagnostic role. Am J Gastroenterol 2000; 95: 705–11.
8. Sharif F, McDermott M, Dillon M, et al. Focally enhanced gastritis in children with Crohn's disease and ulcerative colitis. Am J Gastroenterol 2002; 97: 1415–20.
9. Abdullah BA, Gupta SK, Croffie JM, et al. The role of esophagogastroduodenoscopy in the initial evaluation of childhood inflammatory bowel disease: a 7-year study. J Pediatr Gastroenetrol Nutr 2002; 35: 636–40.
10. Bitton A, Peppercorn MA, Antonioli DA, et al. Clinical, biological, and histologic parameters as predictors of relapse in ulcerative colitis. Gastroenterology 2001; 120: 13–20.
11. Jacobstein DA, Mamula P, Markowitz JE, Leonard M, Baldassano RN. Predictors of immunomodulator use as early therapy in pediatric Crohn's disease. J Clin Gastroenterol 2006; 40: 145–8.
12. Hildebrand HF, Holmquist B, Kristiansson L, et al. Chronic inflammatory bowel disease in children and adolescents in Sweden. J Pediatr Gastroenterol Nutr 1991; 13: 293–7.
13. Faubion WA, Loftus EV, Haremsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population- based study. Gastroenterology 2001; 121: 255–60.
14. Lichtiger S, Present D, Kornbluth A, et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. N Eng J Med 1994; 330: 1841–5.
15. Russell GH, Katz AJ. Infliximab is effective in acute but no chronic childhood ulcerative colitis. J Pediatr Gastroenterol Nutr 2004; 39: 166–70.
16. Berni Canani R, Rapacciuolo L, Romano MT, et al. Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice Dig Liv Dis 2004; 36: 767–70.
17. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. Gut 2006; 55: 1512–20.
18. Corcoran AC, Page IH. A method for the determination of mannitol in plasma and urine. Biol Che 1947; 170: 165–71.
19. Strobel S, Brydon WG, Ferguson A. Cellobiose/mannitol sugar permeability test complements biopsy histopathology in clinical investigation of the jejunum. Gut 1984; 25: 1241–6.
20. Troncone R, Mayer M, Mugione P, Cucciardi M, Abete A, Greco L. Cellobiose/mannitol sugar permeability test in
children in relation to jejunal morphometry. Ital J Gastroenterol 1995; 27: 489–93.

21. Chong SK, Blackshaw AJ, Boyle S, William C, Walker-Smith JA. Histological diagnosis of chronic inflammatory bowel disease in childhood. Gut 1985; 26: 55–9.
22. Castellaneta SP, Afzal NA, Greenberg M, et al. Diagnostic role of upper gastrointestinal endoscopy in pediatric inflammatory bowel disease. J Pediatr Gastro enterol Nutr 2004; 39: 257–61.
23. Snedecor GW, Cochran WG. Statistical methods. Iowa University Press, Ames, Iowa, USA, 2000.
24. Hyams J, Markowitz J, Lerer T, et al. The natural history of corticosteroid therapy for ulcerative colitis in children. Clin Gastroenterol Hepatol 2006; 4: 1124–9.
25. Cellier C, Sahmoud T, Froguel E, et al. Correlations between clinical activity, endoscopic severity and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre multicentre study of 121 cases. The Group d'Etudes Therapeutiques des Affections Inflammatoires Digestives. Gut 1994; 35: 231–5.
26. Monteleone G, Fina D, Caruso R, Pallone F. New mediators of immunity and inflammation in inflammatory bowel disease. Curr Opin Gastroenterol 2006; 22: 361–4.
27. Modigliani R, Mary JY, Simon JF, et al. Clinical biological and endoscopic picture of attacks of Crohn's disease; evolution on prednisolone. Gastroenterology 1990; 98: 811–8.
28. Landi B, Nguyen Anh T, Cortot A, et al. Endoscopic monitoring of Crohn's disease treatment: a prospective randomized clinical trial. The Group d'Etudes Therapeutiques des Affections Inflammatoires Digestives. Gastroenterology 1992; 102: 1647–53.
29. Jorgensen LGM, Fredholm L, Petersen PH, Hey H, Munkholm P, Brandslund I. How accurate are clinical activity indices for scoring of disease activity in inflammatory bowel disease (IBD)?. Clin Chem Lab Med 2005; 43: 403–11.
30. Costa F, Mumolo MG, Ceccarelli L, et al. Calprotectin is as stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. Gut 2005; 54: 364–8.
31. Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I. Intestinal permeability in Crohn's disease and its relation to disease activity and relapse following treatment with elemental diet. Eur J Gastroenterol Hepatol 1993; 5: 79–84.
32. Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal permeability and the predictor of relapse in Crohn's disease. Lancet 1993; 341: 1437–9.
33. Gross V, Andus T, Caesar I, Roth M, Scholmerich J. Evidence for continuous stimulation of interleukin 6 production in Crohn's disease. Gastroenterology 1992; 102: 514–9.
34. Amit-Romach E, Reifen R, Uni Z. Mucosal function in rat jejunum and ileum is altered by induction of colitis. Int J Mol Med 2006; 18: 721–7.

TABLES

Table 1. Lichtiger colitis activity index scoring

Symptom	Score
Diarrhoea (no. daily stools)	
0–2	0
3 or 4	1
5 or 6	2
7–9	3
10	4
Nocturnal diarrhoea	
No	0
Yes	1
Visible blood (% of movements)	
0	0
Less than 50	1
Greater than 50	2
100	3
Faecal incontinence	
No	0
Yes	1
Abdominal pain or cramping	
None	0
Mild	1
Moderate	2
Severe	3
General well-being	
Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5
Abdominal tenderness	
None	0
Mild and localized	1
Mild to moderate and diffuse	2
Severe or rebound	3
Need for antidiarrhoea drugs	
No	0
Yes	1

Table 2. Baseline characteristics in 33 children with newly diagnosed UC

Characteristic	Value
Sex	
Male	13 (39.4%)
Female	20 (60.6%)
Mean age (year)	
Male	8.4 (range, 1–14)
Female	7.8 (range, 3–11)
Mean duration of symptoms at onset (months)	6.2 (range: 1–17)
Disease location	
Proctosigmoiditis	7 (21.2%)
Left-sided colitis	13 (39.4%)
Pancolitis	13 (39.4%)
Mean duration of steroid exposure (days)	75.7 (range: 44–140)

UC, ulcerative colitis.

Table 3. Serum parameters and faecal calprotectin in early relapsers vs. non-relapsers with UC

	Early relapsers (s.d.)	Non-relapsers (s.d.)	
	Baseline	Baseline	P^*
Mean laboratory markers			
Hematocrit (%)	32.3 (5.1)	34.3 (3.8)	0.122
Albumin (g/dL)	3.9 (0.2)	4.1 (0.4)	0.273
ESR (mm/h)	28.6 (23.2)	22.5 (16.7)	0.181
CRP (mg/dL)	1.4 (0.2)	1.6 (0.4)	0.176
Faecal calprotectin (μg/g)	272 (95.5)	260 (67)	0.719

UC, ulcerative colitis; s.d., standard deviation; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

FIGURES

Figure 1. Relationship between cellobiose/mannitol ratio at diagnosis in patients with ulcerative colitis early and non-relapsers (lines represent the mean value). Higher cellobiose/mannitol ratio is related to earlier relapse ($P < 0.013$).

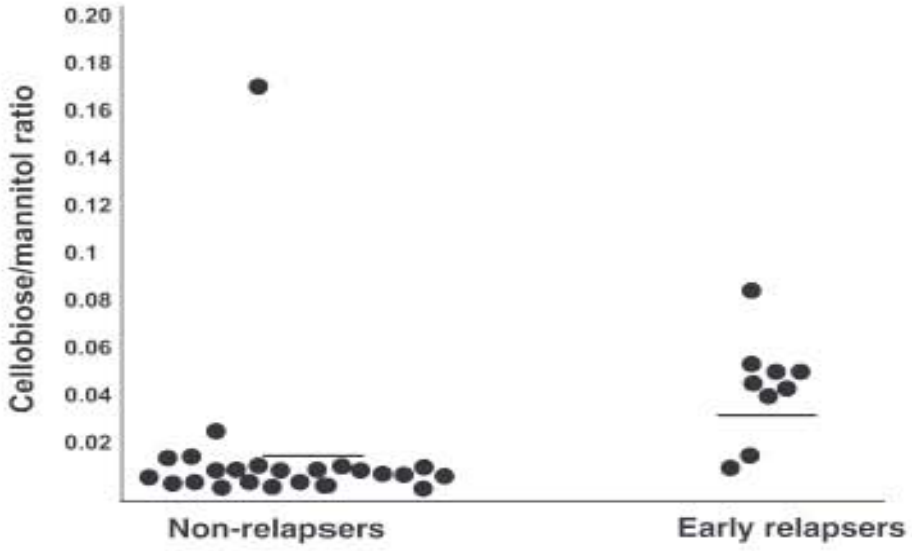
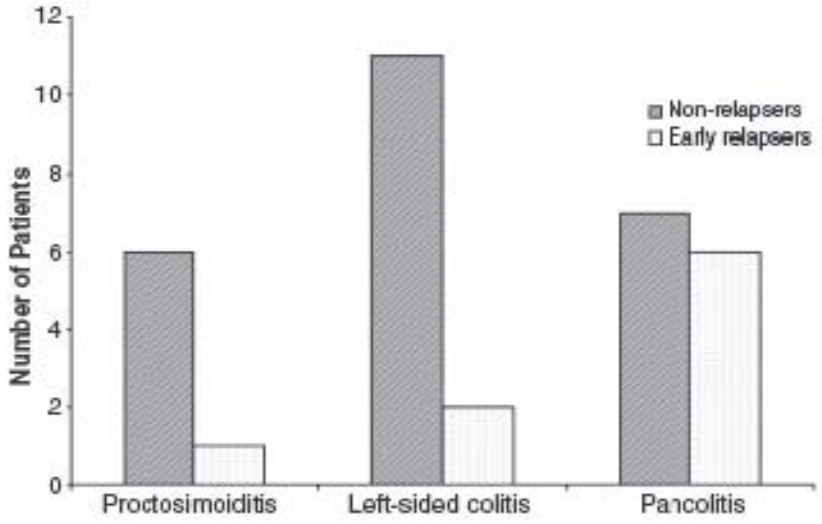


Figure 2. Relationship between colonic involvement and time to relapse (P = 0.145).



3.2 Immunohistochemical markers of small bowel inflammation in children with ulcerative colitis

ABSTRACTS

Aim: We recently demonstrated that abnormal cellobiose/mannitol small intestinal permeability study is a predictive marker of early relapse in children with ulcerative colitis (UC). The purpose of this study was to evaluate in the jejunum of children with UC, by immunohistochemistry, subtle inflammatory changes, even in presence of normal histopathology.

Methods: From February 2008 to February 2009, 24 pediatric patients (M: F = 11:13, mean age: 124 months, age range: 30-191 months) with new diagnosis of UC, based on accepted historical, endoscopic, histological and/or radiological criteria, were evaluated. The Pediatric Ulcerative Colitis Activity Index (PUCAI) was used to measure disease activity. All patients underwent upper gastrointestinal (GI) endoscopy and duodenal cryostat sections were stained for CD3+, γδ + T cells, CD25+ mononuclear cells, ICAM-1, HLA-DR. Twenty-nine children with functional dyspepsia, who underwent upper GI endoscopy, represented the control group.

Results: CD3 in the intraepithelial compartment revealed that UC patients presented a significative higher density of intraepithelial CD3 + cells compared to controls (mean ± SEM: 31.96 ± 2.184 vs 21.24 ± 1.608: $p < 0.001$).

In addition, as a group UC children presented a significant higher density of lamina propria CD25 + cells compared to controls (mean ± SEM: 9.75 ± 1.477 vs 3.138 ± 0.2148: $p < 0.001$) Among these children, 3/12 (18.7%) presented a PUCAI value ≥ 20 (moderate disease activity) and 13/12 (81.2%) patients showed a PUCAI value ≥ 35 (severe disease activity). Finally, the jejunal lamina propria density of CD25+ mononuclear cells was higher in 11/24 (45,8%) of early relapsers (within 6 months).

No significant differences were noted as regard ICAM-1 and HLA-DR between study population and controls.

Conclusion: The majority of UC children present inflammatory signs at level of the jejunal mucosa even in the absence of gross histopathological changes. The extent of upper GI tract involvement is wider than previously thought. Upper GI tract inflammation is related to the activity of the disease and is probably a predictive marker of early relapse.

INTRODUCTION

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission with episodes of clinical relapse, involving an increase in symptoms (1, 2).

It was previously thought that involvement of the small bowel in patients with UC was rare and limited to backwash ileitis or pouch-related conditions.

A number of recent studies have reported the presence of chronic active inflammation in the small bowel in patients with UC. A striking pattern that has been noted in a number of case reports is diffuse mucosal inflammation with crypt and villous architectural distortion, basal plasmocytosis and crypt abscesses involving duodenum and occasionally the jejunum and ileum. *Rubenstein et al* (3) in a review proposed the term "UC-associated enteropathy (UCAE)" to describe these category of patients. Fifthy percent of these UC patients reported in literature presented a diffuse inflammation of the small bowel within one month of total colectomy. More recently *Corporaal et al* (4) seems to confirm this view: the authors reported two cases of UC with small bowel inflammation and even perforation within one month after colectomy. The cause of inflammation in the upper GI tract of UC patients is not clear yet. It could be due to the patient's overall poor health or could be a response to yet-unknown immunological factors associated with the disease.

Recently, we demonstrated that abnormal intestinal permeability could be predictive of early relapse in paediatric patients affected by UC. Abnormal permeability to cellobiose at diagnosis was found in 77.8% of early relapsed patients, even though histopathological evaluation of small bowel biopsy was normal in two-thirds of our cases (5). These data suggest that there is a subtle alteration which can manifest as in increase in paracellular permeability and that small bowel involvement in UC is not simply a rare or colectomy-related event.

Limited efforts have been made to determine changes in the upper GI tract in UC. In this study we aimed to evaluate in the jejunum of children with newly diagnosed UC, by immunohistochemistry, subtle inflammatory changes, even in presence of normal histopathology. To our knowlwdge it is the first study in which immunohistochemical inflammatory signs at level of the jejunal mucosa of patients with UC are investigated as markers of early relapse and correlated to severity and extention of disease.

MATERIALS AND METHODS

The study was a prospective, single-centre, 1-year study of children with newly diagnosed UC consecutively enrolled at the Department of Pediatrics of the University of Naples ''Federico II'', Italy. Patients were recruited to participate in this study if they had a new diagnosis of UC established based on accepted historical, endoscopic, histological and/or radiological criteria.(6)

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 examination for 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 Pediatric Ulcerative Colitis Activity Index (PUCAI) was used to measure disease activity (7). Individual scores for each section of the test including symptoms, characteristics of stool and physical examination were computed. The extension of disease involvement was evaluated using the Montral Classification (8).

Laboratory studies including complete blood count, albumin, ESR and CRP were performed at first visit. Celiac and infectious diseases at diagnosis were ruled out.

At baseline, all patients were asked to provide a single stool sample for the determination of faecal calprotectin levels according to the methods (9). In addition, some children underwent cellobiose/mannithol permeability test study (CMPS). Cellobiose and mannitol are oligosaccharides and small intestinal permeability probes. They are degraded by the bacterial flora of the large intestine and yield no information regarding colonic permeability characteristics (10). The CMPS was performed after an overnight fast, prior to commencement of any treatment and in close temporal proximity to the endoscopic procedures. Each subject drank a solution containing 2 g mannitol, 5 g cellobiose and water to make 100 mL (osmolarity 270 mmol/L). Urine was collected

for the next 5 h and stored at -20 °C. The subjects went without food during the test but were allowed to drink water after the first hour. Mannitol in urine was measured by the method of Corcoran and Page whereby it is oxidized to formaldehyde by periodic acid. (11) Urine cellobiose was measured using the method described by Strobel et al. in which the compound is digested by D-glucosidase to glucose (12). The final ratio of percentage recovery of cellobiose to percentage recovery of mannitol was calculated. A cellobiose:mannitol ratio >0.022 was considered abnormal, as the value exceeded 2 s.d. over the mean derived from age- and sex-matched normal children (13).

At diagnosis, all children underwent ileocolonoscopy and upper GI endoscopy with mucosal biopsy. Biopsies were taken from the distal oesophagus, the gastric body and antrum, the duodenal bulb and / or third / fourth part of the duodenum. All children had a barium meal and small bowel follow-through.

All histological 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 histological criteria of Chong et al. (14) Helicobacter pylori was systematically sought in gastric antral biopsies using Giemsa staining and CLO colorimetric urease test. The Montreal classification, used to define the extention of UC, is reported in Table 1 (15).

Crohn's disease was excluded on the basis of the absence of: classical macroscopical lesions (e.g. skip lesions, snail track ulcers); non-caseating epithelioid and giant cell granulomas in any part of the gastrointestinal tract; histological findings of focal inflammation, submucosal or transmural inflammation, lymphocyte aggregates (without germinal centres) and mucous retention in the presence of more than minimal inflammation; fistulae and / or perianal abscesses; typical structuring small bowel disease on barium follow-through (16). Twethy-nine age- and sex-matched children with dyspeptic symptoms who underwent upper GI endoscopy represented the control group.

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 for normally distributed variables and the Mann–Whitney U-test and the chi-squared and Fisher 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 of age.

Morphometric and Immunohistochemical Analysis

Fifty children (26 affected by UC and 24 controls) of mean age 124 months (range 30-191) were used to obtain the jejunal biopsy.by upper gastrointestinal endoscopies.One fragment was fixed in 10% formalin,embedded in paraffin wax,sectioned at a thickness of 5 μm and stained with hematoxylin-eosin.The morfometric analysis on 10 well oriented villi,arranged like finger,and 10 corrispondent crypts,arranged perpendicular to the muscolaris mucosae.Villous to crypt height ratio (Vh/Cd) and intraepithelial lymphocytes (IEL) count were evaluated. The overall architecture of the smoll-intestinal mucosa was also evaluated according to the Marsh-Oberhuber classification*.The other fragment was immediately embedded in OCT coumpound(Bio-Optica,Italy) for the immunohistochemical study.Biopsy section of 4-μm were fixed in acetone for 10 min. after incubation with normal rabbit serum (1:200, Dako, Copenhagen, Denmark) for 20 min, section were covered with anti-CD3 (1:200; Dako), anti-CD25 (1:20; Dako), or anti-CD54 (ICAM 1) (1:200; Dako),anti-HLA DR (1:200,Dako),monoclonal antibodies for 1 h, and then with rabbit anti-mouse immunoglobulins for 30 min. monoclonal antibodies were diluited in Tris pH 7.4. all incubations were conducted at room temperature in a humid chamber. As a negative control, mouse

IgG1 (Dako) replaced the primary antibody. After washing with Tris pH 7.4, the sections were covered with monoclonal immunocomplex mouse peroxidase-antiperoxidase (PAP; Dako) or monoclonal mouse alkaline phosphatase anti-alkaline phosphatase (APAAP; Dako) for 30 min then H202 with 2-amino-9-ethyl-carbazole (AEC) (Sigma Aldrich. Milan, Italy)and Naphthol AS BI Phosphate with new fuchsin were used as peroxidase and phosphatase alkaline substrate respectively. Finally, sections were counterstained with Mayer's hematoxylin and mounted with Aquamount (BDH, Poole, England).

Morphometric Analysis

The density of cells expressing CD3 and TCRγδ+ in the intraepithelial compartment was determined by counting the number of stained cells per millimeter of epithelium. We counted the number of CD25 mononuclear cells within 1 mm2 of lamina propria using a microscope with a calibrated lens aligned parallel to the muscolaris mucosae. Cut-off values for CD3+ and TCR gd+ IELs are 34 and 3.4 cells/mm of epithelium,respectively; cut-off value for CD25+ cells is 4 cells/mm2 of lamina propria.These value represent the 90th centile of the non celiac patients. Staining of crypt epithelial cells by anti-HLA DR and expression of lamina propria ICAM-1 were evaluated in terms of staining intensity and graded on an arbitrary scale of staining absent (0),mild (1), marked (2). Cell counting was performed in a blinded manner by two independent observers.

RESULTS

Twenty-six patients with newly diagnosed UC were enrolled in the study. Two patients were excluded because one resulted affected by Helicobacter pylori infection and one by Celiac Disease. Demographic data for the study group are listed in Table 2. In all patients, the diagnosis of UC was confirmed by a median follow-up of 8 months (6-12 months). A t diagnosis, all patients received oral methylprednisolone (1 mg/kg/day, max 40 mg/day per 4 weeks). After 4 weeks, all patients were in remission and began tapering off CS on a weekly basis (25%/week) according to the prescribed schedule on the basis of the PUCAI. All patients received mesalamine maintenance therapy. Oral mesalamine 50 mg/kg was used in 17 patients (70.8%), one 2 g mesalamine enema every one to three nights was used in three patients (12.5%) and oral and topical mesalamine was used in 4 patients (16.6%).

Overall, fourtheen patients (58.3%) relapsed within 6 months of diagnosis, 10 (41.6%) patients (69.7%) did not relapse during the follow-up. Gender and age were not associated with early relapse (p=0.1). Linear growth and weight at diagnosis were normal in all children. No significant differences in weight or height at diagnosis were observed in early relapsers and non-relapsers. Laboratory parameters at diagnosis including haematocrit, albumin, ESR, CRP and faecal calprotectin were not predictive of early relapse. No significant differences in formulation of mesalimine used among early relapsers and non-relapsers were found ($\chi 2$: 0.15; P = 0.96).

Eighteen patients (75%) underwent CMPS; the test was abnormal in five patients (20.8%). Full ileo-colonoscopy was completed in 24 children (100%). Lower endoscopic findings included mucosal granularity, friability, aphthoid ulcers, superficial and deep ulcers and inflammatory polyps. UGI endoscopy, performed in all patients did not show any jejunal abnormalities. The

observed mucosal abnormalities included oesophageal erythema and /or erosions (n = 3) and chronic gastritis (n = 2).

The profile of the inflammatory pattern of surface antigens, analyzed by immunohistochemistry, did not show a significant incrased number of cells expressing ICAM-1 and HLA-DR in jejuna mucosa of children newly diagnosed with UC. Instead, the immunohistochemical evaluation of jejunal density of cells expressing CD3 in the intraepithelial compartment revealed that UC patients presented a significative higher density of intraepithelial CD3 + cells compared to controls (mean ± SEM: 31.96 ± 2.184 vs 21.24 ± 1.608: $p < 0.001$), as reported in Figure 1. In addition the evaluation of jejunal lamina propria density of CD25+ mononuclear cells showed that, as a group, UC children presented a significant higher density of CD25 + mononuclear cells compared to controls (mean ± SEM: 9.75 ± 1.477 vs 3.138 ± 0.2148: $p < 0.001$), as reported in Figure 2. Figure 3 shows the immunohistochemical staining for CD25+ mononuclear cells in the jejunal lamina propria of a subject with Ulcerative Colitis.

PUCAI was significatively higher in patients with an increased density of lamina propria CD25+ cells in jejuna mucosa ($p<0.01$): 16/24 (67%) showed an exceedingly high number of CD 25+ mononuclear cells. Among these children, 3/12 (18.7%) presented a PUCAI value ≥ 20 (moderate disease activity) and 13/16 (81.2%) patients showed a PUCAI value ≥ 35 (severe disease activity).

A trend for patients with higher lamina propria density of CD 25+ mononuclear cells to present more extensive disease, evaluated by Montreal classification, was observed but this finding did not meet statistical significance (37.5% vs 17%).

CMPS has been performed in 17/24 (71%) patients at diagnosis. Among these patients, five children (29.4%), showing an abnormal CMPS result (using a test cut-off of 0.022), presented an increased number of CD25+ mononuclear cells.

Using a cut-off of 4 cells/mm2, the jejunal lamina propria density of CD25+ mononuclear cells was higher in 11/24 (45,8%) of early relapsers. Figure 4 shows the mean number ± SEM CD 25 + mononuclear cells at diagnosis (study entry) in patients who went on to relapse within 6 months, as well as in those who did not relapse (mean ±SEM: 12.09±2.387, N =11 vs 7.308±1.283, N=13; p=0.07).

DISCUSSION

In the present study we aimed to evaluate, by immunohistochemistry, the immune profile of inflammatory surface antigens in the jejunum of children with newly diagnosed UC and to correlate these markers to extension and disease activity and to the timing of relapse.

We found that the density of cells expressing CD3 in the intraepithelial compartment was significative higher at diagnosis in children with UC compared to controls. In addition the immunohistochemical staining for CD25+ cells in the jejunal lamina propria showed a significant higher density of CD25 + mononuclear cells.

These findings suggest the presence of subtle inflammatory changes in the jejunum of patients affected by UC, even if the endoscopical and histopathological evaluation did not show any abnormalities and none presented upper GI symptoms when investigated. *Honma J* et al. (15) reported the only one study, to our knowledge, designed to investigate the immunohistochemical characteristics of gastroduodenalmucosa in patients with UC. The authors reported a nodular, histologically mild duodenitis in patients with UC, involving CD8+ cell infiltration, the severity of which positively correlates with the extent of disease.

The inflammatory surface antigens changes in patients with UC, lead to recruitment and activation of several immune cell types, to an increased production of cytokines and other proinflammatory mediators at the mucosal site, to the amplification of the inflammatory reaction and are ultimately responsible for IBD tissue damage. In particular, several subset of helper T cells display enhanced responsiveness to IL-2, a heightened state of activation, and a higher survival capability, in IBD affected mucosal tissue (16-18).

Although, we reported that PUCAI was significatively higher in jejunal mucosa of patients with an increased density of lamina propria CD25+ cells ($p<0.01$), suggesting a correlation between the density of CD25 + cells and the disease activity. In addition a trend for patients with higher lamina

propria density of CD 25+ mononuclear cells to present more extensive disease, evaluated by Montreal classification (8), was observed.

Actually we don't know which kind of cells expressing the CD25 + surface antigen are involved and increased in jejunal mucosa of children evaluated. We could hypothesize, as in colonic lamina propria of adult IBD patients (19, 20), that those cells are FOXP3+CD4+CD25+Tr cells increased in jejunal mucosa of patients with active UC disease to suppress uncontrolled immune response to specific triggers, as reported in several experimental mice models of IBD. To clear up this aspects, bioptic specimens of jejunal mucosa need to be stained for FOXP3 and CD4 or, maybe the FOXP3 mRNA expression needs to be investigated in frozen section of jejunal mucosa of children with IBD.

A number of recent studies have reported the presence of chronic active inflammation in the duodenum or stomach or both in patients with UC (21, 22). These studies are more common in children, because upper GI endoscopy is a routine part of the initial workup for inflammatory bowel disease in the paediatric population. We recently demonstrated that abnormal CMPS is a predictive marker of early relapse in children with UC, even though histopathological evaluation of small bowel biopsy was normal in two-thirds of cases (5). The cause of inflammation in the upper GI tract of UC patients is not clear yet. It could be due to the patient's overall poor health or could be a response to yet-unknown immunological factors associated with the disease. In our population CMPS has been performed in 17/24 (71%) patients at diagnosis. Interestingly, among these patients, five children (29.4%), showing an abnormal CMPS result, presented an increased number of CD25+ mononuclear cells, without any histopatholgical abnormalities. This finding could be related, in part, to a patchy distribution of enteropathy in agreement with previous studies which demonstrated that abnormal sugar permeability can occur in the presence of unequivocally abnormal small bowel histology.

As in Crohn's disease, it is conceivable that small bowel involvement represent a previously unrecognized primary phenomenon in UC children with involvement of the proximal GI tract. Alternatively it is possible they represent a secondary response to unknown factors associated with the illness.

Regarding the timing of relapse, jejunal lamina propria density of CD25+ mononuclear cells was higher in 11/24 (45,8%) of early relapsers (within 6 months): upper GI tract inflammation is not only related to the activity of the disease but could be also considered a predictive marker of early relapse.

There is a commonly agreed view that the disorder in children has peculiarities both in term of underlying mechanisms and of clinical management. Moreover, the profile of the immune response andd the relative importance of different pathogenic mechanisms likely change durin the course of IBD disease., thus rendering the comprehension of the mechanisma contributing to the onset and to the perpetuation of the damage more difficult. In conclusion, limited efforts have been made so far to determine changes in the upper GI tract in pediatric UC. To our knowlwdge this is the first study in which immunohistochemical inflammatory signs at level of the jejunal mucosa of patients with UC are investigated as markers of early relapse and correlated to severity and extention of disease.

TABLES AND FIGURES

Items	*Points*
Abdominal pain	
No pain	0
Pain can be ignored	5
Pain cannot be ignored	10
Rectal bleeding	
None	0
Small amount only, in less than 50% of stools	10
Small amount with most stools	20
Large amount (_50% of the stool content)	3
Stool consistency of most stools	
Formed	0
Partially formed	5
Completely unformed	10
Number of stools per 24 hours	
0-2	0
3-5	5
6-8	10
>8	15
Nocturnal stools (any episode causing wakening)	
No	0
Yes	10
Activity level	
No limitation of activity	0
Occasional limitation of activity	5
Severe restricted activity	10
Sum of PUCAI (0-85)	

Table 1.. Pediatric Ulcerative Colitis Activity Index

Characteristics	***Value***
*Sex	
Male	11 (45.8)
Female	13 (54.2)
Mean age (months)	
Male	105 (range, 30-189)
Female	143 (range, 98-191)
*Montreal Classification	
Proctosigmoiditis (E1)	6 (35)
Left-sided colitis (E2)	5 (21)
Pancolitis (E3)	13 (54)
*PUCAI	
Inactive disease	1 (4)
Moderate disease activity (≥ 20)	9 (38)
Severe disease activity (≥ 35)	14 (58)

Table 2.. Baseline characteristics in 24 children with newly diagnosed UC

* The values are indicated as number of patients and percentage (%)

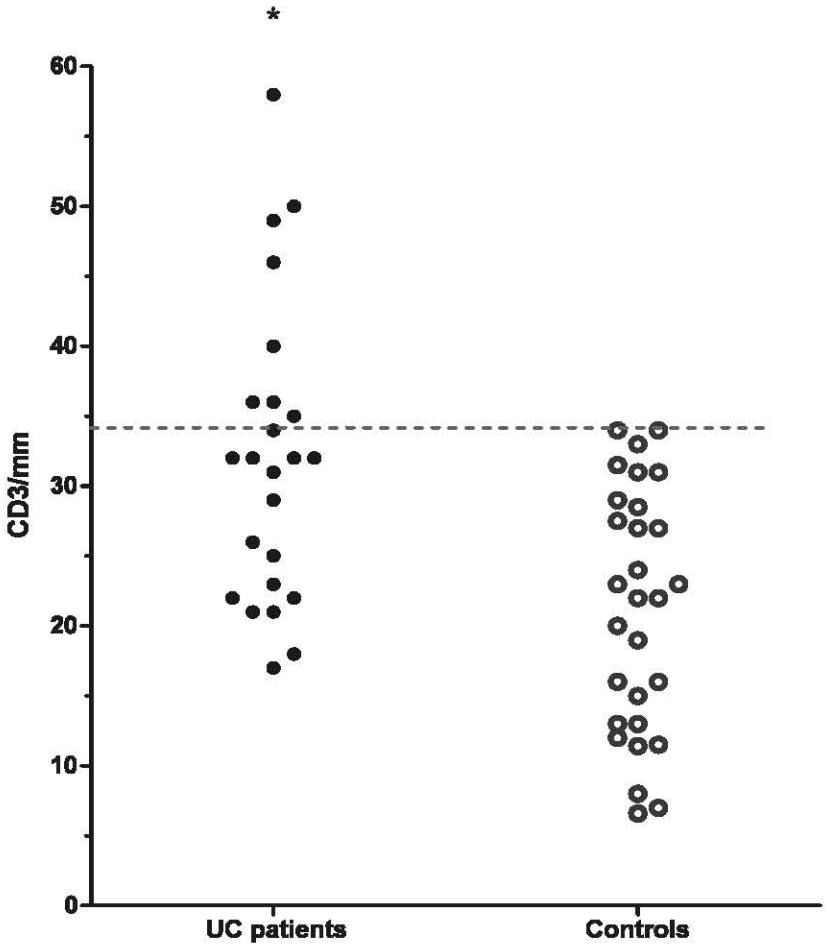


Figure 1. Density of cells expressing CD3+ cells in the intraepithelial compartment of jejunal mucosa in 24 patients affected by Ulcerative Colitis and in controls ($p<0.001$).

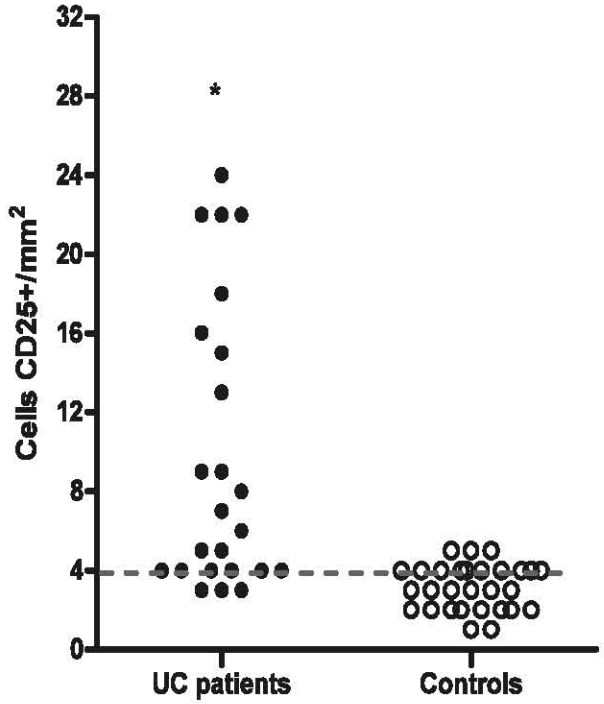


Figure 2. Jejunal lamina propria density of CD25+ mononuclear cells in 24 patients affected by Ulcerative Colitis patients and controls ($p < 0.001$).

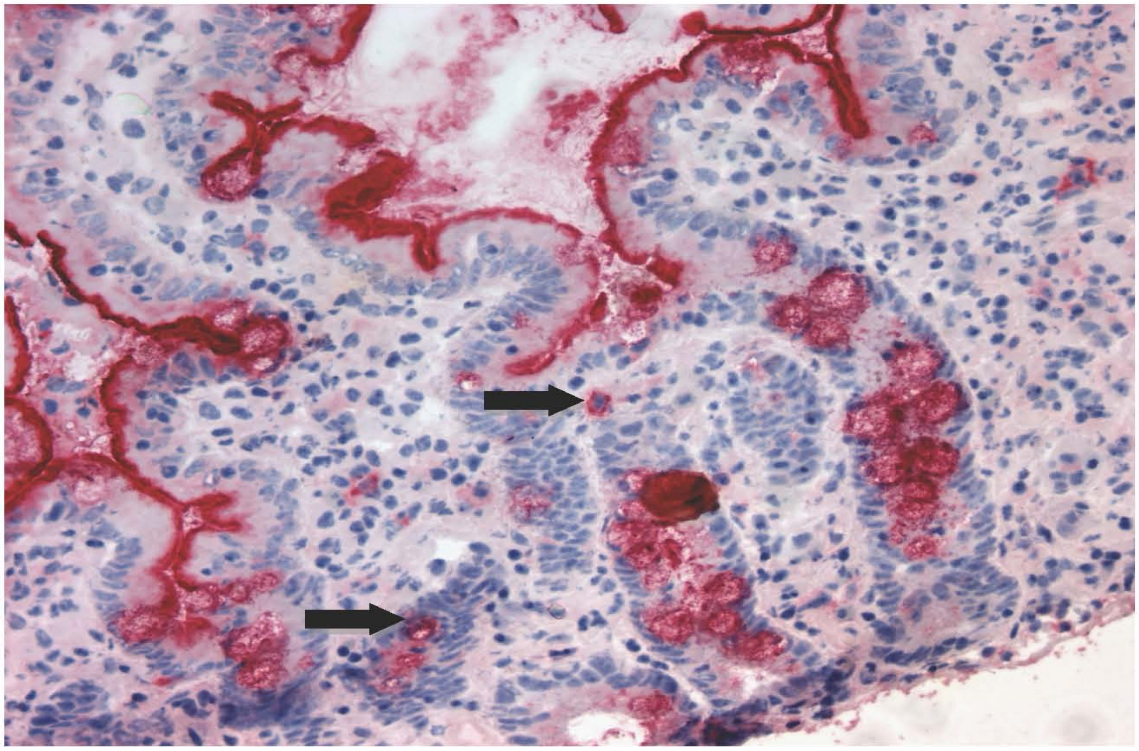


Figure 3. Immunohistochemistry for CD25 positive mononuclear cells in the jejunal lamina propria of a subject with Ulcerative Colitis.

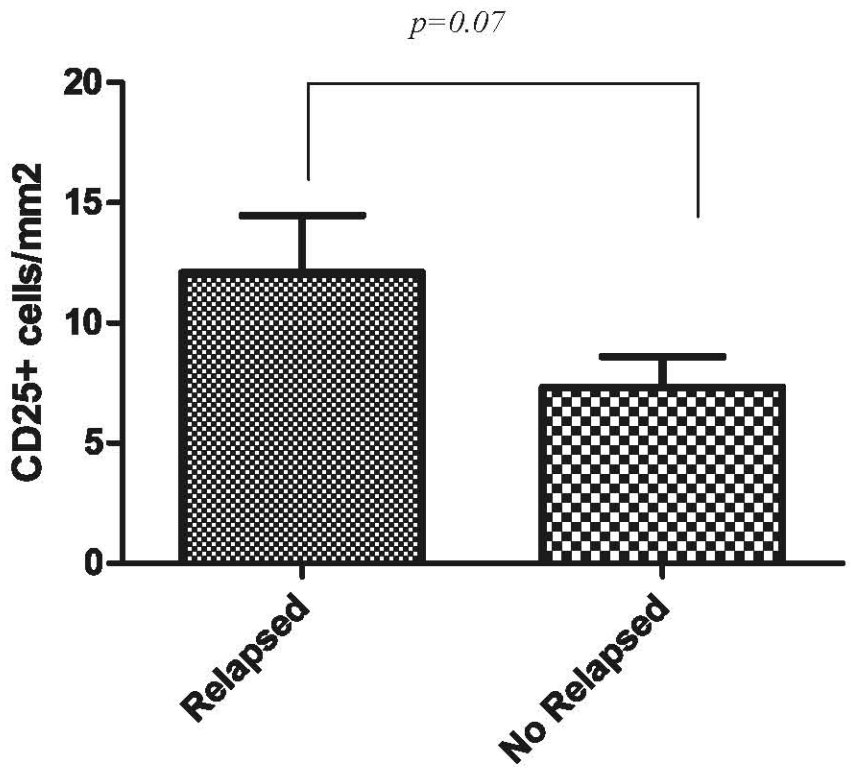


Figure 4. CD25 positive mononuclear cells in 11/24 patients with Ulcerative Colitis who went on to relapse within 6 months and in 13/24 patients who did not relapse.

REFERENCES

1. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. Gastroenterol Clin North Am 1999; 28: 445–58.
2. Tibble JA, Sigthorsson G, Bridger B, Fagerhol MK, Bjarnason I.Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroenterology 2000; 119: 15–22.
3. Rubenstein J, Sherif A, Appelman H, Chey WD Ulcerative colitis associated enteritis: is ulcerative colitis always confined to the colon? J Clin Gastroenterol. 2004 Jan;38(1):46-51.
4. Corporaal S, Karrenbeld A, van der Linde K, Voskuil JH, Kleibeuker JH, Dijkstra G. Diffuse enteritis after colectomy for ulcerative colitis: two case reports and review of the literature. Eur J Gastroenterol Hepatol. 2009 Jun;21(6):710-5.
5. Miele E, Pascarella F, Quaglietta L, Giannetti E, Greco L, Troncone R, Staiano A. Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis. Aliment Pharmacol Ther. 2007 Apr 15;25(8):933-9.
6. Hildebrand HF, Holmquist B, Kristiansson L, et al. Chronic inflammatory bowel disease in children and adolescents in Sweden. J Pediatr Gastroenterol Nutr 1991; 13: 293–7.
7. D. Turner, A. R. Otley, D. Mack, J Development, Validation, and Evaluation of a Pediatric Ulcerative Colitis Activity Index: A Prospective Multicenter Study Gastroenterology 2007;133:423–432
8. Satsangi J, Silverberg MS, Vermiere S et al. The Montreal Classification of Inflammatory bowel disease: controversies, consensus and implications. Gut 2006; 55: 749-753
Berni Canani R, Rapacciuolo L, Romano MT, et al. Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice Dig Liv Dis 2004; 36: 767–70.
9. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. Gut 2006; 55: 1512–20
10. Corcoran AC, Page IH. A method for the determination of mannitol in plasma and urine. Biol Che 1947; 170: 165–71.
11. Strobel S, Brydon WG, Ferguson A. Cellobiose / mannitol sugar permeability test complements biopsy histopathology in clinical investigation of the jejunum. Gut 1984; 25: 1241–6.
12. Troncone R, Mayer M, Mugione P, Cucciardi M, Abete A, Greco L. Cellobiose / mannitol sugar permeability test in
children in relation to jejunal morphometry. Ital J Gastroenterol 1995; 27: 489–93.
13. Chong SK, Blackshaw AJ, Boyle S, William C, Walker-Smith JA. Histological diagnosis of chronic inflammatory bowel disease in childhood. Gut 1985; 26: 55–9.
15. J Satsangi, M S Silverberg, S Vermeire, J-F Colombel. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications Gut 2006;55:749–753
14. Castellaneta SP, Afzal NA, Greenberg M, et al. Diagnostic role of upper gastrointestinal endoscopy in pediatric inflammatory bowel disease. J Pediatr Gastro enterol Nutr 2004; 39: 257–61.
15. Honma J, Mitomi H, Murakami K et al. Nodular duodenitis involving CD8+ cell infiltration in patients with ulcerative colitis. Hepatogastroenterology 2001; 48(42):1606-10
16. Mcdonald TT, Monteleone G. immunity, inflammation and allergy in thee gut. Science 2005; 307: 1920-25
17. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat rev Immunol. 2008; 3: 521-33
18. Bouma G, Strober w. The immunological and gnetical basis of inflammatory bowel disease. Am J gastroenterol 2007; 102:2058-69

19. Makita S, Kanai T, Oshima S et al. CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. J immunol 2004; 173: 119-30.
20. Yu Qt, Saruta M, Avanesyan A et al. Expression and functional characterization of FOXP3+CD4+ regulatory T cells in Ulcerative colitis. Inflamm bowel Dis 2007; 13: 191-99
21. Kaufmann SS, Vanderhoof JA, Young R et al. Gastroenteric Inflammation in children with Ulcerative colitis. Am J gastroenterol 1997; 92:1209-12
22. tobin MJ, Sinha B, Ramanni P et al Upper gastrointestinal Mucosal Disease in Pediatric Crohn Disease and Ulcerative colitis: A blinded Controlled study. J Ped gastroentrol Nutr 2001; 32: 433-448.

CHAPTER 4:

CLINICAL AND THERAPEUTICAL ASPECTS OF INFLAMMATORY BOWEL DISEASE (IBD)

4.1 Effect of a probiotic (VSL#3) on Induction and Manteinance of Remission in Children with Ulcerative colitis.

Abstract

Background & Aims: Several probiotic compounds have shown promise in the therapy of ulcerative colitis (UC). However, a strong sustained benefit remains to be seen. Uncontrolled pilot studies suggest that a probiotic preparation (*VSL#3*) maintains remission in mild to moderate UC and reduces active inflammation in adult patients. Aims of our prospective, one-year, placebo-controlled, double-blind study were to assess the efficacy of *VSL#3* on induction and maintenance of remission and to evaluate the safety and tolerability of the probiotic preparation therapy in children with active UC. **Methods:** Twenty-nine consecutive patients (mean age: 9.8, year; range: 1.7-16.1, year; F/M: 13/16) with newly diagnosed UC were randomised to receive either *VSL#3* (weight based dose, range: 450-1800 billion bacteria/day) (n= 14) or an identical placebo (n= 15) associated to concomitant steroid induction and mesalamine maintenance treatment. Children were prospectively evaluated at four time points: within 1 month , 2 months , 6 months and 1 year after diagnosis or at the time of relapse. Lichtiger colitis activity index and a physician's global assessment were used to measure disease activity. At baseline, within 6 months and 12 months or at the time of relapse all patients were assessed endoscopically and histologically. **Results:** All 29 patients responded to the IBD induction therapy. Remission was achieved in 13 patients (92.8%) treated with *VSL#3* and IBD therapy and in 4 patients (36.4%) treated with placebo and IBD therapy ($p<0.001$). Overall, 3 of 14 (21.4%) patients treated with *VSL#3* and IBD therapy and 11 of 15 (73.3%) patients treated with placebo and IBD therapy relapsed within one year of follow-up ($p=0.014$; RR= 0.32; CI= 0.025-0.773; NNT=2). All 3 patients treated with *VSL#3* and 6 of 11

(54.5%) patients treated with placebo relapsed within 6 months of diagnosis. At 6 months, 12 months or at time of relapse, endoscopic and histological scores were significantly lower in the *VSL#3* group than in the placebo group ($p<0.05$). There were no biochemical or clinical adverse events related to *VSL#3*. **Conclusions:** This is the first pediatric, randomised, placebo-controlled trial that suggests the efficacy and safety of a highly concentrated mixture of probiotic bacterial strains (*VSL#3*) in active UC and demonstrates its role in maintenance of remission.

Introduction

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission with episodes of clinical relapse, involving an increase in symptoms (1, 2). The disease is characterized by diffuse mucosal inflammation limited to the colon. Current medical management consists of aminosalicylates, steroids and immunosuppressant therapies such as azathioprine, 6-mercaptopurine and more recently tumor necrosis factor-alpha antibody. A significant proportion of patients do not tolerate existing treatments due to their adverse effects. In addition, failure to induce remission with current treatments occurs in 20-30% of pediatric patients with a (certain, significant?) proportion of these patients eventually requiring colectomy (3, 4). Consequently, new alternatives for the treatment of UC are constantly being sought.

One of the latest additions to the vast therapeutic armamentarium is probiotics defined as live microbial feed supplements, which beneficially affect the host by improving intestinal microbial balance, blocking adhesion sites on the colonocytes thus enhancing gut barrier function and improving local immune response (5, 6). The indication of probiotics in IBD is grounded on a number of human and animal studies indicating that the enteric flora is centrally involved in the pathogenesis of Crohn's disease and UC (7). Compared with the healthy gut, an increased number of mucosa-associated bacteria, quantitative and qualitative differences, and instability of flora composition have been reported (7). It is still unclear how these findings relate to IBD pathogenesis.

Several probiotic compounds have shown promise in the therapy of UC. However, a strong sustained benefit remains to be seen (6).

The data from experimental studies indicate that a probiotic preparation (VSL#3) combining 8 different probiotic bacteria has immunomodulatory effects. Attenuation of severity of disease activity by means of improvement of histologic grading has been described in various animal

models. A decrease of neutrophil tissue influx and activity has been shown. A diminished pro-inflammatory IL-1β, TNF-α, IFN-γ, IL-12, and IL-8 cytokine production and an enhanced production of the anti-inflammatory cytokine IL-10 have been reported (8). Furthermore, it has been demonstrated that oral administration of the VSL#3 probiotic formula to rats increases MUC2 gene expression as well as mucin protein accumulation in the colonic lumen and up-regulates alkaline sphingoymielinase activity (9, 10)

In vivo, small controlled studies in adult patients suggest that VSL#3 can be effective in preventing pouchitis (11-13). Furthermore, uncontrolled pilot studies suggest that VSL#3 maintains remission in mild to moderate ulcerative colitis in 75% of patients and reduces active inflammation in 87% (14, 15). A recent open-label study suggests a 53% remission rate in ambulatory adult patients with active disease who received VSL#3 (15).

The purposes of this prospective, one-year, placebo-controlled, double-blind study were to assess the efficacy of *VSL#3* on induction and maintenance of remission and to evaluate the safety and tolerability of the probiotic preparation therapy in children with active UC.

Patients and Methods

The study was a prospective, single-center, placebo-controlled, double-blind, one-year study of children with newly diagnosed UC consecutively enrolled over a 24-month period at the Department of Pediatrics of the University of Naples "Federico II", Italy. Patients were recruited to participate in this study if they had a new diagnosis of UC, established on accepted historical, endoscopic, histologic, and/or radiologic criteria, which needed a steroid therapy to induce the remission of the disease (16).

Exclusion criteria were children who had received therapy inducing remission of UC; children who required outpatient antibiotic therapy and/or required surgery for complications related to UC; children with documented history of allergic reaction to *Lactobacillus* or other probiotic compound or with history of endocarditis, rheumatic valvular disease, congenital cardiac malformations, or cardiac surgery; and children who had received *Lactobacillus, Bifidobacterium, Enterococcus, Saccharomyces*, or any other probiotic bacterial supplement within the past 10 days.

Twenty-nine consecutive patients (mean age: 9.8, year; range: 1.7-16.1, year; F/M: 13/16) with newly diagnosed UC were randomised to receive either *VSL#3* (*VSL Pharmaceuticals, Inc, Towson, MD*) (weight based dose, range: 450-1800 billion bacteria/day, Table 1) or an identical placebo associated to concomitant steroid induction treatment (oral methylprednisolon: 1mg/kg/day, max 40 mg/day per 4 weeks) and oral mesalamine maintenance treatment (50 mg/kg/day).

Assignment to therapy or placebo was determined according to a computer-generated randomisation scheme (17). Randomisation was performed by one blind clinical trial investigator, who kept the codes until completion of the study. None of the staff or patients had access to the randomisation codes during the study. The medications were dispensed by the investigator at each visit; compliance was assessed by counting returned bags and questioning the patients. Excellent

compliance was defined as no violation of the protocol with respect to the intake of the study medication.

After four weeks, patients who were in remission began tapering off corticosteroids on a weekly basis (25%/week) according to the prescribed schedule on the basis of the clinical activity index. Upon induction of remission patients continued to receive concomitant therapy (VSL#3 Group: mesalazine and VSL#3; Placebo Group: mesalazine and placebo) for 1 year or until relapse.

VSL#3 was provided in packets, each of which contained 900 billion viable lyophilized bacteria of 4 strains of *Lactobacillus* (*L. paracasei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), 3 strains of *Bifidobacterium* (*B. longum*, *L. breve*, and *B. infantis*), and 1 strain of *Streptococcus salivarius* subsp. *thermophilus* (designated hereafter as *S. thermophilus*). Placebo was provided in identical bags containing 3 g of corn starch. VSL#3 and placebo were administered once daily. The taste and smell of the active drugs were not readily identifiable.

Participants were evaluated at four time points: within 1 month , 2 months , 6 months and 1 year after diagnosis or at the time of relapse. At each visit data were collected including patient questionnaires regarding disease activity (stool frequency, stool consistency, hematochezia, abdominal pain, extra-intestinal manifestations of disease, and overall patient functioning). Additional information collected at the first visit included demographic data, family history, and symptom onset. Physical examination was performed at each visit by a pediatrician and included an abdominal examination and examination for 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 pediatric gastroenterologists of each individual patient exclusively made all decisions regarding therapeutic interventions.

Lichtiger colitis activity index (LCAI), and a physician's global assessment (PGA) were used to measure disease activity (18) (Table 2). Individual scores for each section of the test including symptoms, characteristics of stool and physical examination were computed. A sustained drop in the LCAI to ≤2 after steroid therapy was considered remission. Response was defined by a decrease in LCAI ≥ 3 points, but final score ≥ 3. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in the LCAI to >3 points, such score requiring treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery (2, 19).

Laboratory studies including complete blood count, albumin, erythrocyte sedimentation rate (ESR), and C-reactive protein were performed at each visit and/or at the time of relapse. Celiac and infectious diseases at diagnosis were ruled out. All unfavourable, unexpected symptoms were recorded in the diary kept by patients during the study.

At baseline, within 6 months and 12 months or at the time of relapse, all patients underwent colonoscopy with mucosal biopsy. Colonoscopic grade of inflammation was determined by use of a simple 5-point score reported in table 2 (20). Colonoscopy report system required segmental descriptions (rectum, sigmoid, descending, splenic flexure, transverse, hepatic flexure, ascending colon, and caecum). From the information provided on the colonoscopy report, each segment of the colon was designated a score, and a mean score for that colonoscopy was derived (20). All histologic specimens were reviewed under code by a single pathologist experienced in analysing pediatric intestinal biopsies, blinded to the patients' clinical details, who scored biopsies according to the histologic criteria reported in Table 3 (20). When more than 1 biopsy sample had been taken from a segment of colon, the highest grade of histological inflammation within that segment was recorded and a mean histological score for all segments was calculated for that child (20).

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). The Student's t test for normally distributed variables and the Mann-Whitney U test and the χ^2 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.

Data available were incorporated into analysis irrespective of protocol compliance

Written, informed consent was obtained from 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".

Results

Thirty-three patients with newly diagnosed UC were screened. Four subjects were excluded, because the parents of three refused consent and one child had received probiotic bacterial supplement within the past 10 days. Twenty-nine children resulted eligible and participated to this study. Fourteen subjects were randomly assigned to receive VSL#3 and 15 to receive placebo associated to concomitant steroid induction and oral mesalamine maintenance treatment. Demographic data for the study groups are listed in Table 4. The study groups were well matched with respect to age, sex, extension of colitis and duration of symptoms at onset.

Linear growth and weight at diagnosis were normal in all children. No significant differences in weight or height at diagnosis and during one-year follow-up were observed between VSL#3 group and placebo group.

The mean LCAI performed at diagnosis by the pediatric gastroenterologist of each patients, did not show significant differences between VSL#3 and placebo patients (Mean: 10.9 (range: 10-14) vs 11.1 (range: 10-14), respectively; p=0.533). On the basis of the PGA, the severity of the disease was moderate in 42% of VSL#3 patients vs 33% of placebo patients and severe in 58% of *VSL#3* patients vs 67% placebo group (p=0.586).

At baseline, laboratory parameters at diagnosis including hematocrit, albumin, erythrocyte sedimentation rate (ESR) and C-reactive protein were not significantly different between the two groups and were not predictive of response and/or relapse.

All 29 patients responded to the IBD induction therapy. On the basis of the LCAI, remission was achieved in 13 patients (92.8%) treated with *VSL#3* and IBD conventional therapy and in 4 patients (36.4%) treated with placebo and IBD conventional therapy; response was observed in 1 patient (7.2%) of VSL#3 group vs 11 patients (63.6%) of placebo group ($p<0.001$)

(Figure 1). Mean duration of steroid exposure was not significantly different among VSL#3 group and placebo group (p=0.23).

Overall, 3 of 14 (21.4%) patients treated with *VSL#3* and IBD therapy and 11 of 15 (73.3%) patients treated with placebo relapsed within one year of follow-up (p=0.014; RR= 0.32; CI= 0.025-0.773; NNT=2). All 3 patients treated with *VSL#3* and 6 of 11 (54.5%) patients treated with placebo relapsed within 6 months of diagnosis. Life-table analysis of the relapses in the 2 groups is showed in Figure 2.

At 6 months, 12 months or at time of relapse, endoscopic and histological scores were significantly lower in the *VSL#3* group than in the placebo group ($p<0.05$). The mean colonoscopic and histological scores using the last-observation-carried-forward method are shown in Figure 3.

Compliance with the study probiotic preparation and placebo was excellent in 86% of the children in the VSL#3 group and in 76% of the children in the placebo group.

No side effects or significant changes from baseline values in any of the laboratory parameters examined, attributable to treatment with either VSL#3 or placebo, were registered.

Discussion

UC is a relapsing IBD of the colon of unknown aetiology with a prevalence of about 100 cases per 100.000 children (21). The pathogenesis of IBD involves an interaction between genetically determined host susceptibility, disregulated immune response and the enteric microbiota. Host susceptibility is sometimes favoured by polymorphism in intestinal antimicrobial defences (e. g. defensin deficiency) or in perception of microbial signals in the enterocytes, immune cells or Paneth cells (e. g. NOD2-CARD15 and TLR4 polymorphisms) (22). The deleterious role of some intestinal microorganisms has been established in murine models and is strongly suspected in humans (23). However, other microorganisms seem to be protective (24).

As the microbial environment has been shown to play a role in the development of IBD, targeting of the microbiota presents an option for therapeutic intervention. The use of antibiotics, probiotics, and prebiotics to treat ulcerative colitis, Crohn's disease, and pouchitis has been extensively reviewed (25). Manipulating the abnormal enteric microbiota to decrease the more pathogenic species and enhancing the concentration and metabolic activity of the beneficial species has tremendous potential for therapeutic benefit. However, until now, this rational, physiologic, and non-toxic approach has not yet achieved its potential (25). Studies have shown beneficial effects in children with acute gastroenteritis (26) and in various models of experimental colitis such as interleukin-10 deficient mice (27, 28) and acetic acid-induced colitis in rats (29). There is some evidence that *Escherichia coli* Nissle 1917 may be as effective as 5-ASA for maintenance treatment of UC (30). Uncontrolled studies (15, 31) and some randomised controlled trials (32-35) have suggested that probiotics may be of some benefit for the treatment of active UC.

In addition, the largest body evidence demonstrating efficacy of probiotics in IBD exists for pouchitis. In three double-blind studies, VSL#3 was shown to be significantly superior to placebo

in maintaining remission in patients with chronic pouchitis and preventing the onset of pouchitis (11-13).

