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The Characterization of Lipids and Lipoproteins in Patients with Crohn's Disease

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SOMMAIRE

La maladie de Crohn et la recto-colite hémorrhagique sont des maladies inflammatoires de l'intestin. La recto-colite hémorrhagique n'implique que le rectum et le colon tandis que la maladie de Crohn peut avoir des conséquences sur l'ensemble du tube digestif. L'incidence de la maladie de Crohn semble augmenter progressivement dans les pays industrialisés.

La pathogénèse, les manifestations cliniques et les options thérapeutiques sont plus clairement reconnues, mais l'étiologie de la maladie demeure tout à fait incertaine. De nombreuses hypothèses ont été proposées quand à l'étiologie de la maladie de Crohn, mettant en cause des facteurs génétiques, infectieux , immunologiques et nutritionels.

Les lipoprotéines cellulaires jouent un rôle essentiel dans la mobilisation et la synthèse de triglycérides et de cholestérol ainsi que dans le transport des vitamines liposolubles. Les radicaux libres oxygénés sont des produits instables et hautement réactifs impliqués dans la réaction inflammatoire. Mais, lorsque leur production est excessive et chronique, les radicaux libres épuisent la réserve de substances antioxydantes endogènes. Ce stress oxidatif occasionne la

péroxydation de lipides et de lipoprotéines. De plus, le stress oxydatif contribue aux lésions intestinales.

Nous avons formulé les hypothèses suivantes: 1) le déséquilibre entre les substances antioxydantes et pro-oxydantes est présent chez la population pédiatrique atteinte de maladie de Crohn; 2) il y a une modification du profil lipidique et des apolipoprotéines ainsi que de la composition des lipoprotéines.

Les paramètres de notre étude ont été mesurés chez 22 patients avec des caractéristiques cliniques bien établies et chez 10 contrôles. Nous avons effectué l'analyse des acides gras, la caractérisation des lipoprotéines VLDL, IDL, LDL, HDL_2 et HDL_3 ainsi que la quantification des apolipoprotéines A-I et B. De plus, nous avons determiné le taux plasmatique d'α-tocophérol, de γ-tocophérol, de rétinol, de β-carotène, de malonaldehyde et de glutathion.

C'est ainsi que nous avons démontré la présence de plusieurs modifications dans le taux de lipides et apolipoprotéines ainsi que dans la composition chimique des lipoprotéines des patients atteints de maladie de Crohn. Également, nous avons noté une diminution du retinol et une augmentation de MDA et de glutathion. Ces données

suggèrent qu'il y a réellement un déséquilibre entre substances antioxydantes et pro-oxydantes dans la population pédiatrique souffrant de maladie de Crohn comparée à un groupe contrôle.

En vue de l'importance métabolique des lipides et des lipoprotéines ainsi que de l'association connue des radicaux libres dans la pathogénèse des maladies inflammatoires de l'intestin, nous espérons que nos résultats contribueront au développement de thérapies limitant l'évolution de la maladie de Crohn.

RÉSUMÉ

Les maladies inflammatoires de l'intestin

Les maladies inflammatoires de l'intestin, y compris la maladie de Crohn et la recto-colite hémorragique, sont caractérisées par une inflammation intestinale chronique (1,2,3). La cible d'inflammation dans la recto-colite hémorragique est localisée au rectum et/ou au côlon, tandis que l'inflammation dans la maladie de Crohn peut avoir des conséquences sur l'ensemble du tube digestif mais attaignant, le plus souvent, la portion terminale de l'intestin grêle, le côlon et la région anale (1,4). Plusieurs études ont rapporté une augmentation régulière du nombre de nouveaux cas diagnostiqués chaque année dans les pays industrialisés (5,6). L'incidence de la maladie chez les individus entre 15 et 19 ans est de l'ordre de 16 par 100,000, tandis que chez les enfants en dessous de l'âge de 15 ans l'incidence est de 2.5 par 100,000 (7).

