THE UNIVERSITY OF CALGARY

Crohn's Disease: Investigation of Intestinal Permeability Across Families of Affected Children

by

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Abstract

A subset of first-degree relatives of adults with Crohn's disease have an increased baseline intestinal permeability and/or an exaggerated increase in intestinal permeability after the administration of aspirin. The purpose of this study was to determine intestinal permeability using the lactulose/mannitol (LM) ratio in unaffected first-degree relatives of children with Crohn's disease before and after the administration of ibuprofen. Thirty-six healthy first-degree relatives (from 14 families) and 41 controls (from 14 families) were recruited. Healthy relatives had a baseline LM ratio similar to the control group. Seven relatives (20 %) showed an exaggerated response in LM ratio to ibuprofen. In families with multiple affected relatives there was generally concordance in abnormalities of intestinal permeability. In conclusion a subset of first-degree relatives of children with Crohn's disease have an exaggerated increase in intestinal permeability to ibuprofen. These findings are compatible with a genetic link between abnormalities of intestinal permeability and Crohn's disease.

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List of abbreviations

Å	Angstroms
AD	Adults
5-ASA	5-Aminosalicylate
AZA	azathioprine
CD	Crohn's Disease
CH	Children
95% CI	95% Confidence Interval
[^{51}Cr] EDTA	^{51}Cr -labeled ethylenediaminetetraacetic acid
IBD	Inflammatory bowel disease
Lac	Lactulose
LM	Lactulose-Mannitol
LR	Lactulose-Rhamnose
Man	Mannitol
NSAID	Nonsteroidal anti-inflammatory drug
PEG	Polyethylene glycol
SD	Standard deviation
SEM	Standard error of the mean
UC	Ulcerative colitis

1. Introduction

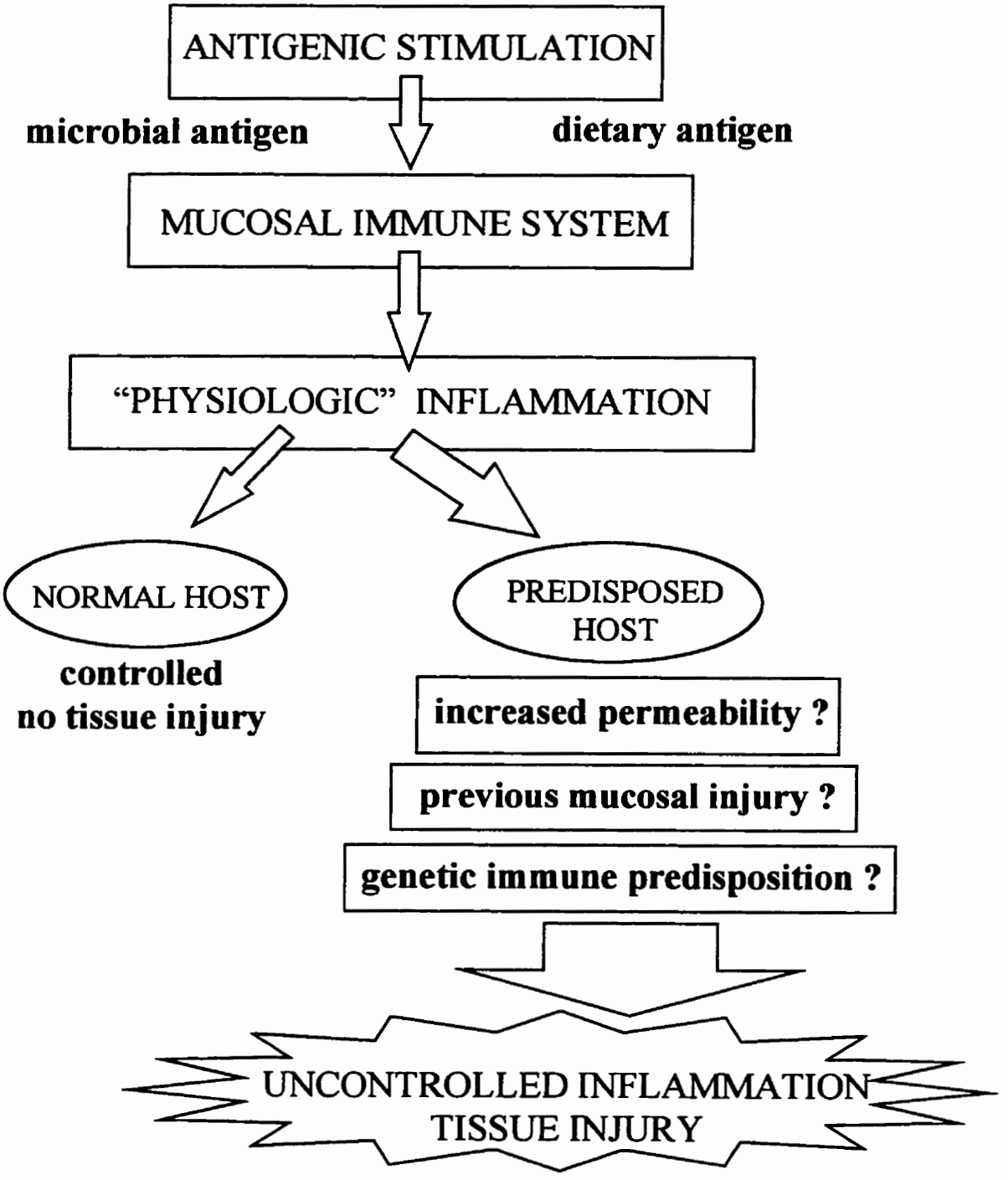
A. Crohn's disease

Crohn's disease and ulcerative colitis are the two most common chronic inflammatory diseases of the bowel. Crohn's disease was first described in 1932 as a pathologic and clinical entity (1). In Crohn's disease the intestinal inflammation is transmural and can affect any segment of the bowel, most frequently the terminal ileum and the colon. Clinically the disease is characterized by recurrent episodes of abdominal pain and diarrhea. Anorexia, vomiting and fever may be associated. One presentation unique to the pediatric population is growth failure or delayed puberty, which can be found in 20 to 40% of cases (2).

The disease has a bimodal age distribution (3). In the majority of affected individuals the disease presents between 15 and 25 years of age. A second peak occurs between the ages of 50 and 80. Women have a slightly higher risk of developing the disease (4). The incidence of Crohn's varies between 0.8 and 11.6 per 100 000 (5,6) and the prevalence between 34 and 106 per 100 000 (7,8). The incidence increased markedly between 1955 and 1975, but current rates have stabilized (7). There is a striking geographical distribution of the disease with a north to south gradient. The disease is most common in North and Western Europe and in North America (3,9,10). In northern Alberta the incidence of Crohn's disease is 10 per 100 000 and the prevalence 44 per 100 000. The prevalence of Crohn's disease is higher in urban than in rural areas and in females than in males (11).

The etiology of Crohn's disease remains unknown. Both genetic and environmental factors contribute to the disease. A genetically determined susceptibility to immune-mediated bowel injury triggered by environmental factors seems to result in the disease (12). An antigenic stimulus, either of microbial or dietary origin, stimulates the immune system of the intestinal mucosa. In a normal host mucosal inflammation occurs in a controlled and protective fashion ("physiologic inflammation") and is self-limited. In a predisposed host, the inflammation cascade is not self-limited, and the continuous production of inflammatory mediators by activated immune cells leads to tissue injury and fibrosis. A genetically determined, defective down-regulation of inflammation may predispose to continuous activation of the intestinal immune system and chronic inflammation. Other factors, which may predispose to uncontrolled inflammation are previous mucosal injuries and/or increased intestinal permeability (Figure 1).

Figure 1
Pathogenesis of Crohn's disease



There are several findings supporting a genetic contribution to the disease (Table 1). Familial aggregation of inflammatory bowel diseases (Crohn's disease and/or ulcerative colitis) is recognized, with a 5 to 20 % likelihood of finding inflammatory bowel disease in relatives of a proband. In the adult population this can be contrasted with a low prevalence in spouses sharing similar environmental factors. The risk of developing Crohn's disease is increased among relatives of affected patients (up to 10 - 35 times increased risk) and having a first-degree relative with Crohn's disease is the strongest risk factor for developing the disease (13-17). A recent survey determining familial empirical risks for inflammatory bowel disease estimated that between 5 and 8 % of first-degree relatives of affected patients will develop Crohn's disease over their lifetime (18).

Familial aggregation of Crohn's disease has been associated with a lower age of onset (19-22) and with clinical characteristics of Crohn's disease (concordance for location and transmural aggressiveness among affected family members) (21,23). Studies of affected parent-child pairs have shown that parents are between 5 and 15 years older than children at onset (20,24,25). These results are compatible with genetic anticipation, which is the tendency for successive generations to develop disease of increasing severity and earlier onset. The molecular basis for genetic anticipation involves the progressive amplification of unstable triplet repeats of DNA.

Studies of monozygotic and dizygotic pairs of twins with an affected proband show high rates of concordance (20 - 58%), especially among monozygotic twins (26,27). The disease is more frequent among whites than non whites. Certain ethnic groups, particularly Ashkenazi Jews have a greater incidence of Crohn's disease (18,28). The

prevalence of inflammatory bowel disease in first-degree relatives of Jewish children with Crohn's disease is particularly high (30 %) (29).

The association of inflammatory bowel disease with other genetically determined conditions (e.g. Turner syndrome) indirectly supports a genetic predisposition (30).

Finally several subclinical markers that may indicate a genetic abnormality predisposing to inflammatory bowel disease have been identified in affected patients and their first-degree relatives. Among these are abnormal production of antibodies against gut epithelial cells (31), phenotypic alteration of circulating B cells (32,33), complement dysfunction (34) and abnormalities of intestinal permeability.

The mode of inheritance of Crohn's disease remains undetermined. Crohn's disease and ulcerative colitis likely represent a number of related polygenic disorders. Segregation analysis has found evidence for a recessive model of transmission in Crohn's disease (35,36). In contrast to ulcerative colitis, the major histocompatibility complex does not seem to contribute to disease susceptibility in Crohn's disease (37,38). Cytokine gene (interleukin-1 receptor antagonist gene, tumor necrosis factor alpha gene) polymorphisms are also unlikely to be important determinants of disease susceptibility (39). Systematic screening of the entire genome in multiply-affected families has identified a putative Crohn's disease susceptibility locus on chromosome 16 (IBD1 locus) (40). This has been confirmed in relative pairs with Crohn's disease by genotyping the region of IBD1 using short tandem repeat markers (41). The IBD1 locus was also found to be associated with Crohn's disease in a case-control study (42).

Table 1

Inflammatory bowel disease

Evidence supporting a genetic contribution

Increased familial aggregation (particularly in Crohn's disease)
Low prevalence in spouses
Clinical concordance of the disease within affected family members
Higher concordance rate in monozygotic twins
Increased prevalence in certain ethnic groups
Association with other genetically determined diseases
Subclinical genetic markers:
Colonic glycoprotein composition (ulcerative colitis)
Antineutrophil cytoplasmic antibodies (ulcerative colitis)
Phenotypic alteration of circulating B lymphocytes
Complement dysfunction
Abnormalities of intestinal permeability

Epidemiological data supports the contribution of environmental factors to the disease. There is an increased risk of developing Crohn's disease with smoking (both active and passive), which is in striking opposition to the protective role of smoking in ulcerative colitis (43-46). There is a modest association between the use of oral

contraceptives and the development of Crohn's disease (47). Less clear associations have been reported with perinatal measles infection (48-50) and diet in infancy (short duration of breast feeding associated with increased risk of subsequently developing the disease) (51,52). Relapses of Crohn's disease have been linked to alcohol binges, infection, and the ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs) (53).

B. Intestinal permeability

Permeability and intestinal barrier

The concept of permeability relates to that property of a membrane that allows passage of a solute by unmediated diffusion. This has to be differentiated from absorption which describes a carrier-mediated transport across the intestine (54). The intestine constitutes a barrier between the outside (intestinal lumen) and internal environment of the body which prevents the entrance of potentially harmful antigenic or toxic substances (55). This protective barrier has several components including the surface mucous layer, the absorptive brush border cell membrane of the epithelial cells, the paracellular junctional areas, the epithelial and subepithelial immune defense mechanisms, and the intestinal lymph nodes. This protective barrier function of the intestine may be imperfectly quantified or measured as intestinal permeability to a variety of probes. The permeation of these probes across the intestinal epithelium depends mainly on their physicochemical properties (molecular size, shape, charge, solubility) and do not reflect the interactions of the immunologic components of the intestinal barrier with external harmful substances.

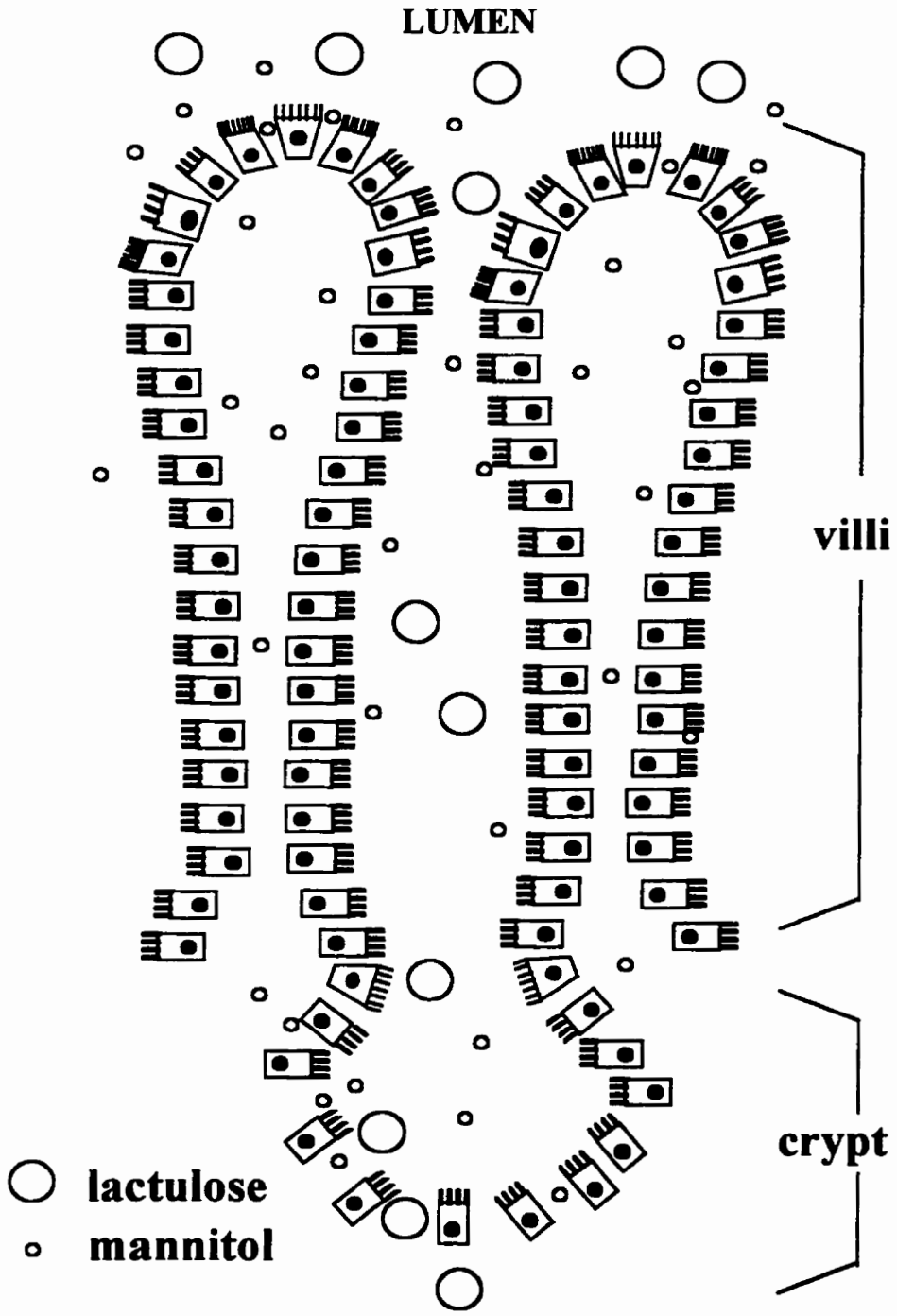
Intestinal epithelium and diffusion of different probes

The microscopic anatomy of the small intestine is composed of villi and crypts. Villi are finger-like structures 0.5 to 1 mm in height pointing towards the lumen, which increase the absorptive and digestive surface of the intestine. Crypts are at the base of the villi, where the epithelium dips in the intestinal wall. The intestinal epithelium is composed of apposed and partially fused cells, the enterocytes. Two components of the barrier function of the epithelium can be differentiated. The first component is the brush border lipid cell membrane of the enterocyte and the second the paracellular space surrounding the brush border surface of the enterocytes and their tight junction (55). These tight junctions can open and close allowing the passage of fluids and nutrients.