To our knowledge, this study represents the first prospective, placebo-controlled, double-blind study assessing the efficacy of *VSL#3* on induction and maintenance of remission and evaluating the safety and tolerability of the probiotic preparation therapy in children with a new diagnosis of active UC.

This study demonstrated a significant efficacy of VSL#3 in the induction and maintenance of remission in pediatric active UC. Patients treated with VSL#3 and concomitant conventional therapy had a significantly higher rate of remission compared to placebo with a significantly lower incidence of relapse within one year of follow-up. There were no biochemical or clinical adverse events related to *VSL#3*.

In contrast to our study, recently, a meta-analysis concluded that there was no evidence that probiotics were superior to placebo or aminosalicylates for the induction of remission and that the use of probiotics as induction therapy for UC could not be recommended (6).

On the basis of the results of our study, one should consider that the efficacy of one probiotic may not be the same in all patients or in the same patient at different stages of disease. Responsiveness to treatment could be dependent on several variables, including the characteristics of the host (age, sex, lifestyle), the lesions (site, extent, type of gross lesions) and risk factors (familial history of IBD). On the other hand, there is no dose-response study available for probiotics. Thus, it is possible that the ineffectiveness of probiotics was due to an inappropriate dose.

In addition, there are many species of probiotic. One type (e.g. VSL#3) might be more effective than another because strain specific properties might influence the efficacy in different cases and situations. The optimal composition, dose, and length of probiotic treatment in various

pediatric IBD clinical settings need to be confirmed by further larger, well designed, placebo-controlled, prospective trials.

In our study, at 6 months, 12 months or at time of relapse, endoscopic and histological scores were significantly lower in the *VSL#3* group than in the placebo group.

Support for a favourable action of VSL#3 in gut inflammation also comes from animal models. Madsen et al. demonstrated that VSL#3 was able to decrease the severity of colitis in IL-10 $^{-/-}$ mice, evidenced by decreasing TNF-α and IFN-γ production, histological scores and barrier integrity (36). In the iodoacetamide model of colitis, pre-treatment with either LGG or VSL#3 significantly decreased the severity of colonic damage, as indicated by a decreased myeloperossidase (MPO) activity and nitric oxide synthase activity (37). The potential health benefits of VSL#3 were also highlighted in a study by Rachmilewitz et al., using the destran sulfate sodium (DSS) model of colitis. VSL#3 was shown to significantly decrease colonic disease activity score, MPO activity and histologic scores in chronic DSS-induced colitis (38). Interestingly, treatment with γ-irradiated probiotics was also shown to decrease these parameters indicating that the probiotic species may not need to be viable to exert its beneficial effects (39). It has been demonstrated that CpG DNA alone, derived from the perobitoci species was sufficient to ameliorate colitis, and that this was dependent on TLR9 signalling (39). In addition, recently, Caballero-Franco et al provided compelling evidence that VSL#3 can enhance colonic mucin gene expression and secretion in *vivo* and in *vitro* (9).

In conclusion, this study suggests the efficacy and safety of a highly concentrated mixture of probiotic bacterial strains (*VSL#3*) in pediatric active UC and demonstrates its role in maintenance of remission.

On the basis of our results, probiotics as natural, safe, and well-tolerated, adjunctive treatment to conventional therapy may provide a simple and attractive way to treat pediatric IBD.

Further well designed randomised controlled trials with higher patient numbers may be justified to confirm results of this first pediatric study.

References

1. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. *Gastroenterol Clin North Am* 1999; 28: 445-458.
2. Tibble JA, Sigthorsson G, Bridger B, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000; 119: 15-22.
3. Turner D, Walsh CM, Benchimol EI, et al. Severe paediatric ulcerative colitis: incidence, outcomes and optimal timing for second-line therapy. *Gut* 2008; 57:331-8.
4. Hyams J, Markowitz J, Lerer T,et al; Pediatric Inflammatory Bowel Disease Collaborative Research Group. The natural history of corticosteroid therapy for ulcerative colitis in children. *Clin Gastroenterol Hepatol* 2006; 4:1118-23.
5. Fuller R. A review: probiotics in man and animals. *J Appl Bacteriol* 1989; 66: 365-378.
6. Mallon P, McKay D, Kirk S, Gardiner K. Probiotics for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007;(4):CD005573.
7. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; 134: 577-94.
8. Gionchetti P, Lammers KM, Rizzello F, Campieri M. VSL#3: An Analysis of Basic and Clinical Contributions in Probiotic Therapeutics. *Gastroenterol Clin N Am* 2005: 34: 499–513
9. Caballero-Franco C, Keller K, De Simone C, Chadee K. The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol. 2007; 292: G315-22.
10. Soo I, Madsen KL, Tejpar Q, et al. VSL#3 probiotic upregulates intestinal mucosal alkaline sphingomyelinase and reduces inflammation. *Can J Gastroenterol* 2008; 22:237-42
11. Gionchetti P, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo controlled trial. *Gastroenterology* 2000;119:305-309.
12. Mimura T, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; 53:108-114.
13. Gionchetti P, et al. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 2003;124:1202-1209.
14. Venturi A, et al. Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol Ther* 1999;13:1103-108.
15. Bibiloni R, Fedorak RN, Tannok G, et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 2005;100:1539-1546.
16. Hildebrand HF, Holmquist B, Kristiansson L, et al. Chronic Inflammatory bowel disease in Children and adolescents in Sweden. *J Pediatr Gastroenetrol Nutr* 1991; 13: 293-297
17. Tiplady B. A basic program for constructing a dispensing list for a randomized clinical trial. *Br J Clin Pharmacol* 1981;11:617–618.

18. Lichtiger S, Present D, Kornbluth A, Irwin Gelerent, Bauer J, Galler Greg, Michelassi F, Hanauer S. Cyclosporine in severe Ulcerative colitis refractory to steroid therapy. *N Eng J Med* 1994; 330: 1841-1845.
19. Russell GH, katz AJ. Infliximab is effective in acute but no chronic childhood ulcerative colitis. J Pediatr Gastroenterol Nutr 2004; 39: 166-170.
20. Rutter M, Saunders B, Wilkinson K, et al. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; 126: 451-459.
21. Heyman MB, Kirschner BS, Gold BD, t al. Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry. *J Pediatr* 2005; 146: 35-40.
22. Wehkamp, J., Stange, E. F., A new look at Crohn's disease: Breakdown of the mucosal antibacterial defense. *Ann. NY Acad. Sci.* 2006; 1072: 321–331.
23. Sartor, R. B., Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: Antibiotics, probiotics, and prebiotics. *Gastroenterology* 2004; 126, 1620–1633.
24. Marteau, P., Shanahan, F., Basic aspects and pharmacology of probiotics: An overview of pharmacokinetics, mechanisms of action and side-effects. *Best Pract. Res. Clin. Gastroenterol* 2003; 17, 725–740.
25. Sartor RB. Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology* 2008;134:577–594.
26. Isolauri E, Juntunen M, Rautanen T, Sillanaukee P, Koivula T. A human Lactobacillus strain (Lactobacillus casei sp strain GG) pro-motes recovery from acute diarrhea in children. *Pediatrics* 1991;88: 90–7.
27. Schultz M, Veltkamp C, Dieleman LA, Grenther WB, Wyrick PB, Tonkonogy SL, et al. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-defcient mice. *Inflamm Bowel Dis* 2002;8:71–80.
28. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. *Gastroenterology* 1999;116(5):1107–14.
29. Fabia R, Ar'Rajab A, Johannsson ML,Willen R, Andersson R,Molin G, et al. The effect of exogenous administration of Lactobacillus reuteri R2LC and oat fiber on acetic acid-induced colitis in the rat. *Scand J Gastroenterol* 1993;28(2):155–62.
30. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, et al. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. *Gut* 2004;53:1617–23.
31. Guslandi M, Giollo P, Testoni PA. A pilot trial of Saccharomyces boulardii in ulcerative colitis. *Eur J Gastroenterol Hepatol* 2003;15:697–8.
32. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT.Non-pathogenic Eshcherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomized trial. *Lancet* 1999;354: 635–9.
33. Kato K, Mizuno S, Umesaki Y, Ishii Y, Sugitani M, Imaoka A, et al. Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. *Aliment Pharmacol Ther* 2004;20:1133–41.
34. Tursi A, Brandimarte G,Giorgetti GM, FortiG,ModeoME,Gigliobianco A. Low dose balsalazide plus a high potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acutemild-to-moderate ulcerative colitis.*Med Sci Monit* 2004;10:126–31.

35. Furrie E, Macfarlane S, KennedyA,Cummings JH,Walsh SV, O'Neil DA, et al. Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* 2005;54: 242–249.
36. Madsen K, Cornish A, Soper P, et al. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 2001; 121: 580-591.
37. Shibolet O, Karmeli F, Eliakim R. Variable response to probiotics in two models of experimental colitis in rats. *Inflam Bowel Dis* 2002; 8: 399-406.
38. Rachmilewitz D, Karmeli F, Takabayashi K, et al. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 2002; 122: 1428-1441.
39. Rachmilewitz D, Katakura K, Karmeli F, et al. Toll-Like Receptor 9 Signaling Mediates the Anti-in.ammatory Effects of Probiotics in Murine Experimental Colitis. *Gastroenterology* 2004;126:520–528

TABLES

Table 1. *VSL#3* Daily Weight Based Dose

Age (yr) **(weight, kg)**	**Daily Dose (packet)** **(bacteria/day)**
4-6 (17-22)	1/2 (450 billion)
7-10 (24-33)	1 (900 billion)
11-14 (34-53)	1+1/2 (1350 billion)
15-17 (54-66)	2 (1800 billion)

Table 2 Lichtiger Colitis Activity Index Scoring

Symptom	Score
Diarrhea (no. of daily stools)	
0-2	0
3 or 4	1
5 or 6	2
7-9	3
10	4
Nocturnal diarrhea	
No	0
Yes	1
Visible blood (% of movements)	
0	0
Less than 50	1
Greater than 50	2
100	3
Fecal incontinence	
No	0
Yes	1
Abdominal pain or cramping	
None	0
Mild	1
Moderate	2
Severe	3
General well-being	
Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5
Abdominal tenderness	
None	0
Mild and localized	1
Mild to moderate and diffuse	2
Severe or rebound	3
Need for antidiarrhea drugs	
No	0
Yes	1

Table 3. Endoscopic and Histological Scores

Colonoscopic Score	
Entirely normal appearance	0
Quiescent disease (mild edema or chronic features, but no active inflammation)	1
Mild active inflammation	2
Moderate active inflammation	3
Severe active inflammation	4
Histological score	
Normal (no inflammatory cells)	0
Chronic inflammation only	1
Mild active (cryptitis but no crypt abscesses)	2
Moderate active (few crypt abscesses)	3
Severe active inflammation (numerous crypt abscesses)	4

Table 3. Baseline Characteristics in 29 Children with Newly Diagnosed UC

Characteristic	VSL#3 Group	Placebo Group
Sex		
Male	8 (57%)	8 (53%)
Female	6 (43%)	7 (47%)
Mean age (yr)		
Male	8.4 (range, 1.7-15)	10.6 (range, 6.6 -16.1)
Female	11.4 (range, 7.8-15)	9 (range, 3-15.6)
Mean duration of symptoms at onset (months)	4.7 (range, 1-20)	3 (range, 1-12)
Disease location (%)		
Proctosigmoiditis	28.6	26.7
Left-sided colitis	35.7	20
Pancolitis	35.7	53.3
Mean duration of steroid exposure (days)	68.5 (range, 52-104)	78.3 (range, 51-114)

FIGURES

Figure 1: Clinical outcome following induction therapy treatment in VSL#3 group and placebo group ($p<0.001$).

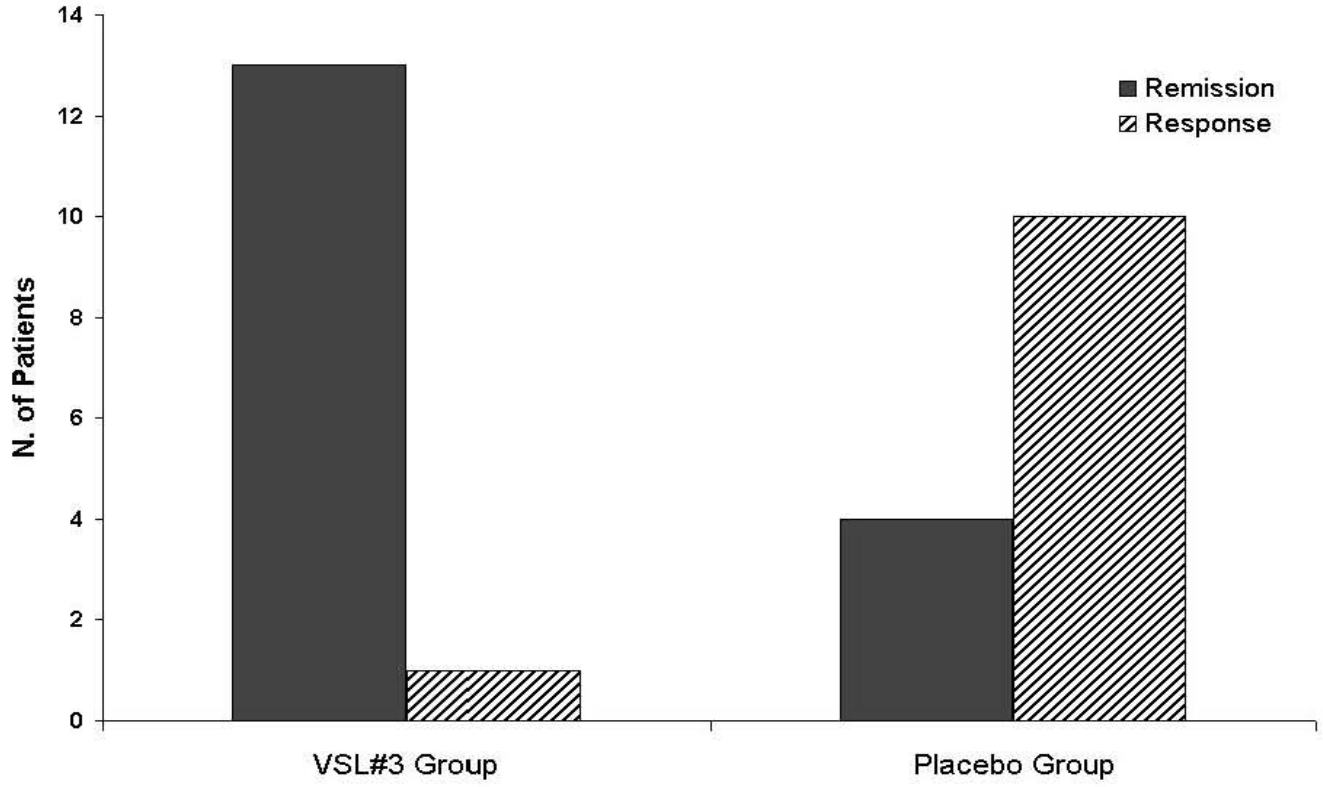


Figure 2: Kaplan–Meier estimates of relapse during treatment with VSL#3 or placebo (log rank test p=0.001).

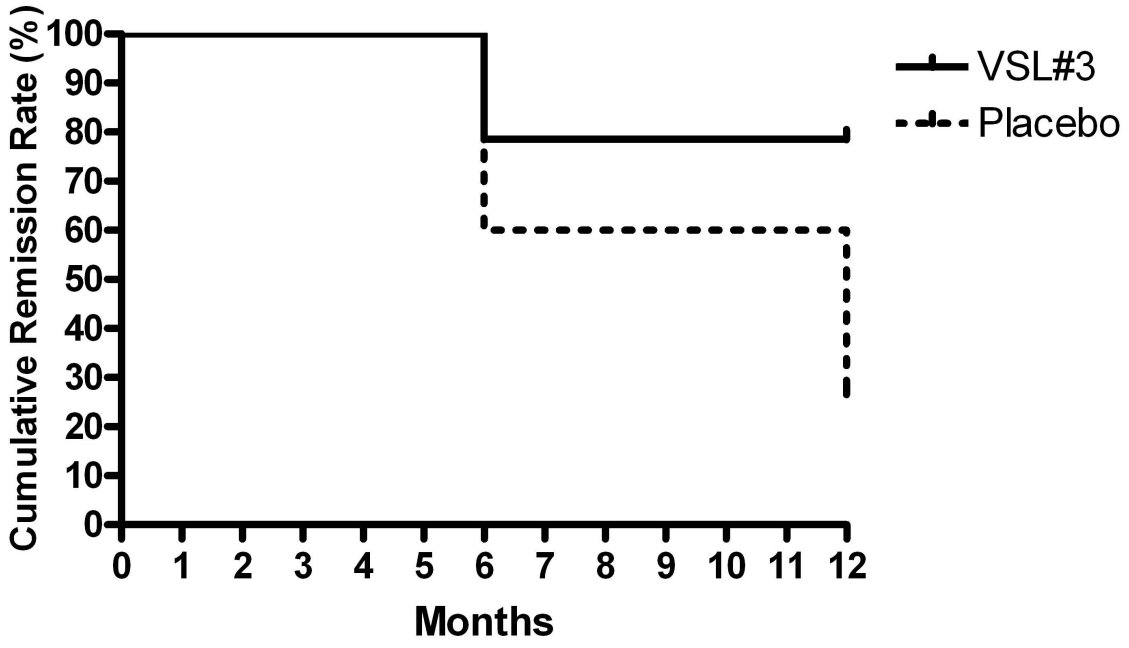
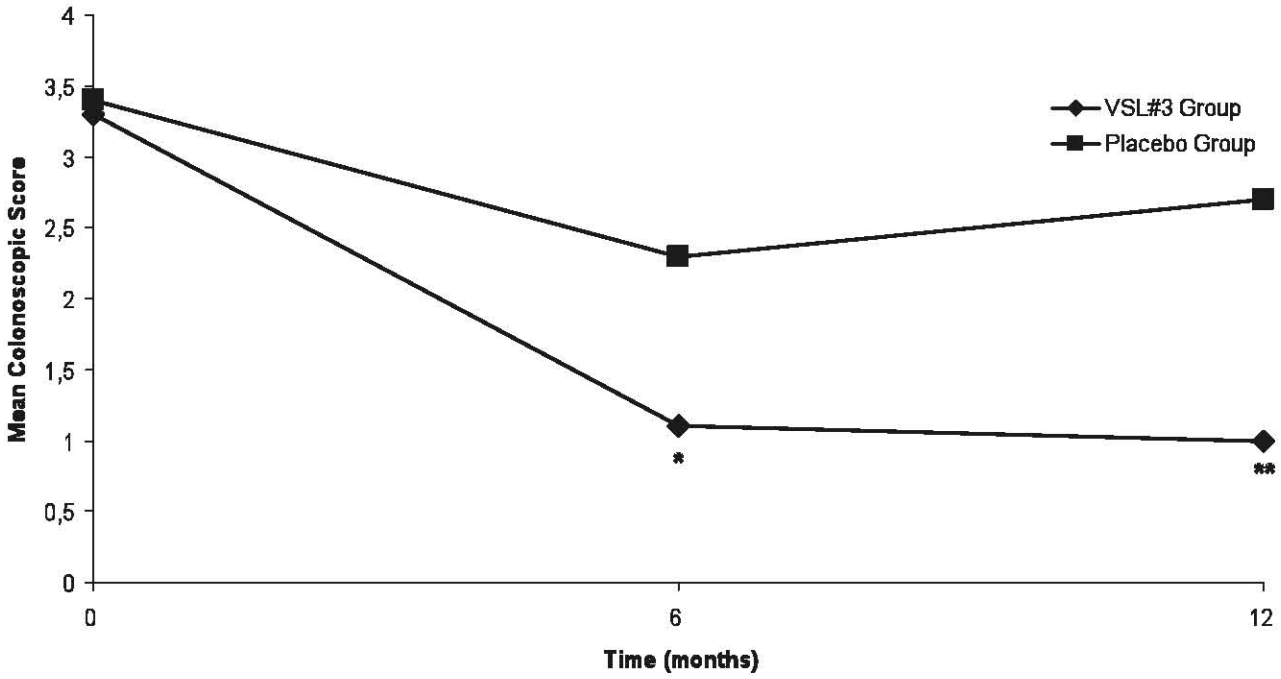


Figure 3: Mean total colonoscopic (A) and histological (B) score over one-year follow-up. The scores in the placebo group were significantly higher to that in the VSL#3 group (*p=0.05; **p=0.01)



CHAPTER 5

A NEWEST ESOPHAGEAL INFLAMMATORY DISEASE: EOSINOPHILIC ESOPHAGITIS

5.1 Indroduction

Over the past decade, esophageal eosinophilia has been increasingly recognized in children and adults who have symptoms similar to those observed in GerD.[1] However, in contrast to patients with GerD, symptoms and histopathology are unresponsive to high-dose acid suppression therapy or surgical fundoplication.[1] This constellation of findings is consistent with the diagnostic features of a newly-emerged disease termed eosinophilic esophagitis (EoE).[1] Evidence points to eoe as an inflammatory disease of the esophagus that is triggered by an antigen resultant dense mucosal eosinophilia and esophageal dysfunction. this review will focus on clinical and pathological features and allergic manifestations of eoe and will summarize effective treatments for the disease.

EoE is a disease characterized by a variety of upper gastrointestinal symptoms that occur in association with dense esophageal eosinophilia (Table 1); its symptoms and histopathology are unresponsive to pharmacological and surgical GerD treatments.[1] Alternative etiologies responsible for the eosinophilia must be ruled out before eoe can be diagnosed.[1,2] EoE has been reported in all age-groups and in all continents except africa.[3, 4] The prevalence of the

disease is not certain, but is estimated to be 1–4 cases per 10,000 individuals.[5,6] The natural history of the disease is as yet unknown;[7] two case reports suggest an association with Barrett esophagus, but this link has not been verified by other studies.[8-10]

Pathogenesis

Mechanisms of esophageal eosinophilic inflammation associated with EoE have been investigated in basic and translational studies. mishra *et al.* conducted a series of studies using

murine models of esophageal eosinophilia in which allergens or cytokines were used to initiate esophageal eosinophilia.[11-14] *Aspergillus fumigatus*, an ubiquitous airborne allergen, was used to sensitize mice, which developed epithelial eosinophilia that was isolated to the esophageal mucosa. Further studies using this model demonstrated that the eosinophilia and colla gen deposition were interleukin 5 (IL-5)-dependent.[15] translational studies have characterized the inflammatory profile of the mucosa revealing increased CD8+ lymphocytes, proinflammatory cytokines, the immunoglobulin e (IgE) receptor, mast cell infiltration, eosinophil degranula tion and, in some cases, basophilia.[16-26] In a landmark study, Blanchard *et al.* discovered an EoE transcriptome.[27] In this study, a genome-wide microarray analysis was performed on esophageal biopsies obtained from 13 children with eoe compared with five children with chronic esophagitis and six healthy controls. The most upregulated gene product was eotaxin-3 (also known as CCl26), a chemokine critical for eosinophil migration; a single-nucleotide polymorphism in this gene was associated with disease susceptibility. In a series of

murine studies, mice deficient in the chemokine receptor CCr3 were shown to be protected from EoE. Other studies have shown increased expression of the gene encoding eotaxin-3 (CCl26) in the mucosa of animals with eoe.[28-31] Together, these findings support a role for eotaxin-3 (CCl26) in the pathogenesis of eoe. the role of acid reflux in the pathogenesis of eoe is a matter of debate. While Sant'Anna *et al.* showed that pH monitoring of the distal esophagus revealed increased periods of alkalinization in children with eoe compared with that of healthy controls, Rosen *et al.* demonstrated that acid and nonacid reflux was not increased in children with EoE using pH-multichannel intraluminal impedance tracings.[32, 33] the coming years will also bring new enlightenment as to the relationships between GerD and EoE.[34] There is probably clinical crossover between these two diseases in some patients that will likely be explained as the esophageal microenvironment becomes better defined.[35-37]

Association with allergic disease

Several lines of evidence support a role for allergic inflammation in the pathogenesis of EoE. The most obvious evidence for such involvement is the central role of the eosinophil. This cell is often considered synonymous with allergic disease because of its accumulation in sputum in asthma, in nasal secretions in allergic rhinitis and in the skin during flares of acute eczema.[38] the majority of patients with EoE have comorbid allergic disease such as food allergy, eczema, allergic rhinitis, or asthma accompanied by IgE-mediated sensitization to food and/or inhalant allergens[1]. An age-related inverse gradient for the presence of coexisiting allergic disease has previously been described in EoE; its frequency is highest in pediatric cohorts, where estimates reach 80%, and lowest in adult cohorts where estimates range from 40% to 70%.[1,39] two studies have demonstrated a potential role for airborne allergens in EoE.[40, 41] Histories of allergic disease in family members are noted in the majority of patients with EoE as exemplified by a report in which 73.5% of 103 patients with EoE had a positive family history of allergic disease.[8] The presence of other atopic diseases in patients with eoe affects peripheral eosinophil counts and total IgE levels making it difficult to ascribe changes in these parameters specifically to EoE.

As could be predicted by the intimate interaction between foods and the esophageal mucosa, food allergy often accompanies eoe and is considered contributory in a substantial proportion of patients.[42] Pediatric studies report coexistent food allergy in up to 73% of patients, whereas adult studies document food allergy in a more widely ranging proportion (25–82%) of patients.[40, 42] Foods most often implicated in EoE-associated allergies include milk, egg, wheat, soy, peanuts, beans, rye and beef, although a variety of other foods may have a role in individual patients.[1] Allergic sensitization to more than one food is common in patients with EoE. when large panels of food allergy skin tests were applied to a cohort of 786 patients with EoE from aggregated case series data, the mean number of food allergies identified per patient varied from 2.7 ± 3.3 to 6 ±

4.2.1 In addition to sensitivity to food allergens, sensitivity to seasonal pollen allergens is common in patients with EoE.[1] Isolated case reports have documented a correlation between pollen allergy skin test results and seasonal changes in eoe symptoms and esophageal eosinophil levels.[43, 44] Allergic reactions to foods are categorized as igemediated, non-IgE-mediated or combined reactions.

According to the immune mechanism involved. in igemediated food allergy, exposure of a genetically predisposed individual to an appropriate food results in the generation of allergen-specific IgE that occupies high affinity receptors for IgE on the surface of mast cells and basophils resulting in allergic sensitization.[45] Re-exposure of the individual to this food results in binding of adjacent cell surface allergen-specific IgE molecules, bridging of their high-affinity receptors, The explosive release of histamine, and the generation of newly formed mediators, some of which are chemotactic for eosinophils.[45] An important translational study demonstrated that eoe represents a tH2-type allergic response. In this report, the investigators demonstrated the presence of IgE-bearing mast cells within the epithelial cell layer of patients with eoe and identified potential allergens inducing the inflammatory response[46] An ige-mediated allergic reaction occurs within minutes to hours of allergen exposure.[45] By contrast, non-IgE mediated allergic reactions typically orchestrated by TH2 lymphocytes are slower in onset, evolving over hours and days after food allergen exposure, resulting in eosinophil accumulation and symptoms localized to the gastrointestinal tract. This delay in symptom onset following food allergen exposure complicates accurate identification of offending foods in non-IgE mediated food allergy. EoE symptoms are often consistent with those seen in non-IgE mediated allergic reactions in that they are localized to the gastrointestinal tract and often delayed rather than immediate in onset.

EoE is considered to be a combined disorder in which both IgE-mediated and non-IgE-mediated immune mechanisms are involved. as a result, methods used to diagnose ige-mediated food allergy,

such as skin-prick testing and the measurement of serum food allergenspecific ige levels are useful in identifying potential culprit foods, but will not detect those causing symptoms through a non-IgE mediated mechanism. atopy patch testing (APT), where food allergen extracts are placed in constant contact with the skin under 12 mm aluminium chambers for 48 h then removed and the sites of exposure examined for a reaction at 72 h, has been proposed as a method for potentially identifying foods causing symptoms of EoE through a non-IgE mediated mechanism. Spergel and colleagues have reported substantial success as evidenced by biopsy-documented improvement in patients placed on elimination diets on the basis of information obtained through a combina tion of allergy skin-prick testing and APT. [39, 47] The most common foods identified through patch testing by spergel *et al.* in their cohort were milk, wheat, corn, beef, egg, potato, chicken, soy, barley, oat and rice. [48] In this cohort the combined negative predictive value of allergy skin-prick testing and APT for milk was considered unacceptably low, leading the authors to recommend consideration of milk elimina tion even when testing to milk was negative. [48] Despite the promising findings reported by Spergel and colleagues, some features have hindered wider use of these tests, including a lack of standardization of the procedure, and the time and expertise required to accurately perform such tests.[49] improvement of EoE following the use of food elimination diets emphasizes the significant causal role that food allergy has in most patients. Symptom resolution and normalization of esophageal biopsies has been achieved in more than 95% of children with eoe on elemental diets.[50] Difficulty in maintaining compliance to an elemental diet in adult patients, and the effect of such a diet on quality of life owing to the poor palatability and expense of these formulas has fostered the exploration of alternative dietary approaches. elimination of the six foods most commonly involved in eoe (dairy products, egg, wheat, soy, peanuts, fish/ shellfish) without the performance of allergy testing led to disease remission in approximately 74% of patients.[51] Food elimination diets based on the combined results of allergy skin-prick testing and APT are known to be effective for inducing

remission in the majority of patients with EoE.[39, 47, 48] The marked improvement in symptoms noted upon removal of culprit foods from the diet underscores the effect that food-allergen exposure has in EoE. A thorough history and assessment of allergic diseases is a vital part of the evaluation of a patient with suspected EoE because of the increased frequency of comorbid allergic disease, and the fact that allergens probably have a role in pathogenesis of this disease. Consultation with an allergist is indicated to aid in identifying, characterizing and treating comorbid allergic disease, in addition to identifying those allergens that are potential contributors to esophageal inflammation.[52] The allergist should aid in the development and monitoring of response to food elimination diets, as well as participating in the patient's long-term management.

Clinical presentation

Significant variation exists in the clinical presentation of children and adults with EoE. Whether this variation is due to differences in the ability of these groups to enunciate discomfort or represents different disease stages is not yet certain. regardless of age, if a patient develops GERD-like symptoms and is not responding to treatment, whether that be pharmacological or surgical, strong consideration should be given to a diagnosis of eoe.[1,53]

Children with EoE present with a broad range of non-specific symptoms that affect the upper gastrointestinal tract and respiratory tree.[54–60] In the largest pediatric study to date of 381 children, Liacouras *et al.* reported that the most common symptoms of EoE were divided into GERD-like symptoms and dysphagia. GERD-like symptoms predominated in this study (312 children) and included vomiting, regurgitation, abdominal pain, heartburn and water brash.[43] of the remaining 69 children with dysphagia, 24 had radiological evidence of esophageal narrowing, but only one had an endoscopic stricture that required dilatation. these observations support the theory that symptoms related to EoE represent two differ ent disease phenotypes. Some patients with eoe experience acute

symptoms due to intermittent esophageal spasm, probably related to smooth-muscle contraction.[61] Nurko and colleagues reported that of eight children with EoE and radio logical evidence of schatzki ring, none showed evidence of esophageal narrowing at the time of endoscopy. [62] Measures of esophageal contraction recorded by 24 h motility monitoring were reported to be disordered in 13 children with EoE compared with children with GERD and healthy controls.[63] By contrast, chronic symptoms of dysphagia might reflect longstanding esophageal remodeling that occurs secondary to chronic eosinophilic inflammation. This tenet is supported by the findings of isolated proximal and distal esophageal strictures, esopha geal rings, long-segment narrowing of the esophagus, and esophageal fibrosis in patients with EoE.[63-–82] An important subgroup of children with EoE have feeding difficulties that include refusal to eat, chewing problems, choking on solids or liquids, or an in ability to tolerate diet beyond certain textures or tastes.[84] Frequently these symptoms are overlooked in the context of a busy practice but they may represent one of the first clues to the diagnosis of EoE. Feeding difficulties may represent acute esophageal dysfunction with pain, or chronic learned behavior from longstanding disease. A family history of EoE, dysphagia or food impaction may be present in children with the disease; it is not unusual for new diagnoses within a family to be uncovered during the evaluation of a child.[85–87]

PEDIATRIC FEATURES GerD-like upper gastrointestinal symptoms unresponsive to pharmacological or surgical management, abdominal pain, vomiting, feeding difficulties
ADULT FEATURES GerD-like upper gastrointestinal symptoms unresponsive to pharmacological or surgical management, dysphagia, food impaction, chest pain
HISTOPATHOLOGICAL FEATURES Dense epithelial eosinophilia of the esophageal mucosa (≥15 eosinophils/high power field), normal gastric and duodenal mucosa, eosinophil microabscesses, superficial layering, rete peg elongation, basal zone hyperplasia
RADIOGRAPHIC FEATURES esophageal stricture (especially proximal), long-segment esophageal narrowing, esophageal rings
ENDOSCOPIC FEATURES Linear furrows, concentric rings, schatzki ring, linear shearing and/or splitting, crepe paper esophagus, white exudates

Table 1: Diagnostic Features of Eosinophilic Esophagitis

Diagnosis

The diagnosis of EoE always begins with the recognition of symptoms; however, the severity and chronicity of EoE symptoms often go overlooked. In a pediatric study, there was a 3-year lag between symptoms and first endoscopy (89). In a recent retrospective German study of mostly adults, there was a 4-year lag between symptoms and diagnosis (90). Food impaction has been a primary presentation of EoE in many adults. Esophageal food impaction occurs with an estimated frequency of 13 cases per 100,000 in adults. Two recent studies found that a significant number of patients who presented with food impaction had underlying EoE (91, 92). Anytime a

food impaction or severe dysphagia occurs in adults, EoE should be considered, and, along with the removal of the impaction, an upper endoscopy with biopsy should be performed. The most important part of diagnosis is upper endoscopy with biopsy. Visual inspection is not sufficient because even a normal appearing esophagus can have significant esophageal eosinophilia. As many as one third of patients with EoE may have a normal appearing esophagus (41). Other findings include esophageal furrows, strictures, mucosal rings (trachealization), and white plaques (7, 41, 52, 63).

Esophageal tissue from patients who have EoE may also demonstrate thickened mucosa with basal layer hyperplasia and papillary lengthening (93). Because there is definite variability in histology within the same patient, multiple esophageal biopsy specimens should be obtained. *Gonsalves* (94) found that the sensitivity and specificity of making the diagnosis of EoE increased by the number of biopsies. Muller (90) reiterated that multiple biopsiesare essential for diagnosis. *Baxi* and colleagues (95) set out to characterize whether the number of eosinophils correlated with the degree of symptoms and found that only dysphagia correlated with higher eosinophil counts. They were unable to find statistical differences in age, gender, or other symptoms in patients with low levels of eosinophils (1 to 5 cells/hpf), moderate levels of esophageal eosinophils (6 to 14 cells/hpf), or higher levels of eosinophils (>15 cells/hpf).

Treatment

Although the natural history of EoE is unknown and despite the fact that EoE is a chronic disease, people can live long with the diagnosis. Currently, the difficulty lies with the fact that it is difficult to identify those individuals who go on to sustain complications versus those who simply have chronic symptoms. In either case, it is important to recognize the symptoms that may interfere with

daily life. Treatment options at this time include esophageal dilatation, dietary management, and pharmacologic options.

Dilatation

Dilation of the esophagus is useful to achieve immediate improvement in symptomatology for food impaction in patients who have significant anatomic esophageal abnormalities. Dilatation is not recommended as first-line therapy secondary to concerns of significant side effects (pain, bleeding, perforation) and does not address the underlying pathogenesis. As an example, a 17-year-old patient who presented with food impaction and long segment esophageal stenosis experienced minor bleeding and perforation of her esophagus postoperatively. Esophageal biopsy at the time revealed more than 100 cells/hpf, and the perforation developed as a complication of dilation (76). Although dilatation has been associated with esophageal perforation, *Schoepfer* (81) recently reported the successful use of esophageal dilatation in a subset of EoE patients. As a rule, whenever possible, other modes of therapy should be used before performing dilatation.

Diet

Recent work from a variety of groups continues to confirm the role of food allergy in the vast majority of patients with EoE. Although aeroallergens were suggested as a cause for EoE in one clinical case,38 food antigens have been definitively demonstrated to be the cause of EoE. Over the past few years, patients have been shown to respond not only to diets directed by the use of skin prick and patch testing but also to empiric diets based on the removal of the most likely food allergies. EoE is thought to be due to a combination of IgE- and non–IgE-mediated food reactions. A recent report found the combination of prick testing (for IgE-mediated reactions) and patch testing (for non–IgE-mediated reactions) to be effective in guiding management (47). The combination of both types of testing had a negative predictive value of 88% to 100% for all foods

except milk, which was low, while the positive predictive value was greater than 74% for the most common foods causing EoE. As a result, when patients with EoE are referred for allergy evaluation, it should include testing for foods via prick and patch testing as well as testing for environmental allergies. Recommendations are to avoid all positive foods by testing and any other food of concern even if negative (known as a restricted or elimination diet) or to begin an elemental diet. *Liacouras* (7) demonstrated that removal of dietary antigens via an elemental diet improved clinical symptoms and esophageal histology in 98% of patients. Others have been successful using elimination diets based on removing the most likely foods known to cause EoE (96). Once an elemental diet is initiated, tissue histology normalizes within a month. Dietary modification (five patients on restricted diets and two on elemental diets) was used in an Italian study with improvement in symptoms and histology (97). In most cases, dietary modification is not easy. Compliance with the diet can be difficult in children as well as adults. Formulas can be very costly, and sometimes insurance will not cover the expense encumbered. Although dietary changes should always be considered as the first-line therapy for children and adults with EoE, many patients may require other treatment modalities. Because of patient compliance and quality of life issues, topical corticosteroids have been prescribed for adults and adolescent patients who have EoE. Because the role of aeroallergens in inducing EoE has been described in the literature, management of environmental allergies is also recommended. One study set out to answer whether aeroallergens that cross react with foods can cause or worsen EoE (98). Grass pollen cross reacts with plant-derived food allergens, namely, wheat and rye. *Simon* (98) studied six adult patients with EoE who had attacks of dysphagia monthly. Radioallergosorbent (RAST) testing was performed on all patients. There was no immediate response on food challenge to either food and overall no change in biopsy or decreased symptoms when wheat and rye were removed for 6 weeks. Interestingly, the

patient who was skin test positive to wheat and rye without grass sensitivity reported clinical improvement on dietary elimination without histologic

Medication

The use of corticosteroids is an effective therapy for patients with EoE. Several reports have shown the efficacy of systemic steroids. In fact, they were the first treatment for EoE patients to show an improvement in both symptoms and histology (99). Unfortunately, the side effects of the long-term use of systemic steroids preclude their regular use, although, at times, short courses are used for patients with severe symptoms. Several investigators have reported success using swallowed steroids in children and adults (100, 20, 101, 77). Besides esophageal symptoms, three patients with subglottic stenosis and laryngeal edema refractory to reflux treatment were found to have EoE which responded to swallowed fluticasone (55). *Konikoff* and colleagues (102) performed a randomized double-blind, placebo-controlled trial of fluticasone given to 36 patients who either refused or failed dietary modification (elemental diet not used) and were placed on inhaled steroids or placebo for 3 months. He reported improved symptom control and less eosinophils per high-power field on biopsy (although not always to normal levels) in a comparison with placebo. These effects were noted more in nonallergic and younger individuals, and, interestingly, a placebo response was noted. *Aceves* (103) reported on two children who failed an elemental formula and were unable to swallow fluticasone who responded to budesonide (mixed with Splenda to form a viscous solution). *Helou* (104) performed a follow-up study on 51 adult patients treated with a swallowed steroid inhaler. Patients who were diagnosed with EoE received 880 mg of fluticasone twice daily for 6 weeks. A questionnaire was then conducted approximately 3 years after the initial dose of fluticasone was administered. At follow-up, more than 90% of patients experienced a recurrence of symptoms within 1 year, and 70% needed repeat therapy with fluticasone. Approximately 20% required the use of systemic steroids, 28% experienced subsequent food

impactions, and 22% underwent repeat esophageal dilatation. Both cromolyn sodium and leukotriene receptor antagonists have been tried but have not been shown to alter esophageal histology. Investigators have tried cromolyn sodium as an adjunct to therapy without success. Cromolyn was used in 14 patients without symptomatic or histologic improvement (41). Leukotrienes attract eosinophils. By blocking the leukotriene receptor, one could potentially decrease the effects of leukotrienes and thereby improve symptoms. *Attwood* and colleagues (105) using montelukast in high dosages decreased symptoms, but either no follow-up endoscopy or no change in endoscopy was noted. An interesting report from *Gupta* (106) compared cysteinyl leukotriene levels in 12 children with EoE, 10 healthy controls, and 5 children with EoG. The levels did not correlate with the degree of eosinophilic inflammation, and there was no statistical difference in the levels in patients with EoE and normals. At times, combination therapy works best. *Pentiuk* and colleagues (107) studied 15 pediatric patients with EoE who were referred to a feeding team and documented responses to treatment (from January 2000 through June 2003). All of the patients received therapy with proton pump inhibitors, swallowed fluticasone, and underwent dietary restriction (if testing was performed and was positive). Using this approach, the endoscopic appearance improved in all patients and completely resolved in 93%. This effect was associated with clinical improvement in 87% as well. *Orenstein* (52) found that 25% to 40% patients relapse. Their approach for pediatric patients was dietary management. Adults swallowed fluticasone for 6 weeks; if symptoms returned, therapy was extended for 12 weeks or treatment with oral steroids begun.

Biologic therapy using antibodies that block IL-5 receptors is currently under evaluation. Anti–IL-5 was shown to be effective in a patient with hypereosinophilic syndrome who had esophageal eosinophilia. In addition, four patients with EoE received three doses of intravenous anti–IL-5 and had biopsy specimens obtained at weeks 8. The percentages of blood eosinophils decreased, and the

number of esophageal eosinophils decreased significantly without side effects. Subsequently, Stein performed an open-label safety and efficacy study of anti–IL-5 in four adult patients with EoE who had chronic dysphagia and esophageal strictures. Patients received three infusions of anti–IL-5. The levels of plasma IL-5, peripheral blood eosinophils, and CCR31 cells in blood, quality of life measurements, and a histologic analysis of esophageal biopsies were determined before and 1 month after treatment. The study showed a decrease in peripheral blood eosinophilia and CCR31 cells as well as a significant decrease in esophageal eosinophils and a reduction in clinical symptoms. Currently, two additional pediatric studies of anti–IL-5 therapy are underway in patients with EoE.

References

1. Furuta, G. T. *et al.* eosinophilic esophagitis in children and adults: a systematic review andnconsensus recommendations for diagnosis and treatment. *Gastroenterology* 133, 1342–1363 (2007).
2. Gonsalves, N. eosinophilic esophagitis:history, nomenclature, and diagnostic guidelines. *Gastrointest. Endosc. Clin. N. Am.* 18, 1–9 vii (2008).
3. Kapel, r. C. *et al.* eosinophilic esophagitis: a prevalent disease in the United states that affects all age groups. *Gastroenterology* 134, 1316–1321 (2008).
4. Gill, r., Durst, P., rewalt, M. & elitsur, Y. eosinophilic esophagitis disease in children from west virginia: a review of the last decade (1995–2004). *Am. J. Gastroenterol.* 102, 2281–2285 (2007).
5. Noel, r. J., Putnam, P. e. & rothenberg, M. e. eosinophilic esophagitis. *N. Engl. J. Med.* 351, 940–941 (2004).
6. Straumann, A. & Simon, H. U. eosinophilic esophagitis: escalating epidemiology? *J. Allergy Clin. Immunol.* 115, 418–419 (2005).
7. Straumann, A. The natural history and complications of eosinophilic esophagitis. *Gastrointest. Endosc. Clin. N. Am.* 18, 99–118 (2008).
8. Mukkada, v., Atkins, D. & Furuta, G. T. Uncertain association of Barrett's esophagus with eosinophilic esophagitis. *Clin. Gastroenterol. Hepatol.* 6, 832–833 (2008)
9. Wolfsen, H. C., Hemminger, L. L. & Achem, s. r. eosinophilic esophagitis and Barrett's esophagus with dysplasia. *Clin. Gastroenterol. Hepatol.* 5, A18 (2007).
10. Francalanci, P. *et al.* eosinophilic esophagitis and Barrett's esophagus: an occasional association or an overlap disease? esophageal 'double trouble' in two children. *Digestion* 77, 16–19 (2008).
11. Mishra, A., Hogan, s. P., Brandt, e. B. & Rothenberg, M. e. An etiological role for aeroallergens and eosinophils in experimental esophagitis. *J. Clin. Invest.* 107, 83–90 (2001).
12. Mishra, A., Hogan, s. P., Brandt, e. B. & rothenberg, M. e. iL-5 promotes eosinophil trafficking to the esophagus. *J. Immunol.* 168, 2464–2469 (2002).
13. Mishra, A. & rothenberg, M. e. intratracheal iL-13 induces eosinophilic esophagitis by an iL-5, eotaxin-1, and sTAT6-dependent mechanism. *Gastroenterology* 125, 1419–1427 (2003).
14. Akei, H. s., Mishra, A., Blanchard, C. & rothenberg, M. e. epicutaneous antigen exposure primes for experimental eosinophilic esophagitis in mice. *Gastroenterology* 129, 985–994 (2005).
15. Mishra, A. *et al.* esophageal remodeling develops as a consequence of tissue-specific iL-5-induced eosinophilia. *Gastroenterology* 134, 204–214 (2008).
16. Desai, T. K. *et al.* Association of eosinophilic inflammation with esophageal food impaction in adults. *Gastrointest. Endosc.* 61, 795–801 (2005).
17. Lucendo, A. J. *et al.* Immunophenotypic characterization and quantification of the epithelial inflammatory infiltrate in eosinophilic esophagitis through stereology: an analysis of the cellular mechanisms of the disease and the immunologic capacity of the esophagus. *Am. J. Surg. Pathol.* 31, 598–606 (2007).
18. Chehade, M., sampson, H. A., Morotti, r. A. & Magid, M. s. esophageal subepithelial fibrosis in children with eosinophilic esophagitis. *J. Pediatr. Gastroenterol. Nutr.* 45, 319–328 (2007).
19. Kirsch, r., Bokhary, r., Marcon, M. A. & Cutz, e. Activated mucosal mast cells differentiate eosinophilic (allergic) esophagitis from gastroesophageal reflux disease. *J. Pediatr. Gastroenterol. Nutr.* 44, 20–26 (2007).

20. Teitelbaum, J. *et al.* eosinophilic esophagitis in children: immunopathological analysis and response to fluticasone propionate. *Gastroenterology* 122, 1216–1225 (2002).
21. Yamazaki, K. *et al.* Allergen-specific *in vitro* cytokine production in adult patients with eosinophilic esophagitis. *Dig. Dis. Sci.* 51, 1934–1941 (2006).
22. *Lucendo, A. J., Bellon, T. & Lucendo, B. The role of mast cells in eosinophilic esophagitis. Pediatr. Allergy Immunol. (in press).*Straumann, A. *et al.* Cytokine expression in healthy and inflamed mucosa: probing the role of eosinophils in the digestive tract. *Inflamm. Bowel Dis.* 11, 720–726 (2005).
23. Genta, r. M., Spechler, s. J. & Souza, r. F. The twentieth eosinophil. *Adv. Anat. Pathol.* 14, 340–343 (2007).
24. Spechler, s. J., Genta, r. M. & Souza, r. F. Thoughts on the complex relationship between gastroesophageal reflux disease and eosinophilic esophagitis. *Am. J. Gastroenterol.* 102, 1301–1306 (2007).
25. Chehade, M. & sampson. H. A. The role of lymphocytes in eosinophilic gastrointestinal disorders. *Immunol. Allergy Clin. North Am.* 29, 149–158 (2009).
26. Wershik, B. K. exploring the role of mast cells in eosinophilic esophagitis. *Immunol. Allergy Clin. North Am.* 29, 189–195 (2009).
27. Dehlink, e. & Fiebiger, e. The role of the highaffinity IgE receptor, Fcεri, in eosinophilic gastrointestinal diseases. *Immunol. Allergy Clin. North Am.* 29, 159–170 (2009).
28. Blanchard, C. *et al.* eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. *J. Clin. Invest.* 116, 536–547 (2006).
29. Bhattacharya, B. et al. increased expression of eotaxin-3 distinguishes between eosinophilic esophagitis and gastroesophageal reflux disease. Hum. Pathol. 48, 1744–1753 (2007).
30. Blanchard, C. et al. eotaxin-3/CCL26 gene expression in intestinal epithelial cells is upregulated by interleukin-4 and interleukin-13 via the signal transducer and activator of transcription 6. Int. J. Biochem. Cell Biol. 37, 2559–2573 (2005).
31. Fujiwara, H. et al. infiltrating eosinophils and eotaxin: their association with idiopathiceosinophilic esophagitis. Ann. Allergy Asthma Immunol. 89, 429–432 (2002).
32. Bullock, J. Z. et al. interplay of adaptive TH2 immunity with eotaxin-3/C-C chemokine receptor 3 in eosinophilic esophagitis. J. Pediatr. Gastroenterol. Nutr. 45, 22–31 (2007).
33. Sant'Anna, A. M., Rolland, s., Fournet, J. C., Yazbeck, s. & Drouin, e. eosinophilic esophagitis in children: symptoms, histology and pH probe results. J. Pediatr. Gastroenterol. Nutr. 39, 373–377 (2004).
34. Rosen, r., Furuta, G., Fritz, J., Donovan, K. & Nurko, s. role of acid and nonacid reflux in children with eosinophilic esophagitis compared with patients with gastroesophageal reflux and control patients. J. Pediatr. Gastroenterol. Nutr. 46, 520–523 (2008).
35. Bonis, P. A. Putting the puzzle together: epidemiological and clinical clues in the etiology of eosinophilic esophagitis. Immunol. Allergy Clin. North Am. 29, 41–52 (2009).
36. Molina-infante, J., Ferrando-Lamana, L., Mateos-rodriguez, J. M., Perez-Gallardo, B. & Prieto-Bermejo, A. B. Overlap of reflux and eosinophilic esophagitis in two patients requiring different therapies: a review of the literature. World J. Gastroenterol. 14, 1463–1466 (2008).
37. Mikhak, Z. & Luster, D. Chemokines in cell movement and allergic inflammation. In Middleton's Allergy Principles & Practice 7th edn (eds Adkinson, N. F. et al.) 181–201 (Mosby elsevier, New York, 2009).
38. Spergel, J. M., Beausoleil, J. L., Mascarenhas, M. & Liacouras, C. A. The use of skin prick tests and patch tests to identify causative foods in eosinophilic esophagitis. J. Allergy Clin. Immunol. 109, 363–368 (2002).

39. Roy-Ghanta, s., Larosa, D. F. & Katzka, D. A. Atopic characteristics of adult patients with eosinophilic esophagitis. Clin. Gastroenterol. Hepatol. 6, 531–535 (2008).
40. Simon, D. et al. Eosinophilic esophagitis is frequently associated with ige-mediated allergic airway diseases. J. Allergy Clin. Immunol. 115, 1090–1092 (2005).
41. Liacouras, C. A. et al. eosinophilic esophagitis: a 10-year experience in 381 children. Clin.Gastroenterol. Hepatol. 3, 1198–1206 (2005).
42. Arora, A. s. & Yamazaki, K. Eosinophilic esophagitis: asthma of the esophagus? Clin. Gastroenterol. Hepatol. 2, 523–530 (2004).
43. Fogg, M. i., ruchelli, e. & spergel, J. M. Pollen and eosinophilic esophagitis. J. Allergy Clin. Immunol. 112, 796–797 (2003).
44. Onbasi, K. et al. eosinophil infiltration of the oesophageal mucosa in patients with pollen allergy during the season. Clin. Exp. Allergy 35, 1423–1431 (2005).
45. Sampson, H. A. & Burks, A. w. Adverse reactions to foods. in Middleton's Allergy Principles & Practice 7th edn (eds Adkinson, N. F. et al.) 1139–1167 (Mosby elsevier, New York, 2009).
46. Straumann, A. *et al.* idiopathic eosinophilic esophagitis is associated with a TH2-type allergic inflammatory response *J. Allergy Clin. Immunol.* 108, 954–961 (2001).
46. Spergel, J. M., Andrews, T., Brown-whitehorn, T. F., Beausoleil, J. L. & Liacouras, C. A. Treatment of eosinophilic esophagitis with specific food elimination diet directed by a combination of skin prick and patch tests. *Ann. Allergy Asthma Immunol.* 95, 336–343 (2005).
47. Spergel, J. M., Brown-whitehorn, T., Beausoleil, J. L., shuker, M. & Liacouras, C. A. Predictive values for skin prick test and atopy patch test for eosinophilic esophagitis. *J. Allergy Clin. Immunol.* 119, 509–511 (2007).
48. Spergel, J. M. & Brown-whitehorn, T. The use of patch testing in the diagnosis of food allergy. *Curr. Allergy Asthma Rep.* 5, 86–90 (2005).
49. Kagalwalla, A. F. *et al.* effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. *Clin. Gastroenterol. Hepatol.* 4, 1097–1102 (2006).
50. Liacouras, C. A. Failed Nissen fundoplication in two patients who had persistent vomiting and eosinophilic esophagitis. *J. Pediatr. Surg.* 32, 1504–1506 (1997).
51. Putnam, P. e. evaluation of the child who has eosinophilic esophagitis. *Immunol. Allergy Clin North Am.* 29, 1–10 (2009)
52. Orenstein, s. r. *et al.* The spectrum of pediatric eosinophilic esophagitis beyond infancy:a clinical series of 30 children. *Am. J. Gastroenterol.* 95, 1422–1430 (2000).
53. Kelly, K. J. *et al.* eosinophilic esophagitis attributed to gastroesophageal reflux: improvement with an amino acid-based formula. *Gastroenterology* 109, 1503–1512 (1995).
54. Cheung, K. M., Oliver, M. r., Cameron, D. J., Catto-smith, A. G. & Chow, C. w. esophageal eosinophilia in children with dysphagia. *J. Pediatr. Gastroenterol. Nutr.* 37, 498–503 (2003).
55. Dauer, e. H., Ponikau, J. U., smyrk, T. C., Murray, J. A. & Thompson, D. M. Airway manifestations of pediatric eosinophilic esophagitis: a clinical and histopathologic report of an emerging association. *Ann. Otol. Rhinol. Laryngol.* 115, 507–517 (2006).
56. Thompson, D. M., Arora, A. s., romero, Y. & Dauer, e. H. eosinophilic esophagitis: its role in aerodigestive tract disorders. *Otolaryngol. Clin. North Am.* 39, 205–221 (2006).
57. Putnam, P. e. eosinophilic esophagitis in children: clinical manifestations. *Gastrointest. Endosc. Clin. N. Am.* 18, 11–23 (2008).
58. Nurko, s. & rosen, r. esophageal dysmotility in patients who have eosinophilic esophagitis. *Gastrointest. Endosc. Clin. N. Am.* 18, 73–89 (2008).

59. Nurko, s. *et al.* Association of schatzki ring with eosinophilic esophagitis in children. *J. Pediatr. Gastroenterol. Nutr.* 38, 436–441 (2004).
60. Nurko, s. *et al.* esophageal motor abnormalities in patients with allergic esophagitis. A study with prolonged esophageal pH/manometry. *J. Pediatr. Gastroenterol. Nutr. Abstr.* 33, 417 (2001).
61. Feczko, P., Halpert, r. & Zonca, M. radiographic abnormalities in eosinophilic esophagitis. *Gastrointest. Radiol.* 10, 321–324 (1985)
62. Vitellas, K. M. *et al.* idiopathic eosinophilic esophagitis. *Radiology* 186, 789–793 (1993).
63. Siafakas, C. G., ryan, C. K., Brown, M. r. & Miller, T. L. Multiple esophageal rings: an association with eosinophilic esophagitis: case report and review of the literature. *Am. J. Gastroenterol.* 95, 1572–1575 (2000).
64. . Bonis, P. A. ringed esophagus: unclear relationship to gastroesophageal reflux disease. *Am. J. Gastroenterol.* 96, 3439–3431 (2001).
65. Vasilopoulos, s. & shaker, r. Defiant dysphagia: small-caliber esophagus and refractory benign esophageal strictures. *Curr. Gastroenterol. Rep.* 3, 225–230 (2001).
66. Batres, L. A., Liacouras, C., schnaufer, L. & Mascarenhas, M. r. eosinophilic esophagitis associated with anastomotic strictures after esophageal atresia repair. *J. Pediatr. Gastroenterol. Nutr.* 35, 224–226 (2002).
67. Fox, v. L., Nurko, s. & Furuta, G. T. eosinophilic esophagitis: it's not just kid's stuff. *Gastrointest. Endosc.* 56, 260–270 (2002)
68. Vasilopoulos, s. *et al.* The small-caliber esophagus: an unappreciated cause of dysphagia for solids in patients with eosinophilic esophagitis. *Gastrointest. Endosc.* 55, 99–106 (2002).
69. Croese, J. *et al.* Clinical and endoscopic features of eosinophilic esophagitis in adults. *Gastrointest. Endosc.* 58, 516–522 (2003).
70. Kaplan, M. *et al.* endoscopy in eosinophilic esophagitis: "feline" esophagus and perforation risk. *Clin. Gastroenterol. Hepatol.* 1, 433–437 (2003).
71. Khan, s. *et al.* eosinophilic esophagitis: strictures, impactions, dysphagia. *Dig. Dis. Sci.*48, 22–29 (2003).
72. Straumann, A. *et al.* Natural history of primary eosinophilic esophagitis: a follow-up of 30 adult patients for up to 11.5 years. *Gastroenterology* 125, 1660–1669 (2003).
73. Potter, J. w. *et al.* eosinophilic esophagitis in adults: an emerging problem with unique esophageal features. *Gastrointest. Endosc.* 59, 355–361 (2004).
74. Cantu, P., velio, P., Prada, A. & Penagini, r. ringed oesophagus and idiopathic eosinophilic oesophagitis in adults: an association in two cases. *Dig. Liver Dis.* 37, 129–134 (2005).
75. Zimmerman, s. L. *et al.* idiopathic eosinophilic esophagitis in adults: the ringed esophagus. *Radiology* 236, 159–165 (2005).
76. Eeisenbach, C. *et al.* Perforation of the esophagus after dilation treatment for dysphagia in a patient with eosinophilic esophagitis. *Endoscopy* 38, e43–e44 (2006).
77. Remedios, M., Campbell, C., Jones, D. M. & Kerlin, P. eosinophilic esophagitis in adults: clinical, endoscopic, histologic findings, and response to treatment with fluticasone propionate. *Gastrointest. Endosc.* 63, 3–12 (2006).
78. Lucendo, A. J. *et al.* endoscopic, bioptic, and manometric findings in eosinophilic esophagitis before and after steroid therapy: a case series. *Endoscopy* 39, 765–771 (2007).
79. Liguori, G., Cortale, M., Cimino, F. & sozzi, M. Circumferential mucosal dissection and esophageal perforation in a patient with eosinophilic esophagitis. *World J. Gastroenterol.* 14, 803–804 (2008).