Manifestations cliniques de la maladie de Crohn

Les symptômes les plus fréquents de la maladie de Crohn sont les douleurs abdominales et la diarrhée. Les douleurs abdominales sont variables dans leur site et leur intensité. Par exemple, une douleur dans le quadrant inférieur droit suggère une atteinte à la portion

terminale de l'intestin grêle et/ou du caecum (5,7). Une asthénie, une anorexie et une légère augmentation de la température corporelle jusqu'à 37.5°C ou une fièvre élevée à 39°C – 40°C surviennent avec les poussées de la maladie (5,7). Une concentration plasmatique élevée du facteur de nécrose tumorale (TNF) est un élément clé dans l'anorexie provoquée par la maladie. Une perte de poids accompagne souvent un retard de croissance, de l'âge osseux et de la maturité sexuelle (8,9). Plusieurs facteurs peuvent contribuer à la perte de poids, tels que, la restriction des apports alimentaires favorisée par les douleurs abdominales, ainsi que les troubles d'absorption en cas d'atteinte de l'intestin grêle, d'entéropathie exsudative et d'ulcérations intestinales. Aussi, la prise médicamenteuse et l'augmentation des besoins énergétiques résultant de la fièvre ou d' infections favorisent la perte de poids (1,7,8). L'occlusion intestinale secondaire à une sténose, les abcès intra-abdominaux et les fistules est une des complications particulières à la maladie de Crohn.

Manifestations extra-intestinales

Les manifestations cliniques de la maladie de Crohn ne restent pas localisées seulement au niveau du système digestif. Les manifestations extra-digestives les plus fréquentes sont le rhumatisme périphérique (affectant les articulations des membres), les

manifestations oculaires telle que l'inflammation de l'iris, les manifestations cutanées comme l'érythème noueux et une faible densité osseuse (5,10).

L'étiologie de la maladie de Crohn

La cause de la maladie de Crohn demeure inconnue mais plusieurs hypothèses étiologiques ont été proposées, mettant en jeu des facteurs génétiques, infectieux, alimentaires et immunologiques.

Il existe des cas familiaux de la maladie, tel que des jumeaux ou des frères et soeurs où la fréquence des cas est nettement supérieure à la population générale. De plus, certaines maladies, comme, la spondylarthrite ankylosante sont associées très souvent avec la maladie de Crohn (5,7). Des sites génétiques de susceptibilité ont été identifiés sur le chromosome 3, 7, 12 et 16 (26-29).

Des facteurs infectieux sont aussi mis en cause. Par exemple, l'infection par le virus de la rougeole lors du troisième trimestre de la grossesse ou pendant la période périnatale pourrait jouer un rôle dans l'apparition de la maladie de Crohn plusieurs années plus tard (13).

Une étude épidémiologique au Japon a rapporté que l'augmentation de l'incidence de la maladie de Crohn est assocociée avec une hausse de la consommation des lipides, surtout des acides gras polyinsaturés de type oméga-6, l'apport de protéines de sources animales et l'augmentation du ratio entre les acides gras polyinsaturés oméga-6 et oméga-3 (33).

Parmi les cellules impliquées dans l'initiation de la réponse inflammatoire les macrophages et les monocytes produisent plusieurs médiateurs tels que le facteur de nécrose tumorale (TNF) et l'interleukine-1 (IL-1). Ces cytokines sont retrouvés à des concentrations élevées chez les individus atteints de la maladie de Crohn et semblent jouer un rôle clé dans la perturbation du système immunitaire de ces patients (18,45,46). TNF et l'IL-1 sont impliqués dans la réponse inflammatoire de phase aiguë, la fièvre et la production de protéines de phase aiguë. De plus, le TNFα induit une perturbation du métabolisme des lipides. Par exemple, le TNFα stimule la secrétion accrue des lipoprotéines de très basse densité (VLDL), et par conséquent, augmente la synthèse hépatique des triglycérides (47-50).

Métabolisme des lipoprotéines

Les lipoprotéines sont des macromolécules composées de triglycérides et de cholestérol estérifié dans le noyau et d'apolipoprotéines, phospholipides et cholestérol non-estérifié en périphérie. Les lipoprotéines jouent un rôle essentiel dans le transport, l'entreposage et la synthèse de triglycérides et de cholestérol ainsi que dans le transport des vitamines antioxydantes liposolubles, par exemple, la vitamine E.

Les principales classes de lipoprotéines sont: les chylomicrons (CM), les lipoprotéines de très basse densité (VLDL), les lipoprotéines de densité intermédiaire (IDL), les lipoprotéines de basse densité (LDL) et les deux sous-types de lipoprotéines de haute densité (HDL_2 et HDL_3). Les apolipoprotéines A (en particulier l'apo A-I) se retrouvent dans les HDL, tandis que les LDL ne contiennent que l'apoprotéine B (apo B).