The routes of permeation of the different compounds used in intestinal permeability testing is controversial. The probes used to measure intestinal permeability are generally water soluble and have cross-sectional diameters of 5 to 15 angstroms (Å). By increasing size, the most frequently used probes are the cylindrical polymer polyethylene glycol (PEG 400 with a cross-sectional diameter of 5.3 Å), the sugars mannitol (6.7 Å diameter), rhamnose (8.3 Å diameter), lactulose (9.5 Å diameter) and cellobiose (10.5 Å diameter), and a radiolabeled probe, ^{51}Cr -labeled ethylenediaminetetraacetic acid ([^{51}Cr] EDTA) (11 Å diameter). Small probes penetrate the barrier more readily than the larger probes. One explanation for this would be the existence of pores of differing dimensions in the brush border lipid membrane with a higher number of small pores explaining the greater permeation of small markers (56). An alternative hypothesis is that the water-soluble probes traverse the epithelium

predominantly through paracellular tight junctions and not through the cell membranes (55). Tight junctions in the crypts would be more permeable than tight junctions in the villi (57). Bigger probes (such as lactulose) would preferentially permeate through the crypt's tight junctions whereas small probes (such as mannitol) would also permeate at the villous level through looser tight junctions. As the surface area of the villi is much greater and more accessible than the crypt's surface, only small amounts of the larger probes would permeate the epithelium compared to the penetration of smaller probes (Figure 2). This last hypothesis could explain why there is increased permeation of large probes and decreased permeation of small probes in intestinal diseases associated with villous damage and atrophy (i.e. celiac disease). In this situation the larger probes would then more easily reach the crypts and penetrate, whereas the loss of villous surface area would decrease the penetration of small probes.

Figure 2
Permeation of small and large sugars



Small sugars permeate through tight junctions in the villi
Large sugars permeate through looser tight junctions in crypts

Intestinal permeability testing

Testing of intestinal permeability has been performed using a variety of markers based on the same principle. After a period of fasting the subject drinks a solution containing the test substances which are non metabolized. A fraction of these substances penetrates the epithelium and reaches the systemic circulation, where they are rapidly cleared by the kidneys and excreted. Over a defined period of time the urine is collected to measure the amount of marker recovered. The fraction of the ingested probe recovered in the urine (fractional excretion) reflects the intestinal permeation of the marker. Among the substances which have been used as markers are polyethylene glycols (PEG) of different molecular weight, [^{51}Cr] EDTA and small sugars resistant to digestion and hydrolysis (58). When only one marker is used to measure intestinal permeability several pre-mucosal and post-mucosal factors can influence the urinary excretion of the orally ingested substance. Among pre-mucosal factors are the completeness of ingestion, gastric dilution and emptying, intestinal dilution and transit, bacterial degradation and the unstirred water layer. Among the post-mucosal factors are tissue distribution, renal function, timing and completeness of urinary collection and bacterial degradation of the sugars in the recovered urine. Considering the multiplicity of factors which can influence the reliability of the test, the principle of differential urinary excretion of two simultaneously ingested markers was formulated. When a non hydrolyzed disaccharide (i.e. lactulose, melibiose, raffinose) and a monosaccharide (i.e. mannitol, L-rhamnose) are ingested simultaneously, all the pre mucosal and post mucosal factors should equally impact on their urinary excretion. The ratio of their fractional excretion in the urine (i.e. lactulose/mannitol ratio) will then reflect

their different routes of permeation across the intestine and become a specific index of intestinal permeability unaffected by the non mucosal factors. Intestinal disease will alter to a different extent the permeation pathways of the two probes and modify the urinary ratio. Under pathological conditions the permeability for large sugars (i.e. lactulose) increases, whereas the permeability for small sugars (i.e. mannitol) does not change or decreases, which results in an increase of the lactulose/mannitol ratio.

The lactulose/mannitol permeability test has been validated in various studies of intestinal permeability (59,60) and is particularly attractive as a non invasive assessment of intestinal permeability in children. Various studies in preterm babies, term neonates, infants and children have documented its safety and tolerability (60-65).

Despite the wide use of permeability testing with non metabolized sugars, no uniform test solution or protocol has become established. Even when using lactulose and mannitol as the two probes, there is marked variation in the volume of the solution ingested, the sugar content, the osmolarity of the test solution and the duration of the urine collection (58). Frequently an additional substance is added to the lactulose-mannitol solution in order to increase osmolarity (osmotic "filler). In order to simplify the preparation of the drink and avoid hyperosmolarity, we have combined two previously described protocols for the lactulose-mannitol test used in adults (66) and children (65). Table 2 illustrates a few of the different protocols using lactulose and mannitol found in the literature. Table 3 gives the values of lactulose/mannitol ratios reported by these investigators in normal adults and children. Normal values are difficult to compare between studies because the osmolarity of the solution influences intestinal permeability.

Furthermore some investigators report mean and standard deviations whereas others describe median and ranges, probably because the distribution of lactulose/mannitol ratios did not appear to be normal.

Table 2

Lactulose/Mannitol Intestinal Permeability Protocols

Author (Ref.)	Lac (g)	Man (g)	Other (g)	Volume (ml)	Osm. (mmol/L)	Collection (hours)
May (66)	5	2	glucose (5)	250	300	overnight
Hilsden (67)	5	2	sucrose (100)	350	908	overnight
Van Elburg (60)	10	2	sucrose (40)	100	1560	5
Hamilton (65)	5	2	glycerol (9)	100 (2 ml/kg)	1510	5
Pearson (68)	5	5	-	65	580	5
Murphy (69)	5	5	-	65	580	5
Dupont (70)	0.1 g/kg	0.1 g/kg	-		1001	5
*	5	2	-	100 (2 ml/kg)	273	overnight

Lac: lactulose; Man: mannitol; Osm: osmolarity.

* present study

Table 3

Lactulose/Mannitol Ratio

Normal Values for Adults (AD) and Children (CH)

Author (Ref.)	Subjects	Mean	SD	Median	Range	Normal upper limit
May (66)	38 AD	0.009	0.006			0.022
Hilsden (67)	40 AD	0.0174	0.007			0.0281
Van Elburg (60)	40 AD	0.027	0.02			0.066
	30 CH	0.034	0.016			0.066
Hamilton (65)	33 CH			0.03	0.008 - 0.54	
Pearsons (68)	31 CH	0.018			0.005 - 0.028	
Murphy (69)	31 CH			0.02	0.01 - 0.03	
Dupont (70)	39 CH	0.0245	0.01			0.045*

*calculated from study data as mean + 2 SD.

Factors influencing intestinal permeability

Increased intestinal permeability has been demonstrated in several conditions affecting the gastrointestinal tract such as infection, active celiac disease or ingestion of alcohol or NSAIDs (54,59). The influence of smoking on intestinal permeability remains

disputed. Finally, when testing intestinal permeability with a sugar solution, the osmolarity of the drink increases permeability (hyperosmolar solutions of 500 mOsm/L or higher).

Several microbial infections have been shown to increase intestinal permeability probably by the mucosal damage associated with the enteritis (71). Interestingly in acute gastroenteritis early nutrition as compared to fasting normalizes intestinal permeability which probably reflects a hastened recovery by provision of nutrition (62).

Intestinal permeability has been shown to be elevated in celiac disease and to return towards normal with a gluten-free diet (72). A gluten challenge transiently increases permeability (73). Ten first-degree relatives of patients with celiac disease, without villous atrophy, have been described as having an abnormal intestinal permeability which may be interpreted as a constitutional factor in people susceptible to the disease (74).

Chronic alcoholics (drinking 80 to 150 g of ethanol daily for at least 6 months) have an increased intestinal permeability which returns to normal within one to two weeks of abstention (75,76). In contrast acute alcohol ingestion (0.8 g/kg of ethanol) does not seem to influence intestinal permeability as measured by the lactulose/mannitol ratio (76).

Non steroidal anti inflammatory drugs (NSAIDs) increase intestinal permeability in humans within 12 hours of ingestion. The increase in permeability seems to be related to the potential to inhibit cyclo-oxygenase. In a study using [^{51}Cr] EDTA as a marker, intestinal permeability showed a stepwise increase after aspirin (1.2 + 1.2 g), ibuprofen (400 + 400 mg) and indomethacin (75 + 50 mg), which provoked the highest increase in permeability (77). Prostaglandins administered concomitantly reduced the changes in permeability induced by NSAIDs (78).

Limited work has been done on the influence of smoking on intestinal permeability. Smoking was reported to decrease intestinal permeability (79,80), whereas other studies suggest no influence of smoking (67,81,82).

The osmolarity of the solution influences intestinal permeability. It has been claimed that hyperosmolar solutions discriminate better between normal and abnormal individuals but this remains debated (54). Moderately hyperosmolar solutions (500 mmol/L) may cause structural damage to the mucosa (subepithelial blebs with eventual loss of cells) (83).

C. Intestinal permeability in Crohn's disease and their first-degree relatives

Using a large variety of markers, intestinal permeability has been shown to be elevated in adults and children with Crohn's disease (69,84-88). Most patients with small intestinal involvement of Crohn's disease have increased intestinal permeability and 50% of patients with colonic Crohn's disease are abnormal (84,85,89). Considering that patients with ulcerative colitis have normal intestinal permeability (84,85), the increase in permeability in patients with Crohn's colitis probably reflects small intestinal involvement which cannot be detected by radiology, endoscopy, or histology. The increase in intestinal permeability correlates with the activity of the disease (84,89-91), returns towards normal with successful treatment (92-94) and may be helpful in predicting relapses (94-97).

Increased intestinal permeability has been demonstrated in healthy relatives of patients with Crohn's disease (66,98-101). This has generated the attractive hypothesis that increased intestinal permeability could be a primary defect leading to the exposure of

the gastrointestinal immune system to excessive antigenic stimulation resulting finally in the inflammatory response affecting the gut (102,103). However several authors have contested this finding and reported normal intestinal permeability among relatives of patients with Crohn's disease, when considered as an homogeneous group compared to healthy controls (104-111). These authors have concluded that the increased intestinal permeability in Crohn's disease is not a primary factor but merely reflects the activity of the disease and is secondary to the inflammation. Table 4 summarizes the studies on intestinal permeability in relatives of Crohn's disease. One explanation for this controversy has been suggested by the Gastrointestinal Research Group of the University of Calgary (66,112). Since only 3% to 10% of the relatives of affected patients will develop the disease, only a small percentage of the relatives screened would be expected to show an increased intestinal permeability. Unless a large number of relatives are examined, studies considering the whole group of relatives as homogeneous will fail to demonstrate a difference of intestinal permeability to healthy controls. This seems likely when examining Table 4; the majority of studies suggest increased permeability in healthy relatives but without reaching statistical significance. A more fruitful approach to the analysis of permeability data is to define a normal range of values in control subjects and then to identify a subgroup of healthy relatives of patients with Crohn's disease in whom intestinal permeability is abnormally elevated. When published studies are reanalyzed by this method the majority show increased intestinal permeability in a subgroup of the healthy relatives (113).

Table 4

Intestinal Permeability in Relatives of Patients with Crohn's Disease

Author (Ref.)	Subjects (relatives)	Probe	Results		
			Relatives mean	Controls (SE)	p value
Hollander (98)	32	PEG	566 (62.4) (mg)	215 (29.6) (mg)	< 0.001
May (66)	38	LM	0.013 (0.001)	0.009 (0.001)	0.14*
Katz (106)	41	LM	= (only figures	presented)	NS*
Peeters (99)	13	LM	> (only figures	presented)	-
Peeters (100)	70	LM	> (only figures	presented)	0.03
Ainsworth (104)	20	EDTA	1.935 % (median)	1.17 % (median)	NS
Howden (105)	10	EDTA	1.35 % (median)	2 % (mean)	-
Munkholm (107)	59	LM	0.0071 (median)	0.0054 (median)	NS
Ruttenberg (108)	20	PEG	23.7 % (median)	25 % (median)	NS
Teahnon (109)	32	LR	0.05 (0.003)	0.039 (0.004)	NS
Valpiani (110)	40	LM	0.04 (median)	0.05 (median)	NS

*when raw data from the two studies combined, p = 0.05.
PEG = polyethylene glycol; LM = lactulose-mannitol; EDTA = ^{51}Cr labelled ethylene-diaminetetraacetic acid; LR = lactulose-rhamnose.

Another explanation for the conflicting data on intestinal permeability among relatives of patients with Crohn's disease is that the inherited abnormality could be an enhanced susceptibility to factors increasing intestinal permeability rather than a constant increase in the "baseline" permeability. An enhanced susceptibility to factors increasing intestinal permeability has been demonstrated in healthy first-degree relatives of affected individuals. After a challenge with aspirin (an agent recognized to increase intestinal permeability), some relatives of patients with Crohn's disease showed an exaggerated increase in intestinal permeability compared to controls (67,114).

On the basis of this interesting finding one can speculate that an exaggerated increase in intestinal permeability in response to external factors would allow an exaggerated antigenic exposure of the gastrointestinal immune system giving rise to inflammation. Inflammation could, in turn, increase intestinal permeability and initiate a prolonged vicious cycle of altered permeability and inflammation.

A genetically determined susceptibility to factors increasing intestinal permeability would provide a plausible explanation for some of the environmental factors associated with Crohn's disease or its relapses. Breast fed neonates show a decline in intestinal permeability but not formula fed babies (61,115) and short duration of breast feeding has been associated with an increased risk of developing Crohn's disease (51,52). NSAIDs (77,116), alcohol (75,76) and infection (62,71) are recognized to induce an increase in intestinal permeability and have all been associated with relapses of Crohn's disease (53,117).

C. NSAID related intestinal damage

NSAIDs are widely used for the treatment of fever and inflammation. Their generally accepted mechanism of action is the blockage of prostaglandin production. Prostaglandins and leukotrienes are inflammatory mediators generated from arachidonic acid through two different pathways, involving the enzymes lipo-oxygenase and cyclo-oxygenase. NSAIDs block cyclo-oxygenase and interfere with the production of prostaglandins (Figure 3).

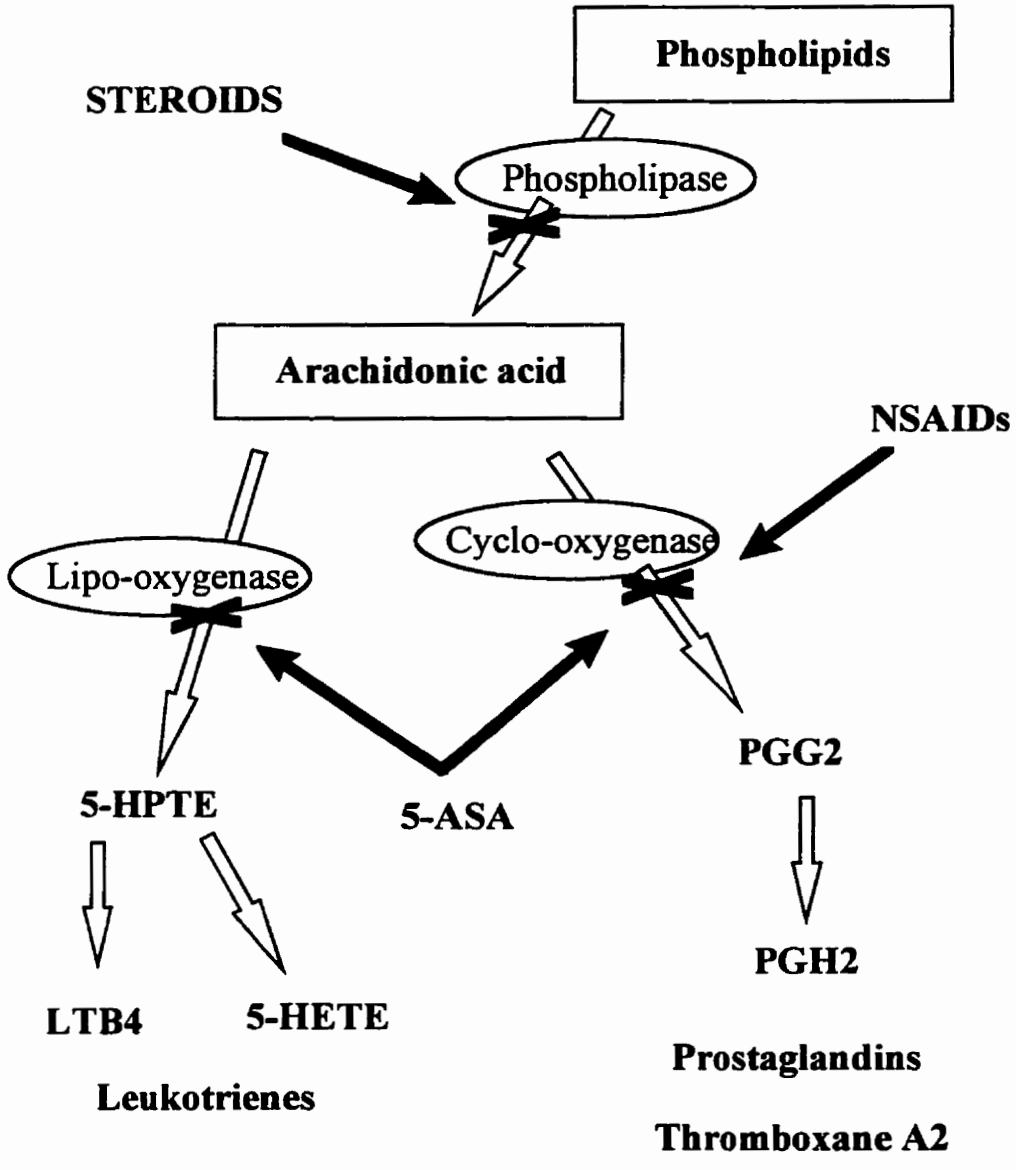
In adults NSAIDs can cause both large and small intestinal damage. When administered for non gastrointestinal diseases, NSAIDs can cause colitis, ulcers, bleeding, and perforation of the large intestine. In diverticulitis NSAIDs have been associated with perforations and strictures. In the small intestine, NSAIDs can cause ulcers, small intestinal perforations, strictures, and most frequently, intestinal inflammation with blood and protein loss. It has been suggested that up to 60 - 70 % of patients on long-term (> 6 months) therapy may have an asymptomatic enteropathy (118). Diaphragm-like small intestinal strictures are highly suggestive of NSAID damage (119).