80. Robles-Medranda, C., villard, F., Bouvier, r., Dumortier, J. & Lachaux, A. spontaneous esophageal perforation in eosinophilic esophagitis in children. *Endoscopy* 40 (suppl. 2), e171 (2008).
81. Schoepfer, A. M., Gschossmann, J., scheurer, U., seibold, F. & straumann, A. esophageal strictures in adult eosinophilic esophagitis: dilation is an effective and safe alternative after failure of topical corticosteroids. *Endoscopy* 40, 161–164 (2008).
82. Haas, A. & Creskoff-Naune, N. Feeding dysfunction in children with eosinophilic esophagitis. *Immunol. Allergy Clin. North Am.* (in press).
83. Collins, M. H. *et al.* Clinical, pathologic, and molecular characterization of familial eosinophilic esophagitis compared with sporadic cases. *Clin. Gastroenterol. Hepatol.* 6, 128 (2008).
84. Meyer, G. w. eosinophilic esophagitis in a father and a daughter. *Gastrointest. Endosc.* 61, 932 (2005).
85. Zink, D. A., Amin, M., Gebara, s. & Desai, T. K. Familial dysphagia and eosinophilia. *Gastrointest. Endosc.* 65, 330–334 (2007)
86. Katzka, D. A. eosinophilic esophagitis: it's all in the family. *Gastrointest. Endosc.* 65, 335–336 (2007).
87. Patel, s. M. & Falchuk, K. r. Three brothers with dysphagia caused by eosinophilic esophagitis. *Gastrointest. Endosc.* 61, 165–167 (2005)
88. D Atkins, R Kramer, K Capocelli, M Lovell and G T. Furuta. Eosinophilic esophagitis: the newest esophageal inflammatory disease *Nat. Rev. Gastroenterol. Hepatol.* 6, 267–278 (2009)
89. Assad AH, Putnam PE, Collins MH, et al. Pediatric patients with eosinophilic esophagitis: an 8 year follow-up. J Allergy Clin Immunol 2007;119:731–8
90. Muller S, Puhl S, Vieth M, et al. Analysis of symptoms and endoscopic findings in 117 patients with histological diagnosis of EE. Endoscopy 2007;39:339–44.
91. Desai TK, Stecevic V, Chang CH, et al. Association of eosinophilic inflammation with esophageal food impaction in adults. Gastrointest Endosc 2005;61:795–801.
92. Byrne KR, Panagiotakis PH,Hilden K, et al. Retrospective analysis of esophageal food impaction: differences in etiology by age and gender. Dig Dis Sci 2006;52:717–21.
93. Mueller S, Aigner T, Neureiter D, et al. Eosinophilic infiltration and degranulation in oesophageal mucosa from adult patients with eosinophilic oesophagitis: a retrospective and comparative study on pathological biopsy. J Clin Pathol 2006;59: 1175–80.
94. Gonsalves N, Policarpio-Nicolas M, Zhang Q, et al. Histopathologic variability and endoscopic correlates in adults with eosinophilic esophagitis. Gastrointest Endosc 2006;64:313–9.
95. Baxi S, Gupta SK, Swigonski N, et al. Clinical presentation of patients with eosinophilic inflammation of the esophagus. Gastrointest Endosc 2006;64(4):473–8
96. Kagawalla A, Sentongo TA, Ritz S, et al. Effect of six food elimination diet on clinical and histologic outcomes of eosinophilic esophagitis. Clin Gastroenterol 2006; 4(9):1097–102.
97. De Angelis P, Markowitz JE, Torroni F, et al. Paediatric eosinophilic oesophagitis: towards early diagnosis and best treatment. Dig Liver Dis 2006;38(4):245–51.
98. Simon D, Straumann A, Wenk A, et al. Eosinophilic esophagitis in adults—no clinical relevance of wheat and rye sensitizations. Allergy 2006;61:1480–3.
99. Liacouras CA. Primary eosinophilic esophagitis in children: successful treatment with oral corticosteroids. J Pediatr Gastroenterol Nutr 1998;26:380–5.
100. Faubion WA, Perrault J, Burgart LJ, et al. Treatment of eosinophilic esophagitis with inhaled corticosteroids. J Pediatr Gastroenterol Nutr 1998;27(1):90–3.

101. Noel RJ, Putnam PE, Collins MH, et al. Clinical and immunopathologic effects of swallowed fluticasone for eosinophilic esophagitis. Clin Gastroenterol Hepatol 2004;2:568–75.
102. Konikoff MR, Noel RJ, Blanchard C, et al. A randomized, double blind, placebo controlled trial of fluticasone proprionate for pediatric eosinophilic esophagitis. Gastroenterology 2006;131(5):1381–91.
103. Aceves S, Bastian J, Newbury R, et al. Topical viscous budesonide for pediatric eosinophilic esophagitis. Gastroenterology 2006;130:A577.
104. Helou EF, Simonson J, Arora AS. 3-Year follow-up of topical corticosteroid treatment for eosinophilic esophagitis in adults. Am J Gastroenterol 2008;103:1–6.
105. Attwood SEA, Lewis CJ, Bronder CS, et al. Eosinophilic oesophagitis: a novel treatment using montelukast. Gut 2003;52:181–5.
106. . Gupta SK, Peters-Golden M, Fitzgerald JF, et al. Cysteinyl leukotriene levels in esophageal mucosal biopsies of children with eosinophilic inflammation: are they all the same? Am J Gastroenterol 2006;101(5):1125–8.
107. Pentiuk SP, Miller CK, Kaul A. Eosinophilic esophagitis in infants and toddlers. Dysphagia 2007;22:44–8

5.2 Eosinophilic Esophagitis and Celiac Disease: Is There an Association?

ABSTRACT

Aim To report a series of 17 children affected by eosinophilic oesophagitis. Six of them also received a diagnosis of coeliac disease. Methods Seventeen children with history of dyspeptic symptoms were investigated. Results Six patients (M/ F:2 / 4; mean age _ s.d.: 5.6 _ 1.3 years, range: 4–7 years; Group A) affected by eosinophilic oesophagitis also received a diagnosis of coeliac disease. The other 11 children (M/ F:10 / 1, mean age _ s.d.:7.5 _ 2.3 years, range: 4–10 years, Group B) were affected solely by eosinophilic oesophagitis. All children underwent a change in dietary regimen. Group A received a gluten-free diet. Group B attempted dietary restriction based on the allergy testing results. After 6 months follow-up, all patients in Group A showed a complete disappearance of symptoms and three of them, who underwent upper gastrointestinal endoscopy, showed histologic remission. Patients from Group B had moderate clinical improvement and in seven of them (64%) a repeated upper gastrointestinal endoscopy showed a statistically significant reduction in eosinophilic infiltration. Conclusions This is the first reported group of patients with an association between coeliac disease and eosinophilic oesophagitis. To date, it is not possible to exclude that in a subgroup of children with coeliac disease the oesophageal eosinophilic infiltration could be caused by coeliac disease itself.

INTRODUCTION

Eosinophilic oesophagitis (EoE) and coeliac disease (CD) are distinct clinical entities with specific clinical, laboratory and histological features. Eosinophilic oesophagitis is a chronic inflammatory disorder of the oesophagus, presenting with dysphagia and with symptoms mimicking those of gastro-oesophageal reflux, including vomiting, regurgitation, nausea and epigastric pain. Patients with EoE are predominantly young males and have a dense and isolated eosinophilic infiltration (>24/high-power field) of the oesophageal mucosa.1 The endoscopic evaluation of the oesophagus commonly reveals furrowing or mucosal rings; the oesophagus could appear only slightly altered in some patients.2, 3 Eosinophilic oesophagitis is a disease emerging throughout the world: until 1995, oesophageal eosinophilia

was routinely associated with reflux oesophagitis; however, recent studies suggest that a large number of isolated eosinophils in the oesophagus may represent a separate diagnosis. Noel et al.4 reported on a specific population of children diagnosed with EoE between the years 2000 and 2003, and calculated a disease incidence of one in 10 000/year and a prevalence of four in 10 000. CD is an immune-mediated enteropathy caused by a permanent sensitivity to gluten in genetically susceptible individuals. The prevalence of CD in children between 3 and 15 years of age in the general population is 3–13 per 1000 children, or approximately 1:300 to 1:80 children.5, 6 Although food antigenes are implicated in both diseases, CD is clearly a food-mediated disorder while the pathophysiology of EoE is still unclear given that food allergy is not always found during the evaluation of affected EoE patients. We report a series of 17 patients with EoE. Six of them were also affected by CD. The unexpectedly high prevalence of CD in this EoE-affected population and the probability of an association between gluten-induced disease and EoE prompted us to report these patients.

SUBJECTS AND METHODS

From April 2005 to December 2006, all children referred for upper gastrointestinal (GI) endoscopy to the Gastrointestinal Endoscopy and Motility Unit of the Department of Pediatrics, University 'Federico II' of Naples, Italy, which received a diagnosis of EoE, were evaluated. In the same period, 315 children were diagnosed with CD. The patients were referred for a long-standing history of dyspeptic symptoms, such as vomiting, regurgitation, abdominal pain and/or dysphagia, in spite of standard antireflux therapies, including H2 receptor antagonist and/or protonic pump inhibitors. These treatments had been stopped at least 2 weeks before enrolment. None of them showed bloating, diarrhoea or weight loss. Because all the patients were under 10 years of age, their parents were given a questionnaire to fill out on behalf of their children: it gathered data regarding age, sex, GI and allergic symptoms, family and personal history of allergic diseases. During initial symptom evaluation, an arbitrary scoring system was used for symptomatologic assessment (Table 1). Furthermore, all children underwent haematologic and blood chemistry analyses, allergy testing and upper GI endoscopy with oesophageal, antral and duodenal biopsies.

Nine patients also underwent 24-h intra-oesophageal pH-monitoring. Gastro-oesophageal reflux disease was excluded by a lack of endoscopic and histological signs of reflux disease and/or normal 24-h intra-oesophageal pH-monitoring, defined by a reflux index (the total percentage of time during which the oesophageal pH is <4) £ 4%, according to previous studies.7, 8 Informed consent was obtained from parents of all patients, and the experimental design was approved by the Independent Ethics Committee of University of Naples, 'Federico II'.

Blood chemistry analysis and allergy testing

Blood leucocytes and a differential count were determined by using an automated blood count analysis. Numbers for peripheral eosinophils were considered as follows: normal, <350 cells/mm3; slightly elevated, 350–1500 cells/mm3; moderately elevated, >1500– 5000 cells/mm3; or severely

elevated, >5000 cells /mm3.3, 9 Liver enzymes, amylase, total protein, albumin, C-reactive protein, erythrocyte sedimentation rate and creatinine values were determined in all

patients using the standard methods. Serum antitissue transglutaminase IgA (anti-tTG Abs) and anti-endomysium IgA (EMA) antibodies were performed. Serum levels of IgA anti-TG2 were determined by ELISA technique using a kit based on human recombinant antigen (Eu-tTg IgA; Eurospital Kit, Trieste, Italy). Values were considered positive if ‡7 UA/ mL. Serum IgA EMA was detected by indirect immunofluorescence on 7-lm thick frozen section of human umbilical cord as source of antigen. Diagnostic criteria for CD included: anti-tTG Abs and EMA antibodies and evidence of typical histologic changes on duodenal biopsies in accordance with Marsh classification modified by Oberhuber.10, 11 All patients were evaluated in an attempt to determine the specific food allergies. They were tested for

total and specific serum IgE levels dietary allergens (cow's milk, alpha-lactoalbumin, beta-lactoglobulin, casein, egg, wheat, rice, soya, chicken, codfish and tomato). Children enrolled were also tested with skin prick tests to the same allergens, performed by one physician who was not aware of the subject's clinical history. Total IgE levels >35 kU/ L were considered positive, as were specific IgE levels >0.70 kU / L, as per reported reference ranges for the commercial test used in our laboratory (Unicap specific IgE allergen immune cap, manufactured by Pharmacia, Uppsala, Sweden).

Skin tests were read as positive if the wheal was at least two millimetres larger the control antigen.12

Prolonged pH measurement

Prolonged 24-h intra-oesophageal pH monitoring was performed with a portable digital recorder (Digitrapper II; Synectics, Medtronic Gastrointestinal, Synectics Medical AB, Stockolm, Sweden) connected to a flexible antimonium probe (Olympus GIF-XP240, Olympus Optical Co. Ltd, Tokyo,

Japan). At the beginning of the study, the pH meter was calibrated with a standard solution of pH 4 and 7. Subsequently, a pH electrode was inserted via the nose, with the tip placed at 87% of the distance from the nares to the lower oesophageal sphincter, according to technique of Strobel.13

Endoscopy and histology

Upper GI endoscopy was performed in all 17 patients by the same endoscopist (EM), using a paediatric fiberoptic

gastroscope (Olympus GIFPQ20, Olympus Optical Co., LTD, Tokyo, Japan). During endoscopy, the overall intensity of the endoscopic alterations was classified as (i) absent, (ii) minimal (e.g. fine nodules, fine whitish reticular structures, furrows), (iii) moderate (e.g. bright whitish scale-like, plaque-like structures, corrugated rings), or (iv) severe (e.g. mucosal lesion, fixed stenosis).14 By using standard forceps, at least two biopsy specimens were taken from each of the following four sites: the mid-oesophagus (7–10 cm from Z-line), the distal oesophagus (2–4 cm from the Z-line), the antrum of the stomach and the duodenum. The samples were immediately fixed in a 4% paraformaldehyde solution and embedded in paraffin. Paraffin blocks were cut in 5-lm section and stained with H&E, Giemsa, and Alcian blue／periodic acid- Schiff for histologic examination. All oesophageal biopsy specimens were examined by an experienced GI pathologist. The eosinophils in the most densely infiltrated area were counted in five consecutive highpower fields (HPF). Data were expressed as the mean number of eosinophils per HPF (Zeiss Axiophot, Oberkochen,

Germany; Plan-Neofluar 40, ocular magnification 10· , area of microscopic field 0.3072 mm2). The diagnosis of EoE was based on the demonstration of oesophageal infiltration with >24 eosinophils in any single 400 · HPF along with normal antral and duodenal biopsies.2, 15 Measurements of the epithelial thickness and papillary height were made in oesophageal specimens

in which at least three well-oriented papillae could be identified. Measurements were considered normal if basal zone hyperplasia was <20% of the epithelial thickness and/or lengthening of the papillae was <75% of the epithelial height according to data reported by Teitelbaum et al.8

Dietary treatment of EoE and CD

Dietary treatment included either dietary restriction, with the exclusion of selected foods based on allergy testing, for patients affected by EoE only, or glutenfree diet for children affected by EoE and CD. A diet counselling for appropriate calories assessment was performed and the nutritional state was monitored every 2 months by a paediatrician and a nutritionist. The response was based on the symptomatic and histologic improvement. Six months after initiating the diet, clinical evaluation and the second endoscopy with biopsies were performed. If the oesophageal histology normalized, a slow re-introduction of foods was begun. Once a week, a food that had previously been excluded was re-introduced. The re-introduction of the excluded foods is still in progress.

RESULTS

Seventeen patients (M / F: 12 / 5; mean age _ s.d.: 6.7 _ 2.3 years, range: 4–10 years) received a diagnosis of EoE. The mean number _ s.d. of eosinophils in the epithelium of middle and distal oesophageal biopsies was 39.9 _ 7.8 and 41.6 _ 9.2, respectively. Six of 17 patients (35%; M/F:2 / 4; mean age _ s.d.: 5.6 _ 1.3 years; Group A) showed villous atrophy of the duodenum (Marsh type 3). A subsequent serologic evaluation revealed that EMA and anti-tTG Abs were present in all six patients, confirming the diagnosis of CD In the other 11 children (65%; M/F:10 / 1, mean age _ s.d.: 7.45 _ 2.3 years, Group B), anti-tTG Abs and EMA were negative and duodenal and antral specimens did not show any abnormalities. By endoscopy 13 / 17 (76%), patients had macroscopic signs of EoE. In particular, 10 (59%) children had moderate oesophageal alterations and three (18%) had minimal lesions. The histological examination of distal oesophageal biopsies from all patients did not reveal any sign of gastrooesophageal reflux disease. Our patients did not show a gradient of eosinophil density from distal to proximal oesophagus or neutrophilic infiltration of oesophageal mucosa. In addition, in all oesophageal histological specimens, the measurements of oesophageal epithelial thickness and papillary height were not suggestive of gastro-oesophageal reflux disease.8 Results of continuous 24-h intra-oesophageal pH monitoring were normal in all nine patients evaluated (two patients from Group A and seven from Group B). Sex and chronological data, family history of atopy, allergy testing results are reported in Table 2. Prevalence of presenting complaints in a group of 315 patients with CD and in 17 patients with EoE is reported in Table 3. All 17 enrolled children underwent a change in their dietary regimen. Group A (six patients) received gluten-free diet. Group B (11 patients) attempted dietary restriction (56%, cow's milk and egg; 22%, codfish and wheat; 33%, cow's milk only), in particular, nine patients for documented food hypersensitivity and two patients as a trial challenge. Patients were assessed every 2 months for therapy adherence and symptom score. All patients were adherent to the dietary

treatment. After a mean follow-up of 6 months, a second endoscopy was performed in three children from Group A and seven children from Group B. All six patients in Group A showed a complete disappearance of symptoms and three of them who underwent upper GI endoscopy showed an histologic remission of EoE, defined as a mean eosinophils count of £ 10 eosinophils / HPF.16 In all these subjects, a significant improvement of duodenal histology (Marsh type 1) was observed. In these three patients, the number of oesophageal eosinophils per HPF in the middle and distal oesophagus was significantly ($P < 0.0001$) reduced compared with the baseline examination. The other 11 patients with EoE (Group B) had moderate clinical improvement and in seven of them (64%) a repeated upper GI endoscopy showed a significant ($P < 0.001$) reduction in eosinophilic infiltration without normalization. Finally, in both group of patients, specimens from the stomach and duodenum did not show at the time of the diagnosis and at follow-up, an increased number of eosinophils.15 Tables 4 and 5 show the symptom score and the number of eosinophils in the oesophageal epithelium, during the baseline evaluation and at follow-up examination, in three patients from group A and seven from Group B. Parents of three patients from Group A and four patients from Group B did not accept to repeat upper GI endoscopy with biopsies in their asymptomatic children.

DISCUSSION

In this study, we reported 17 patients investigated for upper GI symptoms who received a diagnosis of EoE;

six of 17 children (35%) also exhibited features of CD. Eosinophilic oesophagitis and CD seem to have similarities including genetic susceptibility, classification as food hypersensitivity disorders as well as the risk of complications in untreated patients. However, it is far from clear whether this co-existence is to be considered coincidental, or an expression of a real association. To our knowledge, this is the first reported group of patients with an association between EoE and CD. There are several possible explanations for this association, not being reported before. The first, most likely, is that our series for some reason is biased and our group of patients would be atypical. The second is that EoE is a disease of relatively recent recognition, particularly in Europe, and such association is not emerged yet. Very recent reports of association between EoE and CD would support this view.17, 18 Either of our children from Groups A and B seem to show a variety of common symptoms, such as epigastric pain, dysphagia / food impaction and vomiting. However, our patient's most common complaint was dysphagia, suggesting that the pathological involvement of the oesophagus was the main clinical problem in both groups. The cause of EoE is poorly understood, but allergy has been implicated.19 An IgE-dependent mechanism for EoE is supported by clinical observations. For instance, Spergel et al.20 showed that affected patients have IgE sensitization to a wide variety of foods; although not all patients had evidence of food-specific IgE. We observed a high level of IgE and evidence of IgE sensitization to food allergens in 82% Group B

patients; in contrast only 33% patients from Group A showed increased levels of IgE and none showed positive allergy testing results to food allergens. These observations seem to describe two distinct groups of children, e.g. those with EoE and a subset of CD patients who have elevated

oesophageal eosinophils for reasons different from allergy. It is known that activated eosinophils are found within the lamina propria of patients affected by CD, especially in the later

stages after lymphocyte activation.21 They have the capacity to synthesize IL-5, a cytokine produced by Tand

mast cells that is the major actor involved in eosinophils differentiation and activation.22 Therefore, EoE in CD patients could be the expression of glutendependent enteropathy, and the eosinophilic infiltration

may simply represent a less common oesophageal expression of the CD. From an immunological point of view, EoE, as a typical Th2 condition and as an expression of a food hypersensitivity reaction, is not expected to segregate

with Th1 diseases, such as CD. The possibility of a coexistence of Th1 and Th2 type diseases is still being debated.23 Recently, Nilsson et al.24 co-analysed the cytokines pattern in children with Th1 diseases (CD, type-1 diabetes) and allergy and demonstrated a strong combined Th1 and Th2-like response, as a result of an interplay between the two sides of the Th scale. This observation seems to suggest more than a casual association between CD and allergy-associated diseases and a more generalized defect of immuneregulation. Our patients with EoE from Group A were treated with a gluten-free diet, while Group B patients underwent a specific elimination diet according to the results of the allergy testing. After 6 months of follow- up, symptoms disappeared in all Group A children on a gluten-free diet, while the great majors of a gluten-free diet, while the great majors of patients from Group B patients showed an improvement, without complete resolution, in their symptom score. In addition, at follow-up, a normalization in eosinophilic count was noted in mid and distal oesophageal specimens of patients from Group A. In contrast, a significant reduction of the eosinophilic infiltration without complete normalization was found in the oesophageal biopsies from Group B children. The oesophageal histologic remission on gluten-free diet in our atients

suggests that, at least in a subgroup of patients, EoE and CD share the initial pathogenic trigger event. Gluten, by immunological dysregulation, could stimulate both a Th1 and Th2 reaction and be responsible for two different disorders, characterized by a common oesophageal phenotype. In conclusion, we report a series of 17 children affected by EoE. The discrepancy in symptomatic and histological response between the non-coeliac and coeliac groups highlights the importance of precisely identifying the alimentary allergens, the exclusion of which leads to the remission of oesophageal disease. It

is possible that, in our patients affected solely by EoE, all the allergens really involved in the pathogenesis of eosinophilic infiltration were not identified by allergy testing; therefore, these patients could take advantage from a 'complete dietary elimination' with an amino acid-based hypoallergenic formula or from different therapeutic options. In conclusion, the high proportion of patients affected by both diseases, EoE and CD, the lack of IgE sensitization for food allergens in these children as well as the significant clinical and histological remission on gluten-free diet compared to the group of subjects with EoE only suggest that CD itself could cause oesophageal eosinophilic infiltration and dyspeptic symptoms. However, further studies are needed to investigate the relationship between EoE and CD. Immunohistochemical and cytokine analysis of eosophageal biopsies of patients affected by both diseases most likely could improve our knowledge and be useful in understanding the precise immune mechanism(s), leading to

both symptoms and epithelial eosinophilia.

TABLE

Table 1. Grading of upper gastrointestinal symptoms

Symptoms	Evaluation	Score
Epigastric pain (episodes/week)	None	0
	1	1
	2–4	2
	>4	3
Dysphagia/food impaction (episodes/week)	None	0
	1	1
	2–4	2
	>4	3
Regurgitation/vomiting (episodes/week)	None	0
	<7	1
	<14	2
	>14	3

Table 2. Sex, chronological data, family history of atopy and allergy testing in six patients with eosinophilic oesophagitis and coeliac disease (Group A) and in 11 patients with eosinophilic oesophagitis (Group B)

	GROUP A	**GROUP B**
Sex [a] Male Female	2 (33) 4 (67)	10 (91) 1 (9)
Mean age (range)	5.6 (4-7)	7.45 (4-10)
Family history of atopy [b]	1/6 (17)	7/11 (64)
Blood Eosinophilia [b]	3/6 (50)	9/11 (82)
Increased levels of IgE [b]	2/6 (33)	9/11 (82)
Identified Food Allergy [a, b]	0/6 -	9/11 (82)

[a] The values are indicated as number of patients and percentage (%); [b] By skin prick test, radioallergosorbent test.

Table 3. Prevalence of presenting complaints in patients with coeliac disease (CD) and in patients with eosinophilic oesophagitis (EoE)

Presenting complaints	CD (n = 315) %	EoE (n = 17) %
Failure to thrive	61	0
Diarrhoea	41	0
Anaemia	34	0
Dyphagia/food impaction	0	59
Anorexia	22	0
Abdominal pain	15	41
Regurgitation/vomiting	13	35
Protuberant abdomen	5	0
Hypertransaminasemia	4	0
Constipation	4	0

Table 4. Clinical scores at baseline (to) and after dietary treatment (t1) in six patients with eosinophilic oesophagitis (EoE) and coeliac disease (group A) and in 11 patients with EoE (Group B)

	Group A			Group B		
	Number of patients					
Score	T0	T1	*P*	T0	T1	*P*
Dysphagia						
0	0	4	0.039	0	3	0.047
1–2	2	1		3	4	
3	4	1		8	4	
Epigastric pain						
0	1	5	0.039	1	0	0.053
1–2	1	1		2	9	
3	4	0		8	2	
Regurgitation/vomiting						
0	0	5	0.043	0	4	0.057
1–2	3	1		4	2	
3	3	0		7	5	

T0, baseline examination; T1, follow-up examination.

REFERENCES

1 Noel RJ, Putnam PE, Rothenberg ME. Eosinophilic esophagitis. N Engl J Med 2004; 351: 940–1.
2 Nurko S, Teitelbaum JE, Husain K, et al. Association of Schatzki ring with eosinophilic esophaitis in children. J Pediatr Gastroenterol Nutr 2004; 38: 436–41.
3 Rothenberg ME, Mishra A, Collins MH, et al. Pathogenesis and clinical features of eosinophilic esophagitis. J Allergy Clin Immunol 2001; 108: 891–4.
4 Fox VL, Nurko S, Furuta GT. Eosinophilic esophagitis: it's not just kid's stuff. Gastrointest Endosc 2002; 56: 260–70.
5 Fasano A, Berti I, Gerarduzzi T, et al. Prevalence of coeliac disease in at-risk and not at-risk in the United States. Arch Intern Med 2003; 163: 286–92.
6 Hoffenberg EJ, MacKenzie T, Barriga KJ, et al. A prospective study of the incidence of childhood coeliac disease. J Pediatr 2003; 143: 308–14.
7 Cucchiara S, Staiano A, Gobio-Casali L, et al. Value of the 24-hour intraesophageal pH monitoring in children. Gut 1990; 31: 129–33.
8 Teitelbaum JE, Fox VL, Twarog FJ, et al. Eosinophilic esophagitis in children: immunopathological analysis and response to fluticasone propionate. Gastroenterology 2002; 122: 1216–25.
9 Rothenberg ME. Eosinophilia. N Engl J Med 1998; 338: 1592–600.
10 Marsh MN. Gluten, major histocompatibility complex, and the small intestine a molecular and immunobiologic approach to the spectrum of gluten sensitivity ('coeliac sprue'). Gastroenterology 1992; 102: 330–54.
11 Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease:
time for a standardized report scheme for pathologists. Eur J Gastroenterol Hepatol 1999; 11: 1185–94.
12 Troncone R, Merrett TG, Ferguson A. Prevalence of atopy is unrelated to presence
of inflammatory bowel disease. Clin Allergy 1998; 18: 111–7.
13 Strobel CT, Byrne WJ, Ament ME. Correlation of esophageal lengths in children with height: application to the Tuttle test with-out prior esophageal manometry. J Pediatr 1979; 94: 81–4.
14 Straumann A, Spichtin HP, Grize L, et al. Natural history of primary eosinophilic esophagitis: a follow-up of 30 adult patients for up to 11.5 years. Gastroenterology 2003; 125: 1660–9.
15 Lowichik A, Weinber AG. A quantitative evaluation of mucosal eosiniophils in the pediatric gastrointestinal tract. Mod Pathol 1996; 9: 110–4.
16 Ruchelli E, Wenner W, Voytek T, et al. Severity of esophageal eosinophilia predicts response to conventional gastroesophageal reflux therapy. Pediatr Dev Pathol 1999; 2: 15–8.
17 Shah A, McGreal N, Li B, et al. Coeliac disease in association with eosinophilic esophagitis: case series of six patients from two centers. J Pediatr Gastroenterol Nutr 2006; 43: E24.
18 Kagalwalla AF, Shah A, Ritz S. Cow's milk protein-induced eosinophilic esophagitis in a child with gluten-sensitive enteropathy. J Pediatr Gastroenterol Nutr 2007; 44: 386–8.
19 Feighery C. Coeliac disease, auto-immunity and thyroid disease. Ital J Gastroenterol Hepatol 1999; 31: 288–9.
20 Spergel JM, Beausoleil JL, Mascarenhas M, et al. The use of skin prick tests and patch tests to identify the causative foods in eosinophilic esophagitis. J Allergy Clin Immunol 2002; 109:
363–8.
21 Straumann A, Bauer M, Fischer B, et al. Idiopathic eosinophilic esophagitis is associated with a T(H)2-type allergic inflammatory response. J Allergy Clin Immunol 2001; 108: 954–61.

22 Desreumaux P, Janin A, Colombel JF, et al. Interleukin 5 messenger RNA expression by eosinophils in the intestinal mucosa of patients with coeliac disease. J Exp Med 1992; 175: 293–6.
23 Caffarelli C, Cavagni G, Pierdomenico R, et al. Coexistence of IgE mediated allergy and type 1 diabetes in childhood. Int Arch Allergy Immunol 2004; 134: 288–94.
24 Nilsson L, Kivling A, Jalmelid M, et al. Combinations of common chronic paediatric diseases deviate the immune response in diverging directions. Clin Exp Immunol 2006; 146: 433–

CONCLUSIONS

This thesis aimed to define the genetical, immunological and clinical aspects of gastrointestinal inflammatory diseases in pediatrics, with attention to Inflammatory bowel disease (CD and UC) and to Eosinophilic Esophagitis (EoE). The following results were obtained and reported:

1) The characterization of mice with gut specific expression of Interleukin-12 (IL-12) family genes and their susceptibility to experimental colitis;

2) The role of Interleukin-23 receptor (IL-23R) Gene in Pediatric-Onset IBD and Genotype-Phenotype association in a pediatric population affected by IBD;

3) In order to find non invasive and offer the best therapeutical approach to patients affected by IBD we investigated the intestinal permeability test and we found that altered intestinal permeability study is predictive of early relapse in children with steroid responsive UC.

4) Then, we tried to obtain himmunoistochemical markers of small bowel inflammation in children with UC. We demonstrated that the density of CD25+ mononuclear cells is increase in jejunal mucosa of IBD patients, even in absence of gross endoscopic and histopathological abnormalities.

5) We also investigated the effect of probiotics (VSL#3) on Induction and manteinance of remission in Children with UC.

6) Finally, we riserve the last chapter of the thesis to EoE, an emerging disease in childhood: the EoE. We describe patients affected by celiac disease that also show the histological pattern of EoE. The unexpectedly high prevalence of CD in this EoE affected population and the probability of an association between gluten-induced disease and EoE prompted us to report these patients.

PAPERS AND
Alimentary Pharmacology & Therapeutics

Altered intestinal permeability is predictive of early relapse in children with steroid-responsive ulcerative colitis

E. MIELE, F. PASCARELLA, L. QUAGLIETTA, E. GIANNETTI, L. GRECO, R. TRONCONE & A. STAIANO

Department of Pediatrics, University of Naples "Federico II", Naples, Italy

Correspondence to:
Dr A. Staiano, Department of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5, 80131 Naples, Italy.
E-mail: staiano@unina.it

Publication data
Submitted 1 October 2006
First decision 6 October 2006
Resubmitted 8 January 2007
Resubmitted 9 February 2007
Accepted 9 February 2007

SUMMARY

Aim
To determine if small bowel involvement at diagnosis could predict early relapse in children with ulcerative colitis.

Methods
Children with newly diagnosed ulcerative colitis were evaluated prospectively at three time points: within 1 month, 6 months and 1 year after diagnosis. Clinical activity indices were used to measure disease activity. Laboratory studies were performed at each visit and/or at the time of relapse. At diagnosis, all patients underwent colonoscopy and a cellobiose/mannitol small intestinal permeability study. Some children were further investigated with an upper gastrointestinal endoscopy.

Results
Thirty-three patients completed the 1-year study. Overall, nine patients (27.3%) relapsed within 6 months of diagnosis, one patient (3%) within 1 year, whereas 23 patients (69.7%) did not relapse. The mean clinical activity indices, laboratory parameters, extent of colonic involvement, upper and lower gastrointestinal histological features were not predictive of early relapse. Results of the cellobiose/mannitol small intestinal permeability study were significantly higher in children who relapsed within 6 months compared with children who did not relapse ($P < 0.013$). The cellobiose/mannitol small intestinal permeability study was abnormal in 77.8% of early relapsers compared with only 8.3% of non-relapsers.

Conclusion
Abnormal small intestinal permeability in children with ulcerative colitis could predict a more relapsing disease.

Aliment Pharmacol Ther **25**, 933–939

doi:10.1111/j.1365-2036.2007.03291.x

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission with episodes of clinical relapse, which is characterized by an increase in symptoms.[1, 2]

A number of investigators have recently reported that diffuse gastritis is a common finding in patients with UC.[3–6] In contrast, focal gastritis and granulomas are indicative of Crohn's disease (CD), although focal gastritis occasionally has been observed in UC.[7, 8] In addition, other authors have found that the gastroduodenitis in CD and UC may be indistinguishable unless aphthous ulcers and granulomas are found, even if the histological findings in the upper gastrointestinal (UGI) tract appear to be less severe in UC patients.[3, 4, 9] The cause of the inflammation in the UGI tract of UC patients is not clear. It could be due to the patient's overall poor health or could be a response to yet-unknown immunological factors associated with the disease.[9]

Many histological and laboratory parameters [erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), platelet count, white cell count, interleukin 6, tumour necrosis factor α, interleukin 1β, interleukin 15 and antineutrophil cytoplasmic antibody (ANCA)] that reflect the systemic consequences of inflammation, and clinical disease activity indices have been proposed as predictors of clinical relapse of IBD in adults.[10] Faecal calprotectin has been suggested as a predictor of clinical relapse of disease activity in patients with CD and UC, whereas small intestinal permeability as a useful predictor of relapse in patients with small intestinal CD.[2] However, the predictive values of these different parameters in identifying patients at risk of relapse have generally been disappointing.[2, 10] Recently, it has been demonstrated that lower serum albumin levels and haematocrit and elevated ESR in children with CD at diagnosis may predict the need for immunomodulator therapy earlier in the disease course.[11]

The purpose of this study was to determine if UGI involvement, evaluated by small intestinal permeability test and/or histological studies, as well as clinical and laboratory parameters at diagnosis, could predict early relapse in children with UC.

MATERIALS AND METHODS

The study was a prospective, single-centre, 1-year study of children with newly diagnosed UC consecutively enrolled over a 24-month period at the Department of Pediatrics of the University of Naples "Federico II", Italy. Patients were recruited to participate in this study if they had a new diagnosis of UC established based on accepted historical, endoscopic, histological and/or radiological criteria.[12]

Participants were evaluated a three time points: within 1 month, 6 months and 1 year after commencement of treatment. At each visit data were collected including patient questionnaires regarding disease activity (stool frequency, stool consistency, haematochezia, abdominal pain, extra-intestinal manifestations of disease and overall patient functioning). Additional information collected at the first visit included demographic data, family history and symptom onset. Physical examination was performed at each visit by a paediatrician and included an abdominal examination and examination for 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. Individual patients' paediatric gastroenterologists exclusively made all decisions regarding therapeutic interventions.

Patients who failed to respond to corticosteroids (CS) (not showing regression of clinical symptoms to oral methylprednisolone, 1 mg/kg/day, max 40 mg/day or equivalent within 30 days, or intravenous treatment within 7–10 days), defined as CS resistant or CS refractory, and patients who initially responded to CS but then relapse with CS tapering or shortly after CS discontinuation, defined as CS dependent, were excluded from the study.[13]

Lichtiger colitis activity index (LCAI) (Table 1) and a physician's global assessment (PGA) were used to measure disease activity.[14] Individual scores for each section of the test including symptoms, characteristics of stool and physical examination were computed. A sustained drop in the LCAI to ≤ 2 after steroid therapy was considered remission. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in the LCAI to >2, sufficient to require treatment with CS, azathioprine/immunosuppressive agents or surgery.[2, 15] An early relapser was defined as a patient relapsing within 6 months from diagnosis, and a late relapser as a patient relapsing between 6 months and 1 year after diagnosis.

Laboratory studies including complete blood count, albumin, ESR and CRP were performed at each visit and/or at the time of relapse. Celiac and infectious diseases at diagnosis were ruled out.

Table 1. Lichtiger colitis activity index scoring

Symptom	Score
Diarrhoea (no. daily stools)	
0–2	0
3 or 4	1
5 or 6	2
7–9	3
10	4
Nocturnal diarrhoea	
No	0
Yes	1
Visible blood (% of movements)	
0	0
Less than 50	1
Greater than 50	2
100	3
Faecal incontinence	
No	0
Yes	1
Abdominal pain or cramping	
None	0
Mild	1
Moderate	2
Severe	3
General well-being	
Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5
Abdominal tenderness	
None	0
Mild and localized	1
Mild to moderate and diffuse	2
Severe or rebound	3
Need for antidiarrhoea drugs	
No	0
Yes	1

At baseline, before commencement of any treatment, ANCA titres were evaluated and all patients were asked to provide a single stool sample for the determination of faecal calprotectin levels according to the methods previously reported.[16] In addition, all patients underwent CMPS. Cellobiose and mannitol are oligosaccharides and small intestinal permeability probes. They are degraded by the bacterial flora of the large intestine and yield no information regarding colonic permeability characteristics.[17] The CMPS was performed after an overnight fast, prior to commencement of any treatment and in close temporal proximity to the endoscopic procedures. Each subject drank a solution containing 2 g mannitol, 5 g cellobiose and water to make 100 mL (osmolarity 270 mmol/L). Urine was collected for the next 5 h and stored at −20 °C. The subjects went without food during the test but were allowed to drink water after the first hour. Mannitol in urine was measured by the method of Corcoran and Page whereby it is oxidized to formaldehyde by periodic acid.[18] Urine cellobiose was measured using the method described by Strobel *et al.* in which the compound is digested by D-glucosidase to glucose.[19] The final ratio of percentage recovery of cellobiose to percentage recovery of mannitol was calculated. A cellobiose:mannitol ratio >0.022 was considered abnormal, as the value exceeded 2 s.d. over the mean derived from age- and sex-matched normal children.[20]

At diagnosis, all children underwent colonoscopy with mucosal biopsy; some children were further investigated with an UGI endoscopy. Biopsies were taken from the distal oesophagus, the gastric body and antrum, the duodenal bulb and/or third/fourth part of the duodenum. All children had a barium meal and small bowel follow-through.

All histological 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 histological criteria of Chong *et al.*[21] *Helicobacter pylori* was systematically sought in gastric antral biopsies using Giemsa staining and CLO colorimetric urease test.

Crohn's disease was excluded on the basis of the absence of: classical macroscopical lesions (e.g. skip lesions, snail track ulcers); non-caseating epithelioid and giant cell granulomas in any part of the gastrointestinal tract; histological findings of focal inflammation, submucosal or transmural inflammation, lymphocyte aggregates (without germinal centres) and mucous retention in the presence of more than minimal inflammation; fistulae and/or perianal abscesses; typical stricturing small bowel disease on barium follow-through.[22]

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 for normally distributed variables and the Mann–Whitney *U*-test and the chi-squared and Fisher exact tests for categorical variables were used where appropriate. Sample size calculation parameters for CMPS were: relapse in 30% of

cases; evaluation of minimal test group differences = 50%; type 1 error = 0.05; power = 90%. Ratio test group/control group 1:1, 10 subjects in each group were required [23]

Written, informed consent was obtained from 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".

RESULTS

Thirty-eight patients with newly diagnosed UC were enrolled in the study. Five patients were excluded because two (5.3%) children were CS refractory and three (7.9%) were CS dependent. Thirty-three patients completed the 1-year study. Demographic data for the study group are listed in Table 2. In all patients, the diagnosis of UC was confirmed by a median follow-up of 29 months (range: 17–40 months).

At diagnosis, all patients received oral methylprednisolone (1 mg/kg/day, max 40 mg/day per 4 weeks). After 4 weeks, all patients were in remission and began tapering off CS on a weekly basis (25%/week) according to the prescribed schedule on the basis of the LCAI. All patients received mesalamine maintenance therapy. Oral mesalamine 50 mg/kg was used in 20 patients (60.6%), one 2 g mesalamine enema every one to three nights was used in three patients (9.1%) and oral and topical mesalamine was used in 10 patients (30.3%). Overall, nine patients (27.3%) relapsed within 6 months of diagnosis, one patient (3%) within 1 year and 23 patients (69.7%) did not relapse during the follow-up. No significant differences in formulation of mesalimine used among early relapsers and non-relapsers were found (χ^2: 0.14; $P = 0.93$). Gender and age were not associated with early relapse ($P = 0.1$).

Linear growth and weight at diagnosis were normal in all children. No significant differences in weight or height at diagnosis were observed in early relapsers and non-relapsers. The mean LCAI performed at diagnosis by individual patients' paediatric gastroenterologist did not show differences among patients who had early relapse and those who did not (mean: 8.3 [range: 6–12] vs. 8.3 [range: 6–12], respectively; $P = 0.724$). On the basis of the PGA, the disease severity was classified as mild in 13% of non-relapsers vs. 22% of early relapsers, moderate in 49% of non-relapsers vs. 33% of early relapsers and severe in 38% of non-relapsers vs. 45% of early relapsers ($P = 0.647$).

Laboratory parameters at diagnosis including haematocrit, albumin, ESR, CRP and faecal calprotectin were not predictive of early relapse. Table 3 shows mean serum markers before a relapse (study entry) in patients who went on to relapse within 6 months, as well as at baseline and 6 months in those who did not relapse. Although 12-month measurements were available for the non-relapsers, they were not used in the analysis.

At baseline, ANCA was evaluated in 23 children (69.7%). Nine (39.1%) of 23 patients were perinuclear

Table 2. Baseline characteristics in 33 children with newly diagnosed UC

Characteristic	Value
Sex	
Male	13 (39.4%)
Female	20 (60.6%)
Mean age (year)	
Male	8.4 (range, 1–14)
Female	7.8 (range, 3–11)
Mean duration of symptoms at onset (months)	6.2 (range: 1–17)
Disease location	
Proctosigmoiditis	7 (21.2%)
Left-sided colitis	13 (39.4%)
Pancolitis	13 (39.4%)
Mean duration of steroid exposure (days)	75.7 (range: 44–140)

UC, ulcerative colitis.

Table 3. Serum parameters and faecal calprotectin in early relapsers vs. non-relapsers with UC

	Early relapsers (s.d.)	Non-relapsers (s.d.)	
	Baseline	Baseline	P^*
Mean laboratory markers			
Hematocrit (%)	32.3 (5.1)	34.3 (3.8)	0.122
Albumin (g/dL)	3.9 (0.2)	4.1 (0.4)	0.273
ESR (mm/h)	28.6 (23.2)	22.5 (16.7)	0.181
CRP (mg/dL)	1.4 (0.2)	1.6 (0.4)	0.176
Faecal calprotectin (μg/g)	272 (95.5)	260 (67)	0.719

UC, ulcerative colitis; s.d., standard deviation; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

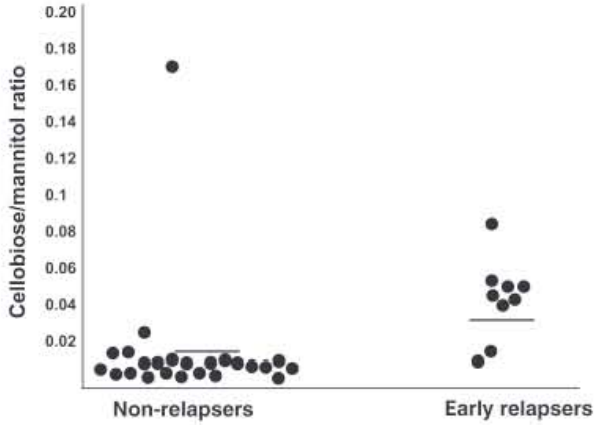


Figure 1. Relationship between cellobiose/mannitol ratio at diagnosis in patients with ulcerative colitis early and non-relapsers (lines represent the mean value). Higher cellobiose/mannitol ratio is related to earlier relapse ($P < 0.013$).

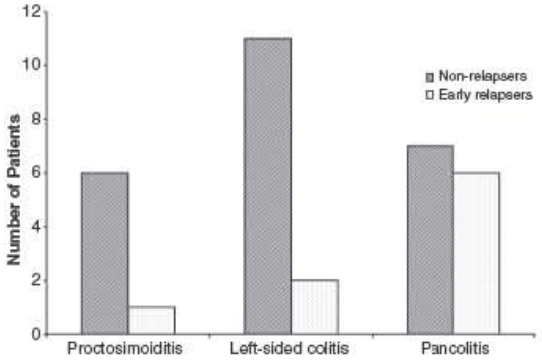


Figure 2. Relationship between colonic involvement and time to relapse ($P = 0.145$).

pANCA positive. ANCA status (i.e. presence/absence) did not change during the follow-up. The pANCA presence was not associated with earlier clinical relapse ($P = 0.666$).

Results of the CMPS were significantly higher for children who relapsed within 6 months (mean: 0.036, range: 0.008–0.1; $P < 0.013$) compared with children who did not relapse (mean: 0.017, range: 0.003–0.17) (Figure 1). Using a test cut-off of 0.022, the CMPS was abnormal in 77.8% of early relapsers compared with only 8.3% of non-relapsers. A cellobiose:mannitol ratio >0.022 gave a sensitivity of 77.7%, a specificity of 91.6%, a positive predictive value of 77.7% and a negative predictive value of 91.6% in predicting early relapse in UC paediatric patients.

Full ileo-colonoscopy was completed in 29 children (88%). All patients had rectal involvement with confluent inflammation. Lower endoscopic findings included mucosal granularity, friability, aphthoid ulcers, superficial and deep ulcers and inflammatory polyps.

There was a trend for early relapsers to present more extensive disease, but this finding did not meet statistical significance ($P = 0.145$) (Figure 2). No mucosal histological colonic feature was identified as a marker of earlier time to relapse. UGI endoscopy was performed in 15 (45.4%) children and revealed abnormalities in 13 (86%). The observed mucosal abnormalities included oesophageal erythema and/or erosions ($n = 7$), gastric oedema and erythema ($n = 7$), antral mucosal nodularity ($n = 2$) and duodenal oedema and hyperaemia ($n = 9$). Two (15.4%) patients had *H. pylori* on Giemsa staining of gastric antral biopsies. Histological examination included oesophagitis ($n = 7$), chronic inflammation of the oesophagus ($n = 1$), acute ($n = 2$) and chronic ($n = 7$) gastritis, duodenitis (erosions) ($n = 3$) and diffuse chronic duodenal inflammation with glandular distortion ($n = 3$). Histological UGI involvement was not significantly different in early relapsers vs. non-relapsers ($P = 0.530$, 0.311 and 0.5 for oesophagus, stomach and duodenum respectively). Barium meal and small bowel follow-through were normal in all patients.

DISCUSSION

Several clinical and laboratory markers have been studied in IBD as diagnostic aids, indicators of disease activity or severity, and to predict the risk of relapse in those patients in remission. In particular, the ability to reliably predict the risk of recurrence would help direct appropriate therapy to those who would most likely benefit from it and avoid the expense and potential toxicity of chronic maintenance therapy in those who have a low risk of recurrence.

To our knowledge, this study represents the first prospective evaluation of indicators for early relapse among paediatric patients with UC.

The natural history of our patients with UC was similar to that recently reported in paediatric patients.[24] Statistical analysis did not identify any differences in patient characteristics between the early relapsing and non-relapsing groups for age and sex. The extent of disease was not predictive of early relapse. This finding suggests that clinical severity may depend more upon deep inflammation than on superficial lesions accessible to the endoscopist, as suggested for adults

with CD.[25] Direct determination of mediators released by gut-associated lymphoid tissue, for example, lymphokines and their soluble receptors, may provide insights into the process taking place in the gut wall.[26] Previous studies in CD have shown that endoscopic findings have no predictive value for either the response to steroid treatment of an acute attack or the clinical course after steroid withdrawal.[27, 28]

The LCAI and the PGA at diagnosis were similar in early relapsers and non-relapsers. Both indices depend almost exclusively on clinical features that are often subjective, such as severity of abdominal pain, and may therefore not be truly representative of the degree of active inflammation and predictive of the risk of relapse.[29, 30] In our study, laboratory parameters at diagnosis, including haematocrit, albumin, ESR and CRP, were not predictive of early relapse, as reported in adult patients with UC.[10, 30]

Our study showed that faecal calprotectin concentration during the acute phase of disease is not useful in identifing early relapsing subjects. (p)ANCA presence was not associated with earlier clinical relapse, as has been reported in adults.[10] Tests of intestinal permeability are sensitive for the detection of patients with active small intestinal CD, are useful for assessing treatment, and may have prognostic implications.[31, 32] In our study, we demonstrated that an abnormal small intestinal permeability could be predictive of early relapse in paediatric patients affected by UC. In fact, abnormal permeability to cellobiose was found in 77.8% of early relapsed patients compared with only 8.3% of non-relapsing subjects, even though histopathological evaluation of small bowel biopsy was normal in two-thirds of our cases. This finding could be related, in part, to a patchy distribution of enteropathy. This observation is in agreement with previous studies which demonstrated that abnormal sugar permeability can occur in the presence of unequivocally normal small bowel histology.[18] This would suggest that there is a subtle alteration in function, independent of inflammation, which can manifest as in increase in paracellular permeability. An intriguing question is whether or not altered intestinal permeability plays a pathogenic role in UC as well as in CD. In CD, it has been hypothesized that increased permeability may lead to the absorption of endotoxin and lipopolysaccharides from the lumen. Both these substances are potent stimulators of acute-phase reactants and liberation of interleukin-6, which has been shown to be an important mediator of inflammation in CD. On the basis of our study, in a subgroup of paediatric patients with UC, a similar process may cause early relapse. The cause of the increase in permeability, however, is still unknown.[31, 33] A recent study suggests that intrarectal administration of trinitro-benzene sulfonate (TNBS) to rats influences not only their colon and terminal ileum, but also the proximal ileum and jejunum. Involvement of the ileum and jejunum in TNBS-induced colitis may be related to the systemic reaction of the immune system and mucosa to colitis.[34] Our study confirms an endoscopic and histological UGI involvement in UC, as previously described, but no significant differences were observed in early relapsers and non-relapsers.[4, 5]

In conclusion, this study strongly suggests that the gut inflammatory reaction in patients with UC is not restricted to the large intestine. Our results show that clinical, endoscopic and biological findings have no predictive value for early relapse. On the other hand, in children with UC an abnormal small intestinal permeability could predict a more relapsing disease. If these data are confirmed on a larger scale, the permeability test in presentation of disease may represent a noninvasive test useful in identifying those patients who will require more targeted treatment including immunomodulators. In addition, further studies on intestinal permeability may lead to a better understanding of the pathogenesis of UC.

ACKNOWLEDGEMENTS

Declaration of personal and funding interests: None.

REFERENCES

1 Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. *Gastroenterol Clin North Am* 1999; 28: 445–58.

2 Tibble JA, Sigthorsson G, Bridger B, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* 2000; 119: 15–22.

3 Ruuska T, Vaajalahti P, Arajarvi P, *et al.* Prospective evaluation of upper gastrointestinal mucosal lesions in children with ulcerative colitis. *J Pediatr Gastroenterol Nutr* 1994; 19: 181–6.

4 Kaufman SS, Vanderhoof JA, Young R, *et al.* Gastroenteric inflammation in children with ulcerative colitis. *Am J Gastroenterol* 1997; 92: 1209–12.

5 Tobin JM, Sinha B, Ramani P, *et al.* Upper gastrointestinal mucosal disease in pediatric Crohn disease and ulcerative colitis: a blinded, controlled study. *J Pediatr Gastroenterol Nutr* 2001; 32: 443–8.

6 Kundhal PS, Stormon MO, Zachos M, *et al.* Gastric antral biopsy in the differentiation of pediatric colitides. *Am J Gastroenterol* 2003; 98: 557–61.

7 Parente F, Cucino C, Bollani S, *et al.* Focal gastric inflammatory infiltrates in inflammatory bowel diseases: prevalence, immunohistochemical characteristics, and diagnostic role. *Am J Gastroenterol* 2000; 95: 705–11.

8 Sharif F, McDermott M, Dillon M, *et al.* Focally enhanced gastritis in children with Crohn's disease and ulcerative colitis. *Am J Gastroenterol* 2002; 97: 1415–20.

9 Abdullah BA, Gupta SK, Croffie JM, *et al.* The role of esophagogastroduodenoscopy in the initial evaluation of childhood inflammatory bowel disease: a 7-year study. *J Pediatr Gastroenetrol Nutr* 2002; 35: 636–40.

10 Bitton A, Peppercorn MA, Antonioli DA, *et al.* Clinical, biological, and histologic parameters as predictors of relapse in ulcerative colitis. *Gastroenterology* 2001; 120: 13–20.

11 Jacobstein DA, Mamula P, Markowitz JE, Leonard M, Baldassano RN. Predictors of immunomodulator use as early therapy in pediatric Crohn's disease. *J Clin Gastroenterol* 2006; 40: 145–8.

12 Hildebrand HF, Holmquist B, Kristiansson L, *et al.* Chronic inflammatory bowel disease in children and adolescents in Sweden. *J Pediatr Gastroenterol Nutr* 1991; 13: 293–7.

13 Faubion WA, Loftus EV, Haremsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; 121: 255–60.

14 Lichtiger S, Present D, Kornbluth A, *et al.* Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Eng J Med* 1994; 330: 1841–5.

15 Russell GH, Katz AJ. Infliximab is effective in acute but no chronic childhood ulcerative colitis. *J Pediatr Gastroenterol Nutr* 2004; 39: 166–70.

16 Berni Canani R, Rapacciuolo L, Romano MT, *et al.* Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice *Dig Liv Dis* 2004; 36: 767–70.

17 Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. *Gut* 2006; 55: 1512–20.

18 Corcoran AC, Page IH. A method for the determination of mannitol in plasma and urine. *Biol Che* 1947; 170: 165–71.

19 Strobel S, Brydon WG, Ferguson A. Cellobiose/mannitol sugar permeability test complements biopsy histopathology in clinical investigation of the jejunum. *Gut* 1984; 25: 1241–6.

20 Troncone R, Mayer M, Mugione P, Cucciardi M, Abete A, Greco L. Cellobiose/mannitol sugar permeability test in children in relation to jejunal morphometry. *Ital J Gastroenterol* 1995; 27: 489–93.

21 Chong SK, Blackshaw AJ, Boyle S, William C, Walker-Smith JA. Histological diagnosis of chronic inflammatory bowel disease in childhood. *Gut* 1985; 26: 55–9.

22 Castellaneta SP, Afzal NA, Greenberg M, *et al. Diagnostic role of upper gastrointestinal endoscopy in pediatric inflammatory bowel disease.* J Pediatr Gastro enterol Nutr 2004; 39: 257–61.

23 Snedecor GW, Cochran WG. *Statistical methods.* Iowa University Press, Ames, Iowa, USA, 2000.

24 Hyams J, Markowitz J, Lerer T, *et al.* The natural history of corticosteroid therapy for ulcerative colitis in children. *Clin Gastroenterol Hepatol* 2006; 4: 1124–9.

25 Cellier C, Sahmoud T, Froguel E, *et al.* Correlations between clinical activity, endoscopic severity and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. The Group d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gut* 1994; 35: 231–5.

26 Monteleone G, Fina D, Caruso R, Pallone F. New mediators of immunity and inflammation in inflammatory bowel disease. *Curr Opin Gastroenterol* 2006; 22: 361–4.