Les radicaux libres et l'oxydation des lipoprotéines

La réponse inflammatoire joue un rôle de défense de l'organsime contre les agressions extérieures et, en cas d'infection vise à éliminer le pathogène. Cependant si un tel processus qui au départ est bénéfique, se prolonge, il devient néfaste et peut entraîner des lésions au niveau

des cellules et des tissus. La production de radicaux libres oxygénés est impliquée dans la réaction inflammatoire et leur présence joue un rôle considérable dans la pathogénèse de la maladie de Crohn (52,57). Lorsque la production de radicaux libres oxygénés est prolongée, telle que dans la maladie de Crohn, la réserve de substances antioxydantes est épuisée et par conséquent, il y une péroxydation des lipides et des lipoprotéines (19).

Les lipoprotéines sont aussi très susceptibles à l'oxydation puisque les triglycérides, le cholestérol estérifié et les phospholipides contiennent un grand nombre d'acides gras polyinsaturés (92). L'oxydation modifie aussi les apolipoprotéines et des répercussions importantes sont observées dans le métabolisme des lipides. Par exemple, si la séquence permettant la liaison entre l'apo B et son récepteur hépatique est modifiée, il y aura une accumulation plasmatique de LDL.

Les objectifs de notre recherche

L'inflammation chronique et la présence prolongée de cellules inflammatoires et de cytokines peuvent épuiser les réserves de substances antioxydantes et occasionner la péroxydation. Nous avons formulé l'hypothèse suivante: les modifications chimiques des

lipoprotéines ont des effets néfastes sur le métabolisme des lipides et par conséquent, jouent un rôle important dans la pathogénèse de la maladie de Crohn. Divers paramètres ont été mesurés tels que la concentration plasmatique des acides gras, des lipoprotéines, des apolipoprotéines, des vitamines antioxydantes liposolubles, du MDA et du glutathion.

Méthodes

Nous avons examiné le profil lipidique, la composition des lipoprotéines et le niveau de substances oxydantes et antioxydantes dans le plasma de 22 patients atteints de la maladie de Crohn et 10 contrôles sains. L'analyse des acides gras a été effectuée par une méthode modifiée par notre laboratoire (123). Les lipoprotéines étaient isolées par ultracentrifugation en fonction de leur densité, et par la suite nous avons déterminé la composition des lipides et des lipoprotéines (10,128,130). Les vitamines étaient mesurées par HPLC (124), et le niveau plasmatique de MDA par une méthode modifiée de Chirico (125). La concentration de glutathion était mesurée par la technique de Tietze (127).

Conclusion

Plusieurs différences ont été observées dans les niveaux de lipoprotéines, d'apolipoprotéines, d'acides gras, de vitamines antioxydantes, de malonaldéhyde (MDA), de glutathion ainsi que dans la composition des lipoprotéines se rapportant aux patients avec la maladie de Crohn.

Premièrement, la composition des acides gras est altérée et documente une augmentation des acides gras saturés et monoinsaturés, accompagnée d'une diminution d'acides gras polyinsaturés. De plus, nous avons observé l'élevation des ratios 16:1(n-7)/18:2(n-6) et ratio 20:3(n-9)/20:4(n-6), indiquant une carence en acides gras essentiels. Deuxièmement, les concentrations plasmatiques des lipoprotéines LDL et HDL ainsi que des apolipoprotéines A-I et B étaient plus faibles, tandis que le niveau de triglycérides était supérieur dans le plasma des patients par rapport au groupe contrôle. Troisièmement, la composition de certaines lipoprotéines était modifiée. Par exemple, la lipoprotéine VLDL montrait une augmentation du pourcentage de protéines et une diminution de pourcentage de triglycérides. Quatrièmement, la présence de péroxydation des lipides s'est manifestée par une augmentation du

MDA. Cinquièmement, l'équilibre entre les substances antioxydantes et oxydantes semblait être perturbé puisque nous avons mesuré une concentration plasmatique élevée de glutathion et une diminution de rétinol.

Compte-tenu de l'importance des acides gras pour maintenir l'intégrité des membranes cellulaires, des lipoprotéines dans la supplémentation des triglycérides et du cholestérol aux tissus périphériques, et des antioxydants pour la protection les cellules et les tissus contre les médiateurs inflammatoires tels que les radicaux libres oxygénés et les cytokines, plus d'études devraient être entreprises afin de nous permettre de développer de nouvelles thérapies qui cibleraient les facteurs qui contribuent à la pathogénèse de la maladie de Crohn.