NSAID related gastrointestinal damage has also been described in children. Peptic ulcer disease or upper gastrointestinal bleeding can be seen after short use of NSAIDs in otherwise healthy children. NSAIDs gastrointestinal damage has been documented in children with rheumatoid diseases receiving long term therapy (120-123). The frequency of gastrotintestinal damage in this population remains undetermined. In retrospective reviews less than 1 % of children with juvenile rheumatoid arthritis receiving NSAIDs were found to have significant gastropathy (esophagitis, gastritis, or peptic ulcer disease)

documented by barium studies or endoscopy (121). This probably represents an underascertainment considering that up to one-third of children on NSAIDs followed in a rheumatology clinic present with abdominal pain (124). Among children with juvenile rheumatoid arthritis referred to pediatric gastroenterogists for evaluation of abdominal pain, anemia or hematemesis, 75 % were found to have gastritis, antral erosions, or ulcers (122).

The pathogenesis of the intestinal damage associated with NSAIDs is incompletely understood. It has been suggested that NSAIDs cause direct intracellular damage to enterocytes by uncoupling oxidative phosphorylation in the mitochondria. This reduces ATP levels and results in the loss of the integrity of intercellular junctions with subsequent increase in intestinal permeability. By increasing intestinal permeability, the mucosa will be exposed to luminal aggressive factors (bile, proteolytic enzymes, bacterial degradation products) which will trigger inflammation. The inhibitory effect of cyclo-oxygenase is postulated to be of secondary importance; the inhibition by NSAIDs of production of reparative prostaglandins would aggravate the initial injury (125). Some support for this theory comes from the fact that intestinal inflammation has been demonstrated in patients with rheumatoid arthritis on NSAIDs using radiolabeled neutrophils scans, whereas untreated patients do not show intestinal inflammation (126). Furthermore metronidazole reduces intestinal inflammation in patients with NSAID enteropathy, probably by reducing the bacterial luminal load (127).

Figure 3
Mechanism of action of NSAIDs, 5-ASA and steroids



NSAIDs block prostaglandins synthesis by inhibiting cyclo-oxygenase.
5-ASA blocks both cyclo-oxygenase and lipo-oxygenase, inhibiting prostaglandins and leukotrienes production.
Steroids block phospholipase interferring with arachidonic acid production.

Aspirin has received special attention in the pediatric literature for its possible links to Reye's syndrome, a virus-associated biphasic disease that causes acute encephalopathy, liver microvesicular steatosis, and liver dysfunction in children (128). Although the evidence linking aspirin to Reye's syndrome is not free of criticism (129,130), a question mark has been hung over the safety of aspirin when given to children with common viral infections. The severity of Reye's syndrome and the availability of alternative effective remedies, has lead in some countries to the recommendation that aspirin should not be given to children under 12 years of age unless specifically indicated for rheumatic conditions (131).

D. NSAIDs and exacerbation of inflammatory bowel disease

NSAIDs are recognized as a cause of relapse in inflammatory bowel disease, although the evidence is based on limited case reports and case control series of adults (53). Four patients with active ulcerative colitis were given indomethacin enemas trying to control the disease and in three the condition worsened following administration (132). It is difficult to know if the deterioration of these three patients was due to indomethacin or was merely the natural history of the flare. Additional case reports document colitis in nine patients with ulcerative colitis and two patients with Crohn's disease after the introduction of NSAIDs (Table 5) (117,133-135). Patients were in clinical remission and received NSAIDs for non-gastrointestinal complaints. Within a few days of NSAID treatment they developed colitis with bloody diarrhea. No patient was re-challanged with the same drug, but at least one subject had brief subsequent relapses when different NSAIDs were tried to

treat his ankylosing spondylitis. Two of the patients reported finally had a colectomy, and the colon showed histologic changes compatible with ulcerative colitis. In most of these case reports the temporal sequence between NSAID introduction and colitis seems established, although it cannot always be excluded that the symptoms motivating the NSAID treatment were not prodromes of a flare up of inflammatory bowel disease (i.e. joint pains). Probably the major interpretation problem comes from the fact that it is not possible to know if the colitis in these patients represented true exacerbation of inflammatory bowel disease or an idiosyncratic NSAID-related colitis that can be seen in people without inflammatory bowel disease. Some additional support for a causal relationship between NSAIDs and exacerbation of inflammatory bowel disease comes from a case control study (136). This study found analgesic intake (paracetamol and NSAIDs) to be the only significant factor more commonly found in patients with ulcerative colitis presenting with a relapse compared to patients presenting in remission. Nevertheless the temporal relationship between NSAIDs and flare up is less clear and some of the patients may have received NSAIDs for prodromal symptoms of relapse (joint pains, headaches, backaches). In addition to possible recall bias, the fact that paracetamol (a drug with practically no anti-inflammatory effect) when considered alone, was significantly related to relapse, weakens, in our opinion, the possibility of a causal relationship between NSAIDs and inflammatory bowel disease. Finally, a prospective cohort study of 92 patients with ulcerative colitis in remission, failed to show an association between analgesic intake and subsequent relapse (137). In summary there is limited support linking use of NSAIDs and relapse of colitis in adult patients with

inflammatory bowel disease (generally ulcerative colitis). However an exacerbation of Crohn's disease after a single dose of NSAID has, to our knowledge never been clearly reported.

Table 5

Exacerbation of Inflammatory Bowel Disease Related to NSAIDs

Case Reports

Ref.	Disease	Drug	Indication	Duration of administration (days)	Lesion
(134)	UC	ibuprofen	arthritis	1	colitis
(133)	UC	indomethacin	ankylosing spondylitis	5	colitis
(133)	UC	benorylate	joint pains	14	colitis
(133)	UC	flurbiprofen	carpal tunnel	5	colitis
(133)	UC	ibuprofen	arthritis	28	colitis
(135)	UC	naproxen	joint pains	42	colitis
(135)	UC	naproxen	joint pains	6	colitis
(117)	UC	piroxicam	arthritis	1	colitis
(117)	UC	naproxen	back pain	7	colitis
(117)	CD	indomethacin	bursitis	"shortly"	rectal ulcer
(117)	CD	indomethacin	sciatica	1	colitis

UC: ulcerative colitis; CD: Crohn's disease.

Interestingly 5-aminosalicylate (5-ASA) derivatives are a standard therapy for inflammatory bowel disease and only rarely seem to exacerbate ulcerative colitis (138,139). The main difference with NSAIDs, probably explaining the beneficial effects of 5-ASA derivatives is that they inhibit both the cyclo-oxygenase and the lipo-oxygenase (blocking the production of protaglandins and leukotrienes), whereas NSAIDs block only the cyclo-oxygenase (Figure 3).

2. Research rationale

When considering the genetic contribution to Crohn's disease a promising area of research is the use of genetic linkage analysis in the investigation of families with multiple affected members, and identification of individual loci that contribute to disease susceptibility (140). However one problem that arises is the fact that relatives of patients with Crohn's disease may have inherited the genetic susceptibility but will not (or not before a long latent period) develop the disease. The study of subclinical markers is a promising field of research, which avoids this problem.

Abnormalities in intestinal permeability have been considered as potential subclinical markers among patients with Crohn's disease and their relatives. Recent data suggest that the abnormal intestinal permeability in healthy first-degree relatives of Crohn's patients may become manifest only after exposure to one of the recognized external factors increasing gut permeability such as NSAIDs (67,114). This limited data also suggests that abnormalities of intestinal permeability seem concordant between the proband and his first-degree relatives, which may indicate a genetic link of abnormal permeability. These studies deserve confirmation.

Because of the higher familial aggregation in the pediatric Crohn's disease population, this group was appropriate to confirm the findings of exaggerated response in intestinal permeability to environmental factors and its eventual genetic distribution. Previous studies in adults have used an aspirin challenge with the lactulose/manitol permeability test in relatives of Crohn's disease patients (67,114). They were based on a study of intestinal permeability in healthy subjects (77) which documented an increase in

intestinal permeability after a challenge with 3 different NSAIDs: aspirin, ibuprofen and indomethacin. Considering the controversy in the pediatric literature regarding the use of aspirin we have chosen ibuprofen in a therapeutic dosage for the NSAID challenge. This drug has been shown to be safe in the treatment of fever in children and is currently recognized as an alternative to acetaminophen (141-143).

3. Objectives

A. Primary

1. To determine whether intestinal permeability is increased in relatives of children with Crohn's disease compared to controls.

2. To determine whether children with Crohn's disease and their relatives show an exaggerated increase in intestinal permeability after the ingestion of ibuprofen.

B. Secondary

Additional information may be gained on whether this abnormal permeability response is consistent within families and conforms to any pattern of genetic transmission among families with a pediatric proband.

4. Methods

The study was submitted to the Conjoint Medical Research Ethics Board of the University of Calgary Medical Faculty and the Alberta Children's Hospital Research and Development Committee and received ethical approval prior to initiation.

A. Study design

Observational cross-sectional study. Considering the fact that a pharmacological agent is given to participants during the permeability testing, this study could also be called an experimental case-control cross-sectional study.

B. Population

Target and Accessible Population

The target population was families (first-degree relatives) of children with Crohn's disease. Parents and siblings of the proband were considered as first-degree relatives. Healthy family units (parents and children) without inflammatory bowel disease represented the control group. The accessible population was families (first-degree relatives) of children with Crohn's disease followed at the Division of Pediatric Gastroenterology of the Alberta Children's Hospital.

Inclusion Criteria

1. Children (less than 18 years of age) with Crohn's disease in clinical remission, previously diagnosed by standard clinical, radiological or histological criteria.

2. Healthy first degree relatives and controls.

3. All participants must have signed the Informed Consent Form (Appendix A).

4. For practical reasons the pediatric participants should be toilet trained in order to collect the urine necessary for the analysis of permeability.

Exclusion Criteria

1. Children with active Crohn's disease.

2. Use of NSAID in the week prior to testing, or alcohol intake in the 3 days prior to testing.

3. Active infection or inflammatory process (as revealed by medical history), renal insufficiency, diabetes, cystic fibrosis or pancreatic insufficiency.

4. Intolerance, allergy, or contraindication to ibuprofen (bleeding diathesis, known thrombocytopenia, anticoagulant treatment, history of hypersensivity reaction).

Recruitment

Recruitment of participants took place between May and December 1996.

Disease population: Families of children with Crohn's disease followed at the Division of Pediatric Gastroenterology of the Alberta Children's Hospital were identified by screening the clinic's charts. In addition Medical Records provided a list of patients admitted in the last ten years with the diagnosis of Crohn's disease or ulcerative colitis. Thirty-one families were identified. All families were sent a letter of information which invited them to participate in the research project. Families were approached again during

their scheduled clinic visits at the Alberta Children's Hospital. For participating families (n =14), compliance with the permeability tests was ensured through phone calls.

Control population: Families with a child seen at the outpatient fracture clinic of the Alberta Children's Hospital and families of hospital staff were approached to participate as controls. Information was provided through posters at the Alberta Children's Hospital and through letters distributed to staff in the different clusters. Fourteen control families were recruited (4 at the fracture clinic and 10 among hospital staff).

C. Study Protocol

Baseline Characteristics

Children with Crohn's disease underwent a clinical assessment (medical history and physical examination) to document previous medical history, activity of the disease and present management. Activity of the disease was determined using a score proposed by Hyams et al (144) but excluding items involving invasive investigations (laboratory) (Appendix B). The score proposed by Hyams has been developed in a population of 133 children with Crohn's disease. The original score ranges from 0 (inactive disease) to 100 (very active disease) and it was suggested that a score above 30 signifies moderate to severe disease. After deleting laboratory investigations, the modified score used in this study had a range from 0 to 80, and by extrapolation scores above 25 could indicate moderate to severe disease. Laboratory investigations were removed from this score in order to simplify the study protocol and increase participation, recognizing that such a

modified score has not been validated. Baseline characteristics of first degree relatives and control families included ethnic background, family history, medical history, medication intake, smoking and alcohol intake. Data collection forms are provided in Appendix C.

Baseline Intestinal Permeability

Following a one or two hour fast, subjects were given the test solution of mannitol (BDH Inc., Toronto, ON, Canada) and lactulose (Technilab, Montreal, Canada) before bedtime. Adults received a standard dose of 5 grams of lactulose and 2 grams of mannitol in 100 ml of water. Children received 0.1 g/kg of lactulose and 0.04 g/kg of mannitol, administered as 2 ml/kg of a solution of 5% lactulose - 2% mannitol, with a maximum dose of 100 ml. Subjects voided prior to drinking the test solution and then collected all urine including the first morning void in containers with 5 ml of 10 % thymol in methanol.

Intestinal Permeability post Ibuprofen

One to five days after the baseline test, intestinal permeability was determined after the ingestion of ibuprofen. One hour before drinking the test solution, subjects took an oral dose of ibuprofen (adults: 800 mg; children 10 mg/kg to a maximum dose of 800 mg) and repeated the lactulose/mannitol test as described under baseline intestinal permeability.

Urine Analysis

Urine samples were kept refrigerated and returned to the lab within 48 hours of test completion. Urine samples (10 ml) were deionized by adding 1 g of a 1:1.5 (wt:wt) mixture of Amberlite IR-120 and IRA-400 resin (BDH Chemicals, Toronto, ON, Canada). Following centrifugation at 3000 rpm for 10 minutes, the supernatant was filtered through a 40-μm/L Millipore filter (Millipore, Bedford, MA). Samples were separated on a Dionex MA-1 anion exchange column in a Dionex HPLC (Dionex, Oakville, ON, Canada) at room temperature using 500 mmol/L NaOH as the isocratic mobile phase. Peak identification was accomplished with the use of authentic standards and detected using pulsed amperometric electrochemical detection on a gold electrode. Samples were diluted as necessary after addition of cellobiose as an internal standard. Quantitation was performed using known standards at multiple concentrations with linear interpolation between concentrations. All samples were diluted so that concentrations of interest fell within the range of the standards.

Lactulose/mannitol (LM) Ratio and Percent Change in LM Ratio

The fractional excretion of each probe was determined by calculating the proportion of the ingested probe that was excreted in the urine. The lactulose/mannitol ratio (LM ratio) was obtained by dividing the fractional excretion of lactulose by the fractional excretion of mannitol. The LM ratio represents the measure of intestinal permeability.

To take into account the baseline level of intestinal permeability in each individual we used the percent change in LM ratio after ibuprofen as the main measure of interest reflecting the response to the NSAID challenge. This ratio was named Change in LM ratio and was calculated as follows:

$$\text{Change in LM ratio} = \frac{(\text{post ibuprofen LM} - \text{baseline LM})}{\text{baseline LM}} \times 100\ (\%)$$

D. Sample size

Hilsden et al. (67) found an exaggerated increase in IP after a challenge with aspirin in about 20% of relatives of patients with CD and in less than 1% of controls. Assuming that 20% of relatives and 1% of controls would have an abnormal response approximately 50 subjects were required in each group to detect a difference between relatives and controls with a power of 80% and an alpha level of 0.05. Enrollment was done over a 7 months period. All families of children with Crohn's disease followed at the Gastroenterology Clinic of the Alberta Children's Hospital were approached on at least two occasions. Twenty-four families of a child with Crohn's disease initially agreed to participate. Ten of them did not complete the tests despite follow up by phone calls. Ultimately a total of 36 first degree relatives of children with Crohn's disease and a total of 41 controls could be enrolled into the study. The discrepancy between planned and final sample size is discussed under limitations and implications of the study.

E. Statistical analysis

Analyses were performed using the statistical software Stata 5.0 (Stata Corporation, College Station, Texas, US). All statistical tests were two sided with an alpha level of 0.05.

Descriptive statistics were first performed on baseline characteristics of children with Crohn's disease, their first degree relatives and controls. Normality of LM ratio data was assessed using graphs and statistical tests (Shapiro-Francia W' test). As the data for baseline and post ibuprofen LM ratios did not appear to have a normal distribution, these data are expressed as median and range.

In order to address the first part of the primary objective (comparison of baseline intestinal permeability) relatives were compared to controls in two ways. First groups were considered homogenous and comparison of baseline LM ratios between relatives and controls was done using the Mann-Whitney U test. Secondly individuals were classified as normal or abnormal using the data from the control population and the proportion of abnormal subjects was compared using the Fisher's exact test. The 95 % percentile in healthy controls served as an upper limit of normal values of baseline LM ratio. Alternatively a logarithm transformation of baseline LM ratio data was done to achieve normality: ln (permeability - k), with k = 0.00598. Then an upper cut off level was defined as the mean + 2 SD and back transformed to the original data.

Similarly in order to address the second part of the primary objective (response to ibuprofen challenge) the Change in LM ratio was compared between relatives and controls using the student t test (comparison of groups) and the Fisher's exact test (comparison of

the proportion of abnormal individuals in both groups). The Change in LM ratio was normally distributed and this data is expressed as mean and standard error. Using the control group an upper cut off level of normality was defined as the mean + 2 SD.

Information on the genetic transmission of abnormal permeability (secondary objective) was provided as descriptive pedigrees.

Limited exploratory analyses were done to determine if adults had different intestinal permeability from children.