27 Modigliani R, Mary JY, Simon JF, *et al.* Clinical biological and endoscopic picture of attacks of Crohn's disease; evolution on prednisolone. *Gastroenterology* 1990; 98: 811–8.

28 Landi B, Nguyen Anh T, Cortot A, *et al.* Endoscopic monitoring of Crohn's disease treatment: a prospective randomised clinical trial. The Group d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gastroenterology* 1992; 102: 1647–53.

29 Jorgensen LGM, Fredholm L, Petersen PH, Hey H, Munkholm P, Brandslund I. How accurate are clinical activity indices for scoring of disease activity in inflammatory bowel disease (IBD)*?. Clin Chem Lab Med* 2005; 43: 403–11.

30 Costa F, Mumolo MG, Ceccarelli L, *et al.* Calprotectin is as stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. *Gut* 2005; 54: 364–8.

31 Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I. Intestinal permeability in Crohn's disease and its relation to disease activity and relapse following treatment with elemental diet. *Eur J Gastroenterol Hepatol* 1993; 5: 79–84.

32 Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal permeability and the predictor of relapse in Crohn's disease. *Lancet* 1993; 341: 1437–9.

33 Gross V, Andus T, Caesar I, Roth M, Scholmerich J. Evidence for continuous stimulation of interleukin 6 production in Crohn's disease. *Gastroenterology* 1992; 102: 514–9.

34 Amit-Romach E, Reifen R, Uni Z. Mucosal function in rat jejunum and ileum is altered by induction of colitis. *Int J Mol Med* 2006; 18: 721–7.

Journal of Pediatric Gastroenterology and Nutrition
44:529–539 © 2007 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

Invited Review

Functional Consequences of *NOD2/CARD15* Mutations in Crohn Disease

*Lucia Quaglietta, †Anje te Velde, *Annamaria Staiano, *Riccardo Troncone, and ‡Daan W. Hommes

*Department of Pediatrics, University Federico II, Naples, Italy, †Departments of Experimental Internal Medicine, and ‡Gastroenterology and Hepatology, Academic Medical Centre, Amsterdam, The Netherlands

ABSTRACT

Crohn disease (CD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract. Its etiology remained obscure until recently, when, through an overwhelming body of research, the main theme of its origin became clear. CD develops in individuals who carry risk alleles for the disease that can cause a loss of physiological tolerance to commensal bacteria. As a consequence, immune responses develop that activate a whole range of immunocompetent cells, resulting in the secretion of proinflammatory mediators that ultimately cause mucosal breaks and the formation of ulceration, edema, and loss of proper function. ***JPGN 44:529–539, 2007.*** **Key Words:** Crohn disease—*NOD2* polymorphisms—Pattern recognition molecules. © 2007 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

CLINICAL PHENOTYPE

Crohn disease (CD) usually develops in the young adult, with the majority of cases diagnosed when the patient is between 15 and 35 years of age. However, CD can affect people at any age and approximately 10% of cases are in patients younger than 18 years of age at presentation. CD predominantly affects white patients, with a prevalence rate of 150/100,000. Inflammatory bowel disease (IBD) is largely a disease of the industrialized world, especially the United States and Europe, and is more common in urban areas and northern climates. No clear-cut Mendelian pattern of inheritance in CD has been established, but CD has a known genetic component, with 25% of patients with CD having a family member with some form of IBD (1).

Signs and symptoms of CD include frequent (bloody) diarrhea, abdominal pain, fatigue, loss of appetite, weight loss, fever, stomatitis, and perianal fistula or fissures. A proportion of patients present with extraintestinal manifestations such as arthritis, erythema nodosum, or pyoderma gangrenosum. In pediatric cases of CD, growth failure is observed in 75% of patients (1).

Clear evidence exists for the activation of the immune response in CD. The lamina propria is infiltrated with lymphocytes, macrophages, and other cells of the immune system. In any immune response a specific antigen serves as a trigger for the response and as a target for the effector arm of the response. During the past 35 years, an intensive search has been conducted for the antigens that trigger the immune response in CD. Immune activation in CD is largely confined to the gastrointestinal tract; therefore, the search for the antigenic trigger has focused on the intestinal lumen. Most of the foreign antigens in the intestinal lumen are of microbial or dietary origin (2).

Three major hypotheses on the antigenic trigger in CD have been postulated. One hypothesis is that the antigenic triggers are microbial pathogens that have not yet been identified because of fastidious culture requirements. According to this hypothesis, the immune response in CD is an appropriate but ineffective response to these pathogens. Various viruses and bacteria have been proposed as candidate organisms, but little evidence has been found to support any of these organisms as having a causative role in CD. The second hypothesis is that the antigenic trigger in CD is some common dietary antigen or usually nonpathogenic microbial agent against which

Received November 29, 2006; accepted January 15, 2007.

Address correspondence and reprint requests to Daniel W. Hommes, Department of Gastroenterology and Hepatology, C2-111, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands (e-mail: d.w.hommes@amc.uva.nl).

the patient mounts an abnormal immune response. In healthy people a finely tuned, low-grade chronic inflammation is present in the intestinal lamina propria. Presumably, this chronic inflammation is a product of chronic exposure of the lamina propria to luminal antigens. Failure to suppress this inflammatory response could result in the uncontrolled immune activation seen in CD. As a result of failure of normal suppressor mechanisms, immune activation in CD may be an inappropriate vigorous and prolonged response to some normal luminal antigens. The third hypothesis for CD is that the antigenic trigger is one expressed on the patient's own cells, particularly intestinal epithelial cells. This is an autoimmune theory for the pathogenesis of CD. In this theory, the patient mounts an appropriate immune response against some luminal antigen, either dietary or microbial; however, because of similarities between proteins on epithelial cells and the luminal antigen, the patient's immune system also attacks the epithelial cells (3).

IMMUNE RESPONSE

Crohn disease is a consequence of a disturbance in the normal immunological unresponsiveness of the mucosal immune system to components of the mucosal microflora. The hyperresponsiveness to these components that ensues gives rise to the T helper type 1 (Th1) cell–mediated inflammation that underlies all forms of the disease. Activated Th1 cells secrete cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-2, and interferon (IFN)-γ. IFN-γ, in turn, activates macrophages, causing them to secrete excess proinflammatory cytokines (eg, IL-1β, TNF-α). Activated macrophages contribute to epithelial injury by secreting TNF-α and reactive oxygen species and by recruiting neutrophils, which also produce free radicals. Neutrophils are also recruited by IL-8 secreted by epithelial cells following activation or injury by bacteria. Neutrophils release reactive oxygen species, and oxygen species injure the epithelial cells. Together, the macrophages and neutrophils produce prostaglandin E2 and leukotriene B4 that contribute to the vasodilation and enhanced vascular permeability characteristic of CD (4). CD bears the immunological stigmata of an exaggerated $CD4^+$ Th1 response. Thus, intestinal $CD4^+$ T cells isolated from patients with CD produce large amounts of the Th1 signature cytokine IFN-γ. Mucosal macrophages from patients with CD also produce large amounts of the Th1-inducing cytokines IL-12, IL-18, and TNF-α. Th1 cell resistance to apoptosis and increased cell cycling in CD inflammation appear to be sustained by these cytokines (5). Blocking the pathways that confer resistance of Th1 cells to apoptotic stimuli and using drugs that enhance mucosal T cell death, such as the immunosuppressive agent azathioprine or the antibody to TNF-α infliximab, are effective in downmodulating intestinal inflammation (6).

Identifying the particular antigen(s) that drive the Th1 inflammatory response in the face of the large amount of potential antigens in the gut has proven difficult. Nevertheless, the likelihood is that bacterial antigens are involved, because stimulation of mucosal CD4 cells from patients with CD with extracts of their own commensal flora can induce IFN-γ production (7). Clinical observations also support a role for antigens derived from the commensal flora. Thus, for example, the antibiotic metronidazole is of therapeutic benefit in CD of the distal colon, eliminating the commensal flora and resulting in decreased inflammation (8).

The cellular and molecular mechanisms of interaction between intestinal mucosal cells and the resident luminal bacteria in healthy individuals and patients with CD is not yet fully understood, but is an area of active investigation. Recently, mutations in *NOD2/CARD15*, encoding an intracellular bacteria-sensing protein expressed mainly by macrophages and dendritic cells, have been associated with CD.

Different theories exist for nuclear factor–κB (NF-κB) activation in *NOD2* variants. Mutations in *NOD2* cause a loss of function of *NOD2*, resulting in a decreased suppression of the Toll-like receptor-2 (TLR2)–driven Th1 response via NF-κB. Conversely, it has been hypothesized that mutated *NOD2* enhances (ie, causes gain of function) the sensitivity of macrophages to the nucleotide-binding oligomerization domain protein (NOD) ligand muramyl dipeptide (MDP), potentiating NF-κB activity. This review focuses on the functional consequences of *NOD2/CARD15* mutations in the pathogenesis of CD.

PATTERN RECOGNITION MOLECULES

The host mucosa is exposed to vast numbers of metabolically active microbial cells and cell wall components, such as lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, and peptidoglycan (PGN), a complex amino sugar present in Gram-negative and Gram-positive bacteria. A unique feature of host–microbial interactions in the intestine is the lack of proinflammatory responses in the mucosa exposed to the resident luminal microflora while retaining the capability to respond to luminal pathogenic bacteria via the recruitment of acute inflammatory cells from the systemic circulation. The gut epithelium itself can also directly sense commensal bacteria and pathogens; integral to this are the mammalian pattern recognition receptors (PRRs), which recognize conserved structures of bacteria and viruses and generally activate proinflammatory pathways alerting the host to infection. Two different classes of PRRs are involved (9,10). The TLRs are usually associated

with cell membranes and have an external leucine-rich repeat (LRR) recognition domain and an intracellular IL-1 receptor (IL-1R)–like signaling domain that activates intracellular signaling pathways (11). To date, 11 members of the TLR family have been identified in mammals. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2, and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast, TLR3, TLR7, and TLR9, all of which are involved in the recognition of nucleic acid–like structures, are not expressed on the cell surface. For example, TLR9 has recently been shown to be expressed in the endoplasmic reticulum (11). After ligand binding TLRs/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signaling molecules (10). These include the adaptor molecule myeloid differentiation primary response protein 88 (MyD88), which activates the serine/threonine kinases of the IL-1R–associated kinase family (IRAK) and TNF receptor–associated factor 6 (TRAF6), subsequently leading to the degradation of the inhibitor of NF-κB activity protein IκB. This results in the activation of NF-κB and its translocation to the nucleus (12). In addition to TLRs, microbial products can be recognized by members of the NOD family.

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN

The NODs are cytosolic proteins that contain a nucleotide-binding oligomerization domain. These proteins include key regulators of apoptosis and pathogen resistance in mammals and plants. A large number of NODs contain leucine-rich repeats (LRRs), hence referred to as NOD-LRR proteins. Genetic variation in several NOD-LRR proteins, including human Nod2, Cryopyrin, and MHC class II transactivator (CIITA), as well as mouse neuronal apoptosis inhibitor protein (NAIP) 5, is associated with inflammatory disease or increased susceptibility to microbial infections. Nod1, Nod2, Cryopyrin, and Ipaf have been implicated in protective immune responses against pathogens. Together with Toll-like receptors, Nod1 and Nod2 appear to play important roles in innate and acquired immunity as sensors of bacterial components. Specifically, Nod1 and Nod2 participate in the signaling events triggered by host recognition of specific motifs in bacterial peptidoglycan and, upon activation, induce the production of proinflammatory mediators. NAIP5 is involved in host resistance to *Legionella pneumophila* through cell autonomous mechanisms, whereas CIITA plays a critical role in antigen presentation and development of antigen-specific T lymphocytes. Thus, NOD-LRR proteins appear to be involved in a diverse array of processes required for host immune reactions against pathogens.

Family of NOD-LRR Proteins

The NOD-LRR proteins are also referred to as the CATERPILLER (CARD, transcription enhancer, R [purine]–binding, pyrin, lots of leucine repeats) family (13). NOD-LRR proteins are expressed primarily in immune cells, although the expression of certain proteins, such as Nod1, is ubiquitous. The majority of animal and plant NOD-LRR proteins are composed of 3 distinct functional domains: an aminoterminal effector domain involved in signaling, a centrally located regulatory NOD-domain, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (Fig. 1).

The effector domains of mammalian NOD-LRR proteins are structurally variable, linking these proteins to multiple signaling pathways and biological functions. These effector domains are involved in homophilic and heterophilic interactions with downstream signaling partners. The diversity of the effector domains allows NOD-LRR proteins to interact with a wide array of binding partners and to activate multiple signaling pathways. Effector domains involved in homophilic association include the caspase-recruitment domain (CARD) (14) and the pyrin domain (PYD, also called DAPIN and PAAD) (15). Both the PYD and the CARD belong to the death domain-fold family characterized by 6 α-helices that are tightly packed and include the death domain (DD) and death effector domain (DED) (16). Only 3 human NOD-LRR proteins possess amino-terminal CARDs, whereas NOD-LRR proteins possessing a PYD are by far the most numerous and include 14 proteins designated as NACHT, leucine-rich repeat, and pyrin domain-containing family proteins (NALPs).

Several NOD-LRR proteins contain amino-terminal sequences that are not involved in homophilic protein interactions, including NAIPs and CIITA (17,18). Instead of a CARD or PYD, the amino termini of NAIPs are composed of amino-terminal baculovirus-inhibitor-of-apoptosis repeats (BIRs) (19). CIITA is a transcriptional coactivator involved in the regulation of major histocompatibility complex class (MHC) genes, especially class II (MHC-II) (17,19). CIITA contains an amino-terminal transcriptional activation domain that is essential for MHC gene transactivation through its interaction with multiple nuclear factors, including CBP/p300, RFX5, NF-Y, and CREB (19) (Fig. 2).

Detection of Microbial Products by NOD-LRR Proteins

Whereas initial studies identified lipopolysaccharide (LPS) as a NOD2 ligand (20), it is now well established that the NOD1 and NOD2 ligands are the peptidoglycan

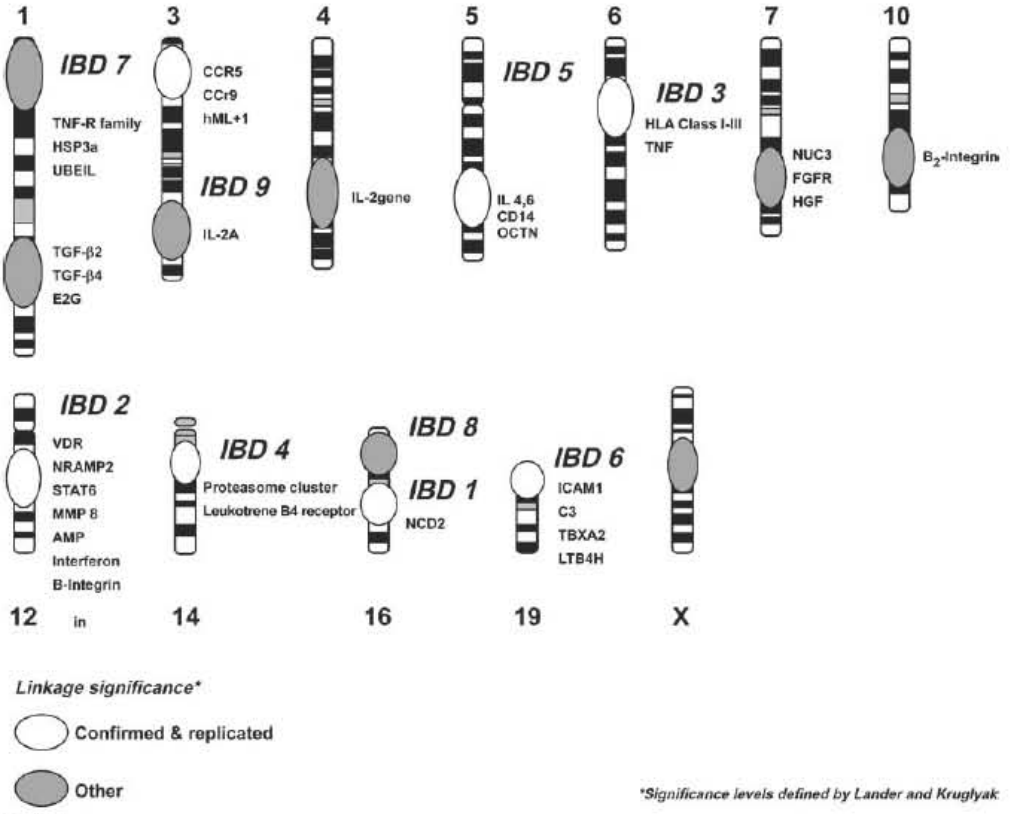


FIG. 1. IBD linkage areas.

(PGN)–derived peptides γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (21,22) and MDP, respectively (23,24). Because PGN from Gram-positive and Gram-negative bacteria contains MDP, NOD2 functions as a general sensor of most, if not all, bacteria. By contrast, because PGNs from Gram-positive bacteria do not contain iE-DAP (except for PGNs derived from specific Gram-positive bacteria such as *Listeria* and *Bacillus* species and from many Gram-positive bacteria in the soil) (25), NOD1 mainly senses products from Gram-negative bacteria. Confirmation of these specificities has come from the finding that macrophages isolated from NOD1- or NOD2-deficient mice are completely unresponsive to their respective ligands (21,26). The NOD protein ligands need to reach the LRR domains of the respective NOD protein for activation of this protein to be initiated. However, information about how this is accomplished is sparse at present, especially in the case of antigen-presenting cells (APCs). One possibility that relates to phagocytic cells such as macrophages or dendritic cells (DCs) is that these cells generate the peptide ligands by ingesting whole bacteria and then digesting them in phagolysosomes (27,28). In epithelial cells a slightly different process may occur in that the apical peptide transporter PEPT1 seems to play a role in the delivery of MDP. This is indicated by the finding that

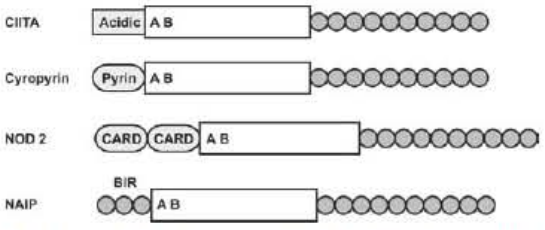


FIG. 2. Domain organization of the CATERPILLER proteins. MHC class II transactivator (CIITA), cryopyrin, NOD2, and NAIP represent the 4 main groups of CATERPILLER proteins, which are distinguished by their distinct amino (N)-terminal domains. The four possible N-terminal domains are the acidic, pyrin, CARD, and baculoviral inhibitory repeat (BIR) domains. All have the NBD-LRR configuration, with A and B depicting the Walker A and B motifs of the NBD. CIITA is the only family member with a documented role as a transcription factor.

MDP that is taken up by PEPT1 into colonic epithelial cells subsequently mediates the activation of NF-κB (29). In addition, it has recently been shown that *Helicobacter pylori* can "inject" PGN into cells through a type IV secretion system, which is encoded by a pathogenicity island (30). This discovery indicates that PGN can enter cells by various mechanisms that involve bacteria–host interactions. After small peptides derived from PGN have been released into the cytosol, they are thought to interact with NOD1 or NOD2 through the LRR domains of these molecules. However, it should be noted that, as is the case for activation of most TLRs by their respective ligands (31), there is as yet no direct evidence for the binding of the NOD1 and NOD2 ligands to these domains. The postulated interaction is then proposed to initiate the activation of NOD1 and NOD2 through the induction of a complex conformational change (32,33). Our understanding of this change comes in part from studies of activation of apoptotic protease-activating factor 1 (APAF1), an NLR-family (*NACHT-LRR* family domain present in *NAIP, CIITA, HET-E,* and *TP1-LRR* families) member that is involved in caspase activation and apoptosis (34,35). Activation of APAF1 is initiated by the interaction of its WD40 domain with its ligand (cytochrome *c*), as well as by the binding of dATP or ATP to an ATP-binding cassette (ABC) or oligomerization cassette in the NOD. The molecule then undergoes self-oligomerization, which enables it to bind its downstream effector molecule, caspase-9, through a CARD–CARD interaction. The large molecular complex that is formed in this way, which is known as the apoptosome, then facilitates activation of the bound caspase-9, possibly by bringing caspase molecules into juxtaposition (34,35). That this activation model applies to NLRs in general (and to NOD1 and NOD2 in particular) is indicated by the presence of structural similarities between NOD proteins and APAF1: the N-terminal region of the central NOD in NLRs contains an ABC and an oligomerization module. At least in the case of NOD2, the introduction of mutations into the ABC region abolishes NOD2 signaling (33). In addition, it has been shown that both NOD1 and NOD2 undergo self-oligomerization following the binding of PGN-derived ligand (32,33). In one model of NOD-protein activation based on the APAF1–caspase-9 pathway, Inohara et al (25) proposed that oligomerization of NOD1 or NOD2 also allows binding to a downstream effector molecule through a CARD–CARD interaction, in this case involving the Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK; also known as RIP2 or CARDIAK), and this, in turn, leads to cross-activation of RICK. However, further work is necessary to establish this possibility.

Signal Transduction Pathways of NOD-LRR

One of the main outcomes of NOD1 and NOD2 activation by their respective ligands is the activation of NF-κB. Whereas such activation was clearly evident in iE-DAP- or MDP-stimulated epithelial cells that had been transfected with constructs encoding wild-type NOD1 or NOD2, it was reduced in cells that were transfected with constructs encoding a mutated NOD2 that had alterations in the LRR domain (23).

Consistent with this, after stimulation with MDP, translocation of NF-κB subunits to the nucleus is observed in human and mouse APCs that have intact NOD2 but not in APCs that are deficient in NOD2 or have a mutation in NOD2 (23,34). The activation of NF-κB by NOD1 and NOD2 occurs exclusively through the downstream effector molecule RICK. This is demonstrated by the finding that transfection of RICK-deficient fibroblasts with constructs encoding NOD1 or NOD2 results in severely defective NF-κB activation (35). However, it should be noted that RICK-deficient macrophages also have reduced cytokine responses following stimulation with LPS, lipoteichoic acid, and PGN, indicating that TLR2 and TLR4 may also use RICK as a downstream effector molecule, although the existence of a TLR4–RICK pathway is controversial (35). RICK is a CARD-containing serine/threonine kinase that physically associates with the CARD(s) of NOD1 and NOD2 through CARD–CARD interactions (36,37). As shown recently by Abbott et al (38), following its activation by NOD2, RICK mediates K63-linked polyubiquitylation of inhibitor of NF-κB (IB)-kinase-γ (IKK-γ; also known as NEMO; the key member of the IKK complex) at a unique ubiquitylation site (the lysine residue at position 285). As shown previously, K63-linked polyubiquitylation is associated with activation of the NF-κB pathway (39), and in the case of the RICK–IKK-γ interaction, it is indeed followed by phosphorylation of IKK-γ and downstream activation of NF-κB, leading to the translocation of transcriptional components of NF-κB to the nucleus. So, in activating the IKK complex, RICK activates an E3 ubiquitin ligase that promotes K63-linked polyubiquitylation or inhibits an enzyme (eg, cylindromatosis protein) that de-ubiquitylates proteins that are modified with K63-linked polyubiquitin, so RICK does not require its own kinase activity for this function. Recently, it has been shown that activated NOD2 (but not NOD1) also interacts with the intracellular molecule GRIM19 (gene associated with retinoid-IFN–induced mortality 19) and that such an interaction may be required for optimal NF-κB activation. However, neither the structural basis of this interaction nor the mechanism of its relation to NF-κB activation is known (40). Another outcome of NOD1 and NOD2 activation is the activation of the mitogen-activated protein kinase (MAPK) pathway. Thus, stimulation of wild-type macrophages, but not NOD2-deficient macrophages, with MDP leads to activation of p38MAPK and extracellular signal–regulated kinase (ERK) (41,42). In addition, activation of NOD1 by its ligand leads to the

activation of JUN N-terminal kinase (JNK) (43). The mechanism of such NF-κB-independent signaling is unknown at present. Finally, it has been shown in transfection and immunoprecipitation studies that NOD2 binds procaspase-1, and when cells are transfected with constructs encoding NOD2 and procaspase-1, NOD2 induces IL-1β secretion (44). Because caspase-1 is required for processing pro-IL-1β (45) into mature IL-1β, NOD2 may bind procaspase-1 through a CARD–CARD interaction in the same way that it binds RICK and, in doing so, convert the procaspase into a caspase. However, whether NOD2 has this function under physiological conditions remains to be seen.

GENETIC NOD2 POLYMORPHISMS

A role for genetic factors in CD was first suggested by epidemiological studies showing familial aggregation of disease and by twin studies that reported greater concordance for disease in monozygotic twins compared with dizygotic twins (46). During the past 8 years, this evidence has been supplemented by molecular data from genome-wide linkage studies of multiple affected IBD families. These studies have been remarkably successful in identifying a number of susceptibility loci, with convincing replication shown for at least 7 loci (IBD1–7; Fig. 1). Some loci have been shown to be specific to ulcerative colitis (eg, IBD2) or CD (IBD1), whereas others confer common susceptibility on IBD. Collectively, these genome scans reaffirm the concept that IBDs are complex genetic disorders with several predisposing genes (47).

In 2001, 3 independent groups reported the identification of the first CD susceptibility gene, *NOD2* (renamed *CARD15* by the international nomenclature committee), on chromosome 16q12 (IBD1) (47). This major breakthrough firmly established a role for genetics in determining susceptibility to CD and has provided proof of principle that model-free linkage analyses may be used successfully to identify disease susceptibility gene loci. Recent studies have highlighted a number of associations between genotype and phenotype. These studies suggest that genetics also may influence the clinical manifestations of CD including disease location, behavior, natural history and response, and side affects of drug therapy (46). These discoveries may allow more accurate prediction of disease, permitting the implementation of highly specific therapy tailored to an individual's genotype.

NOD2/CARD15 has gained recent prominence through its association with increased susceptibility to CD. Thirty nonconservative mutations, also called variants or polymorphisms, associated with CD have been identified within the *NOD2/CARD15* gene, but only 3 are common. Lesage et al (48) showed that the 3 common mutations—Arg702Trp, Gly908Arg, and Leu1007fsinsC—account for 82% of the mutated alleles. The relative risk to develop the disease when carrying 1 mutation is 2 to 3, but increases dramatically to 20 to 40 in case of 2 mutations (49). Approximately 40% to 50% of patients carry at least 1 mutation in the *NOD2/CARD15* gene but heterogeneity has been reported, and in at least 3 populations (Japanese, Korean, and black), the gene is not implicated in CD (50–54). The prevalence of CD in western Europe is 1 to 2/1000, so it is possible to deduce from these relative risks that probability of developing the disease is 4% to 8% in the group with 2 mutations. However, the penetrance is modest: <10% of all people carrying 2 CARD15 risk alleles will develop CD, which means that other genes and environmental stimuli are needed for disease expression (55). Structure of the *CARD15* gene and location of the CD-associated variants are shown in Figure 3.

FUNCTIONAL CONSEQUENCES

The mechanism by which *CARD15* mutations cause susceptibility to CD is poorly understood: 3 main views are being considered at present (Fig. 4).

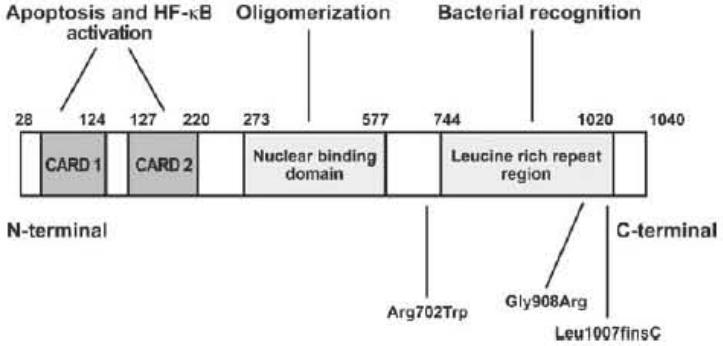


FIG. 3. Structure of the CARD15 gene and location of the CD-associated variants. The numbers represent the amino acid position.

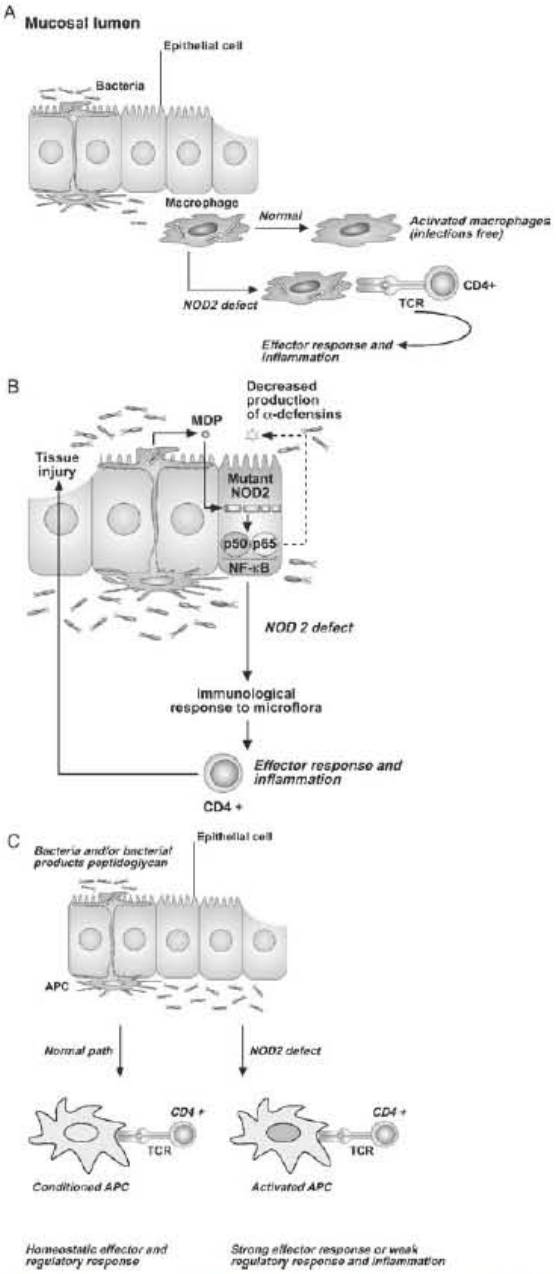


FIG. 4. Possible mechanisms of Crohn's disease caused by *NOD2* mutations. A, Defective function of macrophages leads to persistent intracellular infection of macrophages and chronic stimulation of T cells by macrophage-infecting organism. B, Defective epithelial-cell responses lead to loss of barrier function and increased exposure to the mucosal microflora. C, Defective "conditioning" of APCs leads to inappropriate activation of APCs and disruption of the homeostatic balances of effector and regulatory cells.

Because signaling via mutated NOD2 proteins leads to defective activation of the transcription factor NF-κB, one proposal is that mutations cause deficient NF-κB–dependent Th1 responses and increased susceptibility to infection. Two recent studies used genetically altered mouse models to address this hypothesis and have come to surprisingly different conclusions.

In the first study, Meada et al (56) introduced into mice a mutation of *CARD15* homologous to the major mutation in human CD (Leu007fsinsCys), which resulted in a truncated protein lacking the last 33 amino acids. This model theoretically mimics the genetic defect in CD. They examined the effect of the *NOD2* mutation on NF-κB activation in bone marrow–derived macrophages after stimulation with various TRL ligands or TRL2 ligand (PGN) plus MDP. No differences between wild-type and mutated mice were seen after stimulation, except in the case of stimulation with MDP alone, where the authors found that macrophages of these mutant mice had enhanced sensitivity to MDP relative to WT counterparts with increased production of IL-1β, a target of the proinflammatory mediator NF-κB. These data, together with the finding that knock-in mice were more susceptible to dextran sulfate sodium–induced colitis and showed increased amounts of IL-1β, IL-6, and cyclooxygenase-2 protein in colons relative to wild-type counterparts, indicated that the frameshift mutation associated with CD is a gain of function that results in disease associated with IL-1β (and perhaps IL-6) production. Thus, the authors argue that individuals with *NOD2* mutations may have an enhanced responsiveness to bacterial PGN, resulting in high levels of production of proinflammatory cytokines by intestinal macrophages.

This model cannot explain in vitro data showing that epithelial cells transfected with *CARD15* that contains CD-associated mutations have defective NF-κB activation in response to stimulation with MDP. More important, it also cannot explain why peripheral blood mononuclear cells (PBMCs) isolated from patients with CD who have a frameshift mutation in *CARD15* show a marked defect in IL-1β production rather than increased IL-1β production. Finally, these knock-in mice did not have any abnormality in the production of Th1 cytokines, which is an almost universal finding in CD (57).

To investigate this gain of function hypothesis in human, Zelinkova et al (58) used monocyte-derived DCs from patients with CD who carried double-dose *NOD2* mutations and wild-type controls. Mature DCs were stimulated with MDP and the production of the proinflammatory cytokines TNF-α and IL-12 was measured. Mature DCs from *NOD2* mutants showed significantly increased IL-12p70 and TNF-α production upon stimulation with MDP compared with wild-type controls. These first observations in humans support the hypothesis of *NOD2* variants associates with CD acting

as a gain-of-function allele supporting the regulatory role of *NOD2* as the basic cause of disease.

Conversely, Kobayashi et al (42) proposed a loss-of-function mutation in *NOD2* in vivo that affects mainly epithelial cells rather than macrophages. They generated NOD2$^{-/-}$ mice using a targeting construct to replace the NOD, which is essential for the activation of the protein. The mutant animals displayed no symptoms of intestinal inflammation when observed for as long as 6 months, and there was no significantly enhanced susceptibility to colitis in the dextran sulfate sodium model. Their mutant mice showed a lack of responsiveness to MDP in several assays. The mice also developed a more severe infection with *Listeria monocytogenes* when given orally versus systematically, indicating a loss of control over intestinal infection, but not an overall suppressed ability to defend against the pathogen in NOD2-deficient mice. To investigate potential genes that may be induced by NOD2 during intestinal infection, they isolated RNA samples from wild-type and NOD2$^{-/-}$ terminal ileum Paneth cells before and after *Listeria* infection and screened them by microarray analysis. The most significant difference was in the expression of a subgroup of cryptdins (analogous to α-defensins in human). Cryptidins are antimicrobial peptides that are produced in intestinal Paneth cells of mice, and their antimicrobial activity is important in suppressing infection with pathogenic bacteria. Their results indicate that NOD2 is essential in the detection of bacterial MDP and the regulation of cryptdin (α-defensins in human) expression in Paneth cells. Paneth cells are located at the (terminal) ileum, the side where patients with CD who have *NOD2* gene mutations are mostly affected. These data support the concept that *NOD2* mutations produce a kind of immunodeficiency state that predispose humans to a type of bacteria.

A third resolution emerged from a study by Watanabe et al (34) in which a second mouse strain was also made completely deficient in NOD2 (target deletion of exon 1); these mice had a reduced response to MDP, yet had enhanced responses to PGN inducing elevated levels of IL-12. To explain this finding, the authors invoked an additional signal delivered through TLR2, which was supported by studies with a second TRL2 ligand and purified MDP. Thus, activation of normal NOD2 inhibited signals codelivered through TLR2. A loss of function mutation of *NOD2* together with TLR2 signals delivered by other bacterial products will result in enhanced cytokine responses by macrophages (or DCs) to commensal bacteria and result in inflammation. The data from Watanabe and colleagues (34) emphasize the importance of innate immunity in driving a chronic inflammatory disease. Targeting TLR2 signaling may therefore be a useful approach in the treatment of individuals with CD who carry the *NOD2* mutation. Another outcome of this study is the possibility of using NOD2 ligands as anti-inflammatory agents. This may explain the anti-inflammatory effects of certain Gram-positive bacteria used in the treatment of IBD.

In addition to the results in mice, Wehkamp et al (59) suggest that the expression of α-defensins is diminished in humans with CD, particularly those who have *NOD2* gene mutations. They compared mucosal levels of the human α-defensins—epithelial human defensin 5 (HD5) and epithelial human defensin 6 (HD6)—in patients with CD with respect to *NOD2* genotypes and in healthy controls. They found diminished HD5 and HD6 expression in the ileum of patients carrying a *NOD2* mutation compared with controls or those with nonileal CD. In addition to the NOD2$^{-/-}$ mouse model, they suggest that the defensin deficiency secondary to *NOD2* mutations in humans could lead to a breakdown of the mucosal barrier with a secondary inflammation, leading to the development of CD.

Recent reports have shown synergy between NOD2 activation and several TLR ligands in cellular responses. van Heel et al (60) demonstrated, using primary human cells of differing *NOD2* genotypes, that *NOD2* stimulation normally synergistically enhances TRL9 responses (TNF-α and IL-8 secretion) and that synergy is lost in CD-associated NOD2 homozygotes, with implications for TRL-mediated intestinal homeostasis and inflammation. PBMCs were stimulated with CpG DNA (TRL9 ligand) and MDP. MDP stimulation of PBMCs from normal individuals results in a 2- to 3-fold enhancement of CpG DNA stimulation of PBMC production of TNF-α and IL-8, whereas such enhancement is not seen in PBMCs from patients with CD bearing *NOD2* mutations. Concomitant studies of IL-12 secretion were not reported in this study or in a previous study of normal individuals and patients with CD with *NOD2* mutations (61), perhaps because secretion of IL-12 by PBMCs is low and thus difficult to assess. It is not known whether the loss of the enhancing effect of NOD2 is counterbalanced by loss of an inhibitory effect and whether the same or similar findings would be obtained if the authors had studied intestinal cells that differ considerably from peripheral cells in response to various stimuli. In any case, based on these findings, van Heel and colleagues propose that synergistic cytokine response between TLR9 and NOD2 may be beneficial in maintaining intestinal homeostasis and that the lack of such synergism is a cause of CD (60).

SUMMARY AND CONCLUSIONS

A tightly regulated response allows the immune system to coexist with the large amount of antigenic material present in the gastrointestinal tract in the form of commensal microorganisms and food antigens while retaining the ability to respond to pathogens. However, in genetically susceptible individuals, alterations in

responses to the resident luminal bacteria may lead to the development of CD, a complex multifactorial disease whose pathogenesis is still not well understood. Errors in interpretation or regulation of immune perception and responsiveness disrupt mucosal homoeostasis and predispose the individual to uncontrolled or pathological inflammation. Tissue damage in most patients with CD can be accounted for by the downstream effects of activated Th1 cells. Th1 cell differentiation takes place when T cells interact with APCs that produce proinflammatory cytokines in response to exposure to bacteria. In CD the type 1 activation is exaggerated and results in the secretion of excess proinflammatory cytokines, such as IL-1β and TNF-α. At the same time, anti-inflammatory cytokines such as IL-10 and TGF-β, which are responsible for downregulating the Th1 response, are not produced to counteract this misbalance in CD (61).

Recent studies have begun to define the mechanisms through which crucial protein recognition receptors may regulate intestinal innate immunity. Cooperative and competitive interactions may occur between different bacterial and nonbacterial ligands via TLRs and NODs leading to differential proinflammatory and anti-inflammatory immune responses in different cell types. TLRs and NOD2 are significantly involved in host defense and tissue repair responses, thus crucially maintaining mucosal homeostasis. TLR signaling protects intestinal epithelial barrier and maintains tolerance, whereas NOD2 signaling exerts antimicrobial activity and prevents bacterial invasion. Thus, both receptors collectively exhibit distinct features that ensure commensal as well as mucosal homeostasis. Imbalance of the complex interactions between commensal microorganisms and PRRs may result in tissue injury and subsequent inflammation of the intestinal mucosa. Aberrant TLR and/or NOD signaling may stimulate diverse inflammatory responses, leading to the chronic intestinal inflammation seen in CD.

The discovery of an association of NOD2 variants with CD in humans has led to intensive research in this field for the last few years. Unraveling the signaling transduction cascade of NOD2 has proven difficult, and the link to the development of CD has been demonstrated by epidemiological and linkage studies, but the exact cellular mechanism responsible for the CD phenotype in NOD2 variants needs further analysis. Wild-type NOD2 signaling by its specific ligand MDP leads to NF-κB activation and the subsequent production of proinflammatory cytokines. However, a loss-of-function mutation of *NOD2* variants would lead to deficient NF-κB activation and subsequent decrease of Th1 responses. Kobayashi et al (42) and Wehkamp et al (34) showed that normal NOD2 function is responsible for the production of antibacterial peptides (ie, defensins) in Paneth cells. In NOD2 variants, deficient defensin production leads to diminished bacterial clearance at the epithelial surface and enhanced activation of macrophages or DCs within the intestine. Together these effects would heighten the cellular immune responses mediated by high levels of IL-12 and TNF-α. This theory is particularly interesting because Paneth cells are concentrated most highly in the terminal ileum, which is the most common site of inflammation in CD. Furthermore, *NOD2* mutations have been consistently associated with ileal involvement in CD. Conversely, Meada et al (56) and Zelinkova et al (57) found that macrophages and dendritic cells, respectively, with *NOD2* mutations, had enhanced responsiveness to MDP, releasing excess proinflammatory cytokines compared with wild-type cells. This surprising finding of a gain of function is at odds with previously generated data. Despite some reservations, it remains possible that more complex interactions between mutated *NOD2* and downstream signaling complexes result in enhanced NF-κB activation in macrophages and DCs in response to MDP. Finally, Watanabe et al (34) propose that intact NOD2 signaling prevents the development of CD by controlling PGN-mediated Th1 responses via TLR2. A loss-of-function mutation of *NOD2* together with TLR2 signals delivered by other bacterial products will result in enhanced cytokine responses by macrophages or DCs to commensal bacteria and result in inflammation.

Inflammation not only results from an upregulation of proinflammatory proteins by APCs or effector T cells but also is balanced by regulatory cells that secrete IL-10 or TGF-β, which are cytokines known to inhibit T cell proliferation. Netea et al (62) demonstrated that *NOD2* mutations in mononuclear cells produce less IL-10 in response to various bacterial ligands compared with wild-type cells. The IL-10/TNF-α ratio showed only half of the patients bearing the WT allele, favoring mucosal inflammation. Finally, linkage studies show an association between the TLR4 polymorphism Asp299Gly and the development of CD. However, other research groups were not able to significantly reproduce these findings.

Further studies of the physiological and pathophysiological mechanisms within this network of possible cell–cell, ligand–ligand, and PRR–PRR signaling interactions that may favor or prevent CD could lead to promising, novel approaches that may differentially exploit the TLR/NOD pathways and lead to novel therapeutic strategies. It is likely that differential therapeutic strategies will need to include agonists as well as antagonists of PRRs, taking into account differences of PRR pathophysiology at different stages of disease as well as phenotypic and genotypic heterogeneity between distinct subgroups of patients with CD. Prophylactic application of selective TLR/NOD2 ligands could enhance desired commensal-mediated tissue-protective processes to prevent disease. When an acute inflammatory episode breaks out, some of the untoward effects of intestinal inflammation could be

stopped by blocking uncontrolled signal transduction by specific TLR/NOD2 inhibitors, thus dampening the tissue-destructive effects. One key element to this disease-modifying approach may be to stop rather than entirely eliminate the dysregulated innate responses in CD. In this context, careful assessments of adverse effects will be critical when modulating such fundamental host defense pathways of innate immunity. Given the rapid and exciting advancements of research in this field over the last few years, it is reasonable to presume that more immunological evidence and concrete directions for the potential value of these PRRs as therapeutic targets in CD will emerge in the near future.

REFERENCES

1. Head K, Jurenka J. Inflammatory bowel disease part II: Crohn's disease pathophysiology and conventional and alternative treatment options. *Altern Med Rev* 2004;9:360–401.
2. Philpott DJ, Viala J. Towards an understanding of the role of NOD2/CARD15 in the pathogenesis of Crohn's disease. *Clin Gastroenterol* 2004;18:555–68.
3. Yamada T, Alpers DH, Laine L, et al. Textbook of Gastroenterology, Vol 1. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 1999.
4. Ohkusa T, Nomura T, Sato N. The role of bacterial infection in the pathogenesis of inflammatory bowel disease. *Intern Med* 2004;43:534–9.
5. MacDonald TT, Monteleone G. IL-12 and Th1 immune responses in human Peyer's pathes. *Trends Immunol* 2001;22:244–7.
6. Dhillon S, Loftus EV. Medical therapy of Crohn's disease. *Curr Treat Options Gastroenterol* 2005;8:19–30.
7. Duchmann R, Kaiser I, Hermann E, et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995;102:448–55.
8. Greenberg GR. Antibiotics should be used as first-line therapy for Crohn's disease. *Inflamm Bowel Dis* 2004;10:318–20.
9. Akira S, Takeda K. Toll-like receptor signaling. *Nat Immunol* 2004;4:499–511.
10. Russell RK, Wilson DC, Satsangi J. Unraveling the complex genetics of inflammatory bowel disease. *Arch Dis Child* 2004;89:598–603.
11. Cario E. Bacterial interactions with cells of the intestinal mucosa: toll-like receptors and NOD2. *Gut* 2005;54:1182–93.
12. Dunne A, O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2003;171:re3.
13. Harton JA, Linhoff MW, Zhang J, et al. Cutting edge: CATERPILLER: a large family of mammalian genes containing CARD, pyrin, nucleotide-binding, and leucine-rich repeat domains. *J Immunol* 2002;169:4088–93.
14. Hofmann K, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. *Trends Biochem Sci* 1997;22:155–6.
15. Bertin J, DiStefano PS. The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death Differ* 2000;7:1273–4.
16. Liepinsh E, Barbals R, Dahl E, et al. The death-domain fold of the ASC PYRIN domain, presenting a basis for PYRIN/PYRIN recognition. *J Mol Biol* 2003;332:1155–63.
17. Steimle V, Otten LA, Zufferey M, et al. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell* 1993;75:135–46.
18. Roy N, Mahadevan MS, McLean M, et al. The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 1995;80:167–78.
19. Reith W, Mach B. The bare lymphocyte syndrome and the regulation of MHC expression. *Annu Rev Immunol* 2001;19:331–73.
20. 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.
21. Chamaillard M, Hashimoto M, Horie Y, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 2003;4:702–7.
22. Girardin S, Boneca I, Carneiro LAM, et al. Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 2003;300:1584–7.
23. Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* 2003;278:5509–12.
24. Girardin SE, Boneca I, Viala J, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 2003;278:8869–72.
25. Inohara N, Chamaillard M, McDonald C. NOD-LRR proteins: role in host–microbial interactions and inflammatory disease. *Annu Rev Biochem* 2004;74:355–83.
26. Pauleau AL, Murray PJ. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. *Mol Cell Biol* 2003;23:7531–9.
27. Gupta DK, Theisen N, von Figura K, et al. Comparison of biosynthesis and subcellular distribution of lysozyme and lysosomal enzymes in U937 monocytes. *Biochim Biophys Acta* 1985;847:217–22.
28. Araki Y, Nakatani T, Makino R, et al. Isolation of glucosaminyl-α(1–4)-muramic acid and phosphoric acid ester of this disaccharide from acid hydrolysates of peptidoglycan of *Bacillus cereus* AHU 1356 cell walls. *Biochem Biophys Res Commun* 1971;42:684–90.
29. Vavricka SR, Musch MW, Chang JE, et al. hPepT1 transports muramyl dipeptide, activating NF-κB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. *Gastroenterology* 2004;127:1401–9.
30. Viala J, Chaput C, Boneca IG, et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* 2004;5:1166–74.
31. Bell JK, Mullen GE, Leifer CA, et al. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol* 2003;24:528–33.
32. Inohara N, Koseki T, Lin J, et al. An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signalling pathways. *J Biol Chem* 2000;275:27823–31.
33. Tanabe T, Chamaillard M, Ogura Y, et al. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. *EMBO J* 2004;23:1587–97.
34. Watanabe T, Kitani A, Murray PJ, et al. W. NOD2 is a negative regulator of Toll-like receptor 2- mediated T helper type 1 responses. *Nat Immunol* 2004;5:800–8.
35. Kobayashi K, Inohara K, Hernandez LD, et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002;416:194–9.
36. Ogura Y, Inohara N, Benito A, et al. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* 2001;276:4812–8.
37. Inohara N, Koseki T, del Peso L, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem* 1999;274:14560–7.
38. Abbott DW, Wilkins A, Asara JM, et al. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr Biol* 2004;14:2217–27.
39. Zhou H, Wertz I, O'Rourke K, et al. Bcl10 activates the NF-κB pathway through ubiquitination of NEMO. *Nature* 2004;427:167–71.
40. Bamich N, Hisamatsu T, Aguirre JE, et al. GRIM-19 interacts with nucleotide oligomerization domain 2 and serves as downstream effector of anti-bacterial function in intestinal epithelial cells. *J Biol Chem* 2005;280:19021–6.

41. Pauleau AL, Murray PJ. Role of Nod2 in the response of macrophages to Toll-like receptor agonists. *Mol Cell Biol* 2003;23:7531–9.
42. Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005;307:731–4.
43. Girardin SE, Tournebize R, Mavris M, et al. CARD4/Nod1 mediates NF-κB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep* 2001;2:736–42.
44. Damiano JS, Oliveira V, Welsh K, et al. Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. *Biochem J* 2004;381:213–9.
45. Martinon F, Tschopp J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 2004;117:561–74.
46. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;411:599–603.
47. Ahmad T, Tamboli CP, Jewel D, et al. Clinical relevance of advances in genetics and pharmacogenetics of IBD. *Gastroenterology* 2004; 126:1533–49.
48. Lesage S, Zouali H, Cezard JP, et al. NOD2/CARD15 mutational analysis and genotype- phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 2002;70:845–57.
49. Economou M, Trikalinos TA, Loizou KT, et al. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 2001;99:2393–404.
50. Sugimura M, Kinouchi Y, Takahashi S, et al. NOD2/CARD15 mutational analysis in Japanese patients with Crohn's disease. *Clin Genet* 2003;63:160–2.
51. Inoue N, Tamura K, Kinoudhi Y, et al. Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 2003;123:86–91.
52. Leong RWL, Armuzzi A, Ahmad T, et al. NOD2/CARD15 gene polymorphism and Crohn's disease in the Chinese population. *Aliment Pharmacol Ther* 2003;17:1465–70.
53. Bonen DK, Niclolae DL, Moran T, et al. Racial differences in NOD2 variation: characterization of NOD2 in African-Americans with Crohn's disease. *Gastroenterology* 2002;122 (Suppl):A-29.
54. Croucher PJ, Mascheretti S, Hampe J, et al. Haplotype structure and association to Crohn's disease of CARD15 mutations in two ethnically divergent population. *Eur J Hum Genet* 2003;11:6–16.
55. Vermeire S. NOD2/CARD15: relevance in clinical practice. *Best Pract Res Clin Gastroenterol* 2004;18:569–75.
56. Maeda S, Hsu L, Liu H, et al. Nod 2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing. *Science* 2005; 307:734–8.
57. Strober W, Murray PJ, Kitani A, et al. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* (6):2006:9–20.
58. Zelinkova Z, de Kort F, Pronk I, et al. Functional consequences of NOD2 deficiency in Crohn's disease patients peripheral blood monocytes derived dendritic cells. *Gastroenterology* 2005;128: A510.
59. Wehkamp J, Harder J, Weichenthal M, et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal0020-defensin expression. *Gut* 2004;53:1658–64.
60. van Heel DA, Ghosh S, Hunt KA, et al. Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. *Gut* 2005;54:1553–7.
61. Netea MG, Ferwerda G, de Jong DJ, et al. Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* 2005;174:6518–23.
62. Netea MG, Kullberg BJ, de Jong DJ, et al. NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur J Imuunol* 2004;34:2052–9.

Alimentary Pharmacology & Therapeutics

Eosinophilic oesophagitis and coeliac disease: is there an association?

L. QUAGLIETTA, P. COCCORULLO, E. MIELE, F. PASCARELLA, R. TRONCONE & A. STAIANO

Department of Pediatrics, University of Naples, 'Federico II', Italy

Correspondence to:
Dr A. Staiano, Department of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5, 80131 Napoli, Italy.
E-mail: staiano@unina.it

Publication data
Submitted 23 March 2007
First decision 2 April 2007
Second decision 25 April 2007
Third decision 13 May 2007
Resubmitted 12 April 2007
Resubmitted 3 May 2007
Resubmitted 18 May 2007
Accepted 19 May 2007

ABSTRACT

Aim
To report a series of 17 children affected by eosinophilic oesophagitis. Six of them also received a diagnosis of coeliac disease.

Methods
Seventeen children with history of dyspeptic symptoms were investigated.

Results
Six patients (M/F:2/4; mean age ± s.d.: 5.6 ± 1.3 years, range: 4–7 years; Group A) affected by eosinophilic oesophagitis also received a diagnosis of coeliac disease. The other 11 children (M/F:10/1, mean age ± s.d.:7.5 ± 2.3 years, range: 4–10 years, Group B) were affected solely by eosinophilic oesophagitis. All children underwent a change in dietary regimen. Group A received a gluten-free diet. Group B attempted dietary restriction based on the allergy testing results. After 6 months follow-up, all patients in Group A showed a complete disappearance of symptoms and three of them, who underwent upper gastrointestinal endoscopy, showed histologic remission. Patients from Group B had moderate clinical improvement and in seven of them (64%) a repeated upper gastrointestinal endoscopy showed a statistically significant reduction in eosinophilic infiltration.

Conclusions
This is the first reported group of patients with an association between coeliac disease and eosinophilic oesophagitis. To date, it is not possible to exclude that in a subgroup of children with coeliac disease the oesophageal eosinophilic infiltration could be caused by coeliac disease itself.

Aliment Pharmacol Ther **26**, 487–493

doi:10.1111/j.1365-2036.2007.03388.x

INTRODUCTION

Eosinophilic oesophagitis (EoE) and coeliac disease (CD) are distinct clinical entities with specific clinical, laboratory and histological features.

Eosinophilic oesophagitis is a chronic inflammatory disorder of the oesophagus, presenting with dysphagia and with symptoms mimicking those of gastro-oesophageal reflux, including vomiting, regurgitation, nausea and epigastric pain. Patients with EoE are predominantly young males and have a dense and isolated eosinophilic infiltration (>24/high-power field) of the oesophageal mucosa.[1] The endoscopic evaluation of the oesophagus commonly reveals furrowing or mucosal rings; the oesophagus could appear only slightly altered in some patients.[2, 3]

Eosinophilic oesophagitis is a disease emerging throughout the world: until 1995, oesophageal eosinophilia was routinely associated with reflux oesophagitis; however, recent studies suggest that a large number of isolated eosinophils in the oesophagus may represent a separate diagnosis. Noel *et al.*[4] reported on a specific population of children diagnosed with EoE between the years 2000 and 2003, and calculated a disease incidence of one in 10 000/year and a prevalence of four in 10 000. CD is an immune-mediated enteropathy caused by a permanent sensitivity to gluten in genetically susceptible individuals. The prevalence of CD in children between 3 and 15 years of age in the general population is 3–13 per 1000 children, or approximately 1:300 to 1:80 children.[5, 6] Although food antigenes are implicated in both diseases, CD is clearly a food-mediated disorder while the pathophysiology of EoE is still unclear given that food allergy is not always found during the evaluation of affected EoE patients.

We report a series of 17 patients with EoE. Six of them were also affected by CD. The unexpectedly high prevalence of CD in this EoE-affected population and the probability of an association between gluten-induced disease and EoE prompted us to report these patients.

SUBJECTS AND METHODS

From April 2005 to December 2006, all children referred for upper gastrointestinal (GI) endoscopy to the Gastrointestinal Endoscopy and Motility Unit of the Department of Pediatrics, University 'Federico II' of Naples, Italy, which received a diagnosis of EoE, were evaluated. In the same period, 315 children were diagnosed with CD.

The patients were referred for a long-standing history of dyspeptic symptoms, such as vomiting, regurgitation, abdominal pain and/or dysphagia, in spite of standard antireflux therapies, including H_2 receptor antagonist and/or protonic pump inhibitors. These treatments had been stopped at least 2 weeks before enrolment. None of them showed bloating, diarrhoea or weight loss. Because all the patients were under 10 years of age, their parents were given a questionnaire to fill out on behalf of their children: it gathered data regarding age, sex, GI and allergic symptoms, family and personal history of allergic diseases. During initial symptom evaluation, an arbitrary scoring system was used for symptomatologic assessment (Table 1). Furthermore, all children underwent haematologic and blood chemistry analyses, allergy testing and upper GI endoscopy with oesophageal, antral and duodenal biopsies. Nine patients also underwent 24-h intra-oesophageal pH-monitoring. Gastro-oesophageal reflux disease was excluded by a lack of endoscopic and histological signs of reflux disease and/or normal 24-h intra-oesophageal pH-monitoring, defined by a reflux index (the total percentage of time during which the oesophageal pH is <4) ≤4%, according to previous studies.[7, 8]

Informed consent was obtained from parents of all patients, and the experimental design was approved by the Independent Ethics Committee of University of Naples, 'Federico II'.

Blood chemistry analysis and allergy testing

Blood leucocytes and a differential count were determined by using an automated blood count analysis.

Table 1. Grading of upper gastrointestinal symptoms

Symptoms	Evaluation	Score
Epigastric pain (episodes/week)	None	0
	1	1
	2–4	2
	>4	3
Dysphagia/food impaction (episodes/week)	None	0
	1	1
	2–4	2
	>4	3
Regurgitation/vomiting (episodes/week)	None	0
	<7	1
	<14	2
	>14	3

Numbers for peripheral eosinophils were considered as follows: normal, <350 cells/mm^3; slightly elevated, 350–1500 cells/mm^3; moderately elevated, >1500–5000 cells/mm^3; or severely elevated, >5000 cells/mm^3.[3, 9] Liver enzymes, amylase, total protein, albumin, C-reactive protein, erythrocyte sedimentation rate and creatinine values were determined in all patients using the standard methods.