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α	alpha
Å	angstrom
Apo	apolipoprotein
β	beta
γ	gamma
CETP	cholesteryl ester transfer protein
Chylomicron	chylomicron
CD	Crohn's disease
CDAI	Crohn's disease activity index
SDS	dodecyl sodium sulfate
EFA	essential fatty acid
EDTA	ethylenedinitrilotetraacetic acid
FMLP	formyl-methionyl-leucyl-phenylalanine
FA	free acid
FC	free cholesterol
γ	gamma
HDL_2	high density lipoprotein subtype 2
HDL_3	high density lipoprotein subtype 3
HPLC	high-performance liquid chromatography
IFNγ	interferon gamma
IL	interleukin
IDL	intermediate density lipoprotein
LCAT	lecithin:cholesterol acyltransferase
LDL	light density lipoprotein
MDA	malonaldehyde
μ	micro
Min	minute
MUFA	monounsaturated fatty acid
PL	phospholipid
PUFA	polyunsaturated fatty acid
PR	protein
Rpm	rotation per minutes
SEM	standard error in the mean
TC	total cholesterol
Triglyceride	triglycerides
TNFα	tumour necrosis factor alpha
VLDL	very light density lipoprotein
IL-1RA	interleukin-1 receptor agonist

Bless the Lord, O my soul,
And forget not all his benefits:
Who forgives all your iniquities,
Who heals all your diseases,
Who redeems your life from destruction,
Who crowns you with lovingkindness and tender mercies,
Who satisfies your mouth with good things,
So that your youth is renewed like the eagle's.

Psalm 103:2-5

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Inflammatory bowel disease

Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, is characterized by chronic intestinal inflammation (1,2,3). The target of inflammation in ulcerative colitis is mainly the mucosa of the colon, whereas in Crohn's disease any region along the entire gastrointestinal tract may be affected, from the mucosa through to the serosa (1,4). Investigators have noted that the incidence of Crohn's disease in the adult population of Western nations has increased steadily over the past four decades; the frequency of this disease in the pediatric population has followed a similar trend (5,6). The incidence of Crohn's disease in individuals between 15 and 19 years of age is estimated at 16 per 100,000, and for children below the age of 15 years it is approximately 2.5 per 100,000 (7).

Clinical features of Crohn's disease

Primary symptoms

Abdominal pain, chronic diarrhea and prolonged weight loss are the most common gastrointestinal manifestations. However, fever, growth retardation and anal involvement are very frequently associated

with the disease (5,7). The location of the abdominal pain generally reflects the site of bowel involvement. For example, pain in the right lower quadrant is usually indicative of the disease affecting the terminal ileum and/or cecum (5,7). Diarrhea can range from 2 to more than 6 loose bowel movements. Bloody diarrhea is present when there is involvement of the colon or ulceration in the small bowel (5). Fever can persist for long periods and may be low grade or spiking (5,7). Anal inflammation often leads to fissures, fistulas and tags, and is present in 37% of patients when initially diagnosed (5,7).

Weight loss is frequently associated with growth retardation, delayed bone age and sexual maturation (8,9,10). In 85% of cases when a child is diagnosed with Crohn's disease, weight loss has already occurred (7,11). Growth retardation is observed in 40% of children, and nearly 50% have a weight-for-age measurement inferior to 90% of the expected value (7,12,13). In 20% to 35% of cases, growth failure is permanent when the onset of Crohn's disease was during childhood, resulting in short stature as adults (7). Growth failure is usually due to chronic malnutrition, and sometimes because of exposure to high doses of corticosteroids as well. It may precede the clinical illness by several months or years and not uncommonly continue, during states of remission (5,7). Malnutrition is a common serious complication, and has a multifactorial etiology in children with Crohn's disease (8). High levels

of inflammatory cytokines such as αTNF possibly mediate the most important cause of anorexia. Nutrient intake is often inadequate, since food is associated with abdominal pain and diarrhea (1,8). Also, many patients follow a very restricted diet, which in most cases lacks validity (7,8). Malabsorption of nutrients may occur due to a decreased absorptive surface resulting from the chronic inflammation, bacterial overgrowth, bowel resection, bile acid loss or drug therapy (1,14). Corticosteroids, for example, inhibit the absorption of calcium (1,8). A significant loss of protein, electrolytes, minerals and trace elements can result from the breakdown of the mucosal barrier, intestinal bleeding, diarrhea and drug treatment (1,7,8). A theoretical increase in energy requirements is expected in periods of relapse, but has generally not been substantiated (1).