5. Results

A. Subjects

The 31 families of children with Crohn's disease followed at the Gastroenterology Clinic of the Alberta Children's Hospital who met the inclusion criteria represented the accessible population. Seven families refused to participate principally because the proband did not agree to do the test. Among the 24 families who initially gave informed consent, 14 completed the permeability tests. A similar rate of recruitment was observed in the control group. Fourteen families completed the test out of more than twice as many families that were approached. The ethnic background of controls and participating and non-participating Crohn's families and the proportion with a family history of inflammatory bowel disease (other family members than the index case affected) is given in Table 6. The 14 Crohn's families comprised 50 family members with a median of 4 members per family (range 2 to 5) and the 14 control families 41 family members with a median of 3 members per family (range 2 to 5).

Table 6

Participating Families and Non Participating Crohn's Disease Families

	Control families (n = 14)	Crohn's families (n = 14)	Non-participating Crohn's families (n = 17)
Ethnic background			
Whites	11	13	15
(Ashkenazi Jews)	(1/11 = 9%)	(3/13 = 23 %)	(3/15 = 20 %)
Orientals	2	1	2
African Americans	1	0	0
Family history of IBD	-	3/14 (21 %)	3/17 (18 %)
Family members per family: median (range)	3 (2 - 5)	4 (2 - 5)	4 (2 - 7)

Children with Crohn's disease

Fourteen children with Crohn's disease participated in the study. Table 7 shows the characteristics of these subjects. There were 3 females and 11 males. Their median age was 14 years with a range from 6 to 17. The median number of years since diagnosis was 3.5 with a range from 0.2 to 9. The disease involved the small and large bowel (most frequently ileocolonic disease) in 10 children, only the small bowel in 2 and only the colon in 2. Only one child had had a previous bowel resection for the disease. None of these

children had an exacerbation of Crohn's disease when participating in the study. The median Crohn's disease modified activity score was 5 with a range from 0 to 30. Two subjects had scores which may have indicated active disease (25 and 30 respectively), but their disease was judged under remission by the attending physician seeing the child in clinic. The majority of these children (13 out of 14) were receiving medical treatment. Most frequently a combination of Prednisone and 5-ASA derivatives (6/14) or 5-ASA derivatives alone (5/14). Three children received, in addition, azathioprine (AZA) and one nutritional therapy (continuous night polymeric diet).

Table 7

Characteristics of Children with Crohn's Disease

	N = 14
Female/Male	3/11
Age: median (range)	14 (6 - 17)
Localization of disease	
small + large bowel	10/14
only small bowel	2/14
only colon	2/14
Activity Score: median (range)	5 (0 - 30)
Medical treatment	13/14
steroids + 5-ASA + AZA	3
steroids + 5-ASA	3
steroids + metronidazole	1
5-ASA	5
metronidazole	1

5-ASA: 5 aminosalicylate; AZA: azathioprine

Relatives of children with Crohn's disease

Thirty-six first degree relatives of children with Crohn's disease drawn from 14 different families participated in the study. Table 8 shows the demographic characteristics of this group. There were 23 adults with a median age of 40 years (range: 31 to 64) and 13 children with a median age of 13 years (range: 8 to 17). Among adults four were current smokers (range half to one pack per day) and one regularly drank alcohol (20 to 30 g of ethanol per day) but agreed to withhold alcohol intake for the 3 days prior to testing.

Table 8

Demographic Characteristics of Healthy Relatives

	Parents (n = 23)	**Children (n = 13)**
Age: median (range)	40 (31 - 64)	13 (8 - 17)
Female/Male	13/10	6/7
Current smokers*	4/23 (17 %)	0
Current alcohol drinking*	1/23 (4.3 %)	0
Regular use of NSAID**	0	0

*every day or almost every day

**at least once a week

Healthy controls

Forty-one controls drawn from 14 different families participated in the study. Table 9 shows the demographic characteristics of this group. There were 20 adults with a median age of 40 years (range: 32 to 49) and 21 children with a median age of 13 years (range: 6 to 17). Among adults six were current smokers (range five cigarettes to one pack per day) and one regularly drank alcohol (5 to 10 g of alcohol per day) but not in the 3 days prior to testing.

Table 9

Demographic Characteristics of Controls

	Parents (N = 20)	**Children (N = 21)**
Age: median (range)	40 (32 - 49)	13 (6 - 17)
Female/Male	13/7	8/13
Current smokers*	6/20 (30 %)	0
Current alcohol drinking*	1/20 (5 %)	0
Regular use of NSAID**	0	0

*every day or almost every day

**at least once a week

Control adults and children were similar to adults and children in the healthy relatives group in terms of age and gender distribution, alcohol drinking and use of NSAIDs. Adult controls appeared to smoke more frequently than adult relatives but this

difference was not statistically significant (30 % versus 17 % respectively, Fisher's exact test: p = 0.5).

B. Intestinal permeability

All permeability tests were well tolerated and no secondary effects reported.

Baseline Intestinal Permeability

Table 10 summarizes baseline intestinal permeability data in the control group, first degree relatives and children with Crohn's disease. The data is described in more detail by group.

Table 10

Baseline Intestinal Permeability

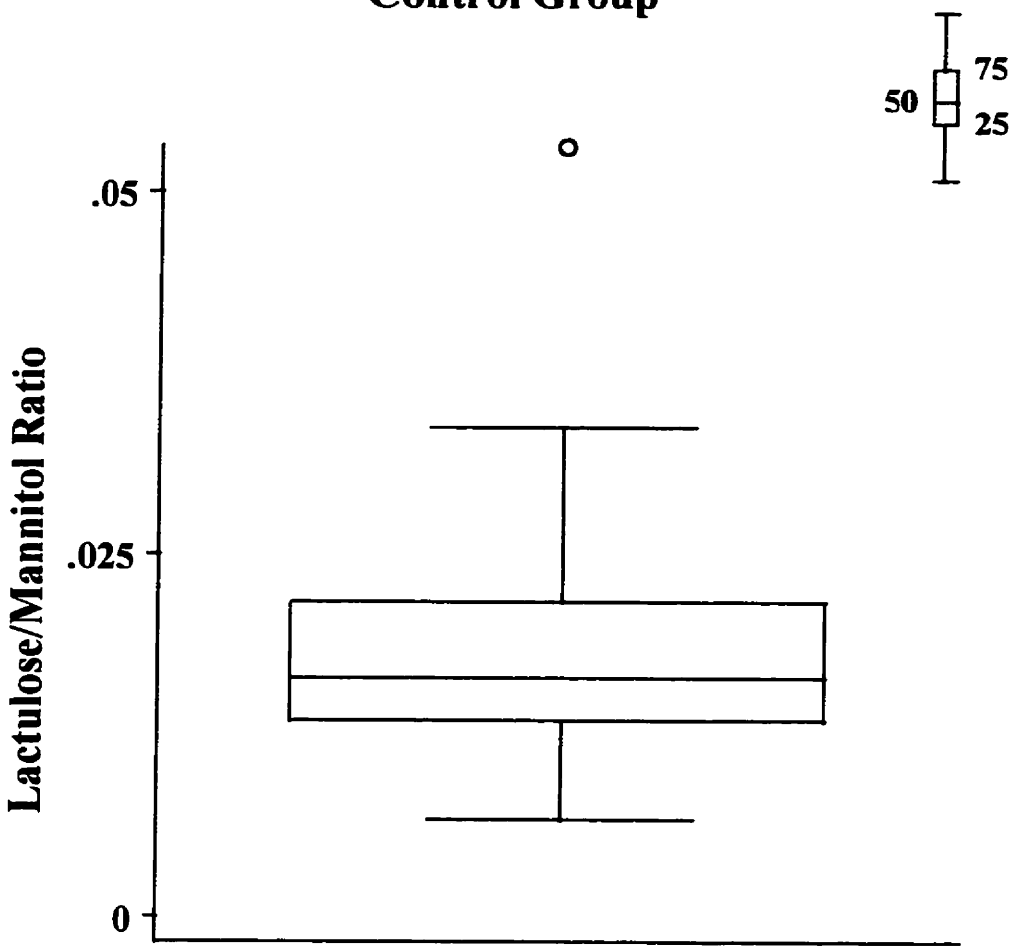
	Controls		**Relatives**		**Crohn's**
	Parents (n = 20)	**Children (n = 21)**	**Parents (n = 23)**	**Children (n = 13)**	**Children (n = 14)**
LM					
median	0.0144	0.0189	0.0155	0.0197	0.0271
range	0.0067 - 0.0289	0.0120 - 0.0532	0.0092 - 0.0349	0.0110 - 0.0549	0.0139 - 0.1735
Abnormal* (%)	0 (0)	1/21 (5)	0 (0)	2/13 (15)	3/14 (21)

*using 0.0468 as the upper cut off level of normality.

1. Control group

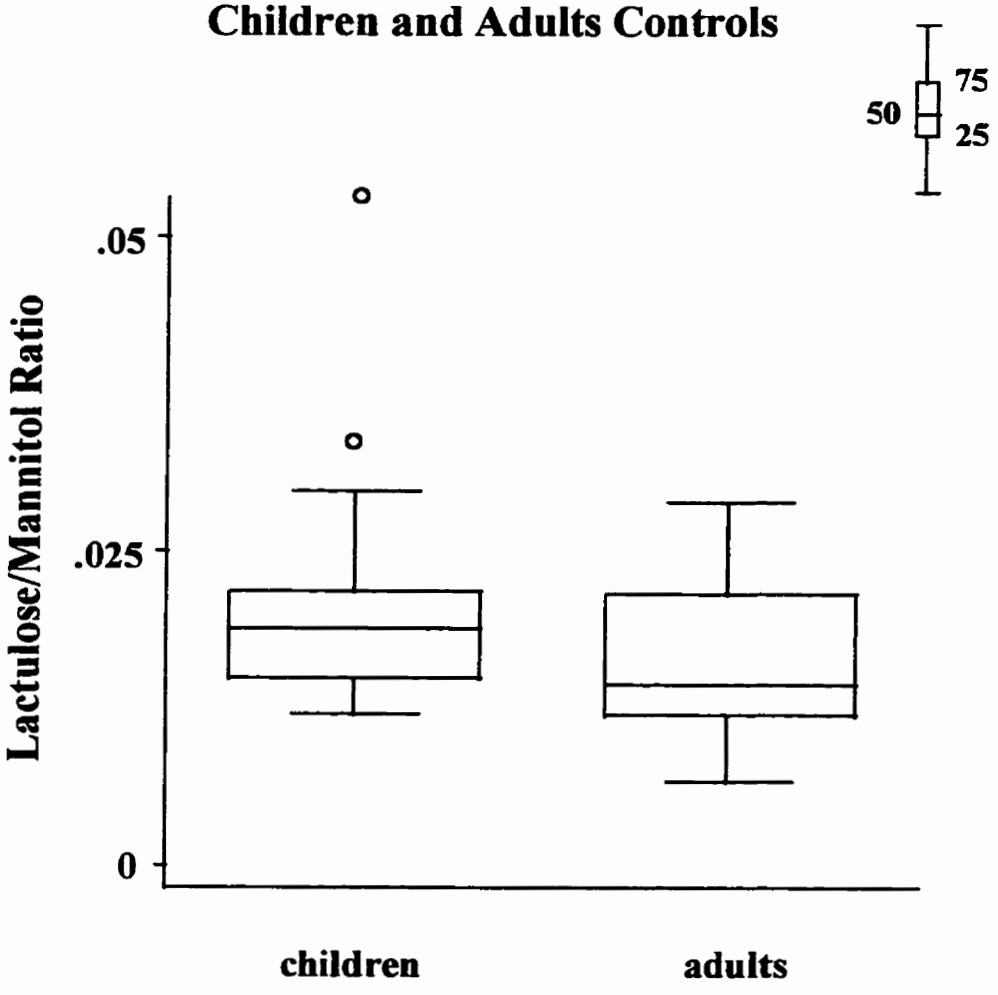
Baseline LM ratio in the control group is illustrated in Figure 4. Baseline LM ratio was not normally distributed (Figure 4 and Shapiro-Francia W' test: $p < 0.001$). The median was 0.0165 with a range from 0.0067 to 0.0532. The upper cut off level for normal values defined using the 95th percentile from the control group was 0.0298. Using a logarithm transformation of the data as described under statistical analysis the upper normal limit was 0.0468 (geometric mean + 2 SD). In the control group two unrelated subjects (a 14 year old girl and a 13 year old boy) had an abnormal baseline LM ratio using 0.0298 as an upper normal limit (2/41 = 4.9 %) and one subject (1/41 = 2.4 %) using 0.0468 as an upper cut off level. As an exploratory analysis the baseline LM ratio was assessed separately for adults and children. As illustrated in Figure 5, children seemed to have higher baseline LM ratios than adults, with a borderline statistical significance (Mann-Whitney U test: $z = 1.8$, $p = 0.07$).

Figure 4
Baseline Lactulose/Mannitol Ratio
Control Group



Box plot of baseline lactulose/mannitol ratio, showing the 25th, 50th, and 75th percentiles of the data. The distribution is positively skewed (Shapiro-Francia W' test for normality: $z = 4.1$, $p < 0.001$)

Figure 5
Baseline Lactulose/Mannitol Ratio
Children and Adults Controls

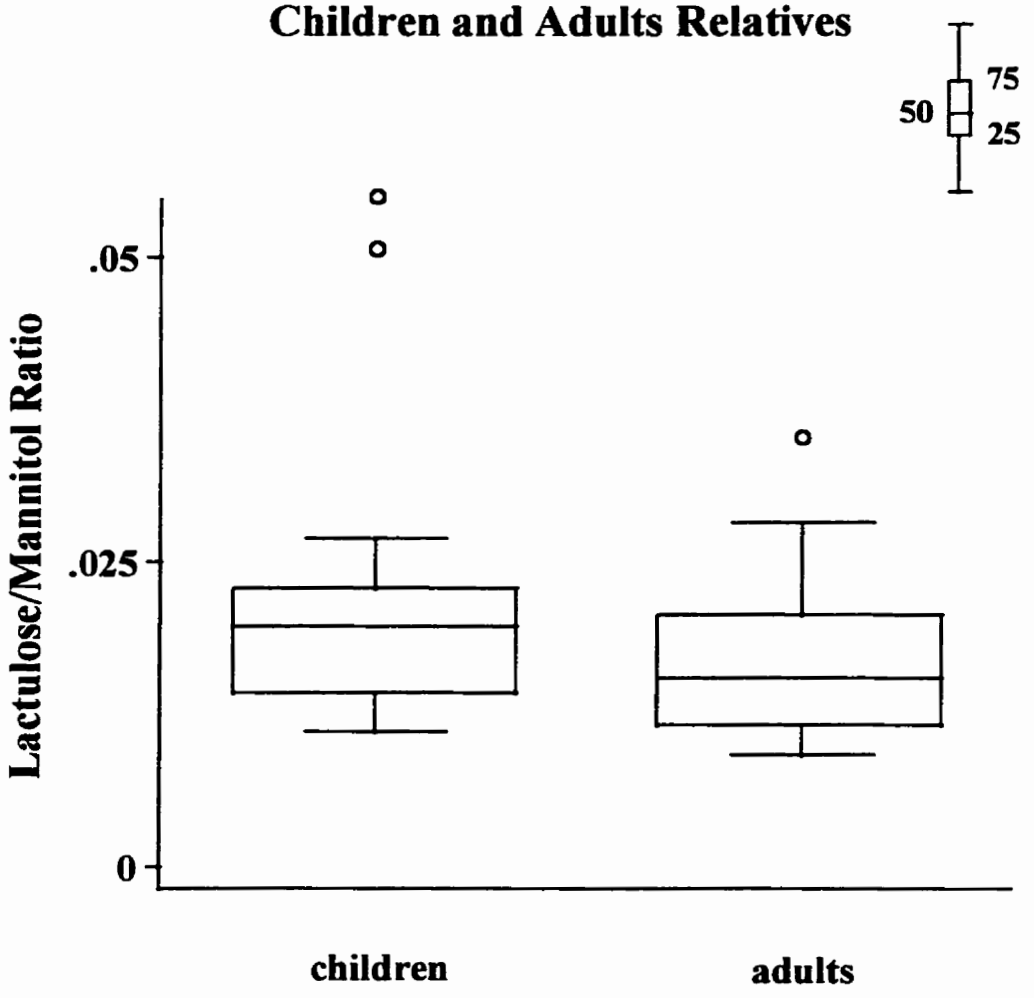


Median lactulose/mannitol ratio in children seems higher than in adults (0.0189 versus 0.0144, Mann-Whitney U test: $z = 1.8$, $p = 0.07$).

2. Healthy relatives

The median baseline LM ratio in healthy relatives was 0.0176 with a range from 0.0092 to 0.0549. Baseline LM ratio in healthy relatives was not significantly different from the control group (Mann-Whitney U test: $z = 0.32$, $p = 0.75$). Three unrelated subjects (40 year old woman, 8 and 15 year old boys) had an abnormal baseline LM ratio using 0.0298 as an upper normal limit (3/36 = 8.3 %). Two subjects (2/36 = 5.6 %) were considered abnormal using 0.0468 as an upper cut off level. With any upper cut off limit the percentage of healthy relatives with abnormal baseline LM ratio did not significantly differ from the controls: 8.3 % versus 4.9 % (Fisher exact test: $p = 0.7$) or 5.6 % versus 2.4 % (Fisher exact test: $p = 0.6$). Using the more conservative normal upper limit the difference in the proportion of abnormal individuals between healthy relatives and controls was of 3.2 % with a 95 % confidence interval of - 5.7 % to 12 %. In the group of healthy relatives baseline LM ratio seemed higher in children than in adults (Figure 6), but this difference did not reach statistical significance (Mann-Whitney U test: $z = 1.6$, $p = 0.11$).