Serum antitissue transglutaminase IgA (anti-tTG Abs) and anti-endomysium IgA (EMA) antibodies were performed. Serum levels of IgA anti-TG2 were determined by ELISA technique using a kit based on human recombinant antigen (Eu-tTg IgA; Eurospital Kit, Trieste, Italy). Values were considered positive if ≥7 UA/mL. Serum IgA EMA was detected by indirect immunofluorescence on 7-μm thick frozen section of human umbilical cord as source of antigen. Diagnostic criteria for CD included: anti-tTG Abs and EMA antibodies and evidence of typical histologic changes on duodenal biopsies in accordance with Marsh classification modified by Oberhuber.[10, 11]

All patients were evaluated in an attempt to determine the specific food allergies. They were tested for total and specific serum IgE levels dietary allergens (cow's milk, alpha-lactoalbumin, beta-lactoglobulin, casein, egg, wheat, rice, soya, chicken, codfish and tomato). Children enrolled were also tested with skin prick tests to the same allergens, performed by one physician who was not aware of the subject's clinical history. Total IgE levels >35 kU/L were considered positive, as were specific IgE levels >0.70 kU/L, as per reported reference ranges for the commercial test used in our laboratory (Unicap specific IgE allergen immuno cap, manufactured by Pharmacia, Uppsala, Sweden). Skin tests were read as positive if the wheal was at least two millimetres larger the control antigen.[12]

Prolonged pH measurement

Prolonged 24-h intra-oesophageal pH monitoring was performed with a portable digital recorder (Digitrapper II; Synectics, Medtronic Gastrointestinal, Synectics Medical AB, Stockolm, Sweden) connected to a flexible antimonium probe (Olympus GIF-XP240, Olympus Optical Co. Ltd, Tokyo, Japan). At the beginning of the study, the pH meter was calibrated with a standard solution of pH 4 and 7. Subsequently, a pH electrode was inserted via the nose, with the tip placed at 87% of the distance from the nares to the lower oesophageal sphincter, according to technique of Strobel.[13]

Endoscopy and histology

Upper GI endoscopy was performed in all 17 patients by the same endoscopist (EM), using a paediatric fiber-optic gastroscope (Olympus GIFPQ20, Olympus Optical Co., LTD, Tokyo, Japan). During endoscopy, the overall intensity of the endoscopic alterations was classified as (i) absent, (ii) minimal (e.g. fine nodules, fine whitish reticular structures, furrows), (iii) moderate (e.g. bright whitish scale-like, plaque-like structures, corrugated rings), or (iv) severe (e.g. mucosal lesion, fixed stenosis).[14] By using standard forceps, at least two biopsy specimens were taken from each of the following four sites: the mid-oesophagus (7–10 cm from Z-line), the distal oesophagus (2–4 cm from the Z-line), the antrum of the stomach and the duodenum. The samples were immediately fixed in a 4% paraformaldehyde solution and embedded in paraffin.

Paraffin blocks were cut in 5-μm section and stained with H&E, Giemsa, and Alcian blue/periodic acid-Schiff for histologic examination. All oesophageal biopsy specimens were examined by an experienced GI pathologist. The eosinophils in the most densely infiltrated area were counted in five consecutive high-power fields (HPF). Data were expressed as the mean number of eosinophils per HPF (Zeiss Axiophot, Oberkochen, Germany; Plan-Neofluar 40, ocular magnification 10×, area of microscopic field 0.3072 mm^2).

The diagnosis of EoE was based on the demonstration of oesophageal infiltration with >24 eosinophils in any single 400 × HPF along with normal antral and duodenal biopsies.[2, 15] Measurements of the epithelial thickness and papillary height were made in oesophageal specimens in which at least three well-oriented papillae could be identified. Measurements were considered normal if basal zone hyperplasia was <20% of the epithelial thickness and/or lengthening of the papillae was <75% of the epithelial height according to data reported by Teitelbaum *et al.*[8]

Dietary treatment of EoE and CD

Dietary treatment included either dietary restriction, with the exclusion of selected foods based on allergy testing, for patients affected by EoE only, or gluten-free diet for children affected by EoE and CD. A diet counselling for appropriate calories assessment was performed and the nutritional state was monitored every 2 months by a paediatrician and a nutritionist. The response was based on the symptomatic and

histologic improvement. Six months after initiating the diet, clinical evaluation and the second endoscopy with biopsies were performed. If the oesophageal histology normalized, a slow re-introduction of foods was begun. Once a week, a food that had previously been excluded was re-introduced. The re-introduction of the excluded foods is still in progress.

Statistical analysis

Predietary and postdietary scores of symptoms were compared by using the non-parametric Wilcoxon's signed rank test. A P-value <0.05 was considered significant. Results of eosinophilic infiltration are expressed as mean ± s.d. Data were compared by Student's t-test.

RESULTS

Seventeen patients (M/F: 12/5; mean age ± s.d.: 6.7 ± 2.3 years, range: 4–10 years) received a diagnosis of EoE. The mean number ± s.d. of eosinophils in the epithelium of middle and distal oesophageal biopsies was 39.9 ± 7.8 and 41.6 ± 9.2, respectively.

Six of 17 patients (35%; M/F:2/4; mean age ± s.d.: 5.6 ± 1.3 years; Group A) showed villous atrophy of the duodenum (Marsh type 3). A subsequent serologic evaluation revealed that EMA and anti-tTG Abs were present in all six patients, confirming the diagnosis of CD In the other 11 children (65%; M/F:10/1, mean age ± s.d.: 7.45 ± 2.3 years, Group B), anti-tTG Abs and EMA were negative and duodenal and antral specimens did not show any abnormalities. By endoscopy 13/17 (76%), patients had macroscopic signs of EoE. In particular, 10 (59%) children had moderate oesophageal alterations and three (18%) had minimal lesions. The histological examination of distal oesophageal biopsies from all patients did not reveal any sign of gastro-oesophageal reflux disease. Our patients did not show a gradient of eosinophil density from distal to proximal oesophagus or neutrophilic infiltration of oesophageal mucosa. In addition, in all oesophageal histological specimens, the measurements of oesophageal epithelial thickness and papillary height were not suggestive of gastro-oesophageal reflux disease.[8]

Results of continuous 24-h intra-oesophageal pH monitoring were normal in all nine patients evaluated (two patients from Group A and seven from Group B).

Sex and chronological data, family history of atopy, allergy testing results are reported in Table 2.

Table 2. Sex, chronological data, family history of atopy and allergy testing in six patients with eosinophilic oesophagitis and coeliac disease (Group A) and in 11 patients with eosinophilic oesophagitis (Group B)

	Group A	Group B
Sex*		
Male	2 (33)	10 (91)
Female	4 (67)	1 (9)
Mean age, year (range)	6 (4–7)	7 (4–10)
Family history of atopy*	1/6 (17)	7/11 (64)
Blood eosinophilia*	3/6 (50)	9/11 (82)
Increased immunoglobulin E levels*	2/6 (33)	9/11 (82)
Identified food allergy*,†	0/6	9/11 (82)

* The values are indicated as number of patients and percentage (%); † By skin prick test, radioallergosorbent test.

Prevalence of presenting complaints in a group of 315 patients with CD and in 17 patients with EoE is reported in Table 3. All 17 enrolled children underwent a change in their dietary regimen. Group A (six patients) received gluten-free diet. Group B (11 patients) attempted dietary restriction (56%, cow's milk and egg; 22%, codfish and wheat; 33%, cow's milk only), in particular, nine patients for documented food hypersensitivity and two patients as a trial challenge.

Patients were assessed every 2 months for therapy adherence and symptom score. All patients were adherent to the dietary treatment. After a mean

Table 3. Prevalence of presenting complaints in patients with coeliac disease (CD) and in patients with eosinophilic oesophagitis (EoE)

Presenting complaints	CD (n = 315) %	EoE (n = 17) %
Failure to thrive	61	0
Diarrhoea	41	0
Anaemia	34	0
Dyphagia/food impaction	0	59
Anorexia	22	0
Abdominal pain	15	41
Regurgitation/vomiting	13	35
Protuberant abdomen	5	0
Hypertransaminasemia	4	0
Constipation	4	0

follow-up of 6 months, a second endoscopy was performed in three children from Group A and seven children from Group B. All six patients in Group A showed a complete disappearance of symptoms and three of them who underwent upper GI endoscopy showed an histologic remission of EoE, defined as a mean eosinophils count of ≤10 eosinophils/HPF.[16] In all these subjects, a significant improvement of duodenal histology (Marsh type 1) was observed.

In these three patients, the number of oesophageal eosinophils per HPF in the middle and distal oesophagus was significantly ($P < 0.0001$) reduced compared with the baseline examination. The other 11 patients with EoE (Group B) had moderate clinical improvement and in seven of them (64%) a repeated upper GI endoscopy showed a significant ($P < 0.001$) reduction in eosinophilic infiltration without normalization. Finally, in both group of patients, specimens from the stomach and duodenum did not show at the time of the diagnosis and at follow-up, an increased number of eosinophils.[15]

Tables 4 and 5 show the symptom score and the number of eosinophils in the oesophageal epithelium, during the baseline evaluation and at follow-up examination, in three patients from group A and seven from Group B. Parents of three patients from Group A and four patients from Group B did not accept to repeat upper GI endoscopy with biopsies in their asymptomatic children.

Table 4. Clinical scores at baseline (to) and after dietary treatment (t1) in six patients with eosinophilic oesophagitis (EoE) and coeliac disease (group A) and in 11 patients with EoE (Group B)

	Group A			Group B		
	Number of patients					
Score	T0	T1	*P*	T0	T1	*P*
Dysphagia						
0	0	4	0.039	0	3	0.047
1–2	2	1		3	4	
3	4	1		8	4	
Epigastric pain						
0	1	5	0.039	1	0	0.053
1–2	1	1		2	9	
3	4	0		8	2	
Regurgitation/vomiting						
0	0	5	0.043	0	4	0.057
1–2	3	1		4	2	
3	3	0		7	5	

T0, baseline examination; T1, follow-up examination.

Table 5. Microscopic findings in seven patients with eosinophilic oesophagitis (EoE) and coeliac disease (Group A) and in three patients with EoE (Group B) at baseline and follow-up examination

	T0	T1	*P*-value
Group A Patients (*n*)	3		
Mean number of eosinophils in the oesophageal epithelium			
Middle part (cells/HPF)	40 (35–45)	6 (4–8)	0.0004
Distal part (cells/HPF)	45 (35–52)	7 (4–10)	0.0022
Group B, patients (*n*)	7		
Mean number of eosinophils in the oesophageal epithelium			
Middle part (cells/HPF)	42 (36–50)	31 (26–40)	0.0012
Distal part (cells/HPF)	44 (33–59)	30 (25–41)	0.006

T0, baseline examination; T1, follow-up examination; HPF, high-power fields.

DISCUSSION

In this study, we reported 17 patients investigated for upper GI symptoms who received a diagnosis of EoE; six of 17 children (35%) also exhibited features of CD.

Eosinophilic oesophagitis and CD seem to have similarities including genetic susceptibility, classification as food hypersensitivity disorders as well as the risk of complications in untreated patients. However, it is far from clear whether this co-existence is to be considered coincidental, or an expression of a real association.

To our knowledge, this is the first reported group of patients with an association between EoE and CD. There are several possible explanations for this association, not being reported before. The first, most likely, is that our series for some reason is biased and our group of patients would be atypical. The second is that EoE is a disease of relatively recent recognition, particularly in Europe, and such association is not emerged yet. Very recent reports of association between EoE and CD would support this view.[17, 18]

Either of our children from Groups A and B seem to show a variety of common symptoms, such as epigastric pain, dysphagia/food impaction and vomiting. However, our patient's most common complaint was dysphagia, suggesting that the pathological

involvement of the oesophagus was the main clinical problem in both groups.

The cause of EoE is poorly understood, but allergy has been implicated.[19] An IgE-dependent mechanism for EoE is supported by clinical observations. For instance, Spergel *et al.*[20] showed that affected patients have IgE sensitization to a wide variety of foods; although not all patients had evidence of food-specific IgE. We observed a high level of IgE and evidence of IgE sensitization to food allergens in 82% Group B patients; in contrast only 33% patients from Group A showed increased levels of IgE and none showed positive allergy testing results to food allergens. These observations seem to describe two distinct groups of children, e.g. those with EoE and a subset of CD patients who have elevated oesophageal eosinophils for reasons different from allergy. It is known that activated eosinophils are found within the lamina propria of patients affected by CD, especially in the later stages after lymphocyte activation.[21] They have the capacity to synthesize IL-5, a cytokine produced by T- and mast cells that is the major actor involved in eosinophils differentiation and activation.[22] Therefore, EoE in CD patients could be the expression of gluten-dependent enteropathy, and the eosinophilic infiltration may simply represent a less common oesophageal expression of the CD.

From an immunological point of view, EoE, as a typical Th2 condition and as an expression of a food hypersensitivity reaction, is not expected to segregate with Th1 diseases, such as CD. The possibility of a co-existence of Th1 and Th2 type diseases is still being debated.[23] Recently, Nilsson *et al.*[24] co-analysed the cytokines pattern in children with Th1 diseases (CD, type-1 diabetes) and allergy and demonstrated a strong combined Th1 and Th2-like response, as a result of an interplay between the two sides of the Th scale. This observation seems to suggest more than a casual association between CD and allergy-associated diseases and a more generalized defect of immunoregulation.

Our patients with EoE from Group A were treated with a gluten-free diet, while Group B patients underwent a specific elimination diet according to the results of the allergy testing. After 6 months of follow-up, symptoms disappeared in all Group A children on a gluten-free diet, while the great majors of patients from Group B patients showed an improvement, without complete resolution, in their symptom score. In addition, at follow-up, a normalization in eosinophilic count was noted in mid and distal oesophageal specimens of patients from Group A. In contrast, a significant reduction of the eosinophilic infiltration without complete normalization was found in the oesophageal biopsies from Group B children. The oesophageal histologic remission on gluten-free diet in our patients suggests that, at least in a subgroup of patients, EoE and CD share the initial pathogenic trigger event. Gluten, by immunological dysregulation, could stimulate both a Th1 and Th2 reaction and be responsible for two different disorders, characterized by a common oesophageal phenotype.

In conclusion, we report a series of 17 children affected by EoE. The discrepancy in symptomatic and histological response between the non-coeliac and coeliac groups highlights the importance of precisely identifying the alimentary allergens, the exclusion of which leads to the remission of oesophageal disease. It is possible that, in our patients affected solely by EoE, all the allergens really involved in the pathogenesis of eosinophilic infiltration were not identified by allergy testing; therefore, these patients could take advantage from a 'complete dietary elimination' with an amino acid-based hypoallergenic formula or from different therapeutic options.

In conclusion, the high proportion of patients affected by both diseases, EoE and CD, the lack of IgE sensitization for food allergens in these children as well as the significant clinical and histological remission on gluten-free diet compared to the group of subjects with EoE only suggest that CD itself could cause oesophageal eosinophilic infiltration and dyspeptic symptoms.

However, further studies are needed to investigate the relationship between EoE and CD. Immunohistochemical and cytokine analysis of eosophageal biopsies of patients affected by both diseases most likely could improve our knowledge and be useful in understanding the precise immune mechanism(s), leading to both symptoms and epithelial eosinophilia.

ACKNOWLEDGMENT

Declaration of personal and funding interests: None

REFERENCES

1 Noel RJ, Putnam PE, Rothenberg ME. Eosinophilic esophagitis. *N Engl J Med* 2004; 351: 940–1.
2 Nurko S, Teitelbaum JE, Husain K, *et al.* Association of Schatzki ring with eosinophilic esophaitis in children. *J Pediatr Gastroenterol Nutr* 2004; 38: 436–41.
3 Rothenberg ME, Mishra A, Collins MH, *et al.* Pathogenesis and clinical features of eosinophilic esophagitis. *J Allergy Clin Immunol* 2001; 108: 891–4.
4 Fox VL, Nurko S, Furuta GT. Eosinophilic esophagitis: it's not just kid's stuff. *Gastrointest Endosc* 2002; 56: 260–70.
5 Fasano A, Berti I, Gerarduzzi T, *et al.* Prevalence of coeliac disease in at-risk and not at-risk in the United States. *Arch Intern Med* 2003; 163: 286–92.
6 Hoffenberg EJ, MacKenzie T, Barriga KJ, *et al.* A prospective study of the incidence of childhood coeliac disease. *J Pediatr* 2003; 143: 308–14.
7 Cucchiara S, Staiano A, Gobio-Casali L, *et al.* Value of the 24-hour intraesophageal pH monitoring in children. *Gut* 1990; 31: 129–33.
8 Teitelbaum JE, Fox VL, Twarog FJ, *et al.* Eosinophilic esophagitis in children: immunopathological analysis and response to fluticasone propionate. *Gastroenterology* 2002; 122: 1216–25.
9 Rothenberg ME. Eosinophilia. *N Engl J Med* 1998; 338: 1592–600.
10 Marsh MN. Gluten, major histocompatibility complex, and the small intestine: a molecular and immunobiologic approach to the spectrum of gluten sensitivity ('coeliac sprue'). *Gastroenterology* 1992; 102: 330–54.
11 Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol* 1999; 11: 1185–94.
12 Troncone R, Merrett TG, Ferguson A. Prevalence of atopy is unrelated to presence of inflammatory bowel disease. *Clin Allergy* 1998; 18: 111–7.
13 Strobel CT, Byrne WJ, Ament ME. Correlation of esophageal lengths in children with height: application to the Tuttle test with-out prior esophageal manometry. *J Pediatr* 1979; 94: 81–4.
14 Straumann A, Spichtin HP, Grize L, *et al.* Natural history of primary eosinophilic esophagitis: a follow-up of 30 adult patients for up to 11.5 years. *Gastroenterology* 2003; 125: 1660–9.
15 Lowichik A, Weinber AG. A quantitative evaluation of mucosal eosiniophils in the pediatric gastrointestinal tract. *Mod Pathol* 1996; 9: 110–4.
16 Ruchelli E, Wenner W, Voytek T, *et al.* Severity of esophageal eosinophilia predicts response to conventional gastroesophageal reflux therapy. *Pediatr Dev Pathol* 1999; 2: 15–8.
17 Shah A, McGreal N, Li B, *et al.* Coeliac disease in association with eosinophilic esophagitis: case series of six patients from two centers. *J Pediatr Gastroenterol Nutr* 2006; 43: E24.
18 Kagalwalla AF, Shah A, Ritz S. Cow's milk protein-induced eosinophilic esophagitis in a child with gluten-sensitive enteropathy. *J Pediatr Gastroenterol Nutr* 2007; 44: 386–8.
19 Feighery C. Coeliac disease, auto-immunity and thyroid disease. *Ital J Gastroenterol Hepatol* 1999; 31: 288–9.
20 Spergel JM, Beausoleil JL, Mascarenhas M, *et al.* The use of skin prick tests and patch tests to identify the causative foods in eosinophilic esophagitis. *J Allergy Clin Immunol* 2002; 109: 363–8.
21 Straumann A, Bauer M, Fischer B, *et al.* Idiopathic eosinophilic esophagitis is associated with a T(H)2-type allergic inflammatory response. *J Allergy Clin Immunol* 2001; 108: 954–61.
22 Desreumaux P, Janin A, Colombel JF, *et al.* Interleukin 5 messenger RNA expression by eosinophils in the intestinal mucosa of patients with coeliac disease. *J Exp Med* 1992; 175: 293–6.
23 Caffarelli C, Cavagni G, Pierdomenico R, *et al.* Coexistence of IgE mediated allergy and type 1 diabetes in childhood. *Int Arch Allergy Immunol* 2004; 134: 288–94.
24 Nilsson L, Kivling A, Jalmelid M, *et al.* Combinations of common chronic paediatric diseases deviate the immune response in diverging directions. *Clin Exp Immunol* 2006; 146: 433–42.

Effect of a Probiotic Preparation (VSL#3) on Induction and Maintenance of Remission in Children With Ulcerative Colitis

Erasmo Miele, MD, PhD[1], Filomena Pascarella, MD[1], Eleonora Giannetti, MD[1], Lucia Quaglietta, MD[1], Robert N. Baldassano, MD[2] and Annamaria Staiano, MD[1]

OBJECTIVES: **Several probiotic compounds have shown promise in the therapy of ulcerative colitis (UC). However, a strong sustained benefit remains to be seen. Uncontrolled pilot studies suggest that a probiotic preparation (VSL#3) maintains remission in mild to moderate UC and reduces active inflammation in adult patients. Aims of our prospective, 1-year, placebo-controlled, double-blind study were to assess the efficacy of VSL#3 on induction and maintenance of remission and to evaluate the safety and tolerability of the probiotic preparation therapy in children with active UC.**

METHODS: **A total of 29 consecutive patients (mean age: 9.8 years; range: 1.7–16.1 years; female/male: 13/16) with newly diagnosed UC were randomized to receive either VSL#3 (weight-based dose, range: 450–1,800 billion bacteria/day; $n=14$) or an identical placebo ($n=15$) in conjunction with concomitant steroid induction and mesalamine maintenance treatment. Children were prospectively evaluated at four time points: within 1 month, 2 months, 6 months, and 1 year after diagnosis or at the time of relapse. Lichtiger colitis activity index and a physician's global assessment were used to measure disease activity. At baseline, within 6 months and 12 months or at the time of relapse, all patients were assessed endoscopically and histologically.**

RESULTS: **All 29 patients responded to the inflammatory bowel disease (IBD) induction therapy. Remission was achieved in 13 patients (92.8%) treated with VSL#3 and IBD therapy and in 4 patients (36.4%) treated with placebo and IBD therapy ($P<0.001$). Overall, 3 of 14 (21.4%) patients treated with VSL#3 and IBD therapy and 11 of 15 (73.3%) patients treated with placebo and IBD therapy relapsed within 1 year of follow-up ($P=0.014$; RR=0.32; CI=0.025–0.773; NNT=2). All 3 patients treated with VSL#3 and 6 of 11 (54.5%) patients treated with placebo relapsed within 6 months of diagnosis. At 6 months, 12 months, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL#3 group than in the placebo group ($P<0.05$). There were no biochemical or clinical adverse events related to VSL#3.**

CONCLUSIONS: **This is the first pediatric, randomized, placebo-controlled trial that suggests the efficacy and safety of a highly concentrated mixture of probiotic bacterial strains (VSL#3) in active UC and demonstrates its role in maintenance of remission.**

Am J Gastroenterol 2009; 104:437–443; doi:10.1038/ajg.2008.118; published online 20 January 2009

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by periods of remission with episodes of clinical relapse, involving an increase in symptoms (1,2). The disease is characterized by diffuse mucosal inflammation limited to the colon. Current medical management consists of

[1]Department of Pediatrics, University of Naples "Federico II", Naples, Italy; [2]The Center for Pediatric Inflammatory Bowel Disease, The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania, USA. **Correspondence:** Annamaria Staiano, MD, Department of Pediatrics, University of Naples "Federico II", Via S. Pansini, 5, Naples 80131, Italy. E-mail: staiano@unina.it
Received 12 June 2008; accepted 7 October 2008

aminosalicylates, steroids, and immunosuppressant therapies such as azathioprine, 6-mercaptopurine, and more recently tumor necrosis factor-α (TNF-α) antibody. A significant proportion of patients do not tolerate existing treatments due to their adverse effects. In addition, failure to induce remission with current treatments occurs in 20–30% of pediatric patients with a significant proportion of these patients eventually requiring colectomy (3,4). Consequently, new alternatives for the treatment of UC are constantly being sought.

One of the latest additions to the vast therapeutic armamentarium is probiotics, defined as live microbial feed supplements, which beneficially affect the host by improving intestinal microbial balance, blocking adhesion sites on the colonocytes thus enhancing gut barrier function and improving local immune response (5,6). The indication for probiotics in IBD is grounded on a number of human and animal studies indicating that the enteric flora is centrally involved in the pathogenesis of Crohn's disease and UC (7). Compared with the healthy gut, an increased number of mucosa-associated bacteria, quantitative and qualitative differences, and instability of flora composition have been reported (7). It is still unclear how these findings relate to IBD pathogenesis.

Several probiotic compounds have shown promise in the therapy of UC. However, a strong sustained benefit remains to be seen (6).

The data from experimental studies indicate that a probiotic preparation (VSL#3) combining eight different probiotic bacteria has immunomodulatory effects. Attenuation of severity of disease activity by means of improvement of histologic grading has been described in various animal models. A decrease of neutrophil tissue influx and activity has been shown. A diminished pro-inflammatory interleukin (IL)-1β, TNF-α, interferon- γ, IL-12, and IL-8 cytokine production and an enhanced production of the antiinflammatory cytokine IL-10 have been reported (8). Furthermore, it has been demonstrated that oral administration of the VSL#3 probiotic formula to rats increases MUC2 gene expression as well as mucin protein accumulation in the colonic lumen and upregulates alkaline sphingoymielinase activity (9,10).

In vivo, small controlled studies in adult patients suggest that VSL#3 can be effective in preventing pouchitis (11–13). Furthermore, uncontrolled pilot studies suggest that VSL#3 maintains remission in mild to moderate UC in 75% of patients and reduces active inflammation in 87% (14,15). A recent open-label study suggests a 53% remission rate in ambulatory adult patients with active disease who received VSL#3 (15).

The purposes of this prospective, 1-year, placebo-controlled, double-blind study were to assess the efficacy of VSL#3 on induction and maintenance of remission and to evaluate the safety and tolerability of the probiotic preparation therapy in children with active UC.

METHODS

The study was a prospective, single-center, placebo-controlled, double-blind, 1-year study of children with newly diagnosed UC consecutively enrolled over a 24-month period at the Department of Pediatrics of the University of Naples "Federico II", Italy. Patients were recruited to participate in this study if they had a new diagnosis of UC, established on accepted historical, endoscopic, histologic, and/or radiologic criteria, which needed a steroid therapy to induce the remission of the disease (16).

Exclusion criteria were children who had received therapy inducing remission of UC; children who required outpatient antibiotic therapy and/or required surgery for complications related to UC; children with documented history of allergic reaction to *Lactobacillus* or other probiotic compound or with history of endocarditis, rheumatic valvular disease, congenital cardiac malformations, or cardiac surgery; and children who had received *Lactobacillus, Bifidobacterium, Enterococcus, Saccharomyces*, or any other probiotic bacterial supplement within the past 10 days.

A total of 29 consecutive patients (mean age: 9.8 years; range: 1.7–16.1 years; female/male: 13/16) with newly diagnosed UC were randomized to receive either VSL#3 (VSL Pharmaceuticals, Towson, MD; weight-based dose, range: 450–1,800 billion bacteria/day; **Table 1**) or an identical placebo associated to concomitant steroid induction treatment (oral methylprednisolon: 1 mg/kg/day, maximum 40 mg/day per 4 weeks) and oral mesalamine maintenance treatment (50 mg/kg/day). Patients were instructed to take VSL#3 or placebo into cold water or any noncarbonated drink.

Assignment to therapy or placebo was determined according to a computer-generated randomization scheme (17). Randomization was performed by one blind clinical trial investigator, who kept the codes until completion of the study. None of the staff or patients had access to the randomization codes during the study. The medications were dispensed by the investigator at each visit; compliance was assessed by counting returned bags and questioning the patients. Excellent compliance was defined as no violation of the protocol with respect to the intake of the study medication.

After 4 weeks, patients who were in remission began tapering off corticosteroids on a weekly basis (25% per week) according to the prescribed schedule on the basis of the clinical activity index. On induction of remission, patients continued to receive concomitant therapy (VSL#3 group: mesalazine and VSL#3; placebo group: mesalazine and placebo) for 1 year or until relapse.

Table 1. VSL#3 daily weight-based dose

Age (year; weight (kg))	Daily dose (packet; bacteria/day)
4–6 (17–22)	1/2 (450 billion)
7–10 (24–33)	1 (900 billion)
11–14 (34–53)	1+1/2 (1350 billion)
15–17 (54–66)	2 (1800 billion)

VSL#3 was provided in packets, each of which contained 900 billion viable lyophilized bacteria of four strains of *Lactobacillus* (*L. paracasei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacterium* (*B. longum*, *B. breve*, and *B. infantis*), and one strain of *Streptococcus salivarius* subsp. *thermophilus* (designated hereafter as *S. thermophilus*). Placebo was provided in identical bags containing 3 g of corn starch. VSL#3 and placebo were administered once daily. The taste and smell of the active drugs were not readily identifiable.

Participants were evaluated at four time points: within 1 month, 2 months, 6 months, and 1 year after diagnosis or at the time of relapse. At each visit data were collected including patient questionnaires regarding disease activity (stool frequency, stool consistency, hematochezia, abdominal pain, extraintestinal manifestations of disease, and overall patient functioning). Additional information collected at the first visit included demographic data, family history, and symptom onset. Physical examination was performed at each visit by a pediatrician and included an abdominal examination and examination for 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 pediatric gastroenterologists of each individual patient exclusively made all decisions regarding therapeutic interventions.

Lichtiger colitis activity index (LCAI), and a physician's global assessment were used to measure disease activity (18) (**Table 2**). Individual scores for each section of the test including symptoms, characteristics of stool, and physical examination were computed. A sustained drop in LCAI to ≤2 after steroid therapy was considered remission. Response was defined by a decrease in LCAI ≥3 points, but final score ≥3. Clinical relapse was defined as the occurrence or worsening of symptoms, accompanied by an increase in LCAI >3 points, sufficient to require treatment with corticosteroids, azathioprine/immunosuppressive agents, or surgery (2,19).

Laboratory studies including complete blood count, albumin, erythrocyte sedimentation rate, and C-reactive protein were performed at each visit and/or at the time of relapse. Celiac and infectious diseases at diagnosis were ruled out. All unfavorable, unexpected symptoms were recorded in the diary kept by patients during the study.

At baseline, within 6 months and 12 months or at the time of relapse, all patients underwent colonoscopy with mucosal biopsy. Colonoscopic grade of inflammation was determined by use of a simple five-point score reported in **Table 2** (20). Colonoscopy report system required segmental descriptions (rectum, sigmoid, descending, splenic flexure, transverse, hepatic flexure, ascending colon, and cecum). From the information provided on the colonoscopy report, each segment of the colon was designated a score, and a mean score for that colonoscopy was derived (20). All histologic specimens were reviewed under code by a single pathologist experienced in analyzing pediatric intestinal biopsies, blinded to the patients' clinical details, who scored biopsies according to the histologic criteria reported in **Table 3** (20). When more than one biopsy sample had been

Table 2. Lichtiger colitis activity index scoring

Symptom	Score
Diarrhea (no. of daily stools)	
0–2	0
3 or 4	1
5 or 6	2
7–9	3
10	4
Nocturnal diarrhea	
No	0
Yes	1
Visible blood	(% of movements)
0	0
Less than 50	1
Greater than 50	2
100	3
Fecal incontinence	
No	0
Yes	1
Abdominal pain or cramping	
None	0
Mild	1
Moderate	2
Severe	3
General well being	
Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5
Abdominal tenderness	
None	0
Mild and localized	1
Mild to moderate and diffuse	2
Severe or rebound	3
Need for antidiarrhea drugs	
No	0
Yes	1

Table 3. Endoscopic and histological scores

Colonoscopic score	
Entirely normal appearance	0
Quiescent disease (mild edema or chronic features, but no active inflammation)	1
Mild active inflammation	2
Moderate active inflammation	3
Severe active inflammation	4
Histological score	
Normal (no inflammatory cells)	0
Chronic inflammation only	1
Mild active (cryptitis but no crypt abscesses)	2
Moderate active (few crypt abscesses)	3
Severe active inflammation (numerous crypt abscesses)	4

taken from a segment of colon, the highest grade of histological inflammation within that segment was recorded and a mean histological score for all segments was calculated for that child (20). Mean and median values were calculated for dimensional variables after controlling for normality of distribution. Statistical analysis was performed using SPSS statistical software package for Windows (13.0; SPSS, Chicago, IL). The Student's *t*-test 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. Survival analysis was used to analyze 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.

Data available were incorporated into analysis irrespective of protocol compliance

Written, informed consent was obtained from 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."

RESULTS

A total of 33 patients with newly diagnosed UC were screened. Four subjects were excluded, because the parents of three refused consent and one child had received probiotic bacterial supplement within the past 10 days. Twenty-nine children were eligible and participated to this study. Of them, 14 were randomly assigned to receive VSL#3 and 15 to receive placebo associated to concomitant steroid induction and oral mesalamine maintenance treatment. Demographic data for the study groups are listed in **Table 4**. The study groups were well matched with respect to age, sex, extension of colitis, and duration of symptoms at onset.

Table 4. Baseline characteristics in 29 children with newly diagnosed UC

Characteristic	VSL#3 group	Placebo group
Sex		
Male	8 (57%)	8 (53%)
Female	6 (43%)	7 (47%)
Mean age (year)		
Male	8.4 (range, 1.7–15)	10.6 (range, 6.6–16.1)
Female	11.4 (range, 7.8–15)	9 (range, 3–15.6)
Mean duration of symptoms at onset (months)	4.7 (range, 1–20)	3 (range, 1–12)
Disease location (%)		
Proctosigmoiditis	28.6	26.7
Left-sided colitis	35.7	20
Pancolitis	35.7	53.3
Mean duration of steroid exposure (days)	68.5 (range, 52–104)	78.3 (range, 51–114)

Linear growth and weight at diagnosis were normal in all children. No significant differences in weight or height at diagnosis and during 1-year follow-up were observed between VSL#3 and placebo groups.

The mean LCAI performed at diagnosis by the pediatric gastroenterologist of each patients, did not show significant differences between VSL#3 and placebo patients (mean: 10.9 (range: 10–14) vs. 11.1 (range: 10–14), respectively; $P=0.533$). On the basis of the physician's global assessment, the severity of the disease was moderate in 42% of VSL#3 patients vs. 33% of placebo patients and severe in 58% of VSL#3 patients vs. 67% placebo group ($P=0.586$).

At baseline, laboratory parameters at diagnosis including hematocrit, albumin, erythrocyte sedimentation rate, and C-reactive protein were not significantly different between the two groups and were not predictive of response and/or relapse.

All 29 patients responded to the IBD induction therapy. On the basis of LCAI, remission was achieved in 13 patients (92.8%) treated with VSL#3 and IBD conventional therapy and in 4 patients (36.4%) treated with placebo and IBD conventional therapy; response was observed in 1 patient (7.2%) of VSL#3 group vs. 11 patients (63.6%) of placebo group ($P<0.001$; **Figure 1**). Mean duration of steroid exposure was not significantly different among VSL#3 group and placebo group ($P=0.23$).

Overall, 3 of 14 (21.4%) patients treated with VSL#3 and IBD therapy and 11 of 15 (73.3%) patients treated with placebo relapsed within 1 year of follow-up ($P=0.014$; RR = 0.32; CI = 0.025–0.773; NNT = 2). All 3 patients treated with VSL#3 and 6 of 11 (54.5%) patients treated with placebo relapsed

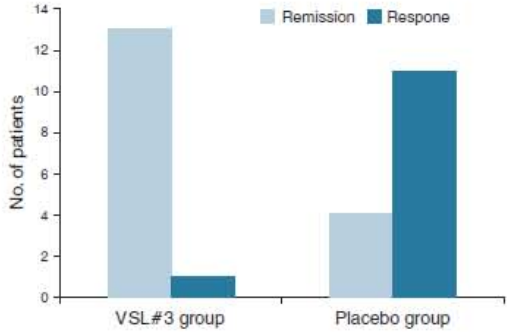


Figure 1. Clinical outcome following induction therapy treatment in VSL#3 group and placebo group ($P<0.001$).

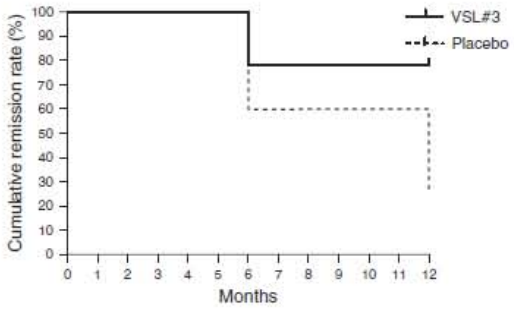


Figure 2. Kaplan–Meier estimates of relapse during treatment with VSL#3 or placebo (log-rank test $P=0.001$).

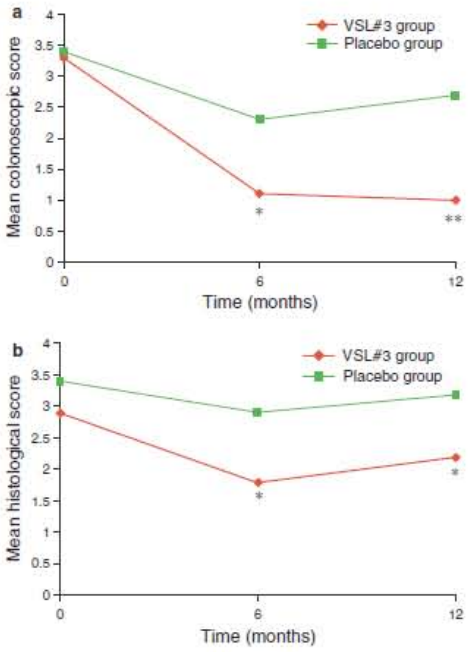


Figure 3. Mean total colonoscopic (**a**) and histological (**b**) score over 1-year follow-up. The scores in the placebo group were significantly higher to that in the VSL#3 group (*$P=0.05$; **$P=0.01$).

within 6 months of diagnosis. Life-table analysis of the relapses in the two groups is shown in **Figure 2**.

The mean endoscopic and histological scores performed at diagnosis were not significantly different between VSL#3 and placebo groups (3.3 vs. 3.4; 2.9 vs. 3.4; $P=0.382$; $P=0.134$, respectively). At 6 months, 12 months, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL#3 group than in the placebo group ($P<0.05$). The mean colonoscopic and histological scores using the last-observation-carried-forward method are shown in **Figure 3**.

Compliance with the study probiotic preparation and placebo was excellent in 86% of the children in the VSL#3 group and in 76% of the children in the placebo group.

No side effects or significant changes from baseline values in any of the laboratory parameters examined, attributable to treatment with either VSL#3 or placebo, were registered.

DISCUSSION

UC is a relapsing IBD of the colon of unknown etiology with a prevalence of about 100 cases per 100,000 children (21). The pathogenesis of IBD involves an interaction between genetically determined host susceptibility, dysregulated immune response, and the enteric microbiota. Host susceptibility is sometimes favored by polymorphism in intestinal antimicrobial defenses (e.g., defensin deficiency) or in perception of microbial signals in the enterocytes, immune cells, or Paneth cells (e.g., NOD2-CARD15 and TLR4 polymorphisms) (22). The deleterious role of some intestinal microorganisms has been established in murine models and is strongly suspected in humans (23). However, other microorganisms seem to be protective (24).

As the microbial environment has been shown to play a role in the development of IBD, targeting of the microbiota presents an option for therapeutic intervention. The use of antibiotics, probiotics, and prebiotics to treat UC, Crohn's disease, and pouchitis has been extensively reviewed (25). Manipulating the abnormal enteric microbiota to decrease the more pathogenic species and enhancing the concentration and metabolic activity of the beneficial species has tremendous potential for therapeutic benefit. However, until now, this rational, physiologic, and nontoxic approach has not yet achieved its potential (25). Studies have shown beneficial effects in children with acute gastroenteritis (26) and in various models of experimental colitis such as IL-10 deficient mice (27,28) and acetic acid-induced colitis in rats (29). There is some evidence that *Escherichia coli*

Nissle 1917 may be as effective as 5-ASA for maintenance treatment of UC (30). Uncontrolled studies (15,31) and some randomized controlled trials (32–35) have suggested that probiotics may be of some benefit for the treatment of active UC.

In addition, the largest body evidence demonstrating efficacy of probiotics in IBD exists for pouchitis. In three double-blind studies, VSL#3 was shown to be significantly superior to placebo in maintaining remission in patients with chronic pouchitis and preventing the onset of pouchitis (11–13).

To our knowledge, this study represents the first prospective, placebo-controlled, double-blind study assessing the efficacy of VSL#3 on induction and maintenance of remission and evaluating the safety and tolerability of the probiotic preparation therapy in children with a new diagnosis of active UC.

This study demonstrated a significant efficacy of VSL#3 in the induction and maintenance of remission in pediatric active UC. Patients treated with VSL#3 and concomitant conventional therapy had a significantly higher rate of remission compared to placebo with a significantly lower incidence of relapse within 1 year of follow-up. There were no biochemical or clinical adverse events related to VSL#3.

According to the study by Hyams *et al.* (4), the remission rate seen in the placebo group was lower than expected (36.4% vs. 60%). The biases that may have led to such a discrepancy could be related to the different activity disease criteria used, to the different time points of outcome evaluation and to the different medication use in addition to corticosteroids during the first year after diagnosis. In the study by Hyams *et al.* (4), in fact, 61% of children on corticosteroid therapy received either azathioprine or 6-mercaptopurine, and 86% received an aminosalicylate.

In contrast to our study, recently, a meta-analysis concluded that there was no evidence that probiotics were superior to placebo or aminosalicylates for the induction of remission and that the use of probiotics as induction therapy for UC could not be recommended (6). We hypothesize that the success of our treatment may be related to the use of a probiotic preparation (VSL#3), characterized by a very high bacterial concentration of 300 billion/g probiotic bacteria consisting of eight strains of viable lyophilized bacteria. Rationale for the use of a cocktail containing large numbers of different strains has been the concept of high efficacy through a synergistic action of the different strains in the mixture (8). On the basis of the results of our study, one should consider that the efficacy of one probiotic may not be the same in all patients or in the same patient at different stages of disease. Responsiveness to treatment could be dependent on several variables, including the characteristics of the host (age, sex, lifestyle), the lesions (site, extent, type of gross lesions), and risk factors (familial history of IBD). On the other hand, there is no dose-response study available for probiotics. Thus, it is possible that the ineffectiveness of probiotics was due to an inappropriate dose.

In addition, there are many species of probiotic. One type (e.g., VSL#3) might be more effective than another because strain-specific properties might influence the efficacy in different cases and situations. The optimal composition, dose, and length of probiotic treatment in various pediatric IBD clinical settings need to be confirmed by further larger, well designed, placebo-controlled, prospective trials.

In our study, at 6 months, 12 months, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL#3 group than in the placebo group.

No endosocopic and histological scores for the evaluation of the pediatric UC have yet been developed and validated. For this reason, we used scores published as a way of correlating colitis with dysplasia risk, believing that these could give us the most representative record possible of each patient's colonic inflammation over time and throughout the colorectum (20).

Support for a favorable action of VSL#3 in gut inflammation also comes from animal models. Madsen *et al.* (36) demonstrated that VSL#3 was able to decrease the severity of colitis in IL-10$^{-/-}$ mice, evidenced by decreasing TNF-α and interferon-γ production, histological scores and barrier integrity. In the iodoacetamide model of colitis, pretreatment with either LGG or VSL#3 significantly decreased the severity of colonic damage, as indicated by a decreased myeloperossidase activity and nitric oxide synthase activity (37). The potential health benefits of VSL#3 were also highlighted in a study by Rachmilewitz *et al.* (38), using the destran sulfate sodium model of colitis. VSL#3 was shown to significantly decrease colonic disease activity score, myeloperossidase activity and histologic scores in chronic destran sulfate sodium-induced colitis. Interestingly, treatment with γ-irradiated probiotics was also shown to decrease these parameters indicating that the probiotic species may not need to be viable to exert its beneficial effects (39). It has been demonstrated that CpG DNA alone, derived from the probiotic species was sufficient to ameliorate colitis, and that this was dependent on TLR9 signaling (39). In addition, recently, Caballero-Franco *et al.* (9) provided compelling evidence that VSL#3 can enhance colonic mucin gene expression and secretion *in vivo* and *in vitro*.

In conclusion, this study suggests the efficacy and safety of a highly concentrated mixture of probiotic bacterial strains (VSL#3) in pediatric active UC and demonstrates its role in maintenance of remission. On the basis of our results, probiotics as natural, safe, and well-tolerated, adjunctive treatment to conventional therapy may provide a simple and attractive way to treat pediatric IBD. Further well designed randomized controlled trials, however, with higher patient numbers may be justified to confirm results of this first pediatric study.

CONFLICT OF INTEREST

Guarantor of the article: Annamaria Staiano, MD.
Specific author contribution: All authors have participated in the concept and design, analysis and interpretation of data, and drafting or revising of the article, and they have approved the article as submitted.
Financial support to the project: None.
Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Experimental studies indicate that a probiotic preparation (VSL#3) combining eight different probiotic bacteria has immunomodulatory effects.
- ✓ In small controlled studies, VSL#3 can be effective in preventing pouchitis.
- ✓ In uncontrolled pilot studies, VSL#3 maintains remission in mild to moderate UC.
- ✓ In an open-label study: VSL#3 induces remission in active UC.

WHAT IS NEW HERE

- ✓ This study represents the first pediatric, randomized, placebo-controlled trial that may suggest a possible efficacy of a highly concentrated mixture of probiotic bacterial strains in active UC and in maintenance of remission.

REFERENCES

1. Baldassano RN, Piccoli DA. Inflammatory bowel disease in pediatric and adolescent patients. Gastroenterol Clin North Am 1999;28:445–58.
2. Tibble JA, Sigthorsson G, Bridger B *et al.* Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroenterology 2000;119:15–22.
3. Turner D, Walsh CM, Benchimol EI *et al.* Severe paediatric ulcerative colitis: incidence, outcomes and optimal timing for second-line therapy. Gut 2008;57:331–8.
4. Hyams J, Markowitz J, Lerer T *et al.* Pediatric Inflammatory Bowel Disease Collaborative Research Group. The natural history of corticosteroid therapy for ulcerative colitis in children. Clin Gastroenterol Hepatol 2006;4: 1118–23.
5. Fuller R. A review: probiotics in man and animals. J Appl Bacteriol 1989;66:365–78.
6. Mallon P, McKay D, Kirk S *et al.* Probiotics for induction of remission in ulcerative colitis. Cochrane Database Syst Rev 2007;(4):CD005573.
7. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577–94.
8. Gionchetti P, Lammers KM, Rizzello F *et al.* VSL#3: an analysis of basic and clinical contributions in probiotic therapeutics. Gastroenterol Clin North Am 2005;34:499–513.
9. Caballero-Franco C, Keller K, De Simone C *et al.* The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am J Physiol Gastrointest Liver Physiol 2007;292:G315–22.
10. Soo I, Madsen KL, Tejpar Q *et al.* VSL#3 probiotic upregulates intestinal mucosal alkaline sphingomyelinase and reduces inflammation. Can J Gastroenterol 2008;22:237–42.
11. Gionchetti P, Rizzello F, Venturi A *et al.* Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo controlled trial. Gastroenterology 2000;119:305–9.
12. Mimura T, Rizzello F, Helwig U *et al.* Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut 2004;53:108–14.
13. Gionchetti P, Rizzello F, Helwig U *et al.* Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. Gastroenterology 2003;124:1202–9.
14. Venturi A, Gionchetti P, Rizzello F *et al.* Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. Aliment Pharmacol Ther 1999;13:1103–8.
15. Bibiloni R, Fedorak RN, Tannok G *et al.* VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol 2005;100:1539–46.
16. Hildebrand HF, Holmquist B, Kristiansson I. *et al.* Chronic inflammatory bowel disease in children and adolescents in Sweden. J Pediatr Gastroenterol Nutr 1991;13:293–7.
17. Tiplady B. A basic program for constructing a dispensing list for a randomized clinical trial. Br J Clin Pharmacol 1981;11:617–8.
18. Lichtiger S, Present D, Kornbluth A *et al.* Cyclosporine in severe ulcerative colitis refractory to steroid therapy. N Eng J Med 1994;330:1841–5.
19. Russell GH, katz AJ. Infliximab is effective in acute but no chronic childhood ulcerative colitis. J Pediatr Gastroenterol Nutr 2004;39:166–70.
20. Rutter M, Saunders B, Wilkinson K *et al.* Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. Gastroenterology 2004;126:451–9.
21. Heyman MB, Kirschner BS, Gold BD *et al.* Children with early-onset inflammatory bowel disease (IBD): analysis of a pediatric IBD consortium registry. J Pediatr 2005;146:35–40.
22. Wehkamp J, Stange EF. A new look at Crohn's disease: breakdown of the mucosal antibacterial defense. Ann NY Acad Sci 2006;1072:321–31.
23. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology 2004;126:1620–33.
24. Marteau P, Shanahan F. Basic aspects and pharmacology of probiotics: an overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract Res Clin Gastroenterol 2003;17:725–40.
25. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577–94.
26. Isolauri E, Juntunen M, Rautanen T *et al.* A human Lactobacillus strain (Lactobacillus casei sp strain GG) promotes recovery from acute diarrhea in children. Pediatrics 1991;88:90–7.
27. Schultz M, Veltkamp C, Dieleman LA *et al.* Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-defcient mice. Inflamm Bowel Dis 2002;8:71–80.
28. Madsen KL, Doyle JS, Jewell LD *et al.* Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 1999;116:1107–14.
29. Fabia R, Ar'Rajab A, Johannsson ML *et al.* The effect of exogenous administration of Lactobacillus reuteri R2LC and oat fiber on acetic acid-induced colitis in the rat. Scand J Gastroenterol 1993;28:155–62.
30. Kruis W, Fric P, Pokrotnieks J *et al.* Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut 2004;53:1617–23.
31. Guslandi M, Giollo P, Testoni PA. A pilot trial of Saccharomyces boulardii in ulcerative colitis. Eur J Gastroenterol Hepatol 2003;15:697–8.
32. Rembacken BJ, Snelling AM, Hawkey PM *et al.* Eshcherichia coli vs. mesalazine for the treatment of ulcerative colitis: a randomized trial. Lancet 1999;354:635–9.
33. Kato K, Mizuno S, Umesaki Y *et al.* Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. Aliment Pharmacol Ther 2004;20:1133–41.
34. Tursi A, Brandimarte G, Giorgetti GM *et al.* Low dose balsalazide plus a high potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acutemild-to-moderate ulcerative colitis. Med Sci Monit 2004;10:126–31.
35. Furrie E, Macfarlane S, Kennedy A *et al.* Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. Gut 2005;54:242–9.
36. Madsen K, Cornish A, Soper P *et al.* Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 2001;121:580–91.
37. Shibolet O, Karmeli F, Eliakim R. Variable response to probiotics in two models of experimental colitis in rats. Inflamm Bowel Dis 2002;8:399–406.
38. Rachmilewitz D, Karmeli F, Takabayashi K *et al.* Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. Gastroenterology 2002;122:1428–41.
39. Rachmilewitz D, Katakura K, Karmeli F *et al.* Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 2004;126:520–8.

Journal of Pediatric Gastroenterology and Nutrition
44:e1–e360 © 2007 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

Abstracts
European Society for Paediatric Gastroenterology, Hepatology, and Nutrition
Annual Meeting
May 9–12, 2007
Barcelona, Spain

PG6-09	**ALTERED INTESTINAL PERMEABILITY IS PREDICTIVE OF EARLY RELAPSE IN CHILDREN WITH STEROID-RESPONSIVE ULCERATIVE COLITIS** MIELE E[1], PASCARELLA F[1], QUAGLIETTA L[1], DE LUCA S[1], GRECO L[1], TRONCONE R[1], STAIANO A[1] (1) Department of Pediatrics, University "Federico II", Naples, ITALY.
	Aims: The predictive values of many clinical disease activity indices and histologic and laboratory parameters in identifying patients at risk of relapse of inflammatory bowel disease have generally been disappointing. The purpose of this study was to determine if small bowel involvement at diagnosis could predict early relapse in children with ulcerative colitis (UC). **Methods**: Children with newly diagnosed UC were prospectively evaluated a three time points: within 1 month, 6 months, and 1 year after diagnosis. Clinical activity indices were used to measure disease activity. Laboratory studies were performed at each visit and/or at the time of relapse. At diagnosis, all patients underwent colonoscopy and a cellobiose/mannitol small intestinal permeability study (CMPS). Some children were further investigated with an UGI endoscopy. **Results:** Thirty-three patients completed one-year study. Overall, 9 patients (27.3%) relapsed within 6 month of diagnosis, one patient (3%) within one year, whereas 23 patients (69.7%) did not relapse. The mean clinical activity indices, laboratory parameters, extent of colonic involvement, upper and lower GI histologic features were not predictive of early relapse. Results of the CMPS were significantly lower in children who relapsed within 6 months compared to children who did not relapse ($p<0.013$). The CMPS was abnormal in 77.8% of early relapsers compared with only 8.3% of non-relapsers. An abnormal CMPS gave a sensitivity of 77.7%, a specificity of 91.6%, a positive predictive value of 77.7% and a negative predictive value of 91.6% in predicting early relapse in UC pediatric patients. **Conclusions:** An abnormal small intestinal permeability in children with UC could predict a more relapsing disease. If these data are confirmed in a larger scale, the CMPS may represent a non-invasive test useful in identifying the patients requiring more targeted treatment.

Journal of Pediatric Gastroenterology and Nutrition
48:E1–E149 © 2009 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

Abstracts

European Society for Paediatric Gastroenterology, Hepatology, and Nutrition Annual Meeting
June 3–6, 2009
Budapest, Hungary

Conclusions: It is suggested that changes in alveolo-capillary diffusion observed in Crohn's disease could be due to a decrease of the pulmonary capillary bed. These changes are likely related to the disease activity.

PG2-18

IMMUNOHISTOCHEMICAL MARKERS OF SMALL BOWEL INFLAMMATION IN CHILDREN WITH ULCERATIVE COLITIS

Presenter: L. Quaglietta. *University of Naples, Naples, Italy.*
Co-authors: E. Giannetti[1], C. Aquino[1], F. Paparo[1], E. Miele[1], C. Friano[1], R. Troncone[1], A. Staiano[1]. [1]*Department of Pediatrics, University of Naples, Naples, Italy.*

Background and Aim: We recently demonstrated that abnormal cellobiose/mannitol small intestinal permeability study is a predictive marker of early relapse in children with ulcerative colitis (UC). The purpose of this study was to evaluate in the jejunum of children with UC, by immunohistochemistry, subtle inflammatory changes, even in presence of normal histopathology.
Methods: From February to December 2008, 18 pediatric patients (M:F 8:10, mean age: 126 months, age range: 30–191 months) with new diagnosis of UC, based on accepted historical, endoscopic, histological and/or radiological criteria, were evaluated. The Pediatric Ulcerative Colitis Activity Index (PUCAI) was used to measure disease activity. All patients underwent upper gastrointestinal (GI) endoscopy and duodenal cryostat sections were stained for CD3+ and gamma/delta + T cells and CD25+ mononuclear cells. Twenty-four children with functional dyspepsia, who underwent upper GI endoscopy, represented the control group.
Results: Although UC patients and controls did not show any jejunal endoscopic lesions, in 3/18 (16%) UC children the histological evidence of mild inflammation of jejunum was noted. As a group UC children presented a significant higher density of lamina propria CD25 + cells compared to controls (mean ± SEM: 9.4/mm^2 ± 1.7 vs 2.9 ± 0.2; $P < 0.0001$). Among these children, 3/12 (75%) presented a PUCAI value ≥20 (moderate disease activity) and 9/12 (69%) patients showed a PUCAI value ≥35 (severe disease activity). No significant differences were noted as regard CD3+ and gamma/delta + T cells between study population and controls.
Conclusions: The majority of UC children present inflammatory signs at level of the jejunal mucosa even in the absence of gross histopathological changes. The extent of upper GI tract involvement is wider than previously thought. Upper GI tract inflammation is related to the activity of the disease and is probably a predictive marker of early relapse.

PG2-19

DOES INFLIXIMAB FAVOURABLY AFFECT GROWTH AND BODY COMPOSITION IN PAEDIATRIC CROHN DISEASE?