Secondary symptoms

The clinical manifestations of Crohn's disease are not restricted to the gastrointestinal system. Extraintestinal manifestations include peripheral arthritis, ocular and dermatologic complications, as well as decreased bone density (5,15). One third of patients appear to have joint involvement, particularly affecting the knees, ankles, hips and shoulders (7,15). Peripheral arthritis is concurrent with the severity of

the colonic involvement in patients with Crohn's disease (7,15). Ocular complications such as uveitis, scleritis or episcleritis are most frequently observed in patients with colitis or ileocolitis (5,15). One half of patients with Crohn's disease who manifest peripheral arthritis, also show ocular complications (5). Therefore, frequent ophthalmologic examinations are strongly recommended (5). Dermatological complications such as erythema nodosum are present in 10% of individuals with Crohn's disease and associated with the severity of the disease activity (5,7,15). Also, 75% of patients with cutaneous manifestations develop arthritis (5).

The decreased bone density observed in patients with Crohn's disease may be the result of several nutritional, metabolic, pharmacologic and immunologic factors (10). Calcium intake is frequently inadequate and its malabsorption due to bowel dysfunction and corticosteroid therapy is common (8). Lipid malabsorption and the loss of bile acids can cause a vitamin D deficiency (8). Abnormal bone metabolism could also be explained by the increased production of cytokines in the inflamed intestinal mucosa (5).

Distribution of gastrointestinal involvement in Crohn's disease

Endoscopic and radiologic evaluation of pediatric patients have revealed certain patterns of intestinal involvement (5,7). In approximately 75% of children the disorder is present in the terminal ileum and either the cecum or the ascending colon (5,7). Between 10% to 15% of patients have disease limited to the colon and 15% to 30% of individuals have anal involvement (5,7). In addition, upper gastrointestinal endoscopies with mucosal biopsies indicate that nearly 30% of patients display involvement of the esophagus, stomach or duodenum (5,7).

The mucosal barrier and inflammation

The mucosal membrane, which consists of an epithelial layer and a subjacent layer of connective tissue, covers the luminal surface of the digestive system (4). The mucosa is not only involved in absorption and secretion, but also in protection against microbial agents, dietary antigens and other noxious agents, which have the potential to cause tissue injury (4,5). The gut-associated lymphoid tissue, including the aggregated lymphoid cells found in Peyer's patches in the ileum, is an

important element of the body's defense mechanisms against microbes and other pathogens (4,7,9).

The principal role of inflammation is to assist in restoring tissue homeostasis by eliminating pathogens, averting their proliferation and providing the necessary components for tissue repair (4). However, if the inflammatory process persists for prolonged periods, as it occurs in inflammatory bowel disease, the mediators generated by these inflammatory cells induce tissue injury, fibrosis and, loss of function to the wounded area (3,5,7). The mediators of such inflammation include eicosanoids, platelet-derived growth factor, biogenic amines, kinins, complement-derived peptides, cytokines, chemokines as adhesion molecules, as well as reactive oxygen and nitrogen species (3,7).

It has been reported that patients with inflammatory bowel disease produce excessive quantities of eicosanoids at sites of tissue inflammation (2). Eicosanoid is a collective term for compounds derived from arachidonic acid, a 20-carbon polyunsaturated fatty acid containing four double bonds (16). Examples of eicosanoids include prostaglandins and leukotrienes (16). Prostaglandins intensify vasodilatation and edema formation, whereas leukotrienes act as chemotactic agents and amplify the production of free radicals (2,4). Platelet activating factor is another phospholipid mediator that also

participates in tissue edema by increasing vascular permeability (2). Colonic mucosal cells derived patients with inflammatory bowel disease have an elevated production of platelet activating factor in vitro (2).

Histamine, a biogenic amine, is released subsequent to tissue injury, resulting in vasodilatation, increased vascular permeability and edema (2, 4). Activation of complement-derived peptides, which is a collective term for approximately 20 proteins involved in the complement system, promotes acute inflammation and elimination of pathogens (2). An increased production and metabolism of these polypeptides has been reported in patients with Crohn's disease (2).