Figure 6
Baseline Lactulose/Mannitol Ratio
Children and Adults Relatives



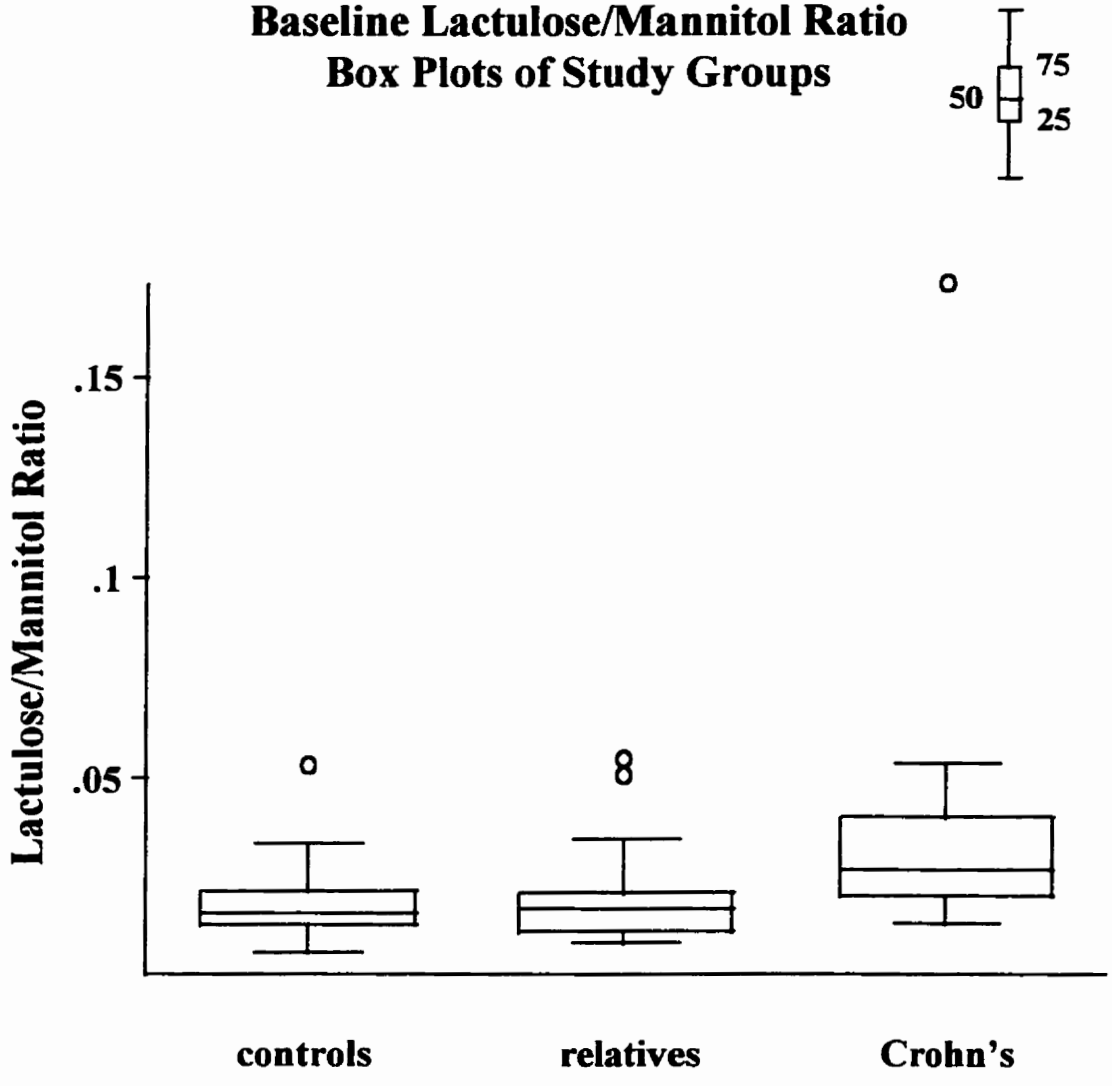
Median lactulose/mannitol ratio in children seems higher than in adults (0.0197 versus 0.0155, Mann-Whitney U test: $z = 1.6$, $p = 0.11$).

3. Children with Crohn's disease

The median baseline LM ratio in children with Crohn's disease was 0.0271 with a range from 0.0139 to 0.1735. The LM ratio in the group of children with Crohn's disease was significantly higher than the control group (Mann-Whitney U test: $z = 3.5$, $p < 0.001$). Six subjects had an abnormal baseline intestinal permeability using 0.0298 as an upper normal limit (6/14 = 43 %). Three subjects (3/14 = 21 %) were considered abnormal using 0.0468 as an upper cut off level; their modified Crohn's activity scores were 0, 5, and 30. Compared to the control group, the percentage of children with Crohn's disease with an abnormal baseline permeability was significantly higher both with the 0.0298 (Fisher's exact test: $p = 0.002$) and the 0.0468 (Fisher's exact test: $p = 0.047$) upper cut off level. One of the three subjects with a LM ratio above the more conservative level relapsed two months after completing the test (intestinal resection); she had a modified activity score of 0 when completing the test.

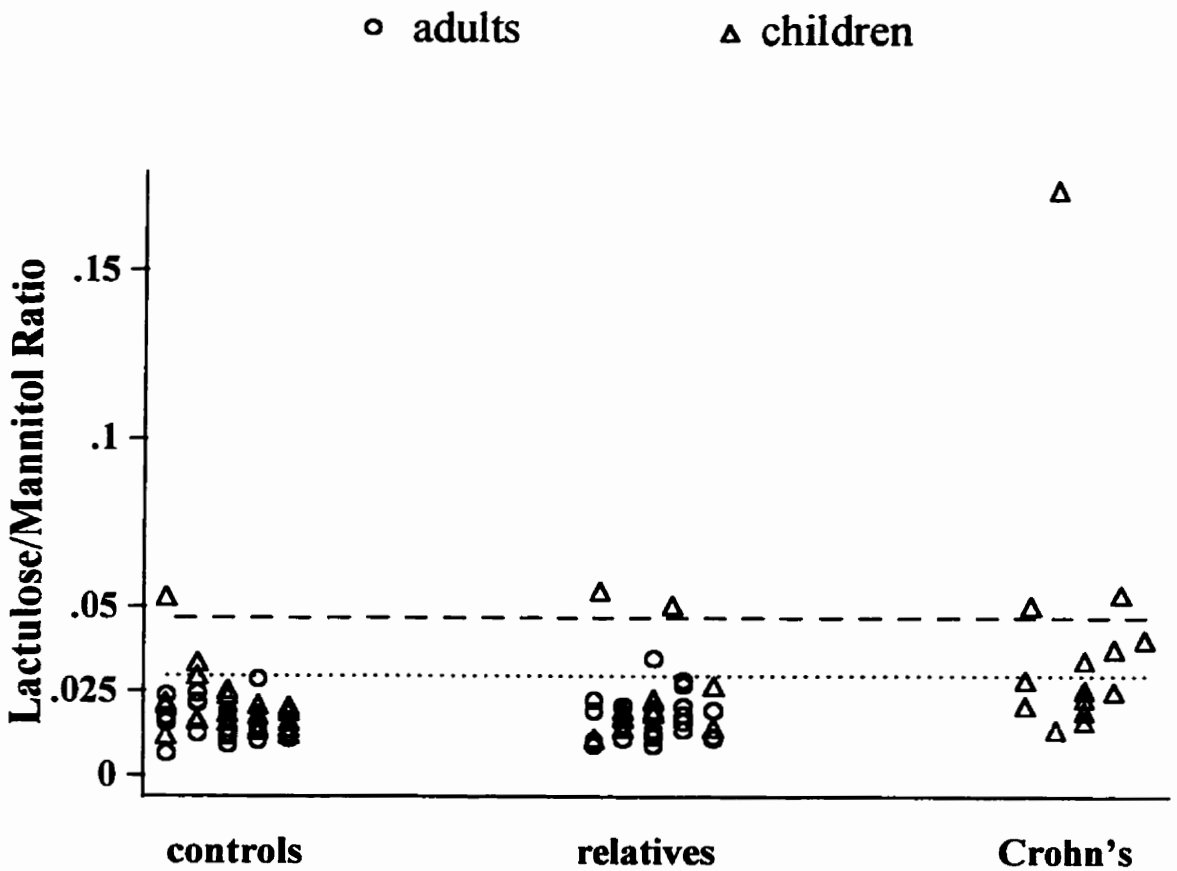
In summary baseline intestinal permeability was similar in healthy relatives and in controls, with a similar percentage of individuals being above the normal range in both groups. Children with Crohn's disease showed an abnormally increased baseline intestinal permeability. Figures 7 and 8 illustrate the data in the three groups with the upper cut off levels defined above.

Figure 7
Baseline Lactulose/Mannitol Ratio
Box Plots of Study Groups



Lactulose/mannitol ratio is similar in controls and in relatives. Crohn's disease children have a median lactulose/mannitol ratio higher than controls (0.0271 versus 0.0165, Mann-Whitney U test: $z = 3.5$, $p < 0.001$).

Figure 8
Baseline Lactulose/Mannitol Ratio
Upper cut off levels of normality
(doted line = 0.0298 ; dashed line = 0.0468)



Using 0.0468 as the upper limit of normality (geometric mean + 2 SD from the control group) for lactulose/mannitol ratio, the proportion of subjects with an abnormal lactulose/mannitol ratio is similar in controls (2.4 %) and relatives (5.6 %), but higher in children with Crohn's disease (21 %).

Response to NSAID Challenge

Intestinal permeability post ibuprofen (LM ratio post ibuprofen, Change in LM ratio) is summarized for controls, healthy relatives and children with Crohn's disease in Table 11. One of the children with Crohn's disease (allergic to ibuprofen) and one healthy relative (intolerant to NSAIDs) did not complete the ibuprofen challenge. The Change in LM ratio is the main measure of interest reflecting the response to the NSAID challenge. The data is discussed in detail by group.

Table 11

Intestinal Permeability

Response to Ibuprofen

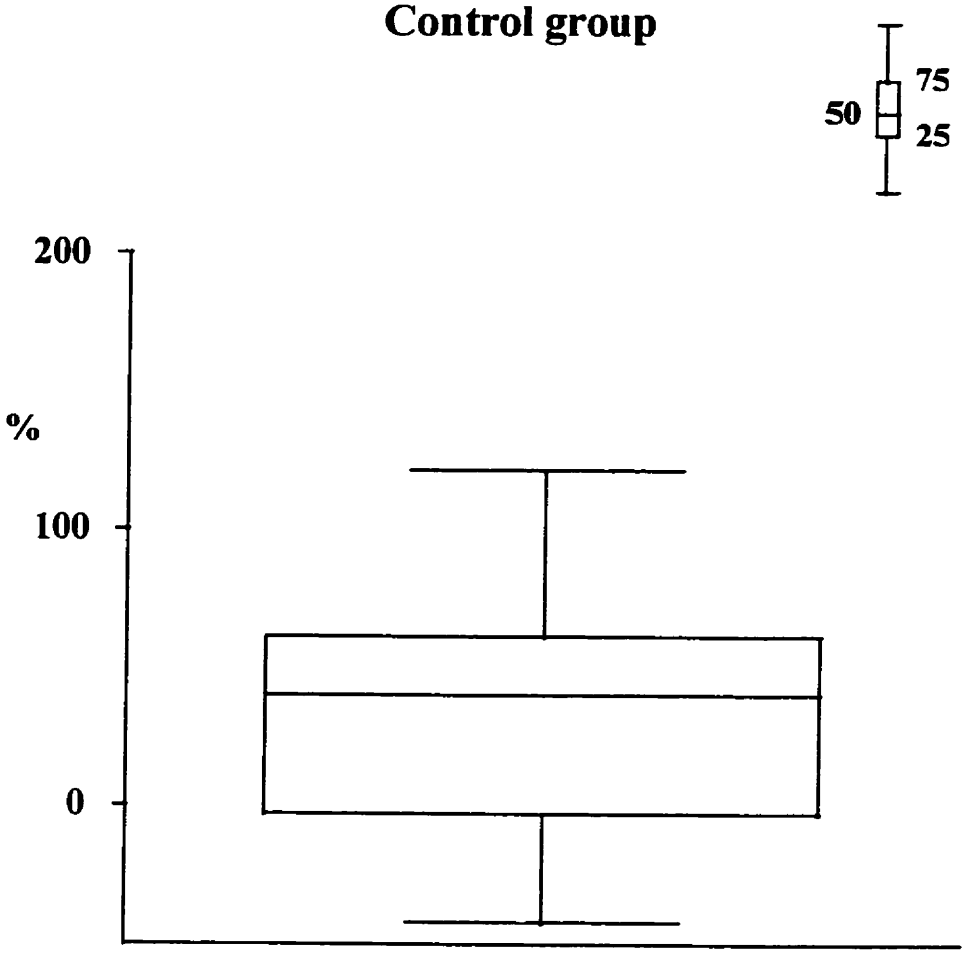
	Controls		**Relatives**		**Crohn's disease**
	Parents (n = 20)	**Children (n = 21)**	**Parents (n = 22)**	**Children (n = 13)**	**(n = 13)**
LM ratio post ibuprofen median (range)	0.0176 (0.0086 - 0.0486)	0.0238 (0.0158 - 0.0475	0.0202 (0.0128 - 0.0338	0.0304 (0.0104 - 0.1953	0.0488 (0.0232 - 0.2024)
% Change in LM mean (SEM)	38 (11)	32 (8)	44 (14)	105 (49)	81 (43)
Abnormal* (%)	0 (0)	0 (0)	3/22 (14)	4/13 (31)	1/13 (7.7)

*using 123 % as the upper cut off level of normality

1. Controls

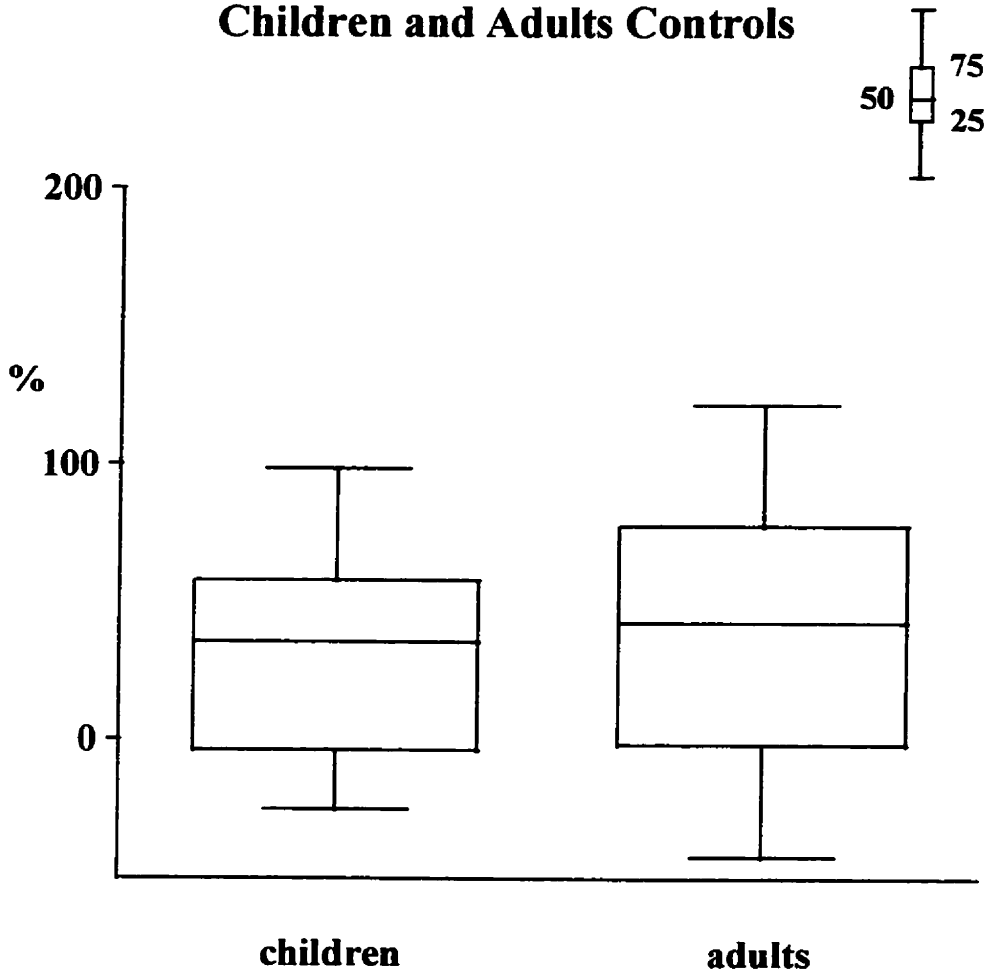
The Change in LM ratio after ibuprofen was normally distributed in the control group (Figure 9 and Shapiro-Francia W' test: $z = -0.29$, $p = 0.6$). In the total control group the mean Change in LM ratio was 35 % (SD = 44 %; SEM = 7 %) giving an upper cut off level for normal values of 123 % (mean + 2 SD). No subject in the control group had a response to ibuprofen above this level. As illustrated in Figure 10, the Change in LM ratio was similar in children and in adults.