Presenter: R. Hill. *University of Queensland, Herston, Australia.*
Co-authors: I. Grange[2], L. Ee[1], G. Withers[1], P. Lewindon[1], F. Connor[1], G. Gleghorn[1], P. Davies[1]. [1]*University of Queensland, Herston, Australia;* [2]*Institut Polytechnique LaSalle Beauvais, Beauvais, France.*

Aim: To determine if infliximab therapy improves growth, weight, body mass index (BMI) and nutritional status in children with Crohn disease (CD).
Methods: In our laboratory, measurements of growth and nutritional status are routinely carried out (6 monthly) on children with CD. Data are presented from a subset of these children (n = 7) who commenced infliximab therapy following their baseline assessments. All children were dosed with infliximab according to body weight and standard protocols. Height and weight were measured and BMI was calculated. Total body potassium (TBK) was used to determine nutritional status. Data were converted to z-scores and changes were assessed in relation to the number of doses of infliximab received and the duration of exposure (6, 12, 18 and 24 months). Relationships were analysed using Pearson's correlation and ANOVA was used to determine differences.
Results: The number of infliximab doses per child ranged from 2 to 13 (mean ± SD = 6.6 ± 3.3). The duration of data available ranged from 6 to 24 months. At baseline, the mean (±SD) age, height, weight, BMI and TBK of the children were 13.5(±2.4) years, 150.2(±14.3) cm, 43.0(±11.2) kg, 18.7(±2.1) kg/m^2, and 74.4(±20.2) g, respectively. The mean z scores for height, weight, BMI and TBK were −1.17(±1.0), −0.79(±1.09), −0.16(±0.97) and −0.84(±0.91), respectively. The mean change in z scores from baseline were positive (height = 0.28(±0.38), weight = 0.37(±0.54), BMI = 0.25(±0.64) and TBK = 0.29(±0.81)), however, no significant differences were found between baseline measurements and subsequent time points. The change in height z score was significantly positively correlated with the number of doses of infliximab ($r = 0.62$, $P = 0.00$) and the duration of exposure ($r = 0.59$, $P = 0.00$). However, a partial correlation between height z score change and number of doses, controlling for duration, was not significant. No significant relations were found for change in weight, BMI and nutritional status z scores.
Conclusions: Change in height z score increased with increasing number of doses of infliximab and increased duration of exposure; however, the lack of significance found after controlling for duration suggests that the positive change in height z score may simply be a function of normal growth over time, irrespective of

ELSEVIER

JCC
JOURNAL OF CROHN'S & COLITIS
SUPPLEMENTS

International Journal Devoted to Inflammatory Bowel Diseases

PIBD 2009

International Symposium on Pediatric Inflammatory Bowel Disease

Abstracts of PIBD 2009

9–12 September 2009, Paris, France

A Journal of the European Crohn's and Colitis Organisation

ECCO

O10
ASSOCIATION BETWEEN IL23R, ATG16L1, IRGM AND NOD2 SUSCEPTIBILITY GENES AND SUBPHENOTYPES ON A LARGE COHORT OF IBD PATIENTS

C. Jung[1]*, J. Colombel[2], L. Beaugerie[3], M. Lemann[4], J. Cezard[5], F. Ruemmele[6], D. Turck[7], H. Zouali[8], G. Thomas[8], J. Hugot[1]. [1]*U843, INSERM, Paris*; [2]*Gastroenterology, Hôpital C. Huriez, Lille*; [3]*Gastroenterology, Hôpital St Antoine*; [4]*Gastroenterology, Hôpital St Louis*; [5]*Pediatric gastroenterology, Hôpital R Debré*; [6]*Pediatric gastroenterology, Hôpital Necker, Paris*; [7]*Pediatric gastroenterology, Hôpital Jeanne de Flandre, Lille*; [8]*Genetics, CEPH, Paris, France*

The aim of the study was to determine the genotype/phenotype correlations for 3 newly reported susceptibility genes on IL23R, ATG16L1 and IRGM genes as well as for the main NOD2 CD associated SNPs in a large cohort of IBD patients with detailed records on disease subphenotypes. 1028 IBD patients (788CD, 192UC, 48IC) have been genotyped for IL23R, ATG16L1 and IRGM SNPs (rs11209026, rs2241180, rs13361189) and for the 3 main NOD2 variants and correlations between genotypes and phenotypes were searched for. The association of ATG16L1 and NOD2 SNPs with CD but not with UC or IC was confirmed ($p=0.021$, $p=0.0001$, respectively). NOD2 rare variants were associated with an earlier age of CD onset ($p=0.0001$) and with an ileal involvement ($p=0.0001$). On CD patients, ATG16L1 at risk allele was associated with a penetrating behavior ($p=0.027$) whereas the protective alleles of ATG16L1 and IRGM were associated with an inflammatory behavior ($p=0.009$ and $p=0.03$ respectively). The IL23R at risk allele was associated with a familial history of IBD ($p=0.02$). No interactions between the analyzed variants were found. NOD2 SNPs are confirmed to be strongly associated with an ileal location and a young age of CD onset while the newly reported IBD genes IL23R, ATG16L1 and IRGM are only modestly associated with CD clinical presentation.

O11
CURCUMIN SUPPRESSES P38 MAPK ACTIVATION, REDUCES IL-1BETA AND MMP-3, AND ENHANCES IL-10 EXPRESSION IN THE MUCOSA OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE

J. Epstein[1]*, T.T. MacDonald[2], I.R. Sanderson[1]. [1]*Digestive Diseases*; [2]*Immunology, Barts and The London, London, United Kingdom*

Background: IBD results from activation of pro-inflammatory, and failure of anti-inflammatory pathways. p38 MAPK is central to the coordination of inflammatory responses and is raised in IBD, suggesting a critical role in pathogenesis and presenting a target for therapy. In IBD there is excess production of pro-inflammatory cytokines including IL-1beta and under-expression of the major anti-inflammatory cytokine IL-10. Fibroblasts over-produce matrix metalloproteinases (MMP), mediating tissue destruction. Curcumin, a component of the spice turmeric, is anti-inflammatory and shows clinical potential in IBD.
Objectives: To assess the effect of curcumin on p38 MAPK activation and on the expression of cytokines and MMP-3 in the gut of children and adults with IBD.
Methods: Colonic mucosal biopsies and myofibroblasts (CMF) from children and adults with active IBD were cultured ex vivo with curcumin. p38 MAPK, NF-kappaB and MMP-3 were measured by immunoblotting and IL-1beta and IL-10 by ELISA.
Results: We show reduced p38 MAPK activation in curcumin-treated mucosal biopsies, enhanced IL-10 expression and reduced IL-1beta. We also demonstrate dose-dependent suppression of MMP-3 in CMF with curcumin, by a mechanism which appears to be p38-independent.
Conclusion: Curcumin, a naturally occurring food substance with no known human toxicity, holds promise as a novel therapy in IBD.

O12
HEMIN ATTENUATES IL-17 PRODUCTION AND EXPERIMENTAL COLITIS

Z. Zhang[1]*, W. Zhong[1], D.J. Hinrichs[2], J.T. Rosenbaum[3]. [1]*Pediatrics*, [2]*Immunology*, [3]*Rheumatology, Oregon Health and Science University, Portland, United States*

Th17 lymphocyte activation and regulatory T cell defect are responsible for the pathogenesis of IBD. Heme oxygenase-1 (HO-1) plays an anti-inflammatory role in many diseases. In this study, we used a dextran sulfate sodium (DSS)-induced colitis model to investigate the effect of up-regulating HO-1 by hemin on the development of colitis. Mice were intraperitoneally administered with hemin for 2 days. Hemin treatment markedly induced HO-1 expression in the colon epithelium. The up-regulation of HO-1 was further correlated with attenuation of DSS-induced colitis. Next, we examined if hemin enhanced the proliferation of FoxP3+ regulatory T cells (Treg) and suppressed the production of interleukin (IL)-17. Flow cytometry analysis revealed that hemin did not notably alter peripheral CD4+CD25+FoxP3+ Treg cell population. In contrast, hemin significantly attenuated IL-17 and its downstream cytokine, IL-6. This coincided with reduced colonic inflammation. Finally, real time-PCR showed that hemin suppressed an array of IL-17-related gene expression, indicating that hemin exerts a broad modulatory effect on IL-17 induction. In summary, these results demonstrate that up-regulation of HO-1 by hemin ameliorated experimental colitis. Moreover, our study suggests that the inhibition of IL-17 is a novel anti-inflammatory mechanism of hemin.

O13
IMMUNOHISTOCHEMICAL MARKERS OF SMALL BOWEL INFLAMMATION IN CHILDREN WITH ULCERATIVE COLITIS

L. Quaglietta[1]*, E. Giannetti[1], C. Aquino[1], F. Paparo[1], E. Miele[1], C. Friano[1], R. Troncone[1], A. Staiano[1]. [1]*Department of Pediatrics, University of Naples Federico II, Naples, Italy*

Background: We recently demonstrated that abnormal cellobiose/mannitol small intestinal permeability study is a predictive marker of early relapse in children with ulcerative colitis (UC).
Aim: The purpose of this study was to evaluate in the jejunum of children with UC, by immunohistochemistry, subtle inflammatory changes, even in presence of normal histopathology.
Methods: From February 2008 to March 2009, 20 pediatric patients (M:F = 8:12, mean age: 126 months, age range: 30–191 months) with new diagnosis of UC were evaluated. All patients underwent upper GI endoscopy and duodenal cryostat sections were stained for CD3+ and γδ+ T cells and CD25+ mononuclear cells. Twenty-four children with functional dyspepsia, who underwent upper GI endoscopy, represented the control group.
Results: UC patients and controls did not show any jejunal endoscopic lesions; in 3/20 (15%) UC children the histological evidence of mild inflammation of jejunum was noted. As a group UC children presented a significant higher density of lamina propria CD25+ cells compared to controls (mean±SD: 9.1/mm2±7 vs 3±1.2; $p<0.0001$). In particular 13/20 (65%) showed an exceedingly high number. No significant differences were noted as regard CD3+ and γδ+ T cells between study population and control group.
Conclusion: The majority of UC children present inflammatory signs at level of the jejunal mucosa even in the absence of gross histopathological changes. The extent of upper GI tract involvement is wider than previously thought.

Poster Communications

P001
T-CELL REGULATION OF NEUTROPHIL INFILTRATE AT THE EARLY STAGES OF A MURINE COLITIS MODEL

P.P.E. van Lierop[1]*, C. de Haar[1], D. Lindenberh-Kortleve[1], Y. Simons-Oosterhuis[1], L. van Rijt[2], B. Lambrecht[2], J.C. Escher[1], J.N. Samsom[1], E.E.S. Nieuwenhuis[1]. [1]*Gastroenterology, Erasmus MC – Sophia Children's Hospital*, [2]*Pulmonary Medicine, Erasmus MC, Rotterdam, Netherlands*

Background: T-cells are a main target for anti-inflammatory drugs in IBD. As the innate immune system is equally implicated in the pathogenesis of IBD, T-cell suppressors may also affect innate immune cell function. Specifically, these drugs may impair innate immune cell recruitment through inhibition of T-cells or act independent of T-cell modulation. We explored the extent of immune modulation by the T-cell inhibitor tacrolimus in a murine colitis model.
Methods: We assessed the effects of tacrolimus on TNBS colitis in wild type and RAG2-deficient mice. Severity of colitis was assessed by means of histological scores. We further characterized the inflammation using immunohistochemistry and by analysis of isolated intestinal leukocytes.
Results: Tacrolimus treated wild type mice were less sensitive to colitis and had fewer activated intestinal T-cells. Inhibition of T-cell function was associated with strongly diminished recruitment of infiltrating neutrophils in the colon. In agreement, immunohistochemistry demonstrated that tacrolimus inhibited production of neutrophil chemoattractants CXCL1 and CXCL2. In T-cell deficient mice, tacrolimus did not affect the severity of TNBS colitis or numbers of intestinal neutrophils.
Conclusion: Both the innate and the adaptive mucosal immune system contribute to TNBS colitis. Tacrolimus suppresses colitis directly through inhibition of T-cell activation and by suppression of T-cell-mediated recruitment of neutrophils.

P002
KIR GENES AS DETERMINANTS OF INNATE RESISTANCE/SUSCEPTIBILITY TO CROHN'S DISEASE

S. Samarani[1]*, Z. Almalte[1], I. Alexandre[1], O. Debbeche[1], D. Amre[2], A. Ahmad[1]. [1]*CHU Ste-Justine-Dep Microbiology and Immunology*, [2]*CHU Ste-Justine-Dep Paediatrics, University of Montreal, Montreal, Canada*

The Killer-cell Immunoglobulin-like Receptor (KIR) family genes are highly polymorphic. They are expressed as receptors on the surface of Natural Killer (NK) cells, NKT cells and different subsets of T cells. The receptors

Approccio diagnostico strumentale in gastroenterologia pediatrica

A. Staiano, L. Quaglietta
Dipartimento di Pediatria, Università di Napoli "Federico II"

Introduzione

La patologia gastrointestinale rappresenta una delle più frequenti cause di consultazione pediatrica, così come risulta essere una delle cause più frequenti di ricovero ospedaliero e consultazione specialistica. L'evoluzione tecnologica nell'approccio diagnostico strumentale alla patologia gastrointestinale si è notevolmente evoluto nell'ultimo decennio, portando frequentemente alla luce patologie prima ritenute rare in età pediatrica e consentendo, in molte situazioni, una diagnosi più precoce. Così come accade nel caso di nuove terapie, anche l'esperienza sull'uso di metodiche innovative in età pediatrica si basa spesso su informazioni mutuate dall'adulto, malgrado la consapevolezza delle specificità e delle particolarità del bambino. Di seguito verranno analizzate nel dettaglio le principali novità strumentali nella diagnosi di patologie gastrointestinali in pediatria, con attenzione ai vantaggi e ai limiti delle metodiche e valutando criticamente il ruolo nella pratica clinica e i benefici derivanti dal loro impiego in tale fascia d'età.

pH-impedenzometria esofagea intraluminale

L'impedenzometria, introdotta nel 1991 come tecnica capace di individuare il flusso di liquidi o gas attraverso un organo cavo, si è presentata fin da subito come una metodica in grado di offrire nuove opportunità per studiare il transito esofageo e per monitorare il reflusso gastroesofageo (RGE) [1-3]. Di seguito ci soffermeremo sull'impedenzometria esofagea combinata con la pH-metria (MII-pH) delle 24 ore e valuteremo aspetti tecnici e applicazioni cliniche in età pediatrica.
La MII-pH esofagea fornisce informazioni sulla presenza di qualunque tipo di bolo che refluisce in esofago (gassoso, liquido, acido o basico) identificando velocità ed estensione prossimale del suo movimento. Il gold standard attuale per l'individuazione del RGE è la pH-metria delle 24 ore ma questo metodo non individua reflussi non acidi, evento diagnosticabile invece con la MII-pH. Il principio base della metodica è identico a quello della pH-metria esofagea: registrare dati riguardanti l'esofago attraverso una sonda posizionata per via trans-nasale e connessa ad un registratore per 24 ore. La tecnica impedenzometrica si basa sulla misurazione, al passaggio del bolo, dell'impedenza elettrica tra elettrodi ravvicinati usando una

sonda intraluminale. L'impedenza elettrica di un volume fisico è la sua resistenza al passaggio di una corrente alternata ed è inversamente proporzionale alla composizione in ioni (conduttività). Elettrodi cilindrici di metallo sono contenuti all'interno di un sottile catetere di plastica ed ogni coppia è connessa ad un trasduttore di voltaggio. Il bolo liquido ha sempre impedenza bassa rispetto alla linea di base mentre l'aria induce aumento di impedenza. In occasione del passaggio di un bolo liquido si osserva un iniziale repentino incremento dell'impedenza seguito da una prolungata riduzione. Questo fenomeno bifasico è legato alla presenza di un bolo gassoso molto veloce che precede quello liquido. L'impedenza rimane bassa nel periodo compreso tra l'entrata del bolo nel segmento misuratore e la sua uscita da quel segmento. La dilatazione del lume esofageo in seguito all'ingresso del bolo procura una caduta di impedenza; al contrario, al termine dell'eliminazione del bolo liquido si osserva un piccolo aumento transitorio di impedenza al di sopra della linea basale, a causa della riduzione del diametro esofageo per la peristalsi [2]. Infine, misurando l'impedenza a diversi livelli si può determinare la direzione anterograda o retrograda del moto del bolo.

La recente pubblicazione di un consensus report ha fornito informazioni dettagliate sulla nomenclatura più appropriata per definire le caratteristiche del reflussato ricavate dalla MII-pH esofagea [4]. Le caratteristiche fisiche dell'evento reflusso (liquido, gas o misto) sono identificate con l'impedenzometria mentre il simultaneo monitoraggio pH-metrico consente di distinguere i reflussi in: *acidi* (caduta di pH < 4), *debolmente acidi* (discesa del pH di almeno 1 punto fino a pH > 4) e *non acidi* (discesa del pH < 1 punto che si arresta prima del pH 4). Il cut-off tra reflussi *debolmente acidi* e reflussi *acidi* è fissato a pH 7. Il documento propone inoltre il termine "reflusso debolmente alcalino" per quegli episodi, peraltro rari, in cui il nadir del pH esofageo non scende < 7. I parametri che vengono presi in considerazione per la valutazione di uno studio pH-impedenzomentrico sono: la percentuale di tempo con pH < 4, il numero totale degli episodi di reflusso, l'esposizione al bolo liquido dell'esofago distale (periodo assoluto e percentuale dell'esame) ed il tempo di clearance del bolo liquido (permanenza media di un bolo liquido in esofago distale). Questi ultimi tre parametri possono essere ulteriormente analizzati in funzione delle caratteristiche chimico-fisiche del bolo (acido, non acido, debolmente acido, sovrapposto, liquido, gassoso o misto) e della posizione del paziente (clino- ed orto-statica). Recentemente uno studio multicentrico condotto su pazienti adulti americani ed europei ha fornito i valori normali per la MII-pH in tale fascia d'età, offrendo dei dati di riferimento sia nella pratica clinica che nella ricerca [5-7]. Studi simili sono stati condotti anche in età pediatrica. In particolare uno studio condotto su neonati pretermine sani, alimentati attraverso sondino nasogastrico e asintomatici, ha messo a disposizione valori di riferimento che potranno essere confrontati con quelli di neonati pretermine in condizioni patologiche [8]. In età pediatrica la sensibilità della MII-pH per valutare il RGE è equiparabile a quella della sola pH-metria delle 24 ore nei bambini non

trattati con farmaci acido-soppressori, ma è superiore alla pH-metria nei pazienti trattati [9,10].

La principale domanda che i ricercatori si sono posti di fronte ai risultati della MII-pH esofagea è la rilevanza clinica dei reflussi non acidi o debolmente acidi messi in luce dalla metodica. È consenso unanime ritenere che la valutazione strumentale (ecografica, radiologica, scintigrafica) del reflusso nel periodo postprandiale per la diagnosi di RGE sia di scarso valore clinico, considerata l'alta prevalenza di reflussi in fase post-prandiale. In uno studio condotto su 16 neonati pretermine alimentati con sondino nasogastrico posizionato oltre il cardias, la MII-pH rilevava un aumento notevole (> 2 volte) del numero degli episodi di reflusso non acido nel periodo post-prandiale e circa l'80% di essi avevano estensione prossimale in esofago [11]. Al contrario Condino et al. [12] hanno riportato in un gruppo di lattanti tra 2 e 11 mesi di vita una distribuzione pressoché equivalente di reflussi acidi e non acidi. È da considerare che l'impatto del reflusso post-prandiale non acido diminuisce con l'età, con la riduzione del numero dei pasti e quindi della durata totale dei periodi post-prandiali [12]. Anche la posizione del bambino è stata valutata come rilevante nell'influenzare presenza e tipo di reflusso. La MII-pH combinata con la manometria esofagea in 10 neonati pretermine sani (età gestazionale: 35-37 settimane) ha mostrato che il numero degli episodi di reflusso erano più frequenti quando i neonati erano posizionati sul lato destro, suggerendo il rapido riempimento gastrico e gli inappropriati rilassamenti transitori dello sfintere esofageo inferiore come principale meccanismo patogenetico che causa episodi di reflusso in questi pazienti [13]. Oltre alla posizione del paziente sono stati oggetto di studio anche gli effetti della formula lattea e dell'alginato su estensione prossimale, frequenza degli episodi e tipo di reflusso. Wenzl et al. [14] hanno condotto un trial clinico in cui venivano valutati gli effetti della formula antireflusso in 14 lattanti utilizzando la MII-pH. Gli autori hanno confermato l'efficacia della formula su frequenza e severità degli episodi di reflusso, e hanno evidenziato un più marcato effetto sui reflussi non acidi, che mostravano per lo più una tendenza all'estensione prossimale. Inoltre l'alginato non era in grado di ridurre il numero di reflussi postprandiali ma ne alterava marginalmente l'estensione prossimale [15].

È piuttosto improbabile che il reflusso non acido svolga un ruolo importante in pazienti con esofagite (endoscopia e biopsia sono il gold standard per la diagnosi del danno mucosale). È noto che la bilirubina è tossica sulla mucosa esofagea tanto quanto l'acido; tuttavia il numero di pazienti con danni mucosali e evidenza di reflussi alcalini o non acidi è limitato [16,17]. Non esistono al momento dati che correlino danno mucosale e risultati della MII-pH.

Molti bambini presentano sintomi respiratori cronici correlabili al RGE: bronchite cronica, wheezing, tosse cronica e apnee. Solo uno studio fino ad ora pubblicato riporta la correlazione tra reflusso acido e non acido e sintomi respiratori. In 22 bambini con rigurgito e con sintomi respiratori, il 90% degli episodi di reflusso erano debolmente acidi. Nello stesso gruppo di pazienti 49/165 episodi di apnea

erano accompagnati da episodi di reflusso e di questi il 77% erano debolmente acidi [16]. Dati simili sono stati riportati in lattanti con apnee, suggerendo l'associazione tra tale sintomo e reflusso debolmente acido di durata superiore a 30 secondi [18]. In ogni caso la relazione tra apnea del prematuro e reflusso debolmente acido resta controversa ed altri studi recentemente pubblicati sembrano non confermare né l'associazione temporale tra il sintomo apnea ed episodio di reflusso né tra sintomo e tipo di reflusso (debolmente acido). Uno studio condotto su 24 pazienti di età compresa tra 5 e 67 mesi affetti da asma, con lo scopo di valutare la proporzione di reflussi acidi e non acidi e determinare l'indice sintomatico, ha dimostrato che i reflussi acidi e non acidi si manifestano con eguale frequenza nei bambini con asma. La maggior parte dei sintomi respiratori si verificherebbe in maniera dissociata dall'evento reflusso [19]. Rosen e Nurko [20] hanno riportato la loro esperienza su 28 bambini affetti da patologia respiratoria cronica e sottoposti a trattamento con farmaci antisecretori. Il tipo di reflusso maggiormente registrato era per lo più di tipo non acido. Inoltre più alta era l'estensione del reflussato e più severi risultavano essere i sintomi presentati.
In conclusione fino ad oggi non sono ancora disponibili dati che suggeriscano un reale beneficio dall'uso della MII-pH delle 24 ore in età pediatrica. Malgrado gli incoraggianti risultati riportati negli studi fino ad ora condotti, la mancanza di forti evidenze scientifiche, l'eterogeneità dei gruppi di pazienti esaminati, il deficit di valori normali per età, rendono tali dati di dubbia interpretazione; accanto a questi limiti strutturali si collocano inoltre i costi elevati della procedura e il tempo necessario per l'analisi e l'interpretazione dei risultati. Nuovi e accurati studi contribuiranno in futuro a chiarire il ruolo della MII-pH in età pediatrica.

Manometria esofagea

Esofago e sfintere esofageo superiore (SES) e inferiore (SEI) agiscono di concerto per garantire il passaggio del bolo deglutito allo stomaco e prevenire il reflusso del contenuto gastrointestinale. A regolare e coordinare tale meccanismo sono impegnati il sistema nervoso centrale e il complesso sistema neuronale della parete esofagea. Per molti anni la manometria esofagea ha fornito al clinico e al ricercatore informazioni riguardanti la funzione motoria dell'esofago e dei suoi sfinteri, giocando un ruolo chiave nell'identificazione di specifiche anomalie motorie. La manometria esofagea viene impiegata per valutare i pazienti con disfagia, dolore toracico non cardiaco e nel work-up pre-operatorio di pazienti destinati alla chirurgia antireflusso. Misura la pressione a livello degli sfinteri esofagei nonché l'efficacia e la coordinazione dei movimenti propulsivi e mette in evidenza contrazioni anomale. È impiegata per la diagnosi di acalasia, spasmi esofagei diffusi, sclerodermia, ipo- e ipertensione dello SEI e per valutare la funzione esofagea dopo determinati procedimenti terapeutici [21]. Viene praticata introducendo un sondino in esofago per via nasogastrica, in grado di registrare le pressioni a

diversi livelli. Durante l'esame sensori intraluminali di pressione convertono il segnale pressorio in segnale elettrico che viene registrato e mostrato graficamente. La pressione intraluminale può essere rilevata da sensori localizzati all'interno dello stesso catetere *(solid-state tecnique)* oppure, attraverso canali a perfusione liquida *(water-perfused tecnique)*, è rilevata da trasduttori esterni che trasmettono il segnale a un registratore. I cateteri utilizzati per la manometria a perfusione liquida sono più resistenti ma più spesso producono artefatti legati alla presenza di bolle d'aria o detriti. I sensori solidi sono in grado di registrare meglio l'attività motoria del faringe e dello SES ma sono più costosi e più fragili [22].

La manometria si è gradualmente evoluta da una procedura che prevedeva l'uso di un catetere con pochi (5-6) rilevatori di pressione alla manometria ad alta risoluzione (HRM), che viene eseguita con cateteri costituiti da più di 20 sensori di pressione (recentemente fino a 36 sensori) e pertanto in grado di fornire una più accurata registrazione delle pressioni esofagee [23-25]. Dal punto di vista pratico la HRM ha come suo maggiore vantaggio la rapidità e semplicità dell'esecuzione e come svantaggio il costo dell'attrezzatura richiesta. Di seguito saranno discussi caratteristiche strumentali e ruolo nella pratica clinica dell'HRM.

Manometria esofagea ad alta risoluzione

La manometria esofagea ad alta risoluzione è stata messa a punto nei primi anni '90 da R. Clouse e A. Staiano. Attraverso un programma computerizzato di plottaggio, lo studio topografico della motilità esofagea trasforma l'onda pressoria misurata durante la manometria convenzionale in immagini tridimensionali, valutando sia relazioni spaziali che temporali degli eventi pressori esofagei. Pertanto, i parametri relativi a tempo, posizione del catetere (come distanza dalle narici) e pressione media vengono utilizzati per ricostruire l'anatomia funzionale dell'esofago [25-27]. Gli elementi fondamentali per la rappresentazione topografica dei segnali manometrici sono due: 1) l'uso di un sistema costituito da molteplici sensori esofagei di pressione posti a breve distanza l'uno dall'altro (in genere a 1 cm), in modo che l'interpolazione assiale risulti più accurata possibile; 2) la disponibilità di un software di plottaggio in grado di mostrare le pressioni assiali in rapporto al tempo, generando così immagini tridimensionali. Un catetere esofageo con 21 punti di registrazione distanziati di un 1 cm l'uno dall'altro, permette una valutazione topografica > 80% del corpo esofageo e SES e SEI.

I tracciati pressori vengono prima allineati su una superficie "planare", in modo che le pressioni tra i diversi siti di registrazione possano essere analizzate. Successivamente i dati pressori sono interpolati tra i sensori applicando colori diversi a specifici livelli di pressione per formare una griglia pressoria. I valori pressori possono essere raffigurati in differenti modi, con immagini plottate delle superficie pressorie o con immagini dei contorni pressori. Il plottaggio dei contorni corrisponde ad una visione dall'alto degli elementi di superficie. I dati

vengono raccolti su 3 specifiche coordinate *x, y, z* ad ogni punto di registrazione, dove *x* è la distanza dallo SEI in cm *y* è il tempo di deglutizione in secondi e *z* è l'ampiezza delle onde in mm Hg, formando griglie tridimensionali, mediante il software di plottaggio. Il plottaggio dei contorni pressori della deglutizione trasforma l'immagine tridimensionale in una mappa caratterizzata da anelli concentrici corrispondenti ad incrementi pressori. L'interpretazione visiva è semplificata dal riempimento degli anelli con colori differenti a seconda dell'ampiezza delle onde (Fig. 1).

L'attrezzatura utilizzata per la HRM è simile a quella della manometria convenzionale, tranne per il software (*Golden Graphic*) che permette la visualizzazione sia delle immagini della manometria convenzionale che le immagini topografiche. I cateteri hanno molti punti di registrazione e possono essere a perfusione lenta, con riduttori oppure, a costi maggiori, esistono più moderni cateteri con microtrasduttori solidi che evitano tutte le difficoltà legate alla perfusione.

HRM e normale motilità esofagea

La dimostrazione topografica della normale peristalsi esofagea rivela informazioni che non possono essere apprezzate con la manometria convenzionale. La peristalsi appare come una catena di segmenti pressori. La Figura 2 mostra un plottaggio di una normale peristalsi esofagea con il catetere a 21 lumi. Il primo segmento pressorio si estende dal SES fino ad un avvallamento pressorio, che dovrebbe corrispondere al passaggio tra muscolo striato e muscolo liscio. Nell'esofago distale la regione esofagea formata da muscolatura liscia sembra essere composta da due segmenti sequenziali, che dividono nettamente l'esofago distale in 2 parti. Un terzo avvallamento pressorio ulteriormente separa il corpo esofageo distale dalla contrazione post-deglutizione dello SEI [24-26 28]. Recentemente è stato dimostrato che i 3 segmenti pressori sequenziali individuati nell'adulto, malgrado non completamente formati, possono essere individuati anche in neonati pretermine e a termine, adattando la metodica a tale fascia d'età. Tuttavia in questi piccoli pazienti la peristalsi è incompleta in circa la metà delle deglutizioni. I meccanismi di controllo della muscolatura liscia e striata non sono ancora completamente sviluppati nei neonati, condizione che potrebbe contribuire alla patogenesi della malattia da reflusso gastroesofageo del bambino. I risultati di questo studio forniscono rilevanti informazioni per la comprensione dell'ontogenesi della motilità esofagea [29]. Successivamente gli stessi autori hanno praticato la HRM in neonati e bambini (età: 1 giorno-14 anni) nei quali vi era indicazione alla manometria esofagea, con lo scopo di determinare la presenza o meno del pattern pressorio segmentale e verificarne l'eventuale variabilità tra gruppi di diversa età o in presenza di patologia esofagea [30]. La presenza del pattern pressorio costituito dai 3 segmenti sequenziali era sempre più evidente man mano che si passava dal neonato al bambino più grande, senza differenze statisticamente significative in caso di patologia esofagea. È fondamentale, dunque, sia nell'adulto che nel

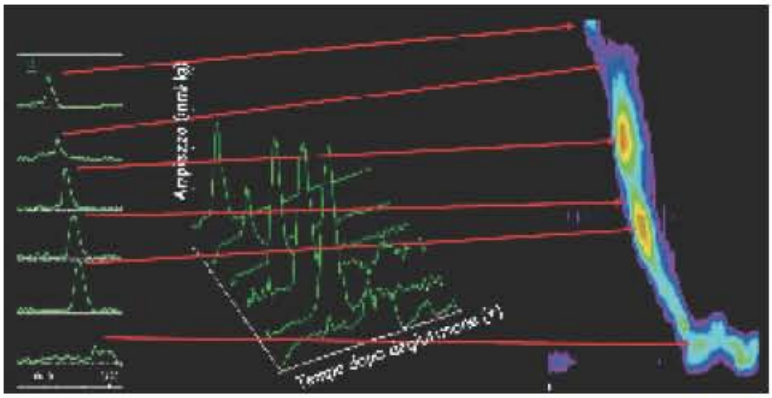


Figura 1.

Esempio di plottaggio dei contorni pressori di una deglutizione grazie alla manometria ad alta risoluzione (HRM). L'immagine tridimensionale viene trasformata in una mappa caratterizzata da anelli concentrici corrispondenti ad incrementi pressori. A ciascun anello di diverso colore corrisponde un diverso valore pressorio (ampiezza delle onde).

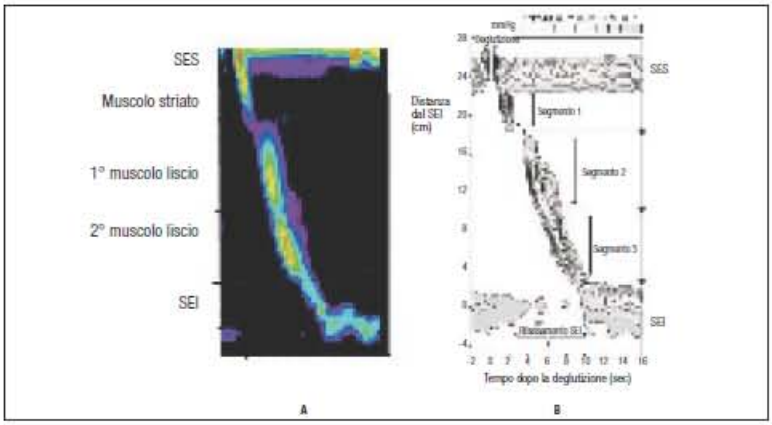


Figura 2.

Caratteristiche tipiche della peristalsi esofagea visualizzata con la manometria ad alta risoluzione (HRM). Sono indicati i tre segmenti pressori separati da tre avvallamenti di pressione. Il primo segmento parte dallo sfintere esofageo superiore (SES) subito dopo la deglutizione. Il primo avvallamento pressorio separa questo segmento dal resto del corpo esofageo costituito da muscolo liscio. Il terzo avvallamento separa questa catena peristaltica dallo sfintere esofageo inferiore (SEI). Gli incrementi pressori sono rappresentati da anelli concentrici in diversi colori (pannello A) o gradazioni di grigio (pannello B), corrispondenti a diverse ampiezze.

bambino tenere conto della presenza della catene dei segmenti pressori durante l'interpretazione di esami manometrici (ad esempio effettuati per testare effetti farmacologici o per valutare la clearance esofagea).

HRM: dalla ricerca alla pratica clinica

La HRM per essere ritenuta utile nella pratica clinica deve: 1) distinguere gli eventi pressori anomali che possono alterare la funzione esofagea e causare sintomi da quelli che non hanno alcun significato clinico; 2) identificare la causa dei sintomi in pazienti nei quali la manometria convenzionale non è risultata diagnostica; 3) incrementare il potere diagnostico e l'accuratezza della metodica [21]. Solo due studi fino ad ora condotti hanno confrontato la HRM con la manometria convenzionale. Entrambi riportano esempi di condizioni patologiche individuabili solo grazie alla HRM, soprattutto in pazienti con disfagia ed endoscopia negativa (Tab. I) [31 32]. In uno studio clinico gli autori mettevano a confronto HRM e manometria convenzionale con tecnica *pull-through* in 220 pazienti con sintomi che indicavano l'esecuzione della manometria esofagea. In questo studio la HRM si rivelava più accurata della manometria convenzionale nella diagnosi di acalasia, individuando come segno altamente specifico e sensibile di tale patologia l'elevato gradiente pressorio esofago-gastrico attraverso lo SEI [31]. In alcuni pazienti con acalasia è possibile osservare con la manometria convenzionale un fallace rilasciamento dello SEI a causa del movimento cefalico dello SEI o di un parziale rilasciamento. Il metodo topografico identifica facilmente il mancato rilasciamento dello SEI con persistenza del gradiente pressorio, rendendo questa metodica particolarmente utile nel differenziare pazienti con acalasia da pazienti con altri disordini motori [31].

La disfagia persistente dopo chirurgia antireflusso rende spesso necessario un nuovo intervento [33 34]. È pertanto utile la valutazione della funzione motoria esofagea prima dell'intervento chirurgico [35 36]. È infatti possibile che una scarsa attività peristaltica possa essere associata con elevato rischio di disfagia post-intervento. Tuttavia, un ampio studio randomizzato e controllato mostra che disfunzioni motorie pre-operatorie identificate con la manometria convenzionale non sono predittive di fallimento dell'intervento chirurgico [37 38]. Sono in corso studi per verificare se le alterazioni della motilità identificabili con la HRM siano predittive di

Tabella I. Indicazioni alla valutazione impedenzometrica del reflusso gastroesofageo.

• Sintomi suggestivi di reflusso gastroesofageo resistenti a terapia con farmaci antisecretori
• Tosse cronica
• Sospetta ruminazione
• Eruttazioni
• Sintomi suggestivi di reflusso gastroesofageo in acloridria
• Apnea

•	Confermare il significato patologico di alterazioni faringee individuate all'*imaging*
•	Identificare alterazioni focali della peristalsi esofagea che alterano la clearance del bolo
•	Incrementare potere diagnostico e accuratezza nella diagnosi di acalasia esofagea
•	Distinguere gli spasmi esofagei dall'incremento della pressione intra-bolo dovuta a ostruzione o alterazioni focali della motilità

Tabella II.
Manometria ad alta risoluzione nella pratica clinica.

disfagia post-operatoria. Per il momento il valore della HRM prima della chirurgia antireflusso resta incerto.

Nuove metodiche di imaging per il piccolo intestino

L'impiego dell'endoscopia in età pediatrica è drammaticamente cresciuto negli ultimi 15-20 anni. Il piccolo intestino, cioè quella parte del tratto digerente che va dall'angolo di Treitz alla valvola ileo-cecale, è una sede nota per essere tecnicamente difficile da esplorare a causa delle condizioni anatomiche e della sua relativa tortuosità. Solo pochi centimetri di ileo sono accessibili per via retrograda attraverso l'ileocolonoscopia. L'enteroclisi del tenue e il tenue seriato consentono di indagare tale tratto in maniera indiretta e si prestano poco o nulla a una diagnosi risolutiva di fronte a casi di sanguinamento intestinale occulto (SIO) [39 40]. In più tali studi contrastografici sono limitati dalla quantità di radiazioni ionizzanti che sono necessarie per ciascun esame. L'enteroscopia e la video capsula endoscopica hanno rivoluzionato il work-up diagnostico del piccolo intestino nell'adulto e stanno acquisendo un ruolo di crescente importanza anche in età pediatrica.

Videocapsula endoscopica

La videocapsula endoscopica rappresenta una metodica di relativa recente introduzione non invasiva che si presta in maniera ottimale all'esplorazione dell'intestino tenue. La procedura si basa sull'uso di una microtelecamera contenuta in una capsula delle dimensioni di una compressa (circa 26 x 11 mm). È monouso, può essere ingerita per via orale o posizionata per via endoscopica direttamente in duodeno (in caso di bambini piccoli o comunque di età < 9 anni, gastroparesi o stenosi del piloro). Essa viene eliminata con le feci entro 24-48 ore dopo aver percorso il tratto digerente. Durante il percorso trasmette immagini video ad un registratore che il paziente porta alla cintura. La durata dell'esame è di circa 8 ore. Al termine il registratore viene collegato ad un computer munito di un software che permette di analizzare le immagini ottenute.
L'utilità diagnostica della videocapsula per l'adulto è stata rapidamente con-

statata, il suo uso rapidamente diffuso e recentemente sono state stilate le linee guida con specifiche indicazioni e confronti con il potere diagnostico di altre metodiche [41]. Una recente metanalisi avente l'obiettivo di valutare il potere diagnostico della videocapsula rispetto ad altre metodiche in pazienti con morbo di Crohn (MC) non stenosante, riporta risultati nettamente favorevoli all'uso della video capsula [42]. I dati relativi all'utilizzo in età pediatrica non sono numerosi [43-48]. Per il MC del piccolo intestino la videocapsula endoscopica è più sensibile di altre indagini strumentali di tipo radiologico, tuttavia specificità e valore predittivo positivo della metodica restano da stabilirsi [49] . Controindicazione assoluta all'esecuzione della videocapsula è la presenza di stenosi o restringimenti del lume intestinale con rischio di incarceramento della capsula. Il working group riunitosi durante la Conferenza Internazionale sulla Videocapsula Endoscopica ha definito la ritenzione della videocapsula come il suo arresto di progressione attraverso il tratto digestivo per un periodo superiore a 2 settimane e con necessità di intervento medico, endoscopico o chirurgico [50]. Uno studio molto ampio condotto su 937 pazienti adulti ha riportato l'incidenza globale di ritenzione della capsula, con successiva necessità di rimozione chirurgica, pari allo 0,75% [51]. In uno studio pediatrico condotto su 45 bambini, il 20% di essi (9/45) ha presentato ritardato transito della capsula (5 a livello gastrico e 4 a livello del piccolo intestino). Dei pazienti con ritenzione nel piccolo intestino, 3 sono stati sottoposti a chirurgia e uno a trattamento steroideo [52]. Per ovviare al rischio di incarceramento della capsula nei casi di sospetta stenosi intestinale è possibile eseguire il "capsula-test" utilizzando una capsula che si autodissolve (*self-dissolving patency capsule*): se è presente una stenosi in cui la capsula viene incarcerata, essa non viene eliminata ma si dissolve entro 40 ore, confermando il dato e controindicando l'esame [53]. Sarebbe consigliabile eseguire uno studio contrastografico prima della videocapsula per evidenziare le alterazioni del calibro intestinale. Tuttavia non sempre la negatività dell'enteroclisi del tenue o del tenue seriato sono risolutive e casi di incarceramento si sono verificati anche in presenza di esami contrastografici negativi [54-56]. La maggior parte delle indicazioni all'uso della videocapsula in pediatria sono riportate nella Tabella III. Il sospetto di MC in età pediatrica rappresenta la più importante delle indicazioni all'esecuzione della videocapsula endoscopica, laddove né l'endoscopia con biopsie ed esame istologico, né le indagini strumentali di tipo radiologico siano state risolutive [57 58]. Nel primo trial controllato condotto in età pediatrica in bambini con SIO, la videocapsula endoscopica ha consentito di fare diagnosi di MC o gastroenteropatia eosinofilica nel 60% dei pazienti esaminati [58]. Lo stesso rate diagnostico viene riportato in altri studi pediatrici (50-86%) [57 59 60]. Le lesioni suggestive di MC comprendono erosioni della mucosa, ulcere e stenosi. L'interpretazione delle immagini video-ricavate è spesso complessa; infatti il 14% dei pazienti sani ha ulcere aftoidi e la maggior parte dei pazienti in terapia antiinfiammatoria presenta erosioni mucosali. Il risultato di tali considerazioni è che il significato di molte delle immagini ottenute è da rivalutare

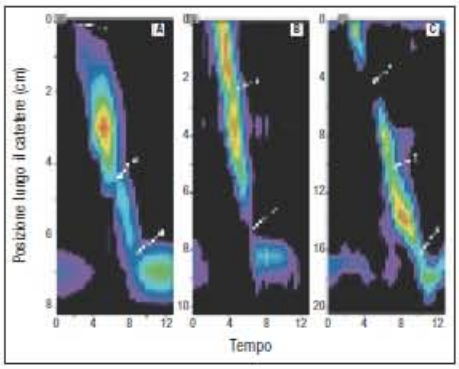


Figura 3.

Esempi di peristalsi esofagea in bambini di diversa età. Pannello A: neonata (20 giorni di vita) studiata con catetere a 9 sensori. È visualizzata la regione dello sfintere esofageo prossimale e distale. Il terzo segmento pressorio non appare ben sviluppato. Pannello B: lattante di 6 mesi studiato con catetere a 11 punti di registrazione. Il segmento prossimale e il primo avvallamento pressorio sono visualizzati solo quando il catetere è riposizionato più prossimalmente. Pannello C: bambino di 6 anni, studiato con catetere a 21 sensori di pressione. Tutti e tre i segmenti pressori e i tre avvallamenti sono ben visualizzati. In ciascun esempio riportato i punti di registrazione sono distanziati di 1 cm l'uno dall'altro. SW, deglutizione.

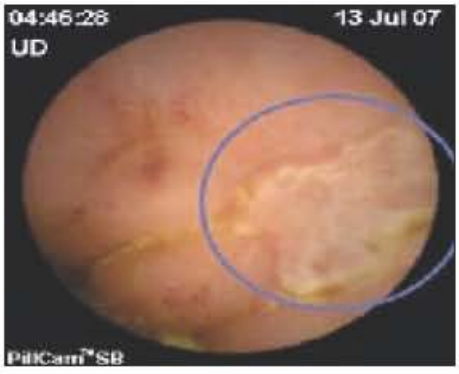


Figura 4.

Videocapsula endoscopica in paziente affetto da morbo di Crohn: ulcera aftoide del piccolo intestino.

nel tempo. Le lesioni mucosali eventualmente utili per la diagnosi possono andare perse in parte o del tutto per presenza di feci da inadeguata pulizia intestinale, per il rapido o ritardato transito intestinale o per lo sfortunato orientamento della microtelecamera lontano dalla lesione. Inoltre l'esatta localizzazione delle lesioni non è sempre del tutto semplice da definire [49]. Infine, come noto, l'impossibilità di praticare biopsie è il più grosso limite alla procedura. Tuttavia, soprattutto nel caso in cui i genitori del bambino di cui si sospetta il MC siano contrari a metodiche invasive oppure contrari a ripetere tali esami, laddove alla prima esecuzione essi siano risultati negativi o non diagnostici, è possibile prendere in considerazione la videocapsula endoscopica. Qualora le immagini derivanti siano tali da orientare fortemente per la diagnosi di MC, il successivo trattamento e il follow-up a lungo termine costituiranno il supporto alla diagnosi ottenuta con la video capsula [57].

Le sindromi poliposiche sono condizioni patologiche che si presentano spesso all'osservazione del pediatra gastroenterologo. I polipi trovati in pazienti affetti da poliposi adenomatosa familiare e sin-

drome di Peutz-Jeghers possono dare sanguinamento intermittente, ostruzione, invaginazione o modificazioni in senso maligno delle lesioni intestinali. Al momento il gold standard della sorveglianza è rappresentato dall'endoscopia. Sono pochi gli studi in cui la videocapsula endoscopica viene utilizzata nel follow-up/sorveglianza di tali lesioni [61-67]. La videocapsula è accurata nel diagnosticare i polipi del piccolo intestino e individua polipi piccoli non visualizzabili con la Risonanza Magnetica Nucleare (RMN) [68]. Inoltre è stato dimostrata una concordanza del 100% con altre metodiche per immagine (endoscopiche o radiologiche) e non espone il paziente a radiazioni [58]. Chiaramente sono necessari più ampi studi al fine di introdurre routinariamente la videocapsula endoscopica nel follow-up e nella sorveglianza di tali patologie. In pazienti adulti con sospetta celiachia, è stato ipotizzato di utilizzare le immagini ricavate dall'impiego della videocapsula endoscopica alle relative alle modificazioni mucosali, unitamente alla positività sierologica per anticorpi antiendomisio e antitransglutaminasi, a fini diagnostici. L'indicazione alla videocapsula in caso di celiachia è al momento l'esclusione di eventuali complicanze, quali i linfomi intestinali [69 70]. I dati in età pediatrica sono scarsi [71]. Sono stati pubblicati alcuni case report sull'uso della videocapsula endoscopica nella linfangectasia intestinale [42]. Le manifestazioni cliniche della linfangectasia intestinale comprendono: edema, diarrea, ascite chilosa, ritardo di crescita e, all'esame istologico, presenza di vasi linfatici dilatati. Il tipico aspetto a spruzzo di calce della mucosa potrebbe essere utile per la diagnosi video-endoscopica della linfangectasia [42].

Enteroscopia con strumento a doppio pallone

L'enteroscopia a "doppio pallone" (Fujinon, Inc, Saitama, Japan, EDP) consiste in una metodica di video-endoscopia ad alta risoluzione, in cui l'enteroscopio viene fatto passare attraverso una sonda di circa 12 mm di diametro che presenta un palloncino di lattice all'estremità. Un altro palloncino è posizionato all'estremità dell'enteroscopio. Questo sistema permette la progressione dell'enteroscopio nel piccolo intestino, che può essere così esplorato per lunghi tratti. Un sistema di pompa a pressione controllata insuffla e toglie aria dai palloncini. L'alternanza di manovre di "push and pull tecnique" ripetute più volte consentono di avanzare ed esaminare ogni volta circa 40 cm di piccolo intestino. L'EDP riduce al minimo i loop dell'endoscopio e richiede mediamente 75 minuti, sia quando l'approccio è orale che transrettale. Rispetto alla videocapsula endoscopica, il maggior vantaggio della EDP è quello di essere, malgrado l'invasività, una metodica sia diagnostica che terapeutica. L'EDP viene praticata, infatti, in anestesia generale ed offre la possibilità di prelievi bioptici e approcci terapeutici quali polipectomia e procedure di emostasi [39-41]. L'uso dell'EDP in età pediatrica è limitato e pochi sono i dati presenti in letteratura. Un piccolo studio condotto in Cina ha valutato l'uso dell'EDP in 14 pazienti

Diagnostiche
• Sanguinamento occulto
• Morbo di Crohn
• Linfangectasia
• Ostruzionale intestinale
• Graft assessment dopo trapianto di piccolo intestino
• Linfoma (sospetto/follow-up dopo trattamento)
Terapeutiche
• Emostasi
• Sorveglianza della poliposi e polipectomia
• Dilatazione
• Posizionamento di tubo per nutrizione naso digiunale/digiunostomia percutanea

Tabella III.
Indicazioni all'enteroscopia e alla videocapsula endoscopica in bambini e adolescenti.

(13 con SIO e uno con diarrea cronica) ottenendo un elevato rate diagnostico (86%) [72]. La Tabella III mostra le indicazioni diagnostiche e terapeutiche all'uso dell'enteroscopia. In età pediatrica e adolescenziale, che peraltro sono comuni alla videocapsula endoscopica. In letteratura è riportato che nel 5% dei pazienti adulti SIO, la gastroscopia e la colonscopia non sono risolutive nell'individuare la causa del sanguinamento [73]. L'uso dell'enteroscopia nel SIO, largamente descritto nell'adulto, è diagnostica nel 12-80% dei casi [73]. Il SIO nei bambini è meno comune; tuttavia l'enteroscopia sembra aver dato risultati sovrapponibili a quanto riportato nell'adulto anche in tale fascia d'età [39]. Per quanto riguarda il MC, l'enteroscopia grazie alla biopsia di ulcere aftoidi, nodularità o pseudo-polipi intestinali, consente di confermare la diagnosi nell'85% dei pazienti di cui si sospetta MC [39], con un rate diagnostico nettamente superiore al tenue seriato [40]. Il potenziale terapeutico dell'enteroscopia nell'ambito delle malattie infiammatorie croniche intestinali si esprime inoltre nella capacità di dilatare tratti intestinali interessati da stenosi infiammatorie [40].

Tomografia Assiale Computerizzata, Risonanza Magnetica Nucleare ed Ecografia dell'ultima ansa ileale nella diagnosi del morbo di Crohn del piccolo intestino

Il MC è più comunemente localizzato a livello del piccolo intestino. Tuttavia proprio per il complesso accesso strumentale, viene riportato in letteratura un ritardo diagnostico, dall'esordio di sintomi, variabile da 1 a 7 anni nei pazienti risultati poi affetti da MC [75,76]. Le opzioni radiologiche a disposizione per la diagnosi sono rappresentate da tenue seriato, Tomografia Assiale Computerizzata (TAC), RMN ed ecografia addominale (US). I dati presenti in letteratura in merito all'accuratezza diagnostica di tali esami è variabile [40,49]. Il tenue seriato fornisce

importanti informazioni sull'estensione di eventuali complicanze quali stenosi o fistole. Inoltre rende evidente il tipico aspetto ad acciottolato, e la distribuzione a "salto" delle lesioni mucosali [77]; la TAC è invece più efficace nel definire lo spessore della parete intestinale e nell'individuare eventuali complicanze ma l'accuratezza nel determinare la presenza di lesioni mucosali non è nota. La RMN con gadolinio si è dimostrata uno strumento diagnostico promettente per identificare il MC del piccolo intestino in età pediatrica. Dati comparativi tra RMN e TAC, in termini di specificità e sensibilità, nella caratterizzazione della patologia del piccolo intestino sono limitati sia nell'adulto che nel bambino. Uno studio relativamente recente condotto in età pediatrica ha mostrato che la RMN, dopo distensione intestinale con polietilene-glicole (PEG) somministrato per via orale (RMN-PEG), è un esame sensibile e specifico nella diagnosi di infiammazione dell'ileo terminale, consentendo di distinguere il MC da altre condizioni infiammatorie dell'intestino attraverso pattern di enhancement e spessore della parete intestinale. La RMN-PEG inoltre consente di distinguere tra rettocolite ulcerosa e MC in pazienti con un quadro di sovrapposizione sintomatologica tra le due condizioni patologiche. Infine la correlazione esistente tra dati ricavati da RMN-PEG, endoscopia, istologia e indice di attività di malattia (*Pediatric Crohn Disease Activity Index*, PCDAI) suggerisce che la RMN-PEG possa essere una metodica preziosa per monitorare il decorso clinico dei pazienti affetti da MC [78]. Più recentemente, l'accuratezza diagnostica della RMN dopo distensione delle anse intestinali con mannitolo si è dimostrata sovrapponibile all'endoscopia e all'ecografia dell'ultima ansa ileale [79].

L'US, visualizzando segni ecografici suggestivi di infiammazione, quali le anomalie di spessore della parete intestinale (estensione e severità), è un utile complemento diagnostico nelle malattie infiammatorie croniche intestinali e nel MC in particolare [80 81]. Per il MC quando è utilizzato come cut-off di normalità un valore pari a 3 mm di spessore della parete intestinale, sensibilità e specificità sono riportati essere rispettivamente dell'88 e del 93%, mentre quando è impostato un cut-off > 4 mm la sensibilità scende al 75% e la specificità del 97% [82]. I falsi negativi sono in genere il risultato della presenza di lesioni molto superficiali quali ulcere aftoidi ed erosioni mucosali. Ulteriore limite di tale metodica è l'esperienza dell'ecografista, fondamentale per escludere del tutto la malattia o confermarne il sospetto e quindi continuare con indagini più invasive. L'US pertanto, presa singolarmente non è risultata sufficientemente accurata in fase diagnostica ed è da ritenersi più utile nel follow-up [83 84]. In ogni caso tali indagini strumentali per *imaging* radiologiche, contrariamente alle metodiche endoscopiche, non consentono di praticare alcuna biopsia, impedendo la valutazione istologica che rappresenta il gold standard per la diagnosi delle malattie infiammatorie croniche intestinali.

Conclusioni

La diagnosi e il trattamento dei pazienti con disturbi gastrointestinali richiede un approccio individualizzato e completo. Le diverse procedure a disposizione, sempre più evolute dal punto di vista tecnologico, assicurano una notevole precisione e accuratezza. In particolare: la MII-pH consente di distinguere accuratamente il tipo di reflussato e il suo transito nell'esofago pur non potendo ancora quantificarlo; la HRM ha aperto nuovi orizzonti allo studio della funzionalità esofagea e, nell'ambito della ricerca, allo studio dell'ontogenesi della motilità esofagea; la videocapsula e l'enteroscopia con strumento a doppio pallone hanno consentito l'accesso a tratti fino ad ora inesplorati del piccolo intestino, aumentando le possibilità diagnostiche e quindi terapeutiche in alcune condizioni patologiche; infine la RMN rappresenta una metodica affascinante il cui utilizzo appare promettente sia nella diagnosi che nel monitoraggio delle malattie infiammatorie croniche intestinali. Ulteriori e più ampi studi sono necessari per incrementare le nostre conoscenze in merito all'utilità clinica di tali procedure nei bambini. A tutt'oggi restano come parte fondamentale della valutazione clinica e diagnostica del paziente pediatrico l'anamnesi e l'esame obiettivo che possono facilitare la diagnosi differenziale e permettere una valutazione strumentale più orientata e accurata.

Bibliografia

1. Silny J. *Intraluminal multiple eletric impedance procedure for measurement of gastrointestinal motility.* J Gastrointest Motil 1991;3:151-62.
2. Silny J. *Verification of the intraluminal multiple electric impedance measurements for recording of gastrointestinal motility.* J Gastrointest Motil 1993;5:107-22.
3. Bradenoord AJ, Tutuian R, Smout AJPM, Castell DO. *Tecnology review: esophageal impedance monitoring.* Am J Gastroenterol 2007;102:187-94.
4. Sifrim D, Castell D, Dent J, Kahrila PJ. *Gastroesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non acid and gas reflux.* Gut 2004;53:1024-31.
5. Shay S, Tutuian R, Sifrim D, Vela M, Wise J, Balaji N, et al. *Twentyfour hour ambulatory simultaneous impedance and pH monitoring: a multicenter report of normal values from 60 healthy volunteers.* Am J Gastroenterol 2004;99:1037-43.
6. Zerbib F, des Varannes SB, Roman S, Pouderoux P, Artigue F, Chaput U, et al. *Normal values and day-to-day variability of 24-h ambulatory oesophageal impedance-pH monitoring in a Belgian-French cohort of healthy subjects.* Aliment Pharmacol Ther 2005;22:1011-21.
7. Zentilin P, Iiritano E, Dulbecco P, Bilardi C, Savarino E, De CS, et al. *Normal values of 24-h ambulatory intraluminal impedance combined with pH-metry in subjects eating a Mediterranean diet.* Dig Liver Dis 2006;38:226-32.
8. Lopez-Alonso M, Moya MJ, Cabo JA, Ribas J, del Carmen MM, Silny J, et al. *Twenty-four-hour esophageal impedance-pH monitoring in healthy preterm neonates: rate and characteristics of acid, weakly acidic, and weakly alkaline gastroesophageal reflux.* Pediatrics 2006;118:e299-308.

9 Rosen R, Lord C, Nurko S. *The sensitivity of multichannel intraluminal impedance and the pH probe in the evaluation of gastroesophageal reflux in children.* Clin Gastroenterol Hepatol 2006;4:167-72.

10 Vandenplas Y, Salvatore S, Vieira MC, Hauser B. *Will esophageal impedance replace pH monitoring?* Pediatrics 2007;119:118-22.

11 López Alonso M, Moya MJ, Cabo JA, Ribas J, Macías MC, Silny J, et al. *Acid and non-acid gastro-esophageal reflux in newborns: preliminary results using intraluminal impedance [in Spanish].* Cir Pediatr 2005;18:121-6.

12 Condino AA, Sondheimer J, Pan Z, Gralla J, Perry D, O'Connor JA. *Evaluation of infantile acid and nonacid gastroesophageal reflux using combined pH monitoring and impedance measurement.* J Pediatr Gastroenterol Nutr 2006;42:16-21.

13 Omari TI, Rommel N, Staunton E, Lontis R, Goodchild L, Haslam R, et al. *Paradoxical impact of body positioning on gastroesophageal reflux and gastric emptying in the premature neonate.* J Pediatr 2004;145:194-200.