Cytokines are pleiotropic small glycosylated peptides, secreted by activated immune cells that modify the immune response (2,7). Cytokine production is a necessary element in establishing immune homeostasis; however, if they are secreted in excessive quantities or persistently, cytokines induce many of the symptoms associated with inflammatory bowel disease (2,7). For example, IL-1 and TNFα provoke fever and anorexia (2,7). IL-1, IL-3, TNFα and IFNγ induce diarrhea by altering the metabolism and regeneration of epithelial intestinal cells (2,7,17,18).

Reactive oxygen and nitrogen species, such as superoxide anion, hydrogen peroxyde, hydroxyl radical and nitric oxide, are products of normal metabolism; however, their concentrations increase during inflammation (3,19).

In inflammatory bowel disease excessive production of reactive oxygen species and nitric oxide exists, creating a pro-oxidative imbalance or "oxidative stress" (3,19-21). These pro-oxidants are toxic to the intestinal mucosa, inhibit essential protein function and lead to tissue damage (3,19,20,22). Hydrogen peroxyde and nitric oxide increase the secretion of water and electrolytes in the inflamed intestinal mucosa and may contribute to diarrhea in inflammatory bowel disease (4). The imbalance between reactive oxygen and nitrogen metabolites and antioxidants induces the oxidation of lipids, proteins, carbohydrates and DNA (19,23).

Etiology of Crohn's disease

There has been considerable progress in the understanding of the pathogenesis, clinical features and management of Crohn's disease. However, the etiology of this disorder remains unknown (5,7,9,24). Expression of the disease is contingent upon a genetically susceptible individual being vulnerable to environmental influences, displaying

abnormalities in the intestinal mucosal barrier and having a dysfunctional immune response (5,7,9,24).

Genetic susceptibility

The genetic basis for Crohn's disease is supported by epidemiological surveys investigating disease frequency between geographical areas, ethnic groups and family history of patients as well as the association of the disorder with other genetically transmitted diseases. The incidence of the disease in certain ethnic groups, for example, the Ashkanazi Jews, is higher than in the general population after controlling for age and gender differences (7,9).

In a study conducted at Hôpital Sainte-Justine, it was predicted that 13% of pediatric patients with Crohn's disease had a first-degree relative affected with inflammatory bowel disease (7). After adjusting for age differences, it was observed that siblings of individuals with Crohn's disease have a 7 to 10% greater risk of developing the disease (7). Furthermore, children with Crohn's disease show a higher frequency of Turner's Syndrome, glycogen storage disease type Ib, Hermansky-Pudlak syndrome and cystic fibrosis (5,7).

Despite the association between Crohn's disease and the factors mentioned above, no single gene locus can be ascribed since the illness is not transmitted by simple Mendelian inheritance but rather involves multiple susceptibility loci and genetic heterogeneity (25,26). Evidence has been provided for the presence of susceptibility loci on chromosomes 3 (marker D3S1573), 7 (marker D7S669), 12 (marker D12S83) and 16 (IBD1 locus in the pericentromeric region) (25-29).

Environmental influences

Irrespective of "susceptible genes", Crohn's disease will not be expressed phenotypically in all individuals. Environmental factors, such as infectious agents and dietary factors have been implicated as essential triggering agents for Crohn's disease (7,9). Among microbes, Mycobacterium paratuberculosis, paramyxovirus (measles) and Listeria monocytogenes have been actively investigated as initiators of Crohn's disease. However, the results of these studies are still inconsistent and inconclusive (7,9,30). Nevertheless, perinatal infections seem to be related to the incidence of Crohn's disease (24). In a Swedish population-based, case-control study, perinatal health incidents were estimated to contribute to 40% of the observed Inflammatory bowel disease cases (13). A greater relative risk for developing inflammatory

bowel disease was observed in individuals with postnatal infections or in which the mother had a prenatal or postnatal infection (such as influenza, measles, varicella and rubella), as compared with individuals with no health events in the perinatal period (13). Evidence has been put forth suggesting that persistent measles virus infection may play a role Crohn's disease (31). An increased incidence of CD has been observed in individuals born successive to measles epidemic (32).

Taken together, these controversial studies suggest that perinatal infections in individuals genetically susceptible to inflammatory bowel disease may alter the immune response and an environmental factor may subsequently trigger the onset of the disease (13,24).