Figure 9
Change in Lactulose/Mannitol Ratio
Control group



Box plot of change in lactulose/mannitol ratio after ibuprofen. The distribution is normal (Shapiro-Francia W' test for normality: $z = -0.29$, $p = 0.6$)

Figure 10
Change in Lactulose/Mannitol Ratio
Children and Adults Controls

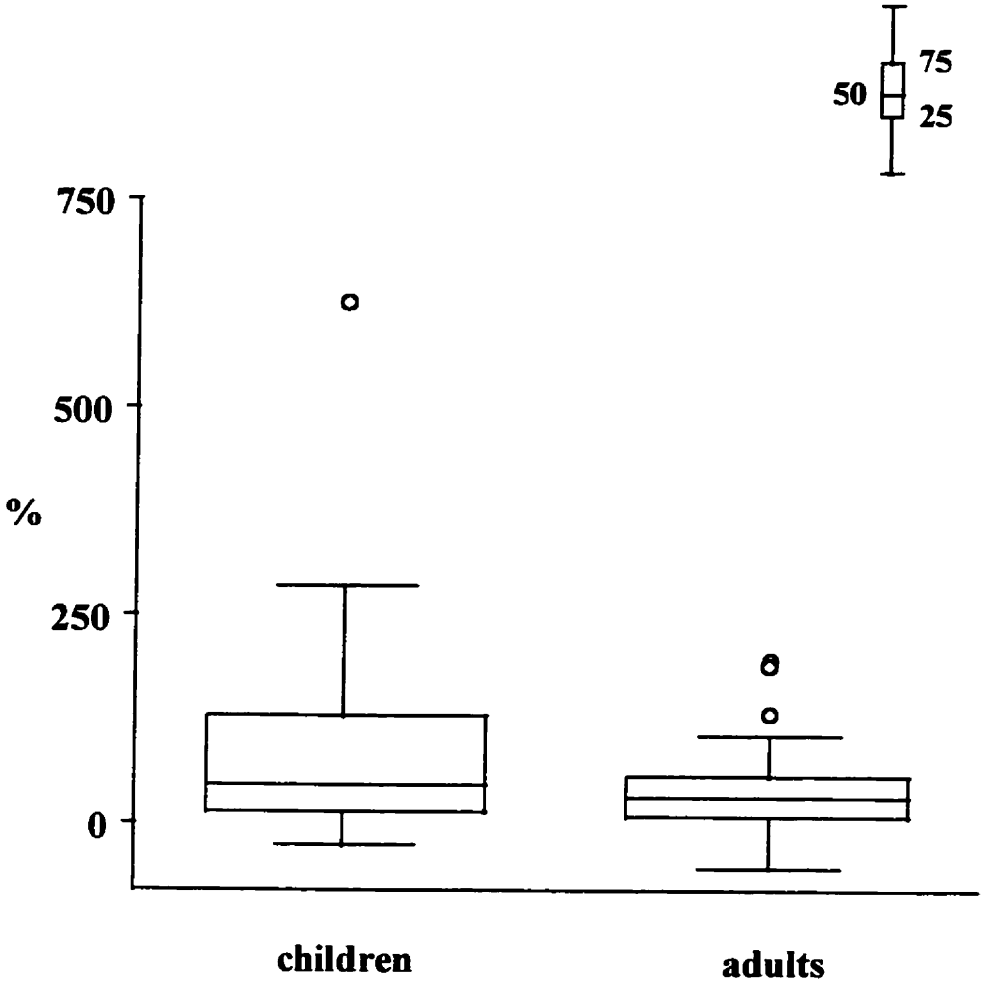


The change in lactulose/mannitol ratio after ibuprofen in children is similar to adults.

2. Healthy relatives

The mean Change in LM ratio after ibuprofen was 66 % (SEM = 20 %). When considered as an homogenous group healthy relatives had a Change in LM ratio similar to the control group (66% versus 35%, t test: t = 1.55, p = 0.13). However when relatives were classified as normal or abnormal using the upper reference level from the control group (mean + 2SD = 123%), seven individuals (3 adults and 4 children) had an abnormal response to ibuprofen challenge (7/35 = 20%, 95% CI: 7 % to 33%). The percentage of relatives with an abnormal response was significantly different from the control group (20% versus 0%, Fisher's exact test: p = 0.003). The difference in the proportion of abnormal individuals between healthy relatives and controls was of 20% with a 95% confidence interval of 7% to 33%. Among healthy relatives the Change in LM ratio seemed higher in children than in adults (Figure 11), but this difference did not reach statistical significance (t test: t = 1.47, p = 0.15).

Figure 11
Change in Lactulose/Mannitol Ratio
Children and Adults Relatives



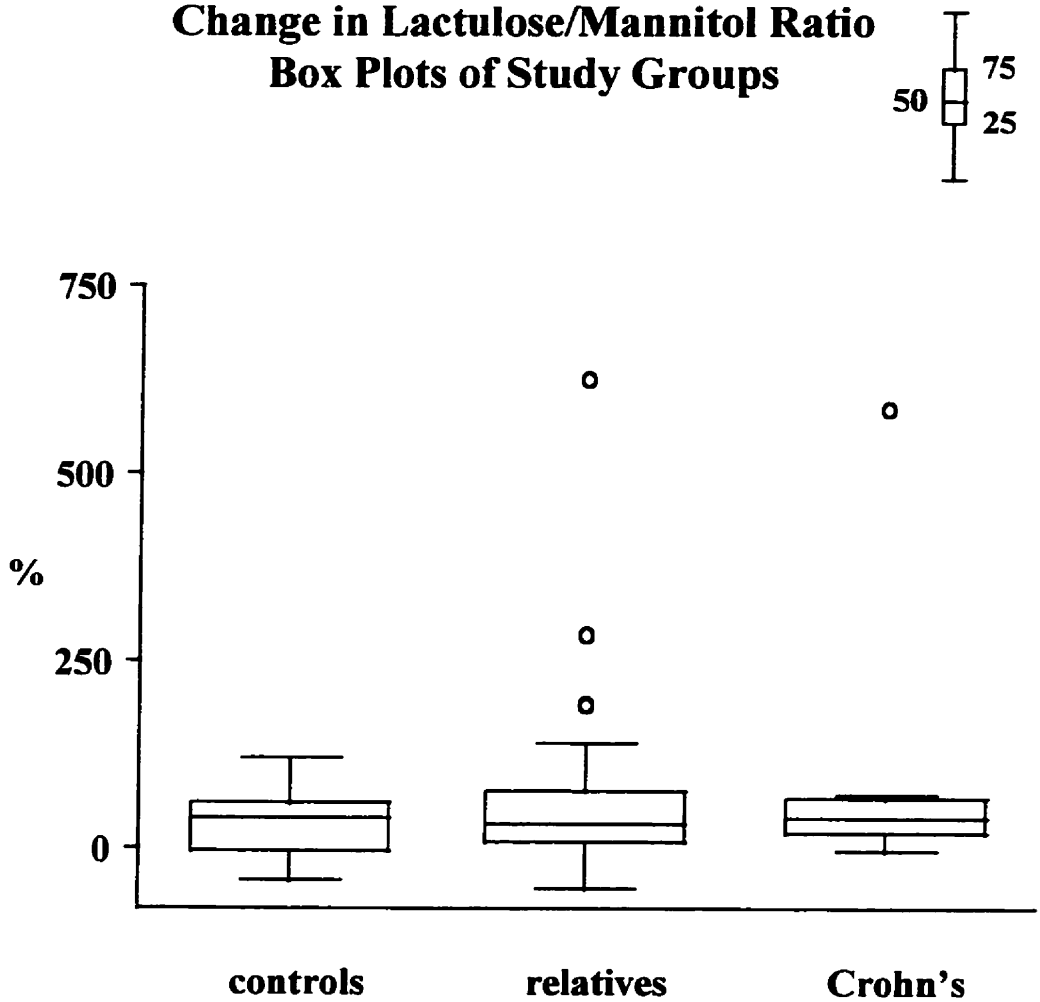
Mean change in lactulose/mannitol ratio after ibuprofen in children seems higher than in adults (105 % versus 44 %, t test: $t = 1.47$, $p = 0.15$).

3. Children with Crohn's disease

The mean Change in LM ratio was 81 % (SEM = 43 %), which was not significantly different from controls (t test: $t = 1.73$, $p = 0.09$). Only one 13 year old boy from this group had a response to ibuprofen above the normal limit (1/13 = 7.7 %). This boy relapsed 2 months after completing the permeability tests (bowel resection); he had a modified activity score of 25 when completing the test. The percentage of individuals with an abnormal response in children with Crohn's disease was not significantly different from controls (7.7 % versus 0 %; Fisher's exact test: $p = 0.26$).

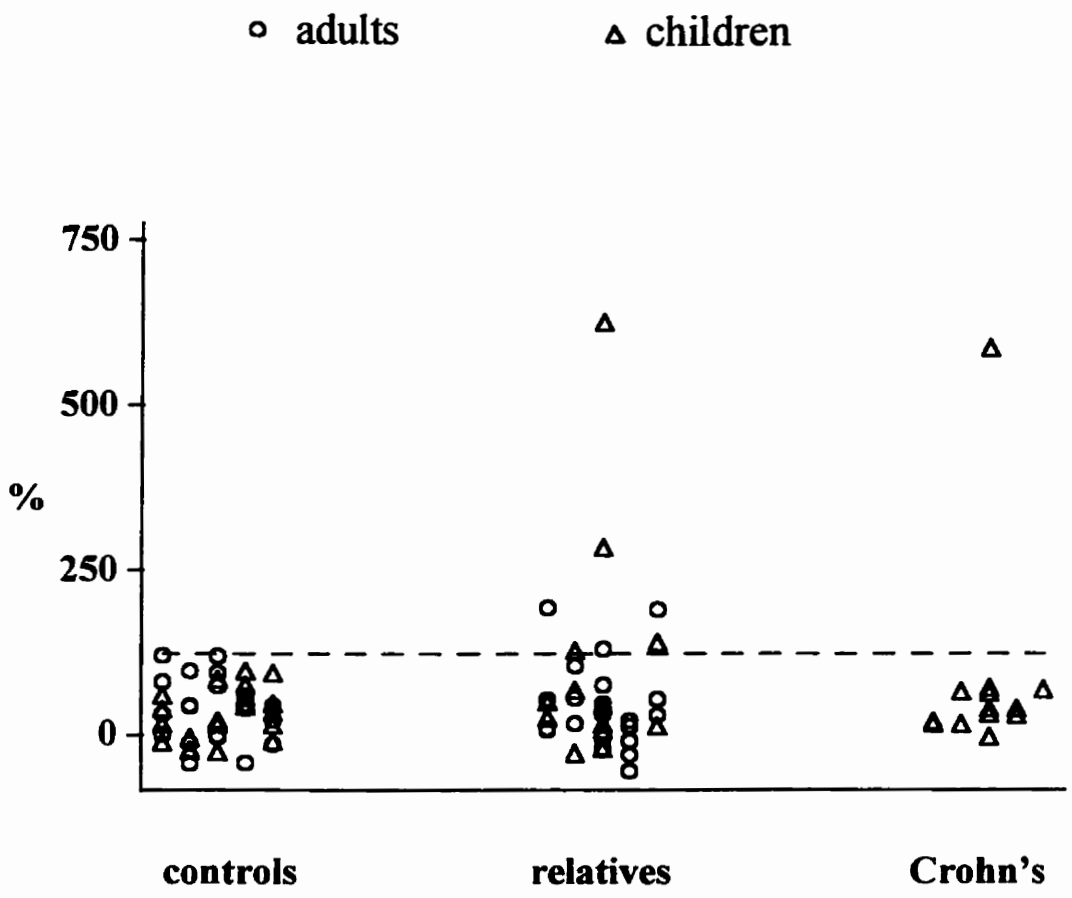
In summary a significantly higher percentage of healthy relatives compared to the control group had an abnormal response in intestinal permeability to the ibuprofen challenge (20 % versus 0 %). Figures 12 and 13 illustrate the data in the three groups with the upper cut off level defined above.

Figure 12
Change in Lactulose/Mannitol Ratio
Box Plots of Study Groups



The change in lactulose/mannitol ratio after ibuprofen is similar in controls, relatives, and children with Crohn's disease.

Figure 13
Change in Lactulose/Mannitol Ratio
Upper cut off level of normality
(dashed line = 123 %)

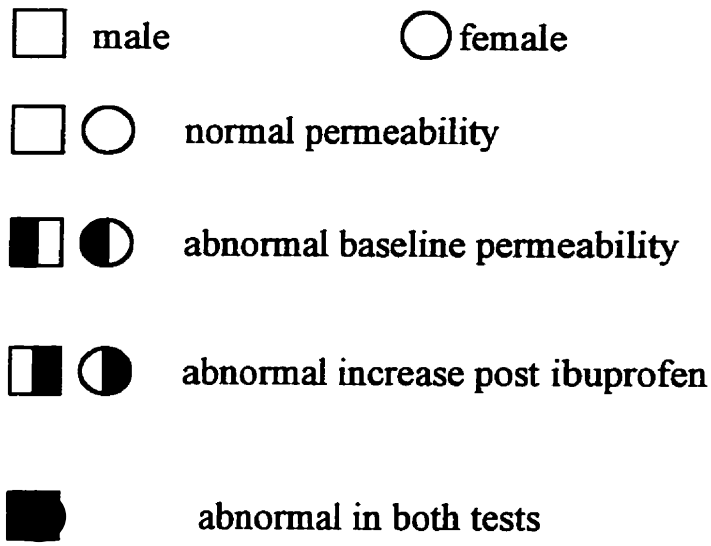


Using 123 % as the upper limit of normality (mean + 2 SD from the control group) for the change in lactulose/mannitol ratio after ibuprofen, the proportion of subjects with an abnormal increase in lactulose/mannitol ratio is higher in relatives than in controls (20 % versus 0 %, Fisher's exact test: p = 0.003).

Family Pedigrees of Abnormal Intestinal Permeability

The total number of families studied and the number of families with abnormalities in intestinal permeability was limited and only descriptive statistics could be done to assess concordance within family members of abnormalities of permeability or pattern of genetic transmission. Figure 14 illustrates abnormalities of intestinal permeability found in one control family (white, non-Jewish family) and six families with Crohn's disease (five white, non-Jewish, and one oriental family). For baseline intestinal permeability data the more conservative upper limit of normality for the LM ratio (0.0468) was used to classified individuals as abnormal. Excluding children with Crohn's disease (in whom abnormalities of intestinal permeability are expected) the proportion of families with abnormalities of intestinal permeability was higher in the Crohn's group (5/14 = 36 %) than in the control group (1/14 = 7.1 %), but this difference did not reach statistical significance (Fisher's exact test: $p = 0.17$).

Figure 14
Pedigrees of Abnormal Permeability



CD = Crohn's disease

FH+ = positive family history for Crohn's disease

Control family

Family # 1

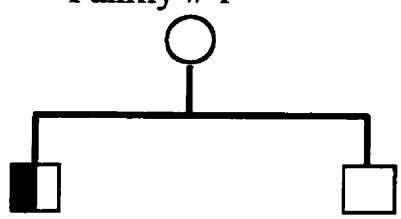
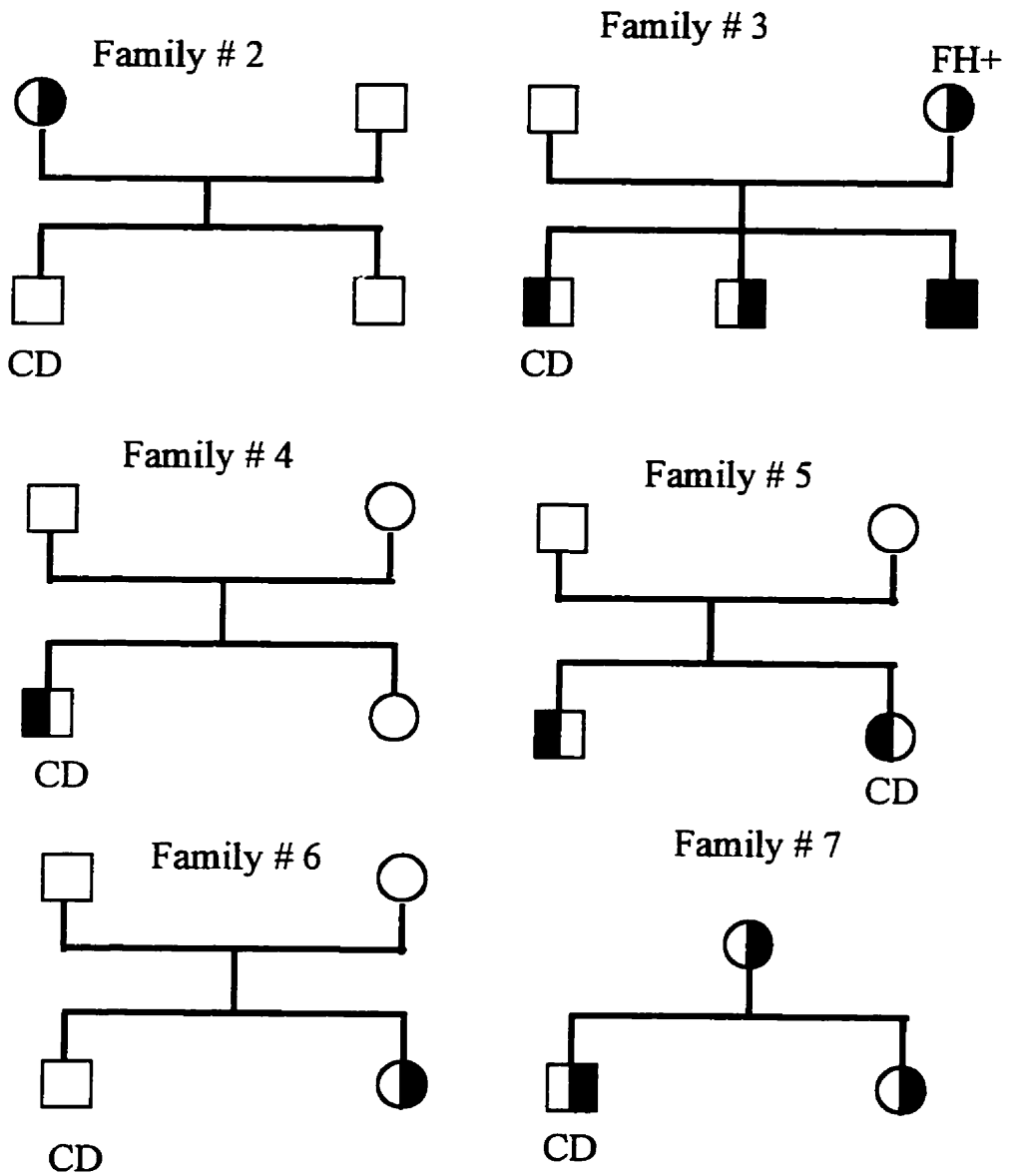


Figure 14
Pedigrees of Abnormal Permeability



6. Discussion

A. Review of Findings

Hollander et al. (98) first suggested in 1986 that healthy first-degree relatives of patients with Crohn's disease have increased intestinal permeability. This generated the attractive hypothesis that abnormal intestinal permeability could be a primary defect in Crohn's disease families leading to the exposure of the gastrointestinal immune system to an excessive antigenic load resulting finally, in some family members, in an inflammatory response affecting the gut and the development of the disease. A few investigators were able to reproduce these findings in the adult population (66,100), but several others have not detected abnormalities of intestinal permeability in healthy first-degree relatives of patients with Crohn's disease (104-109). Two explanations have been put forward to account for this controversy. First, since only 3 to 10 % of the first-degree relatives of affected patients will finally develop the disease, abnormalities of intestinal permeability would be only expected in a minority of healthy relatives studied. If the group of healthy first-degree relatives is considered as an homogenous group the difference in intestinal permeability between relatives and controls will be small and large samples of subjects (larger than in most of the reported studies) would be necessary to detect such differences (66). It has been suggested that a more fruitful approach would be to classify relatives as normal or abnormal using control data and to assess the percentage of abnormal individuals. Second, the increase in intestinal permeability may not be present constantly in an individual, but may only become manifest when this subject encounters factors recognized to increase intestinal permeability. Such an enhanced susceptibility to factors

increasing intestinal permeability has been demonstrated in healthy adult first-degree relatives of patients with Crohn's disease using a provocative test with aspirin (114) and appears in the adult population to improve discrimination of healthy first-degree relatives with an abnormal intestinal permeability (67).