14 Wenzl TG, Schneider S, Scheele F, Silny J, Heimann G, Skopnik H. *Effects of thickened feeding on gastroesophageal reflux in infants: a placebo-controlled crossover study using intraluminal impedance.* Pediatrics 2003;111:355-9.

15 Del Buono R, Wenzl TG, Ball G, Keady S, Thomson M. *Effect of Gaviscon Infant on gastro-oesophageal reflux in infants assessed by combined intraluminal impedance/pH.* Arch Dis Child 2005;90:460-3.

16 Vaezi M, Richter J. *Role of acid and duodenogastroesophageal reflux in gastroesophageal reflux disease.* Gastroenterology 1996;111:1192-9.

17 Wenzl TG, Silny J, Schenke S, Peschgens T, Heimann G, Skopnik H. *Gastro-esophageal reflux and respiratory phenomena in children: status of the intraluminal impedance technique.* J Pediatr Gastroenterol Nutr 1999;28:423-8.

18 Magista AM, Indrio F, Baldassarre M, Bucci N, Menolascina A, Mautone A, et al. *Multichannel intraluminal impedance to detect relationship between gastroesophageal reflux and apnoea of prematurity.* Dig Liver Dis 2007;39:216-21.

19 Condino AA, Sondheimer J, Pan Z, Gralla J, Perry D, O'Connor JA. *Evaluation of gastroesophageal reflux in pediatric patients with asthma using impedance-pH monitoring.* J Pediatr 2006;149:216-9.

20 Rosen R, Nurko S. *The importance of multichannel intraluminal impedance in the evaluation of children with persistent respiratory symptoms.* Am J Gastroenterol 2004;99:2452-8.

21 Fox MR, Bradenoord AJ. *Oesophageal high-resolution manometry: moving from research into clinical practice.* Gut 2008;57:405-23.

22 Bradenoord AJ, Smout AJPM. *Thigh-resolution manometry ring.* Dig Liv Dis 2008;174-81.

23 Pandolfino JE, Shi G, Zhang Q, Ghosh S, Brasseur JG, Kahrilas PJ. *Measuring EGJ opening patterns using high resolution intraluminal impedance.* Neurogastroenterol Motil 2005;17:200-6.

24 Ghosh SK, Pandolfino JE, Zhang Q, Jarosz A, Shah N, Kahrilas PJ, et al. *Quantifying esophageal peristalsis with high-resolution manometry: a study of 75 asymptomatic volunteers.* Am J Physiol Gastrointest Liver Physiol 2006;290:G988-97.

25 Clouse R, Staiano A. *Topography of the esophageal peristaltic pressure wave.* Am J Physiol Gastrointest Liver Physiol 1991;261:G677-84.

[26] Clouse RE, Staiano A. *Topography of normal and high-amplitude esophageal peristalsis.* Am J Physiol 1993;265:G1098-107.

[27] Staiano A, Clouse RE. *The effects of cisapride on the topography of oesophageal peristalsis.* Aliment Pharmacol Ther 1996;10:875-82.

[28] Kahrilas PJ, Lin S, Chen J, Manka M. *The effect of hiatus hernia on gastro-oesophageal junction pressure.* Gut 1999;44:476-82.

[29] Staiano A, Boccia G, Salvia G, Zappulli D, Clouse RE. *Development of esophageal peristalsis in pre-term and term neonates.* Gastroenterology 2007;132:1718-25.

[30] Staiano A, Boccia G, Miele E, Clouse RE. *Segmental characteristics of oesophageal peristalsis in paediatric patients.* Neurogastroenterol Motil 2008;20:19-26.

[31] Clouse RE, Staiano A, Alrakawi A, Haroian L. *Application of topographical methods to clinical esophageal manometry.* Am J Gastroenterol 2000;95:2720-30.

[32] Fox M, Hebbard G, Janiak P, Brasseur JG, Ghosh S, Thumshirn M, et al. *High-resolution manometry predicts the success of oesophageal bolus transport and identifies clinically important abnormalities not detected by conventional manometry.* Neurogastroenterol Motil 2004;16:533-42.

[33] Hunter JG, Swanstrom L, Waring JP. *Dysphagia after laparoscopic antireflux surgery. The impact of operative technique.* Ann Surg 1996;224:51-7.

[34] Hunter JG, Smith CD, Branum GD, Waring JP, Trus TL, Cornwell M, et al. *Laparoscopic fundoplication failures: patterns of failure and response to fundoplication revision.* Ann Surg 1999;230:595-604.

[35] Bodger K, Trudgill N. *Guidelines for oesophageal manometry and pH monitoring. The British Society of Gastroenterology Guidelines 2006.* http://www.bsg.org.uk/ pdf_word_docs/oesp_man.pdf

[36] Pechler SJ, Castell DO. *Classification of oesophageal motility abnormalities.* Gut 2001;49:145-51.13.

[37] Pandolfino JE, Kahrilas PJ. *AGA technical review on the clinical use of esophageal manometry.* Gastroenterology 2005;128:209-24.

[38] Hunt DR, Humphreys KA, Janssen J, Mackay E, Smart R. *Preoperative esophageal transit studies are a useful predictor of dysphagia after fundoplication.* J Gastrointest Surg 1999;3:489-95.

[39] Sidhu R, Sanders DS, McAlindon ME, Thomson M. *Capsule endoscopy and enteroscopy: modern modalities to investigate the small bowel in paediatrics.* Arch Dis Child 2008;93:154-9.

[40] Bruining DH, Loftus EV Jr. *Technology Insight: new techniques for imaging the gut in patients with IBD.* Nat Clin Pract Gastroenterol Hepatol 2008;5:154-61.

[41] Sidhu R, Sanders DS, Morris AJ, McAlindon ME. *Guidelines on small bowel enteroscopy and capsule endoscopy in adults.* Gut 2008;57:125-36.

[42] Triester SL, Leighton JA, Leontiadis GI, Gurudu SR, Fleischer DE, Hara AK, et al. *A meta-analysis of the yield of capsule endoscopy compared to other diagnostic modalities in patients with non-stricturing small bowel Crohn's disease.* Am J Gastroenterol 2006;101:954-64.

[43] Rivet C, Lapalus MG, Dumortier J, Le Gall C, Budin C, Bouvier R, et al. *Use of capsule endoscopy in children with primary intestinal lymphangiectasia.* Gastrointest Endosc 2006;64:649-50.

[44] Seidman EG, Sant'Anna AM, Dirks MH. *Potential applications of wireless capsule endoscopy in the pediatric age group.* Gastrointest Endosc Clin N Am 2004;14:207-17.

45 Arguelles-Arias F, Caunedo A, Romero J, Sanchez A, Rodriguez-Tellez M, Pellicer FJ, et al. *The value of capsule endoscopy in pediatric patients with a suspicion of Crohn's disease*. Endoscopy 2004;36:869-73.

46 Ngo K, Gendy E, Klooster M, Yanni G, Shah M. *Wireless capsule endoscopy (WCE) in pediatric gastroenterology practice*. Gastrointest Endosc 2006;63:AB95.

47 Barth BA, Donovan K, Fox VL. *Endoscopic placement of the capsule endoscope in children*. Gastrointest Endosc 2004;60:818-21.

48 Bizzarri B, Fornaroli F, Cannizzaro R, De' Angelis N, Vincenzi F, Maffini V, et al. *Endoscopic placement of video capsule in a pediatric population*. Gastrointest Endosc 2005;62:991.

49 Hara AK, Leighton JA, Heigh RI, Sharma VK, SILVA AC, De Petris G, et al. *Crohn disease of the small bowel: preliminary comparison among CT enterography, capsule endoscopy, small-bowel followthrough, and ileoscopy*. Radiology 2006;238:128-34.

50 Cave D, Legnani P, de Franchis R, Lewis BS. *ICCE consensus for capsule retention*. Endoscopy 2005;37:1065-7.

51 Barkin J, Friedman S. *Wireless capsule endoscopy requiring surgical intervention: the world's experience [abstract]*. Am J Gastroenterol 2002;97:A907.

52 Moy L, Levine J. *Wireless capsule endoscopy in the pediatric age group: experience and complications*. J Pediatr Gastroenterol Nutr 2007;44:516-20.

53 Boivin ML, Lochs H, Voderholzer WA. *Does passage of a patency capsule indicate small-bowel patency? A prospective clinical trial?* Endoscopy 2005;37:808-15.

54 Pennazio M, Santucci R, Rondonotti E, Abbiati C, Beccari G, Rossini FP, et al. *Outcome of patients with obscure gastrointestinal bleeding after capsule endoscopy: report of 100 consecutive cases*. Gastroenterology 2004;126:643-53.

55 Buchman AL, Miller FH, Wallin A, Chowdhry AA, Ahn C. *Videocapsule endoscopy versus barium contrast studies for the diagnosis of Crohn's disease recurrence involving the small intestine*. Am J Gastroenterol 2004;99:2171-7.

56 Mow WS, Lo SK, Targan SR, Dubinsky MC, Treyzon L, Abreu-Martin MT, et al. *Initial experience with wireless capsule enteroscopy in the diagnosis and management of inflammatory bowel disease*. Clin Gastroenterol Hepatol 2004;2:31-40.

57 Argüelles-Arias F, Caunedo A, Romero J, Sanchez A, Rodriguez-Tellez M, Pellicer FJ, et al. *The value of capsule endoscopy in pediatric patients with a suspicion of Crohn's disease*. Endoscopy 2004;36:869-73.

58 Guilhon de Araujo Sant'Anna AM, Dubois J, Miron MC, Seidman EG. *Wireless capsule endoscopy for obscure small-bowel disorders: final results of the first pediatric controlled trial*. Clin Gastroenterol Hepatol 2005;3:264-70.

59 Seidman EG, Sant'Anna AM, Dirks MH. *Potential applications of wireless capsule endoscopy in the pediatric age group*. Gastrointest Endosc Clin N Am 2004;14:207-17.

60 Thomson M, Fritscher-Ravens A, Mylonaki M, Swain P, Eltumi M, Heuschkel R, et al. *Wireless capsule endoscopy in children: a study to assess the diagnostic yield in small bowel disease in paediatric patients*. J Pediatr Gastroenterol Nutr 2007;44:192-7.

61 Schulmann K, Hollerbach S, Kraus K, Willert J, Vogel T, Möslein Gabriela, et al. *Feasibility and diagnostic utility of video capsule endoscopy for the detection of small bowel polyps in patients with hereditary polyposis syndromes*. Am J Gastroenterol 2005;100:27-37.

62 Soares J, Lopes L, Vilas Boas G, Pinho C. *Wireless capsule endoscopy for evaluation*

of phenotypic expression of small-bowel polyps in patients with Peutz-Jeghers syndrome and in symptomatic first-degree relatives. Endoscopy 2004;36:1060-6.

63 Mata A, Llach J, Castells A, Rovira JM, Pellisé M, Ginès A, et al. *A prospective trial comparing wireless capsule endoscopy and barium contrast series for small-bowel surveillance in hereditary GI polyposis syndromes.* Gastrointest Endosc 2005;61:721-5.

64 Barkay O, Moshkowitz M, Fireman Z, Shemesh E, Goldray O, Revivo M, et al. *Initial experience of videocapsule endoscopy for diagnosing small-bowel tumors in patients with GI polyposis syndromes.* Gastrointest Endosc 2005;62:448-52.

65 Caspari R, von Falkenhausen M, Krautmacher C, Schild H, Heller J, Sauerbruch T. *Comparison of capsule endoscopy and magnetic resonance imaging for the detection of polyps of the small intestine in patients with familial adenomatous polyposis or with Peutz-Jeghers' syndrome.* Endoscopy 2004;36:1054-9.

66 Burke CA, Santisi J, Church J, Levinthal G. *The utility of capsule endoscopy small bowel surveillance in patients with polyposis.* Am J Gastroenterol 2005;100:1498-502.

67 Brown G, Fraser C, Schofield G, Taylor S, Bartram C, Phillips R, et al. *Video capsule endoscopy in peutz-jeghers syndrome: a blinded comparison with barium follow-through for detection of smallbowel polyps.* Endoscopy 2006;38:385-90.

68 Petroniene R, Dubcenco E, Baker JP, Ottaway CA, Tang SJ, Zanati SA, et al. *Given capsule endoscopy in celiac disease: evaluation of diagnostic accuracy and interobserver agreement.* Am J Gastroenterol 2005;100:685-94.

69 Soares J, Lopes L, Vilas Boas G, Pinho C. *Wireless capsule endoscopy for evaluation of phenotypic expression of small-bowel polyps in patients with Peutz-Jeghers syndrome and in symptomatic first-degree relatives.* Endoscopy 2004;36:1060-6.

70 Galmiche JP, Coron E, Sacher-Huvelin S. *Capsule Endoscopy: recent developments.* Gut 2008;57:695-703.

71 Ngo K, Gendy E, Klooster M, Yanni G, Shah M. *Wireless capsule endoscopy (WCE) in pediatric gastroenterology practice.* Gastrointest Endosc 2006;63:AB95.

72 Xu CD, Deng CH, Zhong J, Zhang CL. *[Application of double-balloon push enteroscopy in diagnosis of small bowel disease in children].* Zhonghua Er Ke Za Zhi 2006;44:90-2.

73 GralnekIM. *Obscure-overtgastrointestinalbleeding.*Gastroenterology2005;128:1424-30.

74 Sidhu R, McAlindon ME, Kapur K, Hurlstone DP, Wheeldon MC, Sanders DS. *Push enteroscopy in the era of capsule endoscopy.* J Clin Gastroenterol 2008;42:54-8.

75 Pimentel M, Chang M, Chow EJ, Tabibzadeh S, Kirit-Kiriak V, Targan SR, et al. *Identification of a prodromal period in Crohn's disease but not ulcerative colitis.* Am J Gastroenterol 2000;95:3458-62.

76 Timmer A, Breuer-Katschinski B, Goebell H. *Time trends in the incidence and disease location of Crohn's disease 1980-1995: a prospective analysis in an urban population in Gcrmany.* Inflamm Bowcl Dis 1999;5:79 84.

77 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.

78 Laghi A, Borrelli O, Paolantonio P, Dito L, Buena de Mesquita M, Falconieri P, et al. *Contrast enhanced magnetic resonance imaging of the terminal ileum in children with Crohn's disease.* Gut 2003;52:393-7.

79 Borthne AS, Abdelnoor M, Rugtveit J, Perminow G, Reiseter T, Kløw NE. *Bowel mag-*

netic resonance imaging of pediatric patients with oral mannitol MRI compared to endoscopy and intestinal ultrasound. Eur Radiol 2006;16:207-14.

80 Baud C, Saguintaah M, Veyrac C, Couture A, Ferran JL, Barnéon G, et al. *Sonographic diagnosis of colitis in children.* Eur Radiol 2004;14:2105-19.

81 Bremner AR, Griffiths M, Argent JD, Fairhurst JJ, Beattie RM. *Sonographic evaluation of inflammatory bowel disease: a prospective, blinded, comparative study.* Pediatr Radiol 2006;36:947-53.

82 Fraquelli M, Colli A, Casazza G, Paggi S, Colucci A, Massironi S, et al. *Role of US in detection of Crohn disease: meta-analysis.* Radiology 2005;236:95-101.

83 Quillin SP, Siegel MJ. *Gastrointestinal inflammation in children: color Doppler ultrasonography.* J Ultrasound Med 1994;13:751-6.

84 Ruess L, Blask AR, Bulas DI, Mohan P, Bader A, Latimer JS, et al. *Inflammatory bowel disease in children and young adults: correlation of sonographic and clinical parameters during treatment.* AJR Am J Roentgenol 2000;175:79-84.

CONSENSUS STATEMENT SULL'ESOFAGITE EOSINOFILA IN ETÀ PEDIATRICA

Stato dell'arte e proposte di un panel SIGENP

2009

Coordinatore nazionale

Paola De Angelis *Roma*

Panel

Francesca Aniballi (prof. Catassi) Ancona
Pietro Betalli Padova
Osvaldo Borrelli (prof. Cucchiara) Roma
Tamara Caldaro Roma
Angelo Campanozzi Foggia
Luigi Dall'Oglio Roma
Paola De Angelis Roma
Gian Luigi de' Angelis Parma
Laura Di Iorio (Prof. Paone) Roma
Graziella Guariso Padova
Sandra Lucarelli (prof. Cucchiara) Roma
Alessandro Pane Roma
Lucia Quaglietta, Paola Coccorullo Napoli
Claudio Romano Messina
Silvia Salvatore Varese
Rita Sforza Wietrzykowska, Giuseppe Morino Roma
Filippo Torroni Roma

Con la collaborazione di Jonathan E. Markowitz

University of South Carolina School of Medicine

AREA QUALITÀ

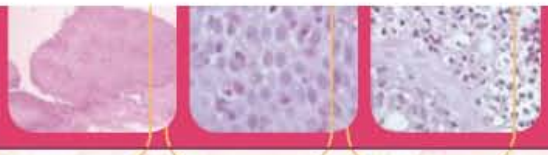
Editore certificato
ISO 9001:2000
Via Comelico 3
20135 Milano
e-mail: info@areaqualita.com

Cod. ISBN 978-88-95394-03-9

Progetto grafico e impaginazione: Il Bozzetto di Patrizia Calla (Milano)

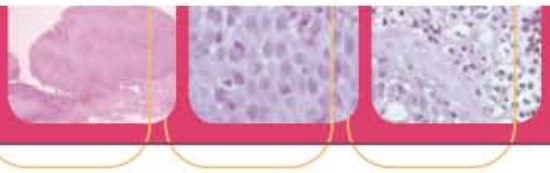
Finito di stampare presso le Tipografie Vigrafica di Monza (Milano) nel mese di Giugno 2009

SIGENP Società Italiana di Gastroenterologia Epatologia e Nutrizione Pediatrica



sommario

introduzione



Jonathan E. Markowitz

Attending Physician, Childen's Center for Digestive Health Greenville Hospital System University Medical Center Greenville, South Carolina; Associate Professor of Clinical Pediatrics University of South Carolina School of Medicine - Member of NASPGHAN, AGA, and ACG

There is an old axiom that says you find only what you seek, and you seek only what you know. Although there are innumerable examples in medicine that support this statement, eosinophilic esophagitis (EE) may be one of the best. There is no doubt that each year we are finding more and more patients with EE and at least one of the reasons is the increased awareness about this disorder among pediatricians, gastroenterologists, and allergists. The rate at which our knowledge on this subject has progressed has been impressive. In just over a decade, there has been an explosion of interest in EE, and the number of publications on the subject continues to increase each year. However, despite the progress we have seen to date, a change in approach is needed if, as a medical community, we are going to unlock the most important secrets to this problem.

There have been reports detailing cases of EE that have originated from 5 of the 6 inhabited continents. Across North America and Europe, several large academic centers have developed multidisciplinary programs for the evaluation and treatment of patients with EE. However, to date the bulk of our knowledge on EE is still derived from case series and descriptive studies. From these case series, experts have been able to generate consensus opinions on diagnostic criteria, and have described some basic characteristics common to patients with EE. Despite this, there remain considerable questions about the true epidemiology of the disease, the etiologic factors, the optimal treatment regimen, and the final treatment goals.

To best answer these questions, there needs to be a collaborative effort between local, national, and international leaders in the field. In October 2007, a meeting of international thought leaders in EE occurred in the United States, coordinated with the meeting of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition Just a few months later, we saw the culmination of a one-year multi-center study conducted across Italy with the goals of forming a diagnostic and therapeutic protocol based on the opinions of the Italian leaders and the best evidence in the medical literature. This study clearly demonstrates the commitment of the Italian leadership towards advancing our understanding EE. It is examples such as these that hopefully will serve as the models for future studies, and will become the seeds of even more far-reaching efforts.

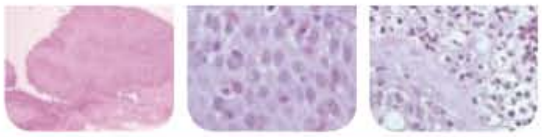
Paola De Angelis

UOC Chirurgia Endoscopia Digestiva, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma

L'esofagite eosinofila (EE) è una malattia infiammatoria cronica localizzata all'esofago, di presumibile eziopatogenesi immuno-allergica, caratterizzata da periodi di remissione clinica ed episodi di attività, durante i quali il paziente presenta sintomi o segni tipici o aspecifici talora simulanti la Malattia da reflusso gastroesofageo (MRGE).

Vi sono numerosi studi effettuati su popolazioni di adulti e bambini affetti da questa patologia che mostrano i complessi aspetti diagnostici e i numerosi tentativi terapeutici compiuti, senza raggiungere ancora un protocollo standardizzato comune.

La definizione più diffusa e convalidata dalla letteratura internazionale dell'EE si riferisce al numero degli eosinofili intraepiteliali e nella lamina propria dell'esofago rilevabili in biopsie esofagee prossimali, medie e distali, da effettuarsi indipendentemente dall'aspetto macroscopico della parete (≥ di 15 eosinofili/campo ad alto potere di definizione - High Power Field: HPF). Un recente

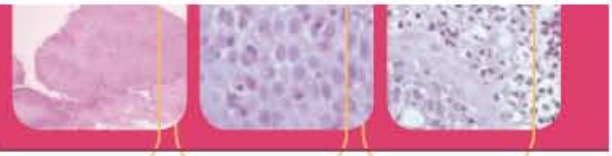


Consensus *dell'American Gastroenterological Association e del NASPGHAN, basato su revisioni sistematiche della letteratura e opinioni di esperti, ha affermato che l'EE è un'entità complessa caratterizzata da un quadro clinico non sempre specifico, da un'istologia ben determinata (*peak count: *il numero massimo di eosinofili in un HPF, eliminate altre cause di eosinofilia esofagea, con normale reperto istologico a livello gastroduodenale) e dall'esclusione della MRGE (assenza di risposta all'inibitore di pompa protonica -IPP- fino a 2 mg/kg/die e pH-metria normale)* ▸ **Fig. 1**.
Negli ultimi anni, anche in età pediatrica, si sta iniziando a diffondere una conoscenza più dettagliata di questa patologia, con la conseguente diagnosi precoce che permette di evitare le complicazioni più gravi, rappresentate dalle stenosi esofagee con inevitabile malnutrizione cronica. Le caratteristiche comuni e le correlazioni presenti tra l'EE e la MRGE impongono la necessità di una diagnosi precisa per distinguere i diversi trattamenti delle due patologie. È possibile, comunque, che vi sia un'interferenza, una dipendenza o una coesistenza tra le due patologie, ancora da definire: tale evidenza è testimoniata da pazienti che presentano entrambe le situazioni e beneficiano di entrambi i trattamenti.
La terapia dell'EE è variegata e complessa; una volta fronteggiata la fase iniziale della patologia diventa indispensabile prevenire gli eventuali possibili episodi di riacutizzazione, spesso molto temuti dai pazienti, soprattutto in età adolescenziale, con vari risvolti di ordine psicologico. Le dilatazioni, i farmaci e la dieta sono le opzioni più comunemente proposte; nell'ambito della terapia medica vengono utilizzati ampiamente gli IPP, i corticosteroidi sistemici e topici, e gli antagonisti dei recettori dei leucotrieni. I composti "biologici" (es. anticorpi anti-IL 5) sono ancora in fase di studio.
Un Panel di soci SIGENP si è riunito a Roma e ha formulato un protocollo diagnostico e terapeutico basato sui dati della letteratura più recente e sull'esperienza personale di ogni centro. Lo scopo del lavoro svolto è stato principalmente quello di dare una risposta alle difficoltà di inquadramento della patologia, alle difformità di comportamento terapeutico e alle necessità di riferimenti diagnostici omogenei.
L'applicazione di una metodologia comune anche in ambito nazionale dal primo approccio al paziente fino a tutto il follow-up, *permetterà diagnosi precoci, un minor numero di complicanze e una gestione completa multidisciplinare più armonica.*

Fig. 1 EE is a clinicopathological disease characterized by:

1. Symptoms including but not restricted to food impaction and dysphagia in adults, and feeding intolerance and GERD symptoms in children
   CLINICAL SYMPTOMS OF ESOPHAGEAL DYSFUNCTION.
2. > or = 15 eosinophils/HPF (HIGH POWER FIELD).
3. Exclusion of other disorders associated with similar clinical, histological, or endoscopic features, especially GERD.
   (Use of high dose proton pump inhibitor treatment up to 2 mg/kg/day or normal pH monitoring).
   Appropriate treatments include dietary approaches based upon eliminating exposure to food allergens, or topical corticosteroids.

Furuta GT, Liacouras CA et al. AGA Institute. Gastroenterology 2007 (3)



capitolo 1

Epidemiologia e quadri clinici

Lucia Quaglietta - Paola Coccorullo - Annamaria Staiano
Dipartimento di Pediatria, Università di Napoli "Federico II"

L'EE è una condizione patologica caratterizzata, a livello istologico, da un'isolata e severa infiltrazione eosinofilica dell'esofago (≥15 eosinofili/HPH), la cui prevalenza ha subìto un netto aumento negli ultimi 10 anni.
Storicamente l'EE è nota da più di 25 anni; tuttavia, prima del 1995, i pazienti con disfagia o sintomi apparentemente compatibili con reflusso gastroesofageo ed evidenza istologica di infiltrazione eosinofilica, ricevevano quasi sempre una diagnosi di MRGE e quindi un trattamento acidosoppressivo a cui si mostravano precocemente resistenti.
Dal 1995 i gastroenterologi pediatri hanno riconosciuto che i bambini con sintomi suggestivi di MRGE e infiltrato eosinofilico alla biopsia esofagea appartengono a un gruppo eterogeneo di individui affetti da un'emergente e ben distinta entità clinica, l'EE.
L'aumento delle casistiche e la maggiore consapevolezza sia dei gastroenterologi sia dei patologi ha reso l'EE più facilmente riconoscibile, con apparente maggior numero di diagnosi nell'ultimo decennio.
È ancora misconosciuta la reale incidenza dell'EE in età pediatrica; i dati di **incidenza** più interessanti presenti in letteratura sono stati mostrati in uno studio demografico basato su una popolazione pediatrica condotto da *Noel et al* [10] tra il 2000-2003.
Gli autori hanno calcolato in questi anni un'incidenza di 1/10.000 casi all'anno, con una prevalenza di 4/10.000. L'EE risultava inoltre più frequente nel **sesso maschile** con una età media di circa 10.5 +/- 5.4 anni.
Molto poco è noto in merito alla **patogenesi** di tale condizione.
Sono tuttavia chiamati in causa diversi meccanismi che suggeriscono una disregolazione immunologica e il contributo di allergeni sia alimentari sia, secondo recenti acquisizioni in letteratura, inalanti.
Gli eosinofili sono spesso aumentati nei tessuti in caso di patologie allergiche, ma al momento non è ben chiaro il ruolo di tali cellule nell'innescare la patologia.
La cellula eosinofilica presenta granuli contenenti mediatori biologicamente attivi, quali i leucotrieni, che sembrano svolgere un ruolo centrale nella contrazione delle cellule muscolari lisce.
Recenti evidenze indicano inoltre l'interleuchina 5 e 13 come molecole chiave nella patogenesi e nel perpetuarsi del danno esofageo e potrebbero rappresentare possibili target per un'eventuale futura terapia biologica.
I pazienti affetti da EE presentano una **storia familiare** o **personale** positiva per allergia nel 50-80% dei casi, con sintomi quali asma, rinite, eczema.
In uno studio condotto da *Orenstein et al* [16], su una casistica di 30 pazienti pediatrici affetti da EE, il 62% di essi presentava frequenti episodi di broncospasmo.
Inoltre, l'eosinofilia periferica e l'aumento dei livelli sierici di IgE sono riportati nel 20-60% dei casi osservati.
Ogni età può essere interessata dall'EE; l'esordio più comune avviene durante l'infanzia e l'adolescenza.
Clinicamente l'EE può presentarsi con una varietà di **quadri sintomatologici** ▸ Tab. I.
I bambini di età inferiore ai 7 anni presentano più comunemente sintomi quali dolori addominali, vomito e/o rigurgito, inappetenza, scarsa crescita.

Tab. I Sintomi e segni nei bambini e negli adulti

BAMBINI	ADULTI
Rifiuto del cibo	Disfagia
MRGE resistente a terapia medica	Ostruzione da bolo alimentare
MRGE resistente a terapia chirurgica	MRGE resistente a terapia medica
Ostruzione da bolo alimentare o da corpo estraneo	MRGE resistente a terapia chirurgica
Dolori addominali epigastrici	
Disfagia	
Scarso accrescimento	

Furuta GT et al, Consensus report of EE.- Gastroenterology 2007

Nei bambini di età >7 anni e negli adolescenti la sintomatologia tipica è rappresentata da disfagia, dolore toracico, *food-impaction*.
Nonostante in letteratura non siano riportati dati in merito alle complicanze a lungo termine dell'EE non trattata, è probabile che i cambiamenti sintomatologici che si registrano al passaggio dall'adolescenza all'età adulta siano correlati all'infiltrazione eosinofilica a tutto spessore della parete esofagea con formazione di stenosi, come recentemente suggerito da *Fox*.

Il **Panel** propone di introdurre nell'iter diagnostico per EE tutti i pazienti con sintomi e segni cronici aspecifici e specifici sospetti: anoressia, rifiuto o difficoltà ad alimentarsi, disfagia, sensazione soggettiva della presenza di cibo che progredisce con difficoltà in esofago, episodi di ostruzione da bolo alimentare acuta o ricorrente, dolore retrosternale, pirosi, dolori epigastrici o toracici, stasi dell'accrescimento, ecc).

capitolo 2

Diagnosi differenziale

Filippo Torroni - Laura Di Iorio*
UOC Chirurgia Endoscopia Digestiva, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma
**Gastroenterologia ed Endoscopia digestiva, Università degli Studi di Roma Tor Vergata*

L'EE può presentare alcuni aspetti clinici sovrapponibili ad altre patologie digestive, in particolare alla **MRGE**. Da qui nasce l'esigenza di una diagnosi clinica e istologica accurata, necessaria per non incorrere in errori metodologici e terapeutici.
La MRGE, così come l'EE, può manifestarsi con vomito, rigurgito, inappetenza, calo ponderale, rifiuto del cibo e dolore retro sternale: quest'ultimo più comune nel bambino più grande. La MRGE "refrattaria" alla terapia con IPP, e soprattutto la comparsa di disfagia ai solidi con *food-impact*, è suggestiva per EE. In questi casi è necessaria un'indagine endoscopico/istologica con biopsie multiple seriate (duodeno, stomaco ed esofago distale, medio ed, eventualmente, prossimale) per dirimere il quadro.
È altresì noto che nella MRGE l'accumulo degli eosinofili in esofago è di comune riscontro anche se in concentrazione minore rispetto all'EE. Nella MRGE la pH-metria è sempre patologica mentre nell'EE risulta sempre negativa o con reflussi di breve durata, prevalentemente post-prandiali. Il riscontro di poliallergie (alimentari o inalatorie), una eosinofilia periferica con sintomi MRGE refrattari al trattamento medico, indirizzano verso una patologia digestiva a screzio eosinofilo più che verso una patologia peptica.
Nella diagnostica differenziale il quadro endoscopico gioca un ruolo importante. Esistono, infatti, quadri endoscopici suggestivi di EE: la presenza di strie longitudinali (*linear furrows*), di fini elementi biancastri (*white specks*) e un esofago ad anelli (*ringed esophagus*) evidenziabili in tutta la lunghezza dell'esofago. Quadri diversi si osservano nell'esofagite peptica, dove sono più frequenti le erosioni e le ulcere dell'esofago distale e medio, fino alle complicanze (stenosi del terzo medio, esofago di Barrett).
Altro argomento dibattuto è relativo alle **stenosi esofagee** che possono risultare di natura peptica o infiammatoria (MICI) ▸ Tab. 1, ▸ Figg. 1, 2 e 3.

Tab. 1 Sintomi e segni nei bambini e negli adulti

MRGE
Stenosi congenite
Malattia di Crohn
Malattie del collagene
Stenosi da caustici

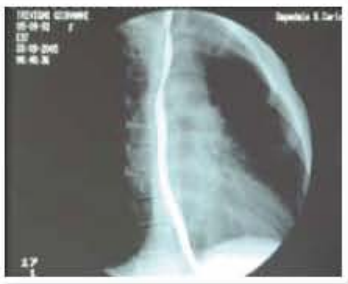


Fig. 1 *Evidenza RX-esofagogramma di stenosi esofagea diffusa in EE*

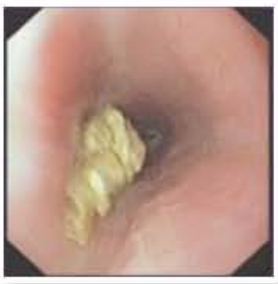


Fig. 2 *Ostruzione da bolo alimentare, con sottostante stenosi esofagea eosinofila*

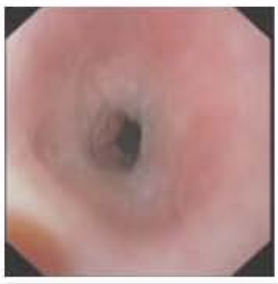


Fig. 3 *Stenosi esofagea eosinofila e rigidità della parete esofagea durante l'insufflazione*

Tra i disordini gastrointestinali con **accumulo di eosinofili** ricordiamo la gastroenterite eosinofila primitiva, la sindrome ipereosinofila con interessamento gastrointestinale, l'eosinofilia associata a "infezioni parassitarie", ad "infiammazione" come nella MRGE, nella malattia celiaca e nella Malattia di Crohn e l'eosinofilia associata a neoplasie ▸ **Tab. 2**.

Tab. 2 Diagnosi differenziale di eosinofilia esofagea

MRGE
EE
Gastroenterite eosinofila
Malattia di Crohn
Malattie del tessuto connettivo
Sindromi ipereosinofile
Infezioni
Ipersensibilità a farmaci

Furuta GT et al, Consensus report of EE, Gastroenterology 2007

Ricordiamo infine l'**esofagite da candida** ▸ **Fig. 4** che - come quadro macroscopico endoscopico - può mimare un'esofagite eosinofila; la presenza di fini elementi biancastri simili ai microascessi eosinofilici, può essere fuorviante ai fini diagnostici. Da qui l'importanza categorica delle biopsie esofagee a livello distale, medio e prossimale, ricercando la presenza di ife sulla biopsia.

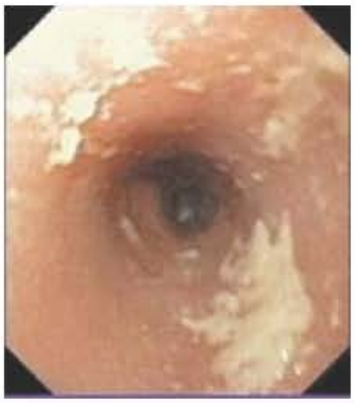


Fig. 4 *Esofagite da Candida, aspetto macroscopico*

capitolo 3

L'endoscopia

Claudio Romano - Gianluigi de' Angelis*
Dipartimento di Scienze Pediatriche Mediche e Chirurgiche, Università di Messina
**Unità di Gastroenterologia Pediatrica, Università di Parma*

La diagnosi presuntiva di EE presuppone l'identificazione di un infiltrato eosinofilo nell'esofago a livello della lamina propria in pazienti sottoposti a EGDS per sintomi dispeptici o per sospetta MRGE e che, talvolta, hanno fallito un tentativo di terapia con farmaci antisecretori o IPP.
L'EGDS con biopsie esofagee può essere quindi considerata la tecnica diagnostica gold-standard.
L'aspetto macroscopico della mucosa esofagea non assume in genere caratteri patognomonici e specifici nel sospetto di EE rispetto a quello evidenziato nella esofagite da MRGE o acido-correlata ▸ **Fig. 1**.

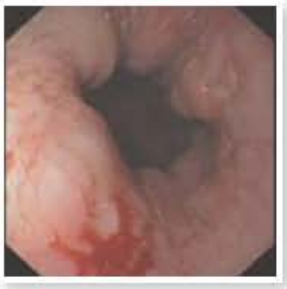


Fig. 1 *Quadro macroscopico aspecifico di esofagite distale/cardite*

In pazienti con sintomi dispeptici e sospetto clinico di EE, l'aspetto macroscopico della mucosa esofagea può apparire normale anche in presenza di un significativo infiltrato eosinofilo o evidenziare un modesto **eritema** e una aspecifica **friabilità** superficiale (compatibile con grado I di Savary o classe A della Classificazione di Los Angeles).
In talune circostanze un'attenta osservazione dell'endoscopista può consentire di evidenziare quadri macroscopici fortemente suggestivi e specifici per EE.
Fox et al [123] segnalano che la presenza di immagini equivalenti a **solchi lineari** o **anelli** nel corpo esofageo con moderata o severa esofagite (esofago trachealizzato) rappresenti un quadro patognomonico nel sospetto di EE ▸ **Figg. 2,3** e **4**.

Tale condizione si accompagna a importanti alterazioni della motilità e peristalsi esofagea e giustifica la disfagia che talvolta rappresenta il "sintomo guida". La presenza di **placche biancastre** aderenti sulla mucosa esofagea, media e distale, sembra correlarsi alla presenza

Figg. 2,3 e **4** *Quadri macroscopici suggestivi di esofagite eosinofila (striature longitudinali, anelli-trachealizzazione, punteggiatura biancastra)*

di ascessi o di significativa infiltrazione di eosinofili a livello della sottomucosa; tali placche si possono estendere in aree circoscritte - o interessare vaste aree - specialmente del terzo distale dell'esofago.
La superficie della mucosa può apparire inoltre iperemica, granulosa, congesta, ma lesioni a carattere ulcerativo sono rare. In genere, la localizzazione del processo infiammatorio interessa sia l'esofago prossimale che quello distale, a differenza dell'esofagite da MRGE che invece interessa prevalentemente la regione distale.

L'esofago di piccolo-calibro rappresenta una condizione associata alle fasi più avanzate di EE, secondario a una stenosi del lume correlato all'infiammazione a prevalente componente eosinofila, alla deposizione di collagene e alla conseguente fibrosi.
Tale condizione si associa al rischio di episodi di **bolo carneo** che talvolta rappresentano una reale complicanza o emergenza.
Non vi è evidenza dell'utilità di una valutazione del tratto digestivo inferiore o di colonscopia in un paziente affetto da EE, se non relativamente alla diagnosi differenziale (es. gastroenterite eosinofila).
È utile eseguire almeno 5 prelievi bioptici (1-2 per porzione esofagea) ▸ **Fig. 5** per raggiungere una sensibilità diagnostica del 90-100%.

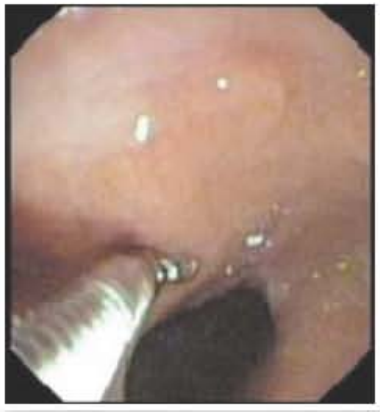


Fig. 5 *Biopsie multiple esofagee*

Il **Panel** propone la seguente classificazione endoscopica di EE*:

- **Grado I** ----> eritema e friabilità della mucosa, accentuazione del disegno vascolare
- **Grado II** ----> solcature lineari verticali
- **Grado III** ----> placche biancastre
- **Grado IV** ----> anelli e trachealizzazione
- **Grado V** ----> ulcere

*NB:
- *alla valutazione del grading bisogna aggiungere il reperto della stenosi (per esempio, grado III + stenosi)*
- *sono raccomandabili biopsie nell'esofago medio, distale e a livello duodenale*
- *sono sufficienti 2 biopsie per sezione*

capitolo 4

L'istologia

Alessandro Pane - Angelo Campanozzi*
UOC Chirurgia Epatobiliare, Ospedale Pediatrico Bambino Gesù, IRCSS di Roma
*Università di Foggia (Completare con istituto)

La diagnosi di EE si basa anche sull'evidenza istologica di eosinofili che infiltrano l'epitelio squamoso della mucosa esofagea e, malgrado non vi sia ancora un *Consensus* sull'argomento, i dati disponibili oggi in letteratura indicano che un numero di eosinofili ≥15 per campo (High Power Field: HPF) rappresenti la diagnosi istologica di EE.
Anche la presenza di microascessi eosinofili, pur non essendo presenti un numero minimo di 20 eosinofili per campo HPF, è considerata sufficiente per effettuare la diagnosi.
Ngo et al [28] hanno recentemente descritto tre pazienti con eosinofilia esofagea >50 eo/HPF risoltasi con terapia medica rivolta alla MRGE.
È comunque importante sottolineare che anche l'esofagite da MRGE si caratterizza per un aumentato numero di eosinofili infiltranti la mucosa ma, in questo caso, il numero di cellule è generalmente inferiore (<10 HPF). Il differente numero di eosinofili in grado di differenziare tra EE ed esofagite da reflusso è molto più evidente quando le biopsie sono prelevate dalla mucosa dell'esofago prossimale: viene quindi raccomandato che le biopsie provengano anche dall'esofago medio. Altre caratteristiche istologiche utili, ma non indispensabili per la diagnosi istologica di EE, sono l'iperplasia della zona basale, l'allungamento delle papille, la stratificazione in superficie degli eosinofili con aggregati o microascessi (aggregati di 4 o più eosinofili contigui) e la fibrosi della lamina propria ▶ **Fig. 1 A-B-C**.

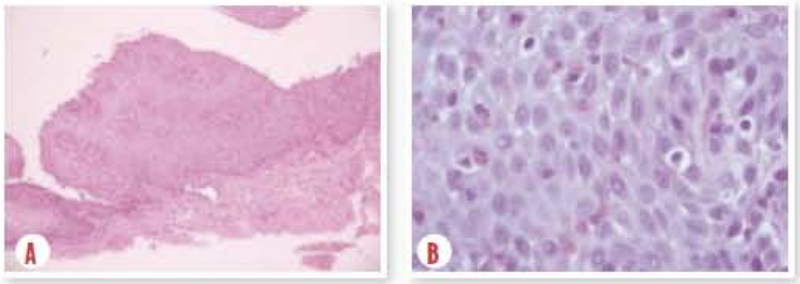


Fig. 1 A-B *Infiltrazione eosinofila a diverso ingrandimento (40X-20X)*

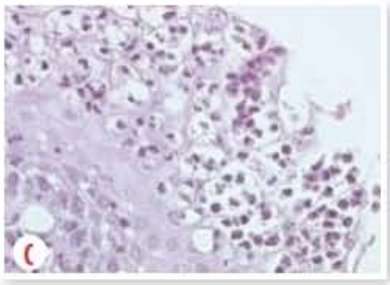


Fig. 1 C *Microascessi eosinofili (20 X)*

Per gentile concessione di Paola Francalanci, Anatomia Patologica, Ospedale Pediatrico Bambino Gesù di Roma

Non esistono dati sufficientemente suffragati che permettano di individuare un'istologia tipica dell'esofagite eosinofila, ovvero il *cut-off* nel conteggio degli eosinofili nella biopsia esofagea. Nella mancanza di prove *evidence-based*, dobbiamo affidarci alla letteratura e, più precisamente, alla più ampia casistica pediatrica attualmente disponibile, pubblicata da *Liacouras et al*, ovvero di 381 pazienti con EE osservati nell'arco di 10 anni (età 9.1 ± 3.1 aa); in 312 erano osservati sintomi da RGE, in 69 disfagia. Venivano eseguite biopsie esofagee multiple osservando la seguente distribuzione nel conteggio degli eosinofili: prossimale: 23.3 ± 10.5 /HPF; distale: 38.7 ± 13.3 /HPF. Ciò viene derivato utilizzando come *cut-off* i valori estremi della curva di distribuzione osservata nel lavoro sopra citato.
Ulteriore elemento istologico di discriminazione nei confronti delle altre forme di esofagite (essenzialmente le forme peptiche) è l'iperplasia delle cellule dello strato epiteliale basale, che viene determinato mediante valutazione quantitativa (ibridazione in sito di anticorpo monoclonale anti-Ki67 o MIB-1).
Secondo quanto rilevato da un recente lavoro di *Steiner SJ et al* [29], in una popolazione pediatrica di 57 soggetti (di cui 27 con riscontro pH-metrico-endoscopico di esofagite peptica e 30 con riscontro istologico-clinico di esofagite eosinofila), era possibile evidenziare una diversa proliferazione dello strato basale.
Questo indice è stato definito come normale per strato basale <25% dello spessore dell'epitelio esofageo, lieve per 26-50%, moderato per 51-75% e grave >75%. Nei pazienti con esofagite eosinofila la severità dell'iperplasia dello strato basale appariva essere significativamente maggiore rispetto ai pazienti con esofagite peptica ($p < 0,001$).
Nei casi di dubbia interpretazione, pertanto, la metodica di quantificazione della proliferazione dello strato basale può essere un utile indice di discriminazione diagnostica.

Sulla base di questi dati, il **Panel** propone il seguente grading istologico relativo all'infiltrazione eosinofila:

Esofago prossimale	Lieve: tra 20 e 30 eosinofili /HPF
	Media: tra 30 e 40 eosinofili /HPF
	Grave: più di 40 eosinofili /HPF
Esofago distale	Lieve: tra 25 e 35 eosinofili /HPF
	Media: tra 35 e 45 eosinofili /HPF
	Grave: più di 45 eosinofili /HPF.

Nota per autore: non c'è riferimento a Liacouras et al in bibliografia di questo capitolo

capitolo 5

Esofagite eosinofila, reflusso gastro-esofageo e pHmetria: quale correlazione?

Tamara Caldaro - Silvia Salvatore*

UOC Chirurgia Endoscopia Digestiva, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma
**Università dell'Insubria di Varese (Completare con istituto)*

Il rapporto tra reflusso gastroesofageo (RGE) ed EE non è ancora completamente chiarito e risulta complicato dall'ampio spettro e dalla possibile sovrapposizione clinica, endoscopica e istologica dei pazienti con EE, dalla variabile risposta terapeutica (in particolare dalla limitata risposta agli PPI) e dal possibile ruolo del reflusso debolmente acido e non acido sul quale i dati sono ancora scarsi. Proprio la presenza a livello delle biopsie esofagee (esofago medio-distale) di un intenso infiltrato eosinofilico, non correlato a pH-metria patologica e associato alla mancata risposta clinico-istologica alla terapia (medica e chirurgica) per RGE sono stati gli elementi che avevano fatto sospettare, circa vent'anni fa, l'esistenza di una "nuova patologia" esofagea nella quali le basi molecolari e il protocollo diagnostico-terapeutico necessitavano di strutturazione. La relazione tra la densità dell'infiltrato eosinofilo esofageo e l'indice di reflusso (acido) (R.I, percentuale di tempo in cui il pH risulta <4 durante la registrazione) è stata valutata da *Steiner et al* [37] in un'ampia casistica di bambini e ragazzi (numero totale 305; età media 7.4 anni; range 1-17.6 anni) sottoposti a biopsie esofagee e pH-metria per la persistenza di vomito, pirosi retrosternale, *food impaction* e disfagia. In base all'entità dell'infiltrato eosinofilo sono stati identificati 5 gruppi di pazienti: gruppo I e II con 0 eosinofili (Eos)/High Power Field (HPF) e, rispettivamente, senza e con alterazioni istologiche; gruppo III con 1-5 Eos/HPF; gruppo IV con 6-20 Eos/HPF e gruppo V con Eos >20/HPF (pazienti con EE). L'esposizione acida è risultata maggiore (con R.I. medio ± DS 5.96 ± 1,53%) nel gruppo III, cioè nei pazienti con modesto numero di eosinofili mentre solo nel 10% dei casi si è riscontrato un R.I. patologico nei pazienti con infiltrato superiore a 20/HPF ed EE. In altri 21 bambini con EE (≥ 15 Eos/HPF), *Lim et al* [38] riportano normali pH-metrie, senza alcuna correlazione con la presenza endoscopica di *white specks*. Una pH-metria normale per esposizione acida, ma con una rilevante alcalinizzazione esofagea (espressa come percentuale di tempo con pH esofageo tra 7 e 8 e >8) e un ridotto numero di reflussi lunghi rispetto ai controlli, è stata riportata da *Sant'Anna et al* [39] in un piccolo numero (nove) di bambini canadesi con EE ▸ **Tab. I**.

Tab. I Risultati della pH-metria in pazienti con EE e in controlli

Parametro pH-metria	EE (n=9)	Controls (n=200)	Matched controls (n=48)
Nº episodi pH <4 >5'	0.4 (0.5)	1.9 (3.5)	1.7 (2.6)
Reflusso più lungo (pH <4)	4.4 (2.7)	10.1 (11.5)	10.6 (12.0)
% Total time pH <4.0	1.8 (1.2)	3.9 (5.1)	3.9 (4.4)
% Total time pH >7.0<8.0	48.4 (14.5)	7.8 (11.1)	15.7 (17.5)
% Total time pH >8.0	19.0 (17.3)	0.5 (2.3)	0.9 (1.6)

Come possibile spiegazione della significativa presenza di reflusso alcalino, gli autori hanno ipotizzato nell'EE sia un'aumentata deglutizione di saliva sia un aumentato stimolo delle ghiandole submucosali esofagee che producono bicarbonato.

Non è attualmente noto se le molecole rilasciate dagli eosinofili (Major Basic Protein -

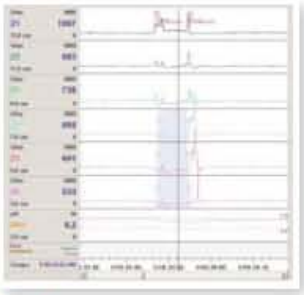


Fig. 1 *Normale pH-metria in paziente con EE*

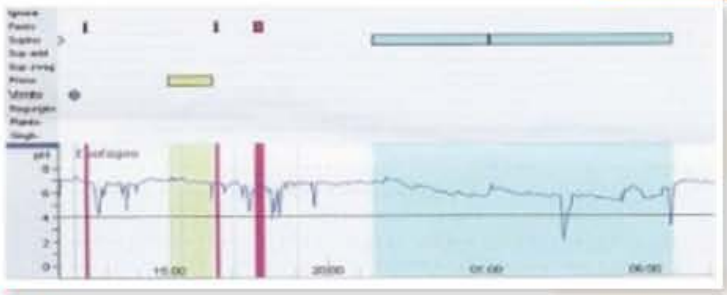


Fig. 2 *pH-impedenzometria dello stesso paziente: RGE debolmente acido, pH 6.2*

MBP - ed *Eosinophilic Cationic Protein* - ECP) possano giocare un ruolo nell'aumento del pH. La presenza di un basso numero di reflussi acidi lunghi e il ridotto valore medio del reflusso più lungo in pazienti con EE suggerirebbe persino la presenza di una miglior (o stimolata) *clearance* esofagea nell'EE rispetto ai controlli. Così l'infiltrato eosinofilico non ridurrebbe la motilità esofagea nonostante la presenza di restringimento del lume, la disfagia e l'impatto del cibo, riportato rispettivamente, in questa casistica, nel 33%, 66% e 50% dei pazienti. Il mantenimento di un'adeguata peristalsi esofagea è stato recentemente confermato da *Remedios et al* [40] in adulti con EE. La pH-metria è risultata normale nel 58% dei casi (14 pazienti), mentre i restanti 10 soggetti con pH-metria patologica (e quindi con EE e concomitante reflusso gastroesofageo patologico), sottoposti a manometria esofagea, hanno mostrato, in 8 casi, un'incontinenza dello sfintere esofageo inferiore e, solo in un caso, aperistalsi probabilmente favorita dall'infiammazione eosinofila evolutasi in fibrosi.
In base ai dati attualmente pubblicati, l'EE non appare pertanto correlata al reflusso acido, pur potendo coesistere le due condizioni nel 10% dei pazienti nei quali si è ipotizzato che il reflusso patologico fosse secondario all'infiammazione determinata dall'intenso infiltrato eosinofilo. Nell'EE la pH-metria risulta quindi utile per individuare i pazienti con coesistente reflusso acido patologico che necessitano di uno specifico protocollo terapeutico (terapia dell'EE associata con PPI) e di *follow-up*. Viceversa, l'EE va sempre sospettata in un paziente con reflusso acido che non risponde alla terapia con PPI. L'avvento della pH-impedenzometria ha permesso di studiare gli episodi di RGE debolmente acidi e alcalini ▶ **Figg. 1** e **2**. *Rosen et al* [41] avevano ipotizzato che, poiché gli antigeni alimentari contribuiscono all'eziopatogenesi dell'EE, si potessero riscontrare in questi pazienti frequenti episodi di reflusso di materiale alimentare nel periodo post-prandiale (visualizzati come *RGE alcalino*). Nella rivalutazione retrospettica di esami pH-impedenzometrici di pazienti con EE, con MRGE e con un gruppo di controllo - al contrario - si è evidenziato che i soggetti con EE non presentavano un aumentato numero di RGE alcalini, né una maggiore percentuale di RGE estesi alla porzione prossimale dell'esofago rispetto ai controlli. Anche la percentuale di tempo di esposizione della mucosa esofagea al materiale refluito risultava simile nei due gruppi. Ulteriori studi pH-impedenzometrici saranno necessari per contribuire a chiarire la fisiopatologia dell'EE e la sua eventuale relazione con il reflusso non acido e alcalino. L'influenza degli eosinofili sulla motilità e clearance esofagea e sul tono dello sfintere esofageo inferiore dovrà essere altresì approfondita.

Il **Panel** propone l'esecuzione della pH-impedenzometria a tutti i pazienti con sospetta EE.

15

capitolo 6

L'iter diagnostico allergologico-autoimmunitario

Sandra Lucarelli - Francesca Aniballi*

UOC Gastroenterologia ed Epatologia Pediatrica, Dipartimento di Pediatria, Università degli Studi "La Sapienza" di Roma
** U.O. di Gastroenterologia Pediatrica, Università Politecnica delle Marche*

L'EE è una malattia infiammatoria cronica localizzata all'esofago la cui eziologia è ancora sconosciuta. Molti studi sembrano confermare come l'**allergia alimentare** giochi un ruolo significativo nella sua patogenesi. L'ipersensibilità agli alimenti può essere mediata sia da un meccanismo immunologico di tipo I, che vede in causa IgE specifiche per un determinato allergene alimentare sia da una reazione di tipo IV, cellulo-mediata, cosiddetta ritardata.

Tuttavia, alcuni autori non la considerano l'unica causa e sottolineano come altri possibili meccanismi allergici e immunologici possano giocare un ruolo importante nello sviluppo dell'esofagite eosinofila.

È importante sottolineare, ad esempio, come l'inalazione di **aereoallergeni** possa contribuire allo sviluppo o alla recrudescenza dell'esofagite eosinofila.

Recenti studi suggeriscono un'associazione tra la risposta infiammatoria eosinofila nelle vie aeree e la risposta infiammatoria a livello della mucosa esofagea.

La teoria più accreditata sembra essere quella che ipotizza che l'antigene a livello intratracheale venga sospinto mediante il movimento ciliare verso l'esofago ove indurrebbe la reazione infiammatoria.

Si è visto che pazienti con anamnesi per asma e/o rinocongiuntivite allergica, mantenuti a dieta costantemente per tutto l'anno, presentavano una ripresa sia clinica sia istologica di malattia nei periodi di esposizione ai pollini verso cui erano sensibilizzati e beneficiavano del simultaneo trattamento per l'allergia alimentare e per l'asma e/o rinite allergica.

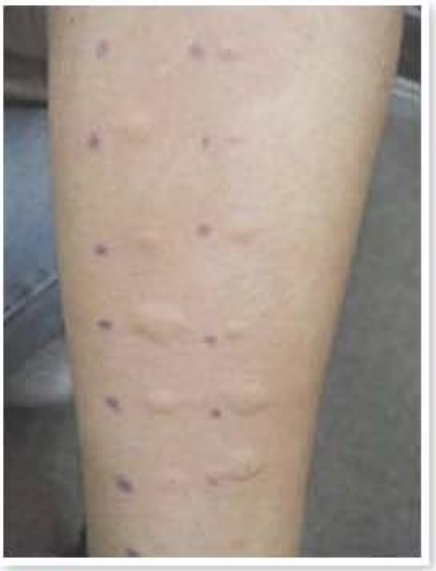


Fig. 1 *Skin prick test*

Fig. 2 *Skin patch test*

Altri autori, inoltre, considerano l'EE un sottogruppo delle enteropatie eosinofile e, come tale, caratterizzato alla base da una **componente immuno-mediata**, causata da un difetto della risposta immunologica ancora sconosciuta.

Il **Panel** propone il seguente iter diagnostico allergologico/autoimmunitario per effettuare le indagini prioritarie in un paziente sospetto:

1. ANAMNESI FAMILIARE PER:

- allergie alimentari
- rinite e/o congiuntivite allergica
- asma
- patologie autoimmuni

2. ANAMNESI PERSONALE PER:

- manifestazioni cliniche (dermatite atopica, sintomi gastrointestinali, sintomi respiratori) in relazione all'assunzione di uno o più alimenti (quantità dell'alimento necessario allo scatenamento della reazione, alimento cotto o crudo, presunto tempo intercorso tra l'assunzione dell'alimento e la comparsa del sintomo)
- patologie autoimmuni

3. ESAMI DI LABORATORIO:

- eosinofilia periferica (ipereosinofilia presente generalmente nel 25-40% dei pazienti affetti)
- livelli di IgE sieriche (iperIgE presente generalmente nel 20-50% dei pazienti affetti)

4. TEST ALLERGOLOGICI:

Skin prick test verso trofo e aereoallergeni con estratti allergenici uniformi per tutti i Centri coinvolti.