Dietary factors have also been postulated as important environmental influences in the development of Crohn's disease. An epidemiological study in Japan reported an association between the increase in Crohn's disease and the rise in dietary fat consumption, particularly of n-6 polyunsaturated fatty acids, with a concomitant decrease in n-3 polyunsaturated fat intake, and an increase in the amount of animal protein consumed (33). In addition, epidemiological studies have proposed a relationship between diets high in refined sugar and lacking fresh fruits and fiber to increased inflammatory bowel disease activity (34-37). Disease activity has also been correlated with

antibody titers against certain food antigens, such as milk proteins (7,9,33,38). However, these antibodies are considered a secondary phenomenon. Recently, antibodies to Saccharomyces cerevisiae, or brewers' and bakers' yeast, have been shown to be specific to CD (17). In this context, it is worthwhile to note that industrialized countries have undergone extreme dietary modifications over the past 50 years. For instance, dietary fat intake has seen a six-fold increase from 1945 to 1985 (33). Although clinical studies linking one particular nutrient to the development of Crohn's disease are not available or feasible, nutritional modifications have a primary role in the management of Crohn's disease (17). Total parenteral nutrition or exclusive elemental diet therapies are effective in inducing remission in individuals with Crohn's disease, while low-residual diets only assist in alleviating symptoms (8,39,40). It is interesting to note that elemental diets that reduce inflammation and control disease activity are low in n-6 polyunsaturated acids (17).

Intestinal permeability

As discussed above, chronic inflammation stimulates the release of mediators that increase mucosal permeability, thus altering the intestinal epithelial integrity (7,30). Heightened permeability of the epithelium in the distal ileum and colon enables microbial agents or their

products, such as, N-formyl-methionyl-leucyl-phenylalanine (FMLP), peptidoglycan-polysaccharide polymers and bacterial endotoxin (lipopolysaccharide), as well as dietary antigens present in the intestinal lumen, to breach the mucosal barrier and contact the immune cells of the gut associated lymphoid (7,9,30). The uptake of antigenic and toxic agents, many of which are chemotactic, provokes and prolongs the inflammation (30). Abnormally, the invulnerable mucosal barrier prevents luminal dietary and bacterial antigens from entering the local circulation, and if antigenic stimulation does occur, inflammation is well-regulated (5). However, individuals with inflammatory bowel disease have shown abnormalities in the immune regulation that consequently results in tissue damage (7,9,30).

Dysfunction of the intestinal immune system

Soluble products of activated immune cells and inflammatory response, such as certain cytokines and reactive oxygen and nitrogen intermediates, precipitate apoptosis and cell death, fibrosis and loss of tissue function (30). Amplification of the inflammatory pathways is determined by immunoregulatory and proinflammatory cytokine levels (7,30,41). In animal model studies it has been observed that fats enriched in n-3 polyunsaturated fatty acids or n-9 monounsaturated fatty

acids or deficient in n-6 polyunsaturated fatty acids lessened the immune's system response to proinflammatory cytokines (42).

The cytokine interleukin-2 (IL-2) is of significant importance in regulating the intestinal immune response to inflammation and it has been proposed that an anomalous regulation of IL-2 may be related to the immunologic dysfunction observed in Crohn's disease (41). Investigators have found an increased expression of the gene coding for IL-2 in intestinal lesions of patients with active Crohn's disease (41,43,44). Furthermore, the serum concentration of the IL-2 receptor has been suggested as a factor reflecting inflammatory disease activity (41).

Proinflammatory cytokines, particularly IL-1, IL-6 and TNFα heighten the level of immune system activity and are increased in the mucosa of individuals with active Crohn's disease (41-43). These three cytokines act as endogenous pyrogens and are involved in the acute-phase response, with the production orosomucoid, alpha-1 and alpha-2 macroglobulins, transferrin and C-reactive protein (8,41,43). Recent reports have suggested that the high production in the intestinal mucosa TNFα, IL-1α and IL-1β induce intestinal dysfunction in the pathogenesis of inflammatory bowel disease (17).

IL-1 functions synergistically with other cytokines to stimulate immune cells (45). In contrast to normal controls, patients with Crohn's disease have an elevated ratio of IL-1 to its endogenous receptor antagonist (IL-1RA), which is a protein that binds to the IL-1 receptor and suppresses inflammation (2,7,30,41).