The present study aimed to assess intestinal permeability in healthy first-degree relatives (adults and children from complete families) from the pediatric population of Crohn's disease and to see if these first-degree relatives had an exaggerated response to ibuprofen. Furthermore it was initially expected that more information could be gained on consistency within families of abnormalities of intestinal permeability by studying complete families of Crohn's disease children.

The present study suggests that the lactulose-mannitol test can be safely used with an ibuprofen challenge, in order to test baseline intestinal permeability and response to environmental factors in adults and children. None of the thirteen children with Crohn's disease completing the ibuprofen challenge had a flare up of the disease attributable to the test. The lactulose-mannitol protocol used in this study represents a simplification of previous investigations (65,66). This was done in order to simplify preparation of the drink, and urine collection (overnight urine collection, then no need for participating subjects to interrupt their routine activities), and avoid hyperosmolarity (no osmotic "fillers" added to the lactulose and mannitol).

This study showed that first-degree relatives of children with Crohn's disease have a baseline intestinal permeability similar to controls both when considered as an homogenous group (median LM ratio: 0.0176 versus 0.0165) and when classified as

normal or abnormal using the data from the control population (5.6 % versus 2.4 %). This is in agreement with several previous studies comparing baseline intestinal permeability between healthy first-degree relatives and controls (104-109). The sample completing the study was small and the power to detect such differences as significant was limited (less than 10 %). Nevertheless based on our best estimates (median values of LM ratio) and independently of power considerations, the difference in baseline intestinal permeability between healthy first-degree relatives and controls does not seem clinically significant.

The ibuprofen challenge showed that a significant proportion (20 %, 95 % CI: 7 % to 33 %) of healthy first-degree relatives had an exaggerated response in intestinal permeability. Healthy relatives were classified as abnormal using an upper cut off level from controls defined as mean + 2 SD. Some authors have used a more conservative upper cut off limit for normality of mean + 3 SD when studying intestinal permeability in healthy relatives of Crohn's disease (67). Even when using this more conservative upper normal limit, the proportion of healthy relatives with an exaggerated response to ibuprofen (4/35 = 11 %, 95 % CI: 0 % to 22 %) remained statistically significantly higher compared to controls (11 % versus 0 %; Fisher's exact test: p = 0.041). The ibuprofen challenge delineated a population of healthy relatives from pediatric Crohn's patients with abnormalities of intestinal permeability which was not evident from the study of baseline intestinal permeability. This confirms the findings reported in healthy first-degree relatives from adult Crohn's patients (67) and supports the hypothesis that an exaggerated response in intestinal permeability to external factors may be genetically determined and play a role in the development of Crohn's disease. The mean increase in LM ratio in healthy relatives

(66 %) was not statistically significantly different from the control group (35 %) reflecting the lack of power from the small sample studied, as it has been previously documented (66); but from a clinical point of view this difference deserves attention.

To our knowledge this is the first study using an ibuprofen challenge (one dose of 800 mg for adults and 10 mg/kg for children) to investigate changes in intestinal permeability in relatives of children with Crohn's disease. Comparison with the previous study using a similar methodology and aspirin (two doses of 1.3 g) in adult relatives of Crohn's disease (67) shows that the changes in LM ratio following ibuprofen are more moderate (Table 12). This was unexpected as an earlier study in healthy adults using similar doses of ibuprofen and aspirin showed a higher increase in intestinal permeability with the former (77). A pilot testing done among seven healthy volunteers (staff collaborating with the project) before the beginning of the study, showed a 50% increase in intestinal permeability after the ibuprofen challenge. For safety reasons a therapeutic dose of ibuprofen was chosen for children, but this does not seem to explain the moderate increase in intestinal permeability after ibuprofen as children responded similarly (Figure 10) or even more (Figure 11) than adults in our study. The sugar solution used with ibuprofen was slightly hypoosmolar whereas the solution used with aspirin was hyperosmolar. As hyperosmolar solutions increase intestinal permeability, this may have contributed to a higher response to NSAID challenge in the aspirin study.

Table 12

Intestinal Permeability

Aspirin and Ibuprofen Studies

NSAID (ref.)		Controls	Relatives	Crohn's
Aspirin (67)	**Subjects**	40	44	32
	Baseline LM mean	0.0174	0.0177	
	% Change in LM mean (SEM)	57 (13)	110 (13)	133 (25)
	Abnormal*: [95% CI]	0	35 % [20 - 53]	-
Ibuprofen	**Subjects**	41	35	13
	Baseline LM median	0.0165	0.0176	0.0271
	% Change in LM mean (SEM)	35 (7)	66 (20)	81 (43)
	Abnormal*: [95% CI]	0	20 % [7 - 33]	7.7 %

*using mean + 2SD of change in LM ratio in the control group as the upper cut off level of normality

Children with Crohn's disease demonstrated increased baseline intestinal permeability. This is in accordance to previous data in adults (84-86) and pediatric Crohn's disease patients (69,87,88). In response to the ibuprofen challenge they did not generally show an exaggerated response. This finding can be partially explained by the fact that almost half of them was receiving steroid treatment which may have blunted the response to the NSAID challenge. Of interest is the observation that 2 of the 4 Crohn's disease patients with abnormalities of intestinal permeability had a relapse in the three months following the test.

One control family and six Crohn's disease families showed abnormalities of intestinal permeability. In families with multiple affected relatives there was generally concordance in the type of abnormality of intestinal permeability (baseline or post ibuprofen abnormalities). The most heavily affected family (4 out of 5 family members with abnormalities of intestinal permeability) had a positive family history for Crohn's disease which is suggestive of a genetic link between abnormalities of intestinal permeability and Crohn's disease. However, as these family members lived together, an alternative explanation would be the influence of a shared environmental factor.

As a result of exploratory analysis there is some suggestion that children may have an increased baseline intestinal permeability compared to adults. Data from a previous study using a different protocol of the LM test also suggested this difference without reaching statistical significance (60). Among healthy relatives of Crohn's disease children seem more likely to have an exaggerated response to ibuprofen than adults (exaggerated response to ibuprofen among healthy relatives: 31 % of children and 14 % of adults).

In conclusion a subset of healthy first-degree relatives of pediatric Crohn's disease patients show an exaggerated response to NSAID challenge. Children with Crohn's disease in stable clinical condition have an abnormal baseline intestinal permeability but not an exaggerated response to NSAID challenge. These findings are compatible with a possible genetic link between abnormalities of intestinal permeability and Crohn's disease.

B. Chance, Bias, and Confounding

Chance

This study had as a primary objective to compare intestinal permeability in healthy first-degree relatives of children with Crohn's disease to controls. A secondary objective was to examine consistency of abnormalities within affected families. This implicated four basic comparisons and related statistical tests (healthy relatives and Crohn's disease children compared to controls both for baseline permeability and for the ibuprofen challenge). As a type I error of 5 % was accepted for the research question when planning the study, an adjustment for these four tests seems necessary setting the significance level at 0.0125. From the literature review it appeared that only the proportion of individuals with abnormal permeability should be compared between groups and not mean values. Even after adjusting for multiple tests the proportion of healthy relatives with an exaggerated response to ibuprofen remained significantly higher than in the control group (original p value = 0.003). The statistical significance of the difference in the proportion of Crohn's disease children with an abnormal baseline intestinal permeability is questionable

as the original p values were 0.001 using an upper cut off level of 0.0289 and 0.047 with the more conservative upper normal limit of 0.0468.

All the remaining analysis reported in the results section should be considered as exploratory and no firm conclusion can be drawn from them.

Selection bias

Selection bias could have been operative if participating families in either the control group or the Crohn's group had a prevalence of abnormal intestinal permeability different from the target population of healthy controls and relatives of children with Crohn's disease respectively.

About half the accessible population of families with an affected child followed at the Gastrointestinal Clinic from the Alberta Children's Hospital participated in the study. These families appeared to be similar to non participating families in terms of ethnic background and family history of inflammatory bowel disease. Furthermore, participating families did not have a significant prevalence of factors recognized to increase intestinal permeability (i.e. intestinal infections, celiac disease, alcoholism, use of NSAIDs). Then we do not have evidence that the increased intestinal permeability found in Crohn's families is explained by the selection of a sample with a particularly high intestinal permeability.

In the control group the possibility of a volunteer bias (145) exists. People volunteering to participate in a study are generally healthier than those refusing to participate. We may suspect that families in the control group are less exposed than the

general population to some factors increasing intestinal permeability (i.e. intestinal infections, alcoholism, use of NSAIDs). They may then represent a sample with particularly low intestinal permeability. However, when defining reference normal data for intestinal permeability it seems appropriate to study a sample free of external factors increasing intestinal permeability.

Informational Bias

Measurement bias in LM ratios is unlikely as urine samples were analyzed with a standard methodology and the status of participants (control or relative) was unknown by the laboratory staff. Misclassification of participants may have occurred to a minor degree in both groups (i.e. families unaffected by Crohn's disease classified in the Crohn's disease group and relatives of Crohn's disease classified as healthy controls). Children with Crohn's disease had been diagnosed using standard clinical, radiological, and endoscopical criteria (2) by experienced Pediatric Gastroenterologists and the possibility that these children may have had a different inflammatory condition of the gut is remote. Families who participated as controls may in fact have had a relative affected with Crohn's disease (although this was looked specifically for at enrollment) and would then have been classified in the Crohn's group. If any misclassification occurred it was unrelated to the results of permeability tests and thus non differential between the study groups. This would result in both groups being more similar and in a dilution of any difference in intestinal permeability.

Confounding

The group of healthy relatives was comparable to the control group in terms of ethnic background, number of members per family, gender and age distribution. These comparisons were done even though there was no evidence that any of these factors can influence directly intestinal permeability (54,59).

Several factors influencing intestinal permeability were recognized as potential cofounders before enrollment of participants (infection, celiac disease, chronic ingestion of alcohol or NSAIDs) and were considered as exclusion criteria. One subject in the control group and one subject in the relatives group regularly ingested moderate amounts of alcohol but not in the three days before testing. Both subjects had normal permeability tests. It has been suggested that smoking can decrease intestinal permeability (79,80) but this remains debated (67,81,82). The proportion of adult smokers in the control group was higher than in the relatives group. Although this difference was not statistically significant, if smoking effectively decreases intestinal permeability this may have confounded the results by lowering intestinal permeability in the control group more than in the relatives group. However intestinal permeability did not appear different between smokers and non smokers. Furthermore when smokers were excluded from the analysis the proportion of relatives with an exaggerated response to ibuprofen remained higher than controls (16 % versus 0 % respectively, Fisher's exact test: $p = 0.06$).

An unexpected problem which may have influenced the results was compliance with the ibuprofen challenge. As far as documented by interview of participants all took the ibuprofen dose as prescribed and permeability tests were well tolerated. Nevertheless

when examining the data of both tests it becomes apparent that a significant proportion of participants (22 %) had an intestinal permeability which decreased after the ibuprofen challenge compared to the baseline intestinal permeability. Although some variability in intestinal permeability is expected in each individual, the NSAID challenge should generally overwhelm this variation and produce an increase in LM ratio. It is thus legitimate to question if individuals showing a decrease after the ibuprofen challenge actually took the medication. Table 13 summarizes the distribution of participants with suspected non compliance (participants in whom the LM decreased after ibuprofen).

Table 13

Participants with Suspected Non Compliance

	Controls (n = 41)	**Relatives (n = 35)**	**Crohn's (n = 13)**
Adults	6	2	-
Children	6	5	1
Total	12	7	1

The proportion of individuals with suspected non compliance in the control group (29 %) appeared higher than in the group of relatives (20 %) although this difference did not reach statistical significance (Fisher's exact test: $p = 0.43$). Nevertheless if non compliance effectively occurred in these individuals, this may have falsely lowered the increase in LM ratio in the control group and then the group of Crohn's relatives (somewhat more compliant) could have falsely appeared as having an exaggerated

response to the NSAID challenge. When only participants with assumed compliance were reanalyzed (29 in the control group and 28 in the group of relatives), the mean percent increase in LM after ibuprofen was 57 % (SEM 6 %) in the control group and 89 % (SEM 24 %) in the group of relatives. One individual in the control group (1/29 = 3.4 %) and 7 subjects in the group of relatives (7/28 = 25 %) had an exaggerated response to the ibuprofen challenge. This difference appeared statistically significant (Fisher's exact test: p = 0.012; difference in proportions = 21.6 % with a 95 % CI from 4.2 % to 39 %). In summary compliance with the ibuprofen challenge did not appear to confound the study findings.

In summary we think that the study had reasonable validity and we do not think that the possible bias or confounding significantly influenced the results. The finding that healthy relatives of children with Crohn's disease have an exaggerated response to ibuprofen remains statistically significant even after considering the problem of multiple testing.

C. Limitations and Implications of the Study

The major limitation of the study comes from the small sample of participating subjects (28 families with 91 family members: 41 controls, 36 relatives of children with Crohn's disease and 14 children with Crohn's disease). The planned sample size (50 relatives and 50 controls) aimed to detect a significant difference between the group of

relatives and controls in the proportion of individuals with an abnormal response to the ibuprofen challenge. The final sample size was below the planned sample size, but still allowed us to detect a significant difference between relatives and controls because the magnitude of this difference was slightly higher than anticipated. The inability to achieve the planned sample size would have been a major handicap from the statistical point of view if the study findings had been negative (i.e. no statistically significant difference between relatives and controls) as then the actual power of the achieved sample size would be lower than planned. The final sample size was sufficiently large to detect clinically important differences in intestinal permeability between controls and relatives of Crohn's disease, and then to confirm in the pediatric Crohn's disease population findings from the adult literature. Nevertheless the new information which may have been expected from the study of complete families of affected individuals, was the eventual genetic distribution of abnormal permeability. Only limited graphical displays of pedigrees with abnormalities resulted from this study and no conclusion can be drawn on inheritance of permeability and its link to Crohn's disease.

Another limitation of the study related to the small sample of participants is the difficulty in establishing limits of normality. It has been demonstrated that reference limits based on sample sizes < 50 are imprecise with 95 % CI around the limits wider than the standard deviation of the observations (146). Studies on intestinal permeability using the LM ratio have generally defined cut off limits of normality using less than 50 subjects (Table 2). Furthermore small samples often fail to detect non normality of the distribution and frequently the upper cut of level for baseline LM ratios have been defined as mean +

2SD (Table 3). This is specifically the case for the two adult studies carried out in Calgary where the upper limits of normality for the baseline LM ratio where 0.022 (66) and 0.028 (67) respectively. As the LM ratios were not normally distributed in our population we preferred to use a more conservative limit (0.0468) obtained after logarithmic transformation. If we had ignored the non normality of our data we could have summarized baseline LM ratios in terms of mean and SD. The values in our control group would be the following: Adults, mean = 0.016 (SD = 0.006); Children, mean = 0.021 (SD = 0.009). Then the corresponding upper limits of normal, defined as mean + 2SD, would have been 0.028 for adults and 0.039 for children. In summary the upper limit of normality for the baseline LM ratio used in this study is higher than those previously used in Calgary because non normality of the data was detected and because children were part of the control group (there is a trend for higher permeability in children than in adults). If the participating sample had been larger, different limits of normality could have been defined for adults and children. Finally, the use of a small control group to define normal limits is also problematic as they then, by definition, have normal permeability. A different control group should ideally be used to validate the limits of normality, but considering the difficulties inherent in recruitment this could not be done.