Allergeni da utilizzare come pannello standard:
- alimenti: caseina, alfa-lattalbumina, beta-lattoglobulina, soia, riso, frumento, mais, uovo (albume e tuorlo), carni (bovina, pollo), pesce (merluzzo), crostacei, vegetali (pomodoro, patata), cacao, arachidi, nocciole;
- inalanti: acari della polvere, graminacee, composite, parietaria, olivo;
- eventuali altri allergeni suggestivi per la storia clinica.

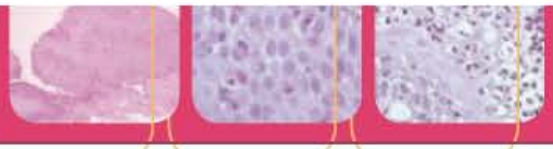
La lettura verrà effettuata dopo 15 minuti valutando il diametro medio del pomfo che verrà considerato positivo se superiore a 3 mm rispetto al controllo negativo.

Prick by prick con l'alimento fresco (latte vaccino, uovo, vegetali eventualmente coinvolti).
La lettura è identica a quella per i prick test.

Dosaggio IgE specifiche (CAP-RAST) verso gli allergeni risultati positivi al prick o se al paziente non è possibile sospendere la terapia antistaminica.

Patch test (dai 6 mesi di vita) **verso aereo e trofoallergeni** (latte vaccino, uovo, frumento, soia, riso, acari della polvere o eventuali altri allergeni sulla base della storia clinica).

I cibi freschi vengono posti su Finn Chambers (Merck o FIRMA) e posizionati nello spazio interscapolo-vertebrale al di sopra della spina della scapola. Il patch viene mantenuto in sede per 48 ore e la lettura viene effettuata dopo 20 minuti dalla sua rimozione.
La reazione verrà considerata positiva e classificata con + per eritema e papule isolate, ++ per eritema e papule, +++ per eritema e vescicole.



capitolo 7

La dietoterapia

Giuseppe Morino - Rita Sforza Wietrzykowska - Graziella Guariso*
Dietologia Clinica, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma
**Università di Padova (Completare con Istituto)*

L'EE rappresenta una patologia di non facile diagnosi e trattamento legata a meccanismi immunoallergici in cui l'alimentazione gioca un ruolo fondamentale: pur non avendo al momento certezze sul ruolo patogenetico dei singoli alimenti nello sviluppo delle lesioni esofagee, la presenza di test cutanei e/o esami ematochimici specifici positivi per allergia per trofoallergeni, pongono questi test alla base di un percorso diagnostico e terapeutico.
Sul piano diagnostico, i vari studi ritengono fondamentale escludere quegli alimenti per i quali vi sia una **positività** ai test: se gli alimenti sono in numero **<10**, si consiglia una dieta di eliminazione oligoantigenica per 3 mesi. Al termine di questo periodo appare utile effettuare una nuova biopsia che definirà l'andamento delle lesioni e, di conseguenza, l'importanza di proseguire la dieta (una normalizzazione del reperto istologico di eosinofili indicherà l'alimento escluso come un fattore importante nello sviluppo della patologia, mentre la persistenza di un reperto patologico escluderà l'alimento presunto come fattore causale principale o indicherà la necessità di una dieta elementare).
Nel caso di **negatività** ai test e/o in presenza di una **positività** a un numero di alimenti **>10**, appare invece utile ricorrere a una dieta elementare per 8 settimane, seguita dalla ripetizione della biopsia.
I fattori implicati nella decisione della dietoterapia sono rappresentati dalla qualità di vita del paziente e della famiglia, dalla diagnosi "reale" di allergia alimentare, dalla fase dello sviluppo del paziente e dal grado di malnutrizione cronica. Nella valutazione nutrizionale, che precede la somministrazione di una dieta di esclusione, devono essere quantificati precisamente i fabbisogni nutrizionali globali per ogni paziente (calorie-vitamine-micro e macronutrienti).

La dieta ipoallergenica

Il riscontro di allergie specifiche a singoli alimenti (se in numero non >10) pone l'indicazione a diete ipoallergeniche prive di tali alimenti. Nel caso di bambini piccoli - di età inferiore a un anno - può essere utile, in ogni caso, ripartire da una formula a ridotta allergenicità (idrolisato "spinto" piuttosto che dieta elementare) per integrare gradualmente con alimenti meno allergizzanti (mais, riso, agnello, coniglio, pera, banana). Nei bambini più grandi in cui vi sia stato il riscontro di singole positività per alimenti di minore importanza nutrizionale (per esempio, frutta e verdura), può essere utile - per il primo periodo - escludere alimenti maggiormente allergizzanti come latte vaccino, uovo, grano.

La dieta elementare

Si caratterizza per un apporto nutrizionalmente adeguato attraverso l'uso di alimenti ad assai ridotta allergenicità: nel caso specifico, data l'elevata importanza dell'allergia nei meccanismi patogenetici e la necessità di avere apporti calorici e in nutrienti adeguati, è preferibile ricorrere a formule a base di aminoacidi, con apporti lipidici (presenza di MCT) e in carboidrati (assenza di lattosio) adeguati e facilmente digeribili. La dieta elementare può essere somministrata per os o per SNG: nel caso di importante disfagia e/o stenosi è preferibile l'uso di quest'ultima via, eventualmente associata a terapia medica (fluticasone

propionato ecc.). L'uso di altre formule (soia, riso, idrolisati) non appare adeguata in questa patologia. La possibilità poi di avere formule elementari liquide, nutrizionalmente complete, a base di aminoacidi di sintesi con un apporto elevato di MCT, senza lattosio e senza glutine, ma maggiormente palatabili in quanto aromatizzate, ne permette l'uso anche nei bambini più grandi.

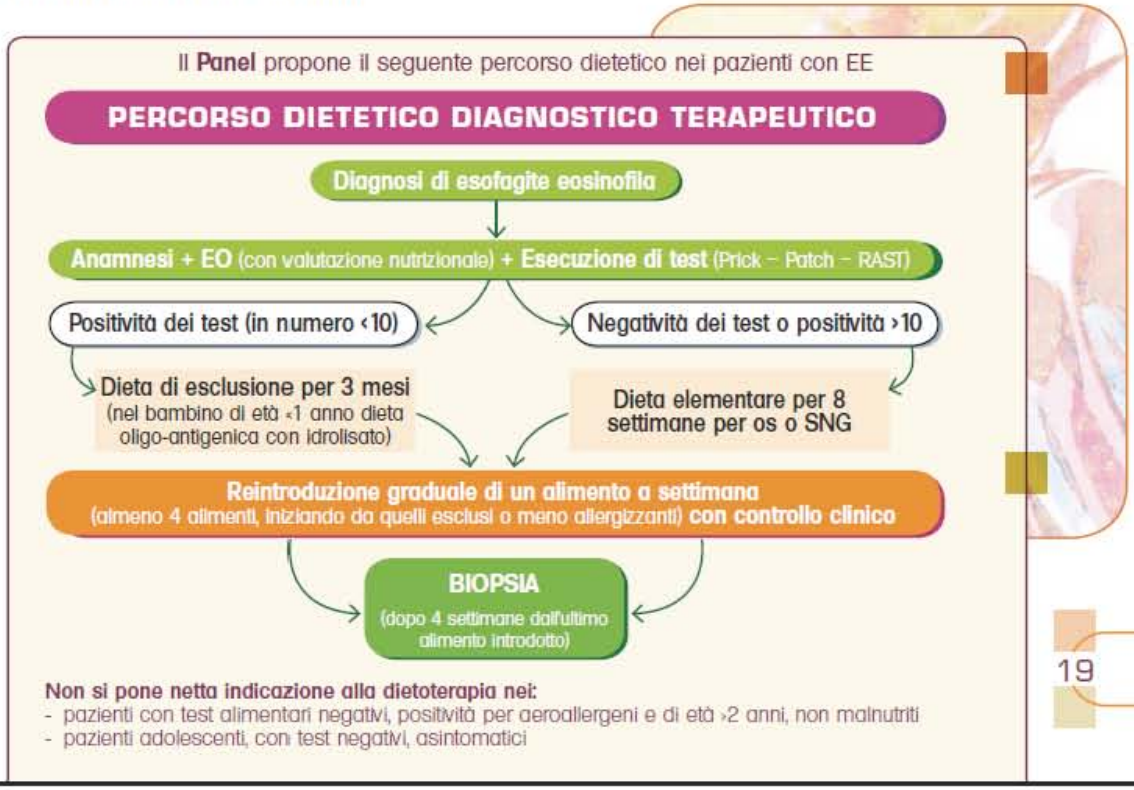
Il challenge

Dopo un periodo di 3 mesi di dieta ipoallergenica, e/o dopo 8 settimane di dieta elementare, in presenza di un reperto bioptico negativo, si potrà effettuare il **Test di provocazione orale**: la reintroduzione può essere effettuata con un alimento a settimana, iniziando da quelli meno allergizzanti (mais, riso, agnello, coniglio, pera, banana) e comunque negativi per tutti i test allergometrici. Dopo l'introduzione di almeno 4 alimenti - e comunque dopo 8 settimane dall'inizio del challenge - è utile ripetere le biopsie esofagee: nel caso di biopsia nuovamente positiva è necessario escludere gli alimenti introdotti per altri 2 mesi e ripetere quindi la biopsia.

In corso di reintroduzione alimentare, e in assenza di sintomatologia, un aumento modesto di eosinofili permette un atteggiamento prudente di controlli clinici, con eventuale anticipo di biopsia, senza escludere gli alimenti introdotti - soprattutto in bambini più grandi - in cui un atteggiamento troppo restrittivo potrebbe provocare reazioni psicologiche negative.

Un atteggiamento egualmente prudente di osservazione clinica, **senza intervento dietetico restrittivo**, può essere indicato in soggetti con forte positività di test per aeroallergeni e/o in adolescenti con scarsa o assente sintomatologia.

Nel caso del paziente negativo a tutti i test allergologici vi è invece l'indicazione per una dieta libera, associata a prednisone, per indurre la remissione, fluticasone dipropionato e/o montelukast nel mantenimento.



capitolo 8

Il ruolo delle dilatazioni esofagee

Nota per autore: Kaplan (nota 57) in bibliografia riporta l'anno 2003 non 2005

Pietro Betalli

Chirurgia Pediatrica di Padova (Completare con Istituto)

Da quanto emerge dalla letteratura, il ruolo delle dilatazioni esofagee nei pazienti adulti e pediatrici affetti da EE non è ancora ben definito e, al momento attuale, non esistono linee guida chiare e standardizzate. Fino a 5-6 anni fa in letteratura si riteneva che nell'EE, pur essendo spesso assenti stenosi esofagee ben visibili all'endoscopia, l'esofago presentasse un calibro ristretto responsabile dei problemi di disfagia segnalati dai pazienti. Per questo motivo veniva suggerito di eseguire sempre e comunque empiriche dilatazioni dell'esofago: queste manovre avrebbero sicuramente determinato un miglioramento della sintomatologia clinica. Nel 2003, *Fox* [55] esprime un concetto che cerca di modificare questo atteggiamento piuttosto invasivo. Secondo l'Autore, infatti, la disfagia nei pazienti affetti da esofagite eosinofila può dipendere dalla presenza di stenosi fibrotiche, anche se in moltissimi pazienti non sono evidenziabili chiare stenosi all'endoscopia. L'Autore sospetta che gli episodi di *food impaction* dipendano più da un problema di dismotilità esofagea che da vere e proprie stenosi organiche del lume esofageo. Sulla scia di queste affermazioni sono stati pubblicati alcuni report nei quali le dilatazioni dell'esofago vengono riferite piuttosto rischiose per l'elevato pericolo di perforazione e di scarsa utilità nella stragrande maggioranza dei casi. *Potter* [56] nel 2004, analizzando la propria casistica di 29 pazienti seguiti nell'arco di 4 anni, conclude che le dilatazioni esofagee siano indicate solo nei pazienti con ripetuti episodi di *food impaction*. Queste devono essere eseguite con massima cautela e con *bougies* di piccolo calibro. Con l'introduzione del trattamento farmacologico, in ogni caso, le dilatazioni hanno assunto un ruolo marginale nel trattamento dell'esofagite eosinofila, e andrebbero effettuate nei pazienti con disfagia persistente nonostante la terapia medica. *Kaplan* [57], nel 2005, ribadisce quanto affermato da Potter: nell'EE esiste un elevato rischio di perforazione dell'esofago durante le dilatazioni. Prima di prendere in considerazione ogni trattamento più invasivo vengono perciò suggerite almeno 8 settimane di terapia medica. Gli autori raccomandano, in conclusione, di eseguire dilatazioni esofagee solo nei pazienti che non rispondono alla terapia medica o che presentano stenosi francamente ostruenti il lume esofageo. Anche *Liacouras* [58], nel 2006, ribadisce l'elevato rischio di perforazione esofagea durante le dilatazione dei pazienti affetti da esofagite eosinofila. Sostiene inoltre che la dilatazione meccanica sia più efficace della dilatazione pneumatica e che, molto spesso, la semplice introduzione dello strumento attraverso la stenosi esofagea sia sufficiente per ottenere benefici nei bambini affetti da esofagite eosinofila. Da quanto emerso dall'analisi della letteratura più recente possiamo affermare che le dilatazioni esofagee non siano sempre necessarie nei pazienti affetti da esofagite eosinofila. Queste manovre dovrebbero essere eseguite solo quando sono presenti stenosi esofagee ben riconoscibili, non transitabili dall'endoscopio, allo scopo di risolvere la disfagia acuta e di permettere l'esecuzione di biopsie diagnostiche. La dilatazione va inoltre eseguita con dilatatori tipo Savary (e filo guida) e non deve essere mai particolarmente "spinta" (calibro massimo consigliato Savary 11) per l'elevato rischio di provocare perforazioni esofagee.

Il **Panel** propone dilatazioni esofagee in caso di stenosi esofagee da EE ben riconoscibili, non transitabili dall'endoscopio, con dilatatori di tipo semirigido (Savary, previo filo guida); calibro massimo consigliato Savary 11 mm di diametro.

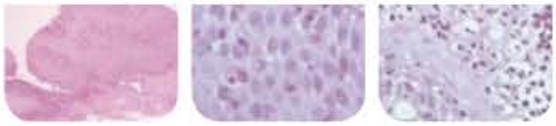
capitolo 9

La terapia farmacologica

Osvaldo Borrelli
UOC Gastroenterologia ed Epatologia pediatrica, Università degli Studi "La Sapienza" di Roma

Terapia Steroidea

I corticosteroidi inibiscono la sintesi e la secrezione di citochine che influenzano la crescita, la differenziazione e l'attivazione degli eosinofili.
I bambini affetti da EE non responsivi alla dieta di eliminazione, o che non possono tollerare i trattamenti dietetici, traggono beneficio dalla somministrazione per via sistemica o topica dei corticosteroidi.
Gli **steroidi sistemici** sono generalmente utilizzati nelle fasi di acuzie, mentre gli steroidi topici sono impiegati nel controllo a lungo termine della sintomatologia.
Liacouras et al [59] hanno trattato 21 bambini affetti da EE con terapia corticosteroidea orale per 1 mese, osservando una significativa risposta clinica già dopo una settimana.
In uno studio retrospettivo su 39 bambini affetti da EE lo stesso gruppo ha successivamente documentato l'efficacia degli steroidi sistemici nell'induzione della remissione sia clinica che istologica.
In corso di terapia con metilprednisolone (1.5 mg/kg/die; dose massima 40 mg) il numero degli eosinofili per HPF si riduceva da una media di 33.5 ± 9.5 a una media di 0.9 ± 0.6. Sei mesi dopo la sospensione della terapia il numero degli eosinofili ritornava ai valori documentati prima della terapia (28.7 ± 5.8).
Clinicamente, 27 dei 29 bambini con sintomi acido-correlati, e tutti i pazienti con disfagia, erano diventati asintomatici in corso di terapia, per ripresentare la stessa sintomatologia in 6 mesi dalla sospensione del trattamento.
In un tentativo di ridurre l'esposizione agli steroidi, l'uso dei **corticosteroidi per via topica** come alternativa al trattamento per l'EE è stato riportato per la prima volta da *Faubion et al* [61]. Il fluticasone dipropionato e il betametasone spray venivano somministrati per os con un dosatore ai pazienti con EE.
I soggetti non inalavano, ma deglutivano lo steroide, così da utilizzare l'azione antinfiammatoria topica sulla mucosa esofagea.
Da allora, molti altri studi (su oltre 70 bambini e adulti) hanno riportato trattamenti riusciti con steroidi topici, in particolare fluticasone dipropionato.
In uno studio retrospettivo su 20 bambini *Noel et al* [62] hanno documentato come quelli non-allergici avessero un'eccellente risposta al fluticasone dipropionato, a differenza dei pazienti in cui era identificabile un'allergia: questi ultimi, infatti, mostravano una parziale risposta nel 20% e nessuna risposta nel 20% dei casi.
Gli autori quindi suggerivano che quei pazienti con EE non responsivi alla terapia dietetica, sebbene in presenza di allergie identificabili, potessero presentare una riduzione della risposta al trattamento con fluticasone.
Questi dati differiscono da quelli riportati da *Teitelbaum et al* [63] in uno studio prospettico in cui tutti i pazienti trattati con fluticasone hanno presentato una risoluzione della sintomatologia, anche se precedentemente non responsivi alla dieta di eliminazione.
Recentemente, *Konikoff et al* [64] hanno valutato l'efficacia del fluticasone dipropionato in uno studio doppio-cieco, placebo-controllato. Trentasei bambini sono stati randomizzati a ricevere fluticasone dipropionato (800 µg/die, in 2 somministrazioni) o placebo,



per un periodo di 3 mesi. Il 50% (10/20) dei pazienti trattati con fluticasone ha mostrato una remissione istologica, rispetto al 9% (1/11) dei soggetti trattati con placebo, con una risposta maggiore nei pazienti non allergici.
Inoltre, la risoluzione della sintomatologia è stata riportata nel 67% degli individui in terapia con fluticasone, rispetto al 27% dei pazienti in terapia con placebo.
La terapia con fluticasone dipropionato è molto attraente, perché solo l'1% del farmaco viene assorbito per via sistemica e si sottopone a un rapido metabolismo epatico.
Il più importante effetto collaterale è l'infezione da Candida a livello orale e esofageo, trattata con successo con fluconazolo.
Inoltre, la deglutizione del fluticasone dipropionato sembra rappresentare un'importante alternativa alla terapia nutrizionale, poiché non prevede formule scarsamente palatabili, e non richiede un'alimentazione attraverso un sondino nasogastrico, mostrando - allo stesso tempo - efficacia e un buon profilo di sicurezza.
Come per i cortisonici sistemici, è importante evidenziare come, in tutti gli studi di *follow-up*, la sospensione della terapia con fluticasone dipropionato si associ alla ricomparsa in un periodo di 6 mesi sia della sintomatologia clinica sia della eosinofilia tissutale a livello della mucosa esofagea.
Paragonando l'efficacia della somministrazione sistemica di corticosteroidi (prednisone) a quella topica (fluticasone) in un trial pediatrico randomizzato che ha interessato 80 pazienti, *Schaefer et al* [69], nel 2008, hanno concluso che non vi siano differenze significative sulla risoluzione dei sintomi, sulla percentuale delle recidive cliniche e sul tempo delle stesse.
Recentemente è stato riportato l'utilizzo della budesonide topica in 2 bambini che non avevano tollerato la terapia con fluticasone dipropionato.
Entrambi i pazienti sono stati sottoposti a terapia con budesonide sciroppo (500 mg; 2 volte/die) miscelata con zuccheri in modo da ottenere una sospensione viscosa. Un paziente ha presentato una completa remissione clinica e istologica, mentre l'altro ha riportato un miglioramento clinico in assenza di una normalizzazione dell'eosinofilia tissutale.

Trattamenti potenziali

La documentazione emersa negli ultimi anni di un gruppo di pazienti non responsivi alla terapia steroidea ha spinto all'utilizzo di altre forme di terapia antinfiammatoria.
Attwood et al [66] hanno descritto l'utilizzo del montelukast nella terapia della EE.
Il montelukast è un inibitore selettivo dei recettori D4 dei leucotrieni ed è utilizzato con successo nella terapia dell'asma.
Attwood et al hanno trattato 8 pazienti adulti iniziando con un dosaggio di 10 mg/die, per incrementare la dose in base alla tolleranza individuale e al controllo della sintomatologia, fino a un massimo di 100 mg/die.
Dopo aver ottenuto il controllo clinico, il dosaggio è stato ridotto a un livello di mantenimento (compreso tra 20 e 40 mg/die).
Sette pazienti hanno mostrato una remissione clinica anche in assenza di una modificazione della eosinofilia tissutale.
Negli ultimi anni evidenze sperimentali hanno chiarito il ruolo chiave della IL5 nella patogenesi dell'EE, suggerendo un suo utilizzo come possibile target terapeutico.
In modelli murini, anticorpi monoclonali anti-IL5 hanno mostrato notevole efficacia nell'inibizione dello sviluppo di una EE sperimentale.

Nell'uomo il mepolizumab, anticorpo umanizzato anti-IL5, è stato utilizzato con successo in 4 pazienti con sindrome ipereosinofilica, suggerendo un sua potenziale efficacia nella EE. Studi su larga scala che prevedono l'utilizzo del mepolizumab sono attualmente in corso.
Infine, *Blanchard et al* [70] hanno documentato in un modello sperimentale murino le potenzialità terapeutiche di un anticorpo anti-IL13 nelle patologia infiammatoria aerea ed esofagea, correlata all'attività eosinofilica, suggerendo un suo potenziale utilizzo nel trattamento della EE nell'uomo.

Il **Panel** propone:

- corticosteroidi per via sistemica (prednisone o metilprednisolone 1.5 mg(kg/die in due somministrazioni per 2-4 settimane, poi a scalare 10 mg/settimana): per indurre la remissione clinica in caso di stenosi esofagee severe e grave flogosi a livello istologico
- corticosteroidi per via topica: fluticasone dipropionato in puff deglutiti (›30 kg 250 mcg per 2 volte/die; ‹30 kg 125 mcg per 2 volte/die) in caso di pazienti in cui non si sia identificata una chiara allergia alimentare, in soggetti positivi ad aero allergeni, in pazienti con test ed anamnesi negativa per allergie. Cicli di 3 - 6 mesi, ripetibili
- inibitori di LT per controllare i sintomi

capitolo 10

Esofagite eosinofila e chirurgi l'importanza di conoscere la patologia

Marco Brunero - Luigi Dall'Oglio*

Divisione di Chirurgia Pediatrica, Azienda Ospedaliera Maggiore della Carità di Novara
**UOC Chirurgia Endoscopia Digestiva, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma*

L'EE è ormai ufficialmente riconosciuta come patologia emergente, ancora sottostimata. È importante, anche per il chirurgo, conoscerne bene le caratteristiche per porre una diagnosi adeguata e non incorrere in errori terapeutici gravi: la patologia va pertanto adeguatamente affrontata in équipe con specialisti dedicati (gastroenterologo, pediatra, allergologo, dietista e chirurgo pediatra).

La diagnosi differenziale va posta attentamente con la MRGE, gastroenterite eosinofila primitiva, infezioni parassitarie, Malattia di Crohn e neoplasie. La diagnosi di EE si pone quando si è in presenza di un quadro clinico talvolta caratteristico, altre volte aspecifico, di biopsie esofagee con eosinofili ≥ a 15/HPF, pH-metria negativa (utile ove possibile impedenzometria per reflussi alcalini), scintigrafia con rallentato svuotamento gastrico e resistenza a un trattamento antiacido (IPP).

Il trattamento è *non chirurgico* e, come precedentemente descritto, si avvale di terapia cortisonica (per os e inalatoria) nonché dietetica (eliminazione di alimenti allergizzanti o dieta semielementare-elementare).

Lo spazio riservato al trattamento chirurgico è limitato solo a casi molto particolari in cui l'infiammazione locale da eosinofili si sovrappone ad altre patologie che ne complicano il decorso.

Sempre più evidente appare inoltre la necessità di individuare con chiarezza la *Sindrome da overlap*: EE e MRGE coesistenti, o MRGE con EE secondaria o anche EE con MRGE conseguente ▶ **Fig. 1**.

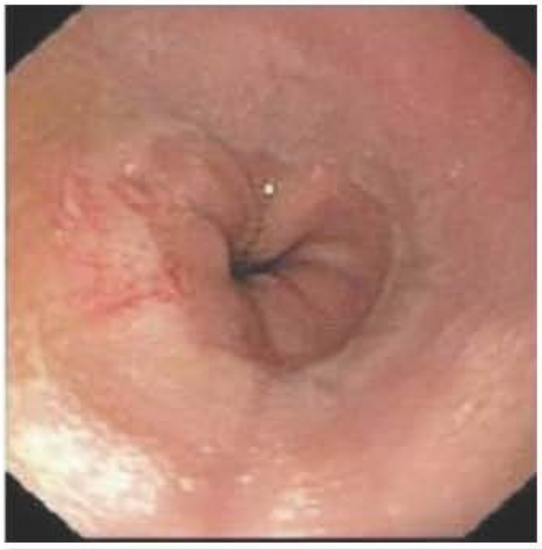


Fig. 1 *Quadro endoscopico overlap EE-MRGE*

Secondo *Liacouras et al* (1998) l'1% dei pazienti pediatrici con MRGE è affetto da EE; secondo *Fox VL et al* (2002), il 6% dei pazienti con esofagite è affetto da EE.

La più evidente difficoltà interpretativa è legata alla presenza contemporanea di EE con eosinofilia mucosale esofagea marcata, ben oltre il *cut off* individuato specifico dell'esofagite peptica, e MRGE concomitante con tutte le sue possibili complicanze.

In letteratura sono presenti varie segnalazioni in cui l'ipereosinofilia è associata a patologie di interesse chirurgico quali diverticolo esofageo, perforazione spontanea dell'esofago, anello di Schatzkis, acalasia esofagea.
L'infiammazione della parete esofagea con infiltrato eosinofilo massivo potrebbe comportare un rallentamento della *clearing* con un aumento del tempo di contatto tra l'acido gastrico e la mucosa esofagea, con incremento del reflusso acido. A volte l'incontinenza dello sfintere esofageo inferiore è primaria, con evidenza di ernia jatale da scivolamento: qualora il paziente fosse resistente al trattamento medico e sviluppasse complicanze gravi (esofago di Barrett, stenosi esofagea recidivante) un trattamento chirurgico del reflusso potrebbe essere risolutivo ▸ **Fig. 2 A-B-C-D**.

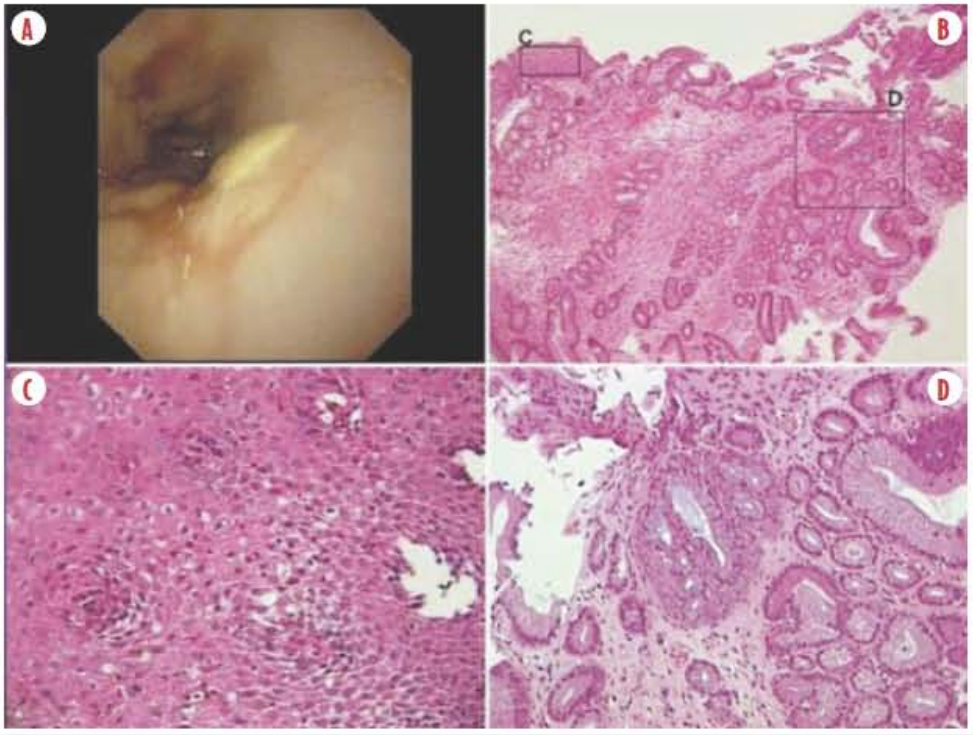


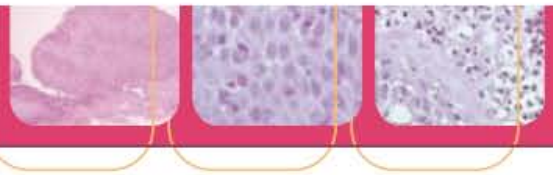
Fig. 2 A-B-C-D *Esofagite eosinofila ed esofago di Barrett*

Per gentile concessione di Paola Francalanci, Anatomia Patologica, Ospedale Pediatrico Bambino Gesù di Roma

Ogni caso, per la sua singolarità, va diligentemente valutato e discusso da un'équipe di specialisti dedicati; lo spazio riservato al trattamento chirurgico è sempre comunque molto ridotto, ma da considerare attentamente in casi selezionati.

Il **Panel** propone la plastica antireflusso a quei pazienti con EE con grave MRGE concomitante, che non migliora con il trattamento medico

25



Paola De Angelis
UOC Chirurgia Endoscopia Digestiva, Ospedale Pediatrico Bambino Gesù, IRCCS di Roma

In conclusione, appare ormai evidente che l'EE rappresenti una patologia complessa, la cui diagnosi non è solo istologica, ma si avvale di valutazioni globali cliniche e strumentali e necessita di essere rivisitata durante tutto il *follow-up*, in funzione delle eterogenee risposte individuali ai trattamenti effettuati. Sintetizzando le proposte del Panel, si pone indicazione all'esecuzione di **EGDS con biopsie multiple** nei pazienti che presentino caratteristiche cliniche sospette di EE; il quadro endoscopico suggestivo di EE comprende friabilità, strie longitudinali, placche biancastre, esofago ad anelli.

Le biopsie devono essere effettuate **a livello di tutto l'esofago** per la diagnosi istologica e per l'esclusione di esofagite peptica, infettiva o autoimmune e **a livello gastroduodenale** per escludere la gastroenterite eosinofila.

Effettuata la diagnosi clinica e istologica di EE - e inquadrato il paziente dal punto di vista anamnestico (familiarità o anamnesi personale immunoallergologica) anche al fine di escludere una MRGE primitiva - è possibile effettuare un ciclo di terapia con IPP per 8 settimane, verificandone la risposta, *Furuta GT et al* [3], oppure una **pH-metria** delle 24 ore (meglio, ormai, la **pH-impedenzometria**). Contemporaneamente, il paziente deve essere introdotto nell'**iter diagnostico allergologico**, per l'identificazione di un possibile allergene implicato (IgE sieriche, eosinofilia periferica; prick test per aeroallergeni e trofoallergeni con estratti allergenici; prick by prick con l'alimento fresco; dosaggio IgE specifiche verso gli allergeni risultati positivi al prick o, se il paziente è in terapia antistaminica; patch test con estratti freschi verso aereo e trofoallergeni).

Se viene identificato l'allergene alimentare presumibilmente in causa si può iniziare una **dieta di esclusione** mirata per 3 mesi, in pazienti con meno di 10 allergeni identificati; nei soggetti con positività superiore ai 10 allergeni, e quando non è stato identificato l'allergene nel paziente con grave malnutrizione o disfagia, può essere proposta una **dieta elementare** per 8 settimane, con successiva reintroduzione graduale di alimenti, seguita da controlli endoscopici seriati per mettere in evidenza eventuali alterazioni esofagee macroscopiche e istologiche. Una **dieta libera** può rimanere un'alternativa nell'ipotesi che non sia implicato nell'etiopatogenesi un trofoallergene, bensì un aeroallergene.

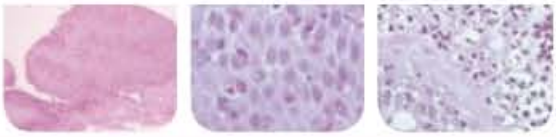
La **dietoterapia** nell'EE è considerata dalla maggioranza degli studiosi un presidio efficace in molti pazienti pediatrici: decidendo di proporre una dieta specifica, bisogna però sempre confrontarsi con la qualità di vita del bambino e della sua famiglia.

La **terapia farmacologica** per l'EE comprende numerose opzioni, da scegliere in base all'andamento clinico, alle alterazioni anatomiche e al grado di interessamento flogistico istologico.

Gli **IPP** vengono usati in fase diagnostica (resistenza a PPI: sospetta EE), in caso di concomitante GERD secondario alla dismotilità causata dalla EE (infiltrazione eosinofila coinvolgente mucosa, sottomucosa fino alla tonaca muscolare) o in corso di corticoterapia sistemica.

I **corticosteroidi** migliorano significativamente la sintomatologia e l'eosinofilia mucosale esofagea, con recidive a distanza dalla sospensione farmacologica.

Gli steroidi **sistemici**, nonostante gli effetti collaterali, conservano un ruolo nell'emergenza: nei pazienti con una disfagia grave, malnutrizione severa, impossibilità ad assumere alimenti per os, scialorrea, intensa flogosi esofagea con stenosi serrate: si consiglia alla diagnosi un ciclo di 2-4 settimane di prednisone o metilprednisolone.



I corticosteroidi **topici** (fluticasone dipropionato spray deglutito, budesonide sospensione orale viscosa) sono stati introdotti nella terapia di mantenimento con interessanti risultati per l'efficacia clinica e istologica, per i limitati effetti collaterali (sovrainfezioni micotiche a livello oro-esofageo, tipo *Candida*), anche se il beneficio tende a scomparire dopo la sospensione.
Allo scopo di alleviare i sintomi senza modificare l'eosinofilia mucosa, nella sintomatologia disfagica persistente sono stati proposti gli inibitori selettivi del recettore D4 dei leucotrieni (**montelukast** 10mg/die la sera).
La terapia biologica ha già offerto promettenti risultati, ma deve essere ancora approvata per protocolli definitivi nell'EE. L'interleuchina 5 (IL-5) è una citochina regolatoria di vari processi associati alla presenza di eosinofili, implicata nella patogenesi dell'EE sperimentale nei topi, al centro dell'interesse scientifico per la realizzazione della terapia con mepolizumab (anticorpi umanizzati contro l'IL-5), ancora in fase di verifica per la terapia iniziale e di mantenimento dell'EE.
Nel *follow-up* il paziente prosegue la dieta di esclusione dell'allergene o agli allergeni responsabili della flogosi, quando identificati. In caso di assenza di presunta causa, rimane utile la programmazione di cicli di fluticasone dipropionato, in puff deglutiti, da associare ad anti-leucotrieni per il controllo dei sintomi.
L'**endoscopia** con biopsie esofagee multiple rappresenta l'indagine diagnostica più utile per verificare la reale remissione della malattia. Non è sempre considerata indispensabile, soprattutto nel paziente asintomatico, salvo durante le fasi di riacutizzazione clinica o nel *follow-up* a lungo termine, per mettere in evidenza eventuali alterazioni causate dalla flogosi cronica. È invece sicuramente importante che venga effettuata un'endoscopia dopo 8 settimane di dieta elementare e dopo 3 mesi di dieta di esclusione/oligoantigenica, per pianificare i successivi passi terapeutici. Se si evidenzia un'istologia negativa per abnorme infiltrato eosinofilo, è possibile una reintroduzione degli alimenti, ripetendo l'EGDS 4 settimane dopo la reintroduzione dell'ultimo, considerando attentamente la sintomatologia clinica. Se l'istologia risulta ancora positiva per infiltrazione eosinofila si dovrebbe proporre nuovamente una dieta elementare per altre 8 settimane, reintroducendo successivamente gli alimenti in base all'andamento clinico.
Le **dilatazioni esofagee**, considerate molto rischiose data la fragilità della parete esofagea affetta dalla malattia, devono essere ormai programmate soltanto per permettere l'esecuzione, in caso di stenosi esofagea serrata, di prelievi bioptici e quindi per formulare una diagnosi istologica, oltre che per risolvere la disfagia acuta e consentire il passaggio, attraverso l'esofago, di saliva e alimenti liquidi prima di iniziare la terapia corticosteroidea di attacco.
L'EE è una malattia cronica che necessita di terapie di lunga durata, che devono essere adattate al singolo paziente in base al quadro clinico, alle condizioni generali e nutrizionali, alla *compliance* personale e familiare e anche agli aspetti psicologici rilevati.
Dieta e **farmaci antinfiammatori** sono al momento i presidi terapeutici più utilizzati, pur non esistendo alcun trial randomizzato controllato che comprovi la maggior efficacia dell'uno rispetto all'altro trattamento. Il ruolo primario della terapia rivolta verso questa patologia rimane l'approccio nei confronti delle **acuzie** e la prevenzione delle **complicanze**, oltre che il mantenimento della remissione clinica. È sempre opportuno definire, durante tutto il decorso della malattia, non soltanto in fase diagnostica, l'entità della **MRGE** coesistente, pre-esistente o conseguente l'EE, per poterla fronteggiare anche, se necessario, con la **chirurgia**.

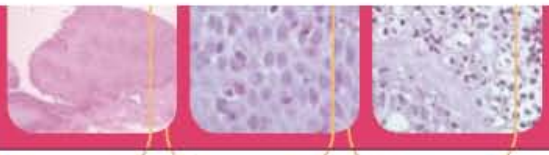
Tab. I Quesiti irrisolti

- Incidenza/prevalenza in Italia
- Etiologia e patogenesi
- Eterogeneità delle manifestazioni cliniche endoscopiche (distinti meccanismi fisiopatologici o diversi stadi della stessa patologia?)
- Correlazione con la malattia da reflusso gastroesofageo (due entità indipendenti o correlate?)
- Metodo di scelta per l'identificazione di allergeni implicati
- Biomarkers non invasivi attendibili di attività
- Prognosi
- Gestione dei pazienti asintomatici con eosinofilia mucosale persistente
- Eticità del *challenge* orale durante il *follow-up*
- Terapia a lungo termine, di mantenimento
- Eosinofilia mucosale legata alla MRGE

La nostra attenzione è diretta verso l'osservazione della storia naturale di cui si conosce troppo poco per trarre conclusioni definitive. In particolare non è noto quanto, nel paziente asintomatico, la flogosi cronica eosinofila persistente possa predisporre a quadri pre-neoplastici. Non è a tal punto possibile definire i tempi esatti del monitoraggio endoscopico-istologico e la sua utilità. La ricerca futura dovrebbe essere focalizzata sulla prevalenza della malattia, sugli esiti a lungo termine e sul migliore approccio diagnostico e terapeutico.

L'EE, oggi sempre meglio diagnosticata e nota nonostante la persistenza di numerosi quesiti irrisolti ▸ **Tab. I**, richiama numerose professionalità mediche - allergologi, gastroenterologi, patologi, internisti, pediatri, otorinolaringoiatri e chirurghi - che dovrebbero essere coinvolte nell'obiettivo comune di raggiungere la gestione multidisciplinare più completa del paziente affetto ▸ **Fig. I**.

Fig. I *Reclutamento dei pazienti da introdurre nella diagnostica per EE*



bibliografia

Introduzione

1 **Markowitz JE, Liacouras CA.** Eosinophilic esophagitis. Gastroenterol Clin N Am 2003;32:949-966

2 **Noel RJ, Tipnis NA.** Evidence-based guidelines for the management of EE are not currently available. Int J Pediatr Otorhinolaryngol 2006 Jul;70(7):1147-53

3 **Furuta GT, Liacouras CA, Collins MH et al.** Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. Gastroenterology 2007;133:1342-1363

4 **Liacouras CA, Spergel JM, Ruchelli E et al.** Eosinophilic esophagitis: a 10-year experience in 381 children. Clin Gastroenterol Hepatol 2005;3:1198-1206

5 **De Angelis P, Markowitz JE, Torroni F et al.** Pediatric eosinophilic esophagitis: towards early diagnosis and best treatment. Digestive and Liver Disease 2006;38:245-51

6 **Sant'Anna A, Rolland S, Fourne JC et al.** Eosinophilic esophagitis in children: symptoms, histology and pH probe results. J Pediatr Gastroenterol Nutr 2004;39:373-377

7 **Gupte AR, Draganov PV.** Eosinophilic esophagitis. World J Gastroenterol 2009 Jan;7;15(1):17-24

Epidemiologia e quadri clinici

8 **Rothenberg ME, Mishra A, Collins MH, Putnam PE.** Pathogenesis and clinical features of eosinophilic esophagitis. J Allergy Clin Immunol 2001; 108:891-89

9 **Winter HS, Madara JL, Stafford RJ et al.** Intraepithelial eosinophils: a new diagnostic criterion for reflux esophagitis. Gastroenterology 1982;83:818-823

10 **Noel RJ, Putnam PE, Rothenberg ME.** Eosinophilic esophagitis. N Engl J Med 2004;351:940-1

11 **Rothenberg ME.** Eosinophilic gastrointestinal disorders (EGID). J Allergy Clin Immunol 2004; 113:11-28

12 **Mishra A, Hogan S, Brandt E, Rothenberg ME.** An etiological role for aeroallergens and eosinophils in experimental esophagitis. J Clin Invest 2001;107:83-90

13 **Mishra A, Rothenberg ME.** Intratracheal IL-13 induces eosinophilic esophagitis by an Il-5, eotaxin-1, and STAT6-dependent mechanism. Gastroenterology 2003;125:1419-1427

14 **Schmid-Grendelmeier P, Altznauer F, Fischer B et al.** Eosinophils express functional IL-13 in eosinophilic inflammatory disease. J Immunol 2002;169:1021-1027

15 **Liacouras CA.** Eosinophilic esophagitis in children and adults. J Pediatr Gastroenterol Nutr 2003 Nov-Dec;37 Suppl 1:S23-8

16 **Orenstein SR, Shalaby TM, Di Lorenzo C et al.** The spectrum of pediatric eosinophilic esophagitis beyond infancy: a clinical series of 30 children. Am J Gastroenterol 2000;95:1422-30

17 **Cheung KM, Oliver MR, Cameron DJ et al.** Esophageal eosinophilia in children with dysphagia. J Pediatr Gastroenterol Nutr 2003;37:498-503

18 **Khan S, Orenstein SR, Di Lorenzo C et al.** Eosinophilic esophagitis: strictures, impactions, dysphagia. Dig Dis Sci 2003;48:22-29

19 **Fox VL, Nurko S, Teitelbaum JE et al.** High-resolution EUS in children with eosinophilic "allergic" esophagitis. Gastrointestinal Endosc 2003;57:30-36

20 **Vanderheyden AD, Petras RE, DeYoung BR, Mitros FA.** Emerging eosinophilic (allergic) esophagitis: increased incidence or increased recognition? Arch Pathol Lab Med 2007 May;131(5):777-9

21 **Prasad GA, Talley NJ, Romero Y et al.** Prevalence and predictive factors of eosinophilic esophagitis in patients presenting with dysphagia: a prospective study. Am J Gastroenterol 2007 Dec;102(12):2627-32

Diagnosi differenziale

22 **De Angelis P, Morino G, Pane A et al.** Eosinophilic esophagitis: management and pharmacotherapy. Expert Opin Pharmacother 2008 Apr;9(5):731-40 Review

L'endoscopia

23 **Fox VL, Nurko S et al.** Eosinophilic esophagitis: it's not just kid's stuff. Gastrointest Endosc 2002;56(2):260-70

24 **N.S. Mann, J.W. Leung.** Pathogenesis of esophageal rings in eosinophilic esophagitis, Medical Hypotheses 2005;64,520-3

25 **Aumed A.** A novel endoscopic appearance of idiopathic eosinophilic esophagitis. Endoscopy 2000;32(6): S33

26 **Gonsalves N, Policarpio-Nicolas M, Zhang Q et al.** Histopathologic variability and endoscopic correlates in adults with eosinophilic esophagitis. Gastrointest Endosc 2006;64:313-319

L'istologia

27 **Dahms BB.** Reflux esophagitis: sequelae and differential diagnosis in infants and children including eosinophilic esophagitis. Pediatr Dev Pathol 2004;7(1):5-16

28 **Ngo P, Furuta GT, Antonioli DA et al.** Eosinophils in the esophagus-peptic or allergic eosinophilic esophagitis? Case series of three patients with esophageal eosinophilia. Am J Gastroent 2006;101:1666-70

29 **Steiner SJ, Kernek KM, Fitzgerald JF.** Severity of basal cell hyperplasia differs in reflux versus eosinophilic esophagitis. J Pediatr Gastroenterol Nutr 2006 May;42(5):506-509

30 **Lee RG.** Marked eosinophilia in esophageal mucosal biopsies. Am J Surg Pathol 1985;9:475-479

31 **Walsh SV, Antonioli DA, Goldman H et al.** Allergic esophagitis in children: a clinicopathological entity. Am J Surg Pathol 1999;23:390-396

32 **Parfitt JR, Gregor JC, Suskin NG et al.** Eosinophilic esophagitis in adults: distinguishing features from gastroesophageal reflux disease. A study of 41 patients. Modern Pathology 2006;19:00 06

33 **Rodrigo S, Abboud G, Oh D et al.** High intraepithelial eosinophil counts in esophageal squamous epithelium are not specific for eosinophilic esophagitis in adults. Am J Gastroenterol 2008 Feb;103(2):435-42

Esofagite eosinofila, reflusso gastroesofageo e pH-metria: quale correlazione?

34 **Winter HS, Madara JL, Stafford RJ et al.** Intraepithelial eosiniphils: a new diagnostic criterion for reflux esophagitis. Gastroenterology 1982;83:818-823

35 **Landres RT, Kuster GG, Strum WB.** Eosinophilic esophagitis in a patient with vigorous achalasia. Gastroenterology 1978;74:1298-1301

36 **Walsh SV, Antonioli DA, Goldman H et al.** Allergic esophagitis in children. Am J Surg Pathol 1999;23:390-396

37 **Steiner SJ, Gupta SK, Croffie JM et al.** Correlation between number of eosinophils and Reflux Index on same day esophageal biopsy and 24 hour esophageal pH monitoring. Am J Gastroenterol 2004;801-805

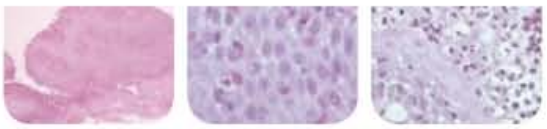
38 **Lim JR, Gupta SK, Croffie JM, Pfefferkorn MD, Molleston JP et al.** White specks in the esophageal mucosa: an endoscopic manifestation of non-reflux eosinophilic esophagitis in children. Gastrointest Endoscop 2004;59(7):835-8

39 **Sant'Anna AM, Rolland S, Fournet JC et al.** Eosinophilic esophagitis in children: symptoms, histology and pH probe results. JPGN 2004;39:373-377

40 **Remedios M, Campbell C, Jones DM et al.** Eosinophilic esophagitis in adults: clinical, endoscopic, histologic findings and response to treatment with fluticasone propionate. Gastrointest Endosc 2006;63:3-12

41 **Rosen R, Furuta GT et al.** Role of acid and nonacid reflux in children with eosinophilic esophagitis compared with patients with gastroesophageal reflux and control patients. JPGN 2008;46:520-523

bibliografia



L'iter diagnostico allergologico-autoimmunitario

42 **Spergel JM, Beausoleil JL, Mascarenhas M, Liacouras CA.** The use of skin prick test and patch tests to identify causative foods in eosinophilic esophagitis. J Allergy Clin Immunol 2002;109:363-368

43 **Spergel JM, Andrews T, Brown-Whitehorn TF et al.** Treatment of eosinophilic esophagitis with specific food elimination diet directed by a combination of skin prick and patch tests. Ann Allergy Asthma Immunol 2005;95:336-343

44 **Markowitz JE, Spergel JM, Ruchelli E, Liacouras CA.** Elemental diet is an effective treatment for eosinophilic esophagitis in children and adolescents. Am J Gastroenterol 2003;98:777-782

La dietoterapia

45 **Assa'ad AH, Putnam PE, Collins MH et al.** Pediatric patients with eosinophilic esophagitis: An 8-year follow-up. J Allergy Clin Immunol 2007

46 **Ferguson DD, Foxx-Orenstein E.** Eosinophilic esophagitis: an update. Diseases of the esophagus 2007;20:2-8

47 **Furuta GT, Straumann et al.** Review article: the pathogenesis and management of eosinophilic esophagitis. Aliment Pharmacol Ther 2006;24:173-82

48 **Kagalwalla AF, Sentongo TA, Ritz S et al.** Effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. Clin Gastroenterol Hepatol 2006 Sep;4(9):1097-102

49 **Liacouras C.A.** Eosinophilic esophagitis: treatment in 2005. Curr Opin Gastroenterology 2006;22:147-152

50 **Markowitz JE, Spergel JM, Ruchelli E, Liacouras CA.** Elemental diet is an effective treatment for eosinophilic esophagitis in children and adolescents. Am J Gastroenterol 2003 Apr;98(4):777-82

51 **Spergel JM, Andrews T, Brown-Whitehorn TF et al.** Treatment of eosinophilic esophagitis with specific food elimination diet directed by a combination of skin prick and patch tests. Ann Allergy Asthma Immunol 2005 Oct;95(4):336-43

52 **Spergel JM, Brown-Whitehorn T, Beausoleil JL et al.** Predictive values for skin prick test and atopy patch test for eosinophilic esophagitis. J Allergy Clin Immunol 2007 Feb;119(2):509-11

Il ruolo delle dilatazioni esofagee

53 **Langdon DE.** Congenital esophageal stenosis, corrugated ringed esophagus, and eosinophilic esophagitis. Am J Gastroenterol 2000;95:1572-75

54 **Vasilopoulos S.** The small-caliber esophagus: an unappreciated cause of dysphagia for solids in patients with eosinophilic esophagitis. Gastrointestinal Endoscopy 2002;55:99-106

55 **Fox V.L.** High-resolution EUS in children with eosinophilic "allergic" esophagitis. Gastrointestinal Endoscopy 2003;57 (1):30-36

56 **Potter JW.** Eosinophilic esophagitis in adults: an emerging problem with unique esophageal features. Gastrointestinal Endoscopy 2004;59:355-61

57 **Kaplan M.** Endoscopy in eosinophilic esophagitis: "feline" esophagus and perforation risk. Clin Gastroenterology and Hepatology 2003;1:433-437

58 **Liacouras CA.** Eosinophilic esophagitis: treatment in 2005. Current Opinion in Gastroenterology 2006;22:147-52

La terapia farmacologica

59 **Liacouras CA, Wenner WJ, Brown K et al.** Primary eosinophilic esophagitis in children: successful treatment with oral corticosteroids. J Pediatr Gastroenterol Nutr 1998;26:380-385

60 **Liacouras CA, Spergel JM, Ruchelli E et al.** Eosinophilic esophagitis: a 10-years experience in 381 children. Clin Gastroenterol Hepatol 2005;3:1198-206

61 **Faubion W, Perrault J, Burgart L et al.** Treatment of eosinophilic esophagitis with inhaled corticosteroids. J Pediatr Gastroenterol Nutr 1998;27:90-93

62 **Noel RJ, Putnam PE, Collins MH et al.** Clinical and immunopathologic effects of swallowed fluticasone for eosinophilic esophagitis. Clin Gastroenterol Hepatol 2004;2:568-575

63 **Teitelbaum et al.** Eosinophilic esophagitis in children: immunopathological analysis and response to fluticasone propionate. Gastroenterol 2002;122:1216-1225

64 **Konikoff MR, Noel RJ, Blanchard C et al.** A randomized, double-blind placebo controlled trial of fluticasone propionate for pediatric eosinofilic esophagitis. Gastroenterol 2006;131:1381-1391

65 **Aceves SS, Dohil R, Newbury RO et al.** Topical viscous budesonide suspension for treatment of eosinophilic esophatitis. J Allergy Clin Immunol 2005;116:705-706

66 **Attwood SEA, Lewis CJ, Bronder CS et al.** Montelukast eosinophilic esophagitis: a novel treatment using. Gut 2003;52;181-185

67 **Garrett JK, Jameson SC, Thomson B et al.** Anti–interleukin-5 (mepolizumab) therapy for hypereosinophilic syndromes. J Allergy Clin Immunol 2004;113:115-9

68 **Blanchard C, Mishra A, Saito-Akei H et al.** Inhibition of human interleukin-13-induced respiratory and esophageal inflammation by anti-human-interleukin-13 antibody (CAT-354). Clin Exp Allergy 2005;35:1096-1103

69 **Schaefer ET, Fitzgerald JF, Molleston JP et al.** Comparison of oral prednisone and topical fluticasone in the treatment of eosinophilic esophagitis: a randomized trial in children. Clin Gastroenterol Hepatol 2008;6(2):165-73

Esofagite eosinofila e chirurgia: l'importanza di conoscere la patologia

70 **Furuta GT, Straumann A.** Review article: the pathogenesis and management of eosinophilic esophagitis. Alimentary Pharmacology and therapeutics 2006;24(2),173-182

71 **Meckelemburg I, Weber C, Folwaczny C.** Spontaneous recovery of disphagia by roptur of an esophageal diverticulum in eosinophilic esophagitis. Dig.Dis.Sci 2006 Jul;51(7):1241-2

72 **Prasad GA, Arora AS.** Spontaneous perforation in the ringed esophagus. Dis. Esophagus 2005;18(6):406-9

73 **Sgouros SN, Bengele C, Mantides A.** Schatzki's rings are not associated with eosinophilic esophagitis. Gastrointest Endosc 2006 Mar;63(3):535-6

74 **Segundo GR**. Esophageal achalasia and eosinophilic esophagitis. J Pediatr (Rio J) 2005Mar-Apr;81-2:185-6

75 **Ngo P, Furuta GT, Antonioli DA, Fox VL.** Eosinophils in the esophagus - peptic or allergic eosinophilic esophagitis? Case series of three patients with esophageal eosinophilia. Am J Gastroenterol 2006 Jul;101 (7):1666-70

76 **Molina-Infante J, Ferrando-Lamana L, Mateos-Rodríguez JM et al.** Overlap of reflux and eosinophilic esophagitis in two patients requiring different therapies: a review of the literature. World J Gastroenterol 2008 Mar 7;14 (9):1463-6

77 **Francalanci P, De Angelis P, Minnei F et al.** Eosinophilic esophagitis and Barrett's esophagus: an occasional association or an overlap disease? Esophageal 'double trouble' in two children. Digestion 2008;77(1):16-9. Epub 2008 Feb 4

LIST OF PAPERS AND ABSTRACTS PUBLISHED DURING THE YEARS 2006-2009 ON DIFFERENT RESEARCH LINES.

A. PAPERS

1. **Quaglietta L**, Strisciuglio C, Staiano A. Gastrointestinal Problems and Nutrition in Neurologically Impaired Children: Guest Editorial. *Int Semin Pediatr Gastroenterol Nutr* 2007;14:1-3.

2. R. Mastroianni, **L. Quaglietta**, E. Miele, D. Simeone, A. Capobianco, A. Tramontano' A. Staiano. Respiratory and Esophageal Function after Operation for Esophageal Atresia. *Ital J Ped*, 2007; 33: 330-335

B. ABSTRACTS

1. M. Galatola, S. Borriello, M. De Rosa, **L.Quaglietta**, A.Staiano, P.Izzo. Identificazione di tre nuove mutazioni del gene LKB1/STK11 associate all'insorgenza di sindrome di Peuts-Jeghers. *Atti delle Giornate Scientifiche della Facoltà di Medicina e Chirurgia,* Napoli-20-21 Settembre 2007; 58

2. M. De Rosa, S. Borriello, **L. Quaglietta**, M. Galatola, A. Staiano, P. Izzo. Una Nuova Mutazione nel gene STK11 è associata all'insorgenza della sindrome di PEUTZ-JEGHERS in una famiglia campana. 63° Congresso Nazionale della Società Italiana di Pediatria (SIP), 26-29 SETTEMBRE 2007, Pisa

3. R. Auricchio, E. Miele, D. Melis, **L. Quaglietta**, Strisciuglio C, Del Mastro A, G. Sebastio, A. Staiano. Clinical and Genetic Characterization of Syndromic Form of Hirschsprung's Disease in Chidren. 4th European Paediaric GI Motility Meeting, 4-6 Ottobre 2007, Londra

4. G. De Simone, M.R. D'Amico, M.G. Scala, **L . Quaglietta**, L. D'Alessandro, M. Ripaldi. G-CSF in children receving allogenic bone marrow transplantation. *Bone Marrow Transplantation* 2008; 41(1): R1319

5. **Quaglietta L**, Fecarotta S, Verrico A, Astarita L, Capasso M, Zanotta G, Cinalli G, Migliorati R, Fiorillo A. Carcinoma dei plessi corioidei: approccio combinato nell'induzione della remissione. *Haematologica* 2008; 93 (S4): P008

6. **Quaglietta L**, Quitadamo P, Masi P, Coccorullo P, Rocco A, Staiano A. delayed gastric emptying in children with post-infectious functional GI disorders (FGIDs). 42nd Annual meeting of the *European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), Budapest June 3-6 2009.*