Investigators have reported that the gene expression of TNFα was greater in patients with active Crohn's disease as compared to healthy subjects (18,46). TNFα induces perturbations in the metabolism of lipids. For instance, there is an upsurge in hepatic triglyceride synthesis and a rise in serum triglyceride levels, which is subsequent to the increase in very-light-density lipoprotein secretion in response to tumour necrosis factor (47-50). TNFα has also been shown to adversely affect intestinal epithelial cell lipid handling (51).

Reactive oxygen intermediates and lipid peroxidation in Crohn's disease

Free radicals play a substantial role in inflammation and immune function since they aid in eliminating pathogens by exposing them to toxic conditions (52). Nonetheless, when the production of reactive oxygen species is chronic and surpasses the body's antioxidant

defenses, significant cellular damage ensues, eventually leading to loss of tissue function (2,52-54). The precise role of reactive oxygen species in recurrent intestinal inflammation is not well defined. However, considerable evidence suggests that free radicals are involved in the pathogenesis of Crohn's disease (19,23,52,55-57).

Free radicals are highly reactive chemical species that contain one or more unpaired electrons (2,20,52). Reactive oxygen intermediates, which include superoxide and hydrogen peroxide, increase the permeability of the mucosa and blood vessels in inflamed areas (23). Hydroxyl radicals are highly reactive species that invade cells and damage proteins, carbohydrates, lipids and nucleic acids (23,52). Hydroxyl and hydroperoxyl radicals target the unsaturated fatty acids and other lipids present in the phospholipid bilayer of cells and consequently initiate lipid peroxydation (52). This then induces structural and functional damage to cellular membranes by decreasing their fluidity and electrochemical potential, enhancing their permeability to hydrogen and other ions, disrupting enzyme activity and fragmenting the membrane (20,52,54). The by-products and decomposition compounds of lipid peroxydation include lipid-conjugated dienes, peroxyl and alkoxyl free radicals, hydroperoxydes, alcohols and aldehydes, such as malonaldehyde (20,52,54). These compounds are extremely toxic to cells and their organelles; they prevent protein synthesis, amplify

inflammatory pathways and have chemotactic and cytotoxic functions, perhaps by controlling the production of cytokines (20,52,54).

Studies suggest that chronic intestinal inflammation may be implicated in the excessive formation of free radical species and oxidized lipid products (19). Inflamed intestinal and colonic cells appear to produce excessive amounts of reactive oxygen species (19,58,59). Investigators have reported that inflamed colonic cells synthesize greater quantities of reactive oxygen intermediates when compared to healthy controls (23,60,61). Biopsy specimens from colonic cells of patients with inflammatory bowel disease revealed high levels of lipid peroxidation compounds (19).

The body's defenses against free radicals include extracellular, membrane and intracellular antioxidants, such as vitamin C, vitamin E and reduced glutathione respectively. However, they may be inadequate when the production of pro-oxidant species is excessive (19,23,53,54). Primary defenses against free radicals include enzymes, transport proteins and other sequestering molecules (52). Superoxyde dismutases and glutathione peroxydase for example, are intracellular antioxidants that convert free radicals into less reactive compounds (52). Specific transport proteins, such as transferrin and ceruloplasmin, bind iron and copper respectively. These transport proteins are extracellular

defenses, which decrease the concentration of free transition metals from circulating in the plasma, thus preventing amplification of free radical reactions (23,52,54). Sequestering molecules include intracellular, extracellular and membrane antioxidants, for instance, reduced glutathione, vitamins C and E respectively, which decrease the quantity of free radicals and protect against oxidative damage (52,54).

Patients with inflammatory bowel disease appear to have decreased concentrations of superoxide dismutase, glutathione dismutase, reduced glutathione, vitamin E and retinol, as well as lower total ascorbic acid, which may be related to the high levels of reactive oxygen species (23,57,62-65).

Lipoprotein metabolism

Lipid transport in the circulation is determined by lipoproteins, lipoprotein receptors, apolipoproteins, lipolytic enzymes and transfer proteins (66). Lipoproteins are macromolecules constituted of lipids and proteins, and they transport insoluble lipid compounds such as triglycerides, cholesterol and cholesteryl ester through the plasma, which is an aqueous milieu (67-69). All lipoproteins have common features; they are comprised of triglycerides and cholesteryl esters in their nonpolar central core, and their surface is