This study suggests that ibuprofen can be safely used both in adults and children to assess the response of intestinal permeability to environmental factors. Nevertheless future studies measuring changes of LM ratios after a provocation test, should take into account the apparent difficulty of completing the two consecutive tests in a population of adults and children (generally adolescents). Even with this relatively simple and non invasive test

of intestinal permeability only about 50 % of subjects initially agreeing to participate finally completed the study. Furthermore in some participants compliance with the ibuprofen challenge may have been an unrecognized problem.

The study of genetic distribution of abnormalities of intestinal permeability and its link to Crohn's disease would probably necessitate segregation analysis involving hundreds of families with an affected pediatric member. It seems unlikely that such a study would be feasible using the permeability tests.

The observation that two Crohn's disease children with abnormalities of intestinal permeability relapsed shortly after the test deserves attention and is in agreement with data form adult Crohn's patients, where abnormalities of intestinal permeability were predictive of relapse. In one study 70 % of patients with an abnormal LM ratio relapsed within one year (97) and in another 27 % within 100 days (147). Further research could explore in the pediatric Crohn's disease population the predictive value of abnormalities of LM ratios. A second open and realistic area of research in the healthy first-degree relatives is to follow children with abnormal permeability to determine if any of them develops Crohn's disease in the future. In that case, ethical issues have to be carefully considered, as these healthy children would be classified as potentially having a chronic disease on the basis of intestinal permeability tests.

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Appendix A

CONSENT FORM

Research project: Crohn's disease: investigation of intestinal permeability across families of affected children.

Investigators: Drs. S. Zamora, B. Scott, D. Butzner, L. Sutherland, J Meddings, R Hilsden.

Funding Agency: Divisional Research Funds.

This consent form, a copy of which has been given to you, is only part of the informed consent. It should give you the basic idea of what the research project is about and what your participation will involve. If you would like more details about something mentioned here, or information not included here, you should feel free to ask. Please take the time to read carefully and to understand any accompanying information.

The cause of Crohn's disease is unknown, but genetic factors are believed to play an important role. The mucosal surface which lines the intestinal tract forms a protective barrier against toxic agents. There is evidence that in Crohn's disease, this barrier may be

more leaky than normal. Some of the healthy relatives of patients with Crohn's disease also have an increased leakiness of their intestine.

Our goal is to determine whether the increased leakiness of the intestinal mucosa is exaggerated in children with Crohn's disease and their families after the ingestion of ibuprofen. Ibuprofen is known to briefly increase the leakiness of the intestinal mucosa in healthy people.

To determine whether there is an exaggerated response to ibuprofen among families of affected children, we will be testing: healthy individuals without a family history of Crohn's disease, children with Crohn's disease and their family members. The test consists of drinking a sugar solution of lactulose and mannitol in the evening and collecting all urine overnight for analysis. One to five days later 1 dose of ibuprofen will be taken and then the test repeated. Limited historical information including name, age, family history, and disease history will be obtained and will be kept confidential.

Your participation in this study would require you to drink a sugar solution and collect your urine overnight on 2 separate occasions (at 1 to 5 days intervals). Prior to the second occasion on which the sugar solution is taken, 1 dose of ibuprofen will be taken (1 hour before drinking the solution).

The leakiness or permeability of the intestine is determined by ingesting a mixture of 2 sugars (lactulose and mannitol), and then measuring their excretion in the urine. This test has been extensively performed in children and adults and has not been associated with any discomfort, short or long term risks.

Ibuprofen is a common pain-killer and anti-inflammatory drug. It can briefly increase intestinal permeability even in healthy subjects. Ibuprofen can cause mild stomach upset. When taken for longer periods, it can occasionally cause stomach ulcers that can bleed, though this would be extremely rare if only 1 dose was taken. Ibuprofen should not be used by people who are allergic to ibuprofen or by those with a history of stomach or duodenal ulcers or kidney disease.

The benefit of this research project will be to determine whether there is a primary defect in intestinal permeability among families with Crohn's disease. This study may lead to a better understanding of the cause of Crohn's disease. However, if increased permeability is found in an individual this does not of itself predict whether that individual will develop the disease. Crohn's disease is probably the result of a multitude of factors and an increased intestinal permeability may be only one of these. It is our intention to publish the results of our study; however, participants will be identified only by initials. Participants wishing to, can request a summary of the results of the investigation once it is complete.

The investigator will, as appropriate, explain to your child the research and his or her involvement and will seek his or her ongoing cooperation through the project.

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to your participation or the participation of your child. In no way does this waive your legal rights nor release the investigators, sponsors or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without

jeopardizing you or your child's health care. Your continued participation should be as informed as your initial consent so you should feel free to ask for clarification or new information throughout your participation. If you have further questions concerning matters related to this research, please contact Dr. Zamora at 229-7211, Dr. Scott at 220-4556 or Dr. Butzner at 2204561.

If you have any questions concerning your rights as a possible participant in this research please contact the office of Medical Bioethics, Faculty of Medicine, University of Calgary, at 220-7990.

_____ _____

(Name) (Signature)

_____ _____

(Name of Witness) (Signature)

_____

(Date)

A copy of this consent form will be given to you. Please keep it for your records and future reference.

Appendix B

Pediatric Crohn's disease activity index (144)

History (recall, 1 week)	
Abdominal pain: none	0
Mild-brief, does not interfere with activities	5
Moderate-severe, longer lasting, affects activities, nocturnal	10
Stools (per day)	
0-1 liquid stools, no blood	0
up to 2 semi-formed with small blood, or 2-5 liquid	5
gross bleeding, or $\geq$ 6 liquid, or nocturnal diarrhea	10
Patient functioning, general well-being (recall, 1 week)	
no limitation of activities, well	0
occasional difficulty in maintaining age appropriate activities	5
frequent limitation of activity, very poor	10
Examination	
Weight	
weight gain or voluntary weight stable/loss	0
involuntary weight stable or weight loss up to 9%	5
weight loss $\geq$ 10%	10
Height	
at diagnosis (new patient)	
< 1 channel decrease	0
$\geq$ 1, < 2 channel decrease	5
$\geq$ 2 channel decrease	10
or	
follow-up	
height velocity $\geq$ -1 SD	0
height velocity < -1 SD, > -2 SD	5
height velocity $\leq$ -2 SD	10

Abdomen

no tenderness, no mass	0
tenderness, or mass without tenderness	5
tenderness, involuntary guarding, definite mass	10

Perirectal disease

none, asymptomatic tags	0
1-2 indolent fistula, scant drainage, no tenderness	5
active fistula, drainage, tenderness, or abscess	10

Extra-intestinal manifestations: fever $\geq$ 38.5 x 3 days over past week, definite arthritis, uveitis, Erythema nodosum, Pyoderma gangrenosum)

none	0
one	5
> two	10

Laboratory

HCT (%)

< 10 years	> 33	0	11-14M	> 35	0
	28-32	2.5		30-34	2.5
	< 28	5		< 30	5
11-19F	> 34	0	15-19M	> 37	0
	29-33	2.5		32-36	2.5
	< 29	5		< 32	5

ESR (mm/hr)

< 20	0
20-50	2.5
> 50	5

Albumin (g/L)

> 35	0
31-34	2.5
< 30	5

Appendix C

DATA COLLECTION CROHN'S PATIENTS

DATE OF CLINIC_____

AGE SEX WT (%) HT (%)

AGE AT DIAGNOSIS:

LOCATION OF DISEASE, PREVIOUS RESECTION (CM)

small bowel:

colon:

ileocolonic

DATE OF LAST REPLAPSE

DATE OF LAST SURGERY

MEDICATIONS OR NUTRITIONAL SUPPLEMENTS:

Drug name	Dose
_____	_____
_____	_____
_____	_____
_____	_____

REGULAR SMOKING (EVERY DAY OR ALMOST EVERY DAY):

- active smoking (at least 1 cigarette/cigar/pipe/day): Y / N
cigarette/cigar/pipe/day:

- passive smoking (are you exposed to at least 1 cigarette/cigar/pipe/day): Y / N
cigarette/cigar/pipe/day:

REGULAR ALCOHOL USAGE (EVERY DAY OR ALMOST EVERY DAY): Y / N

DATA COLLECTION PARENTS

	FATHER		MOTHER	
MEDICAL HISTORY				
cystic fibrosis	Y	N	Y	N
peptic ulcer disease	Y	N	Y	N
bleeding problem:	Y	N	Y	N
kidney disease	Y	N	Y	N
anemia	Y	N	Y	N
other condition	_____		_____	
	_____		_____	
	_____		_____	
SYMPTOMS				
diarrhea (BM/d)	Y	N	Y	N
abdominal pain	Y	N	Y	N
weight loss	Y	N	Y	N
fever	Y	N	Y	N
MEDICATIONS				
anticoagulant treatment	Y	N	Y	N
other	_____		_____	
	_____		_____	
ALLERGY	_____		_____	

HABITS

regular smoking (every day or almost every day):

- active smoking (at least 1 cigarette/cigar/pipe/day)

	Y	N	Y	N

cigarette/cigar/pipe/day:

- passive smoking (are you exposed to at least 1 cigarette/cigar/pipe/day)

	Y	N	Y	N

cigarette/cigar/pipe/day:

regular alcohol use (every day or almost every day)

	Y	N	Y	N

DATA COLLECTION SIBLINGS

name: _____ name: _____

MEDICAL HISTORY

cystic fibrosis	Y	N	Y	N
peptic ulcer disease	Y	N	Y	N
bleeding problem:	Y	N	Y	N
kidney disease	Y	N	Y	N
anemia	Y	N	Y	N

other condition _____ _____

_____ _____

_____ _____

SYMPTOMS

diarrhea (BM/d)	Y	N	Y	N
abdominal pain	Y	N	Y	N
weight loss	Y	N	Y	N
fever	Y	N	Y	N

MEDICATIONS

anticoagulant treatment	Y	N	Y	N

other _____ _____

_____ _____

ALLERGY _____ _____

HABITS

regular smoking (every day or almost every day):

- active smoking (at least 1 cigarette/cigar/pipe/day)

	Y	N	Y	N

cigarette/cigar/pipe/day:

- passive smoking (are you exposed to at least 1 cigarette/cigar/pipe/day)

	Y	N	Y	N

cigarette/cigar/pipe/day:

regular alcohol use (every day or almost every day)

	Y	N	Y	N

DATA COLLECTION PEDIGREES

FAMILIAL PEDIGREE (HEALTHY, AFFECTED RELATIVES, ETHNIC BACKGROUND):

GRAND PARENTS PARENTS SIBLINGS

O_W B O_____

name:_____

age: _____

O_W B O_____

O_W B O____

[]_W B O_____ name:_____

age: _____

O_W B O_____

name:_____

age: _____

* ***INDEX_____

[]_W B O_____

name: _____

age:_____

O_W B O_____

[]_W B O_____

name: _____

[]_W B O_____ age:_____

[]_W B O_____

name: _____

age:_____

W: white; B: black; O: oriental.

Appendix D

MANNITOL/LACTULOSE TEST

This test is simple and without any side effects. The drink is made up of 2 sugars (lactulose and mannitol) mixed with water. In rare conditions this test is not appropriate, please read the exclusion criteria at the back and if you have any of these conditions do not perform the test and contact us.

INSTRUCTIONS

First test date __ __ __

1 No alcohol should be taken in the 3 days before this test. If you need to take a medication for pain choose acetaminophen (Tylenol).

2 No food or drink is to be taken for 1 hours before taking the drink (small amount of water is okay)

3 At bedtime

* empty your bladder and discard urine
* pour the sugar solution from the small bottle into the plastic cup
* fill the same bottle with water and shake and then pour into cup
* repeat this step 2-3 times to ensure that all the orange liquid is emptied from the small bottle
* fill the cup with water up to 100 ml and stir until all of the sugar is completely dissolved
* drink all of the sugar solution (adults) or for children: _____ml of the solution

4 Throughout the night and first thing in the morning, collect all urine that is passed into the container provided and pour into the collecting bottle. Do not pass urine directly into the collecting bottle. Refrigerate the sample until you return it.

PLEASE KEEP THE CONTAINER OUT OF REACH OF CHILDREN.

Second test date __ __ __

Same instructions as above, but 1 hour prior to drinking the sugar solution you take all the medication which has been provided to you (Advil)

If you have any questions contact Dr. S. Zamora at 229 72 11 or the GI lab at 229 72 86.

MANNITOL/LACTULOSE TEST

EXCLUSION CRITERIA

you should not perform the test if you have any of the following conditions:

- allergy to ibuprofen (Advil)
- anticoagulant treatment
- regular use (every day) of alcohol or pain-killers (Tylenol is not a problem)
- gastroenteritis or diarrhea
- bleeding problem (hemophilia, thrombocytopenia)
- active gastric or duodenal ulcer
- cystic fibrosis, kidney disease
- any acute illness at the time of the test

DOSE

Weight	Sugar solution	Ibuprofen (Advil)
CHILDREN		
33 pounds or 15 kg	25 ml	150 mg
44 pounds or 20 kg	50 ml	200 mg
55 pounds or 25 kg	50 ml	250 mg
66 pounds or 30 kg	75 ml	300 mg
77 pounds or 35 kg	75 ml	350 mg
88 pounds or 40 kg	75 ml	400 mg
99 pounds or 45 kg	100 ml	450 mg
110 pounds or 50 kg	100 ml	500 mg
120 pounds or 55 kg	100 ml	550 mg
130 pounds or 60 kg	100 ml	600 mg
(or above) ADULTS	100 ml	800 mg

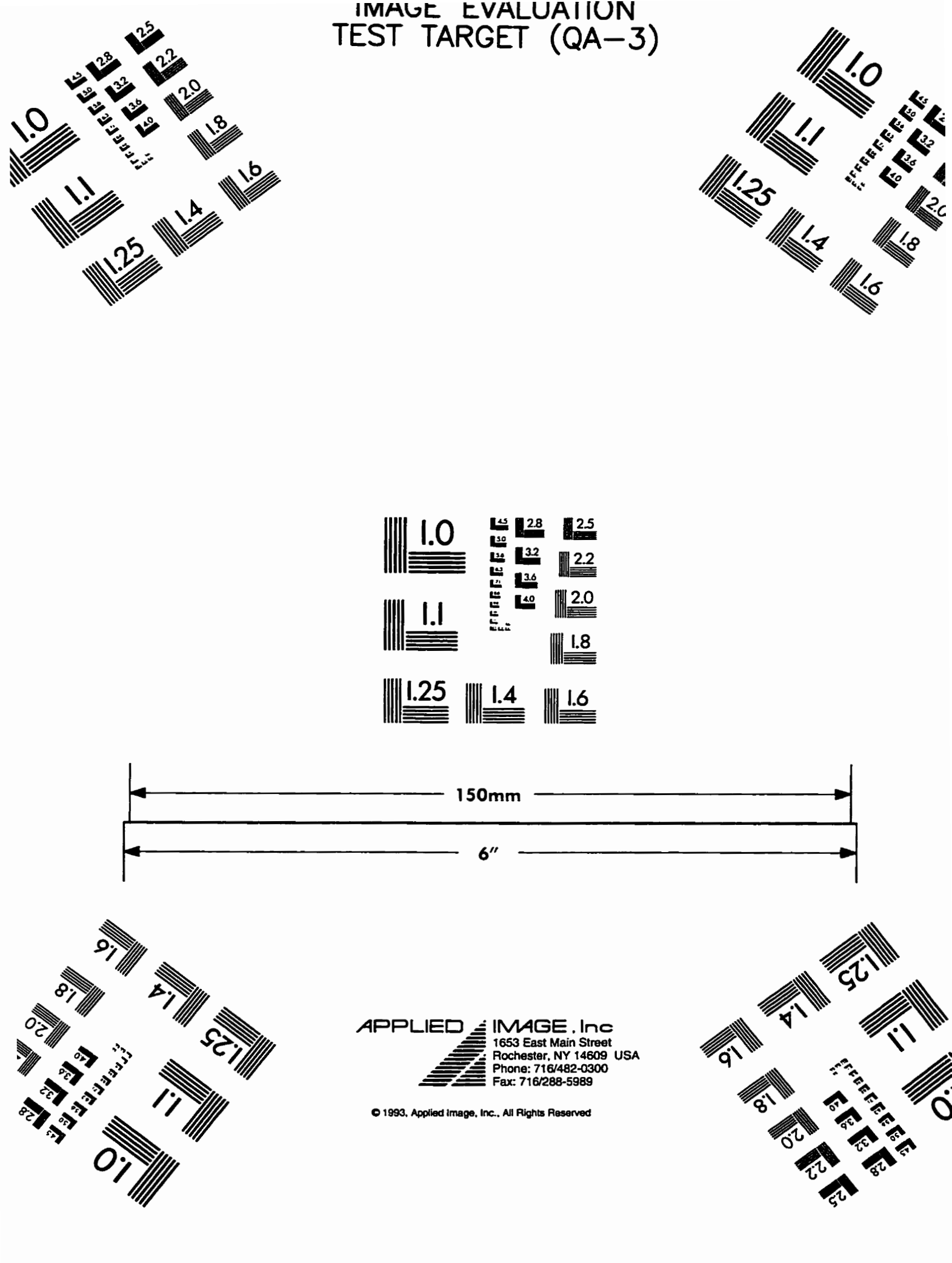
IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
150mm
6"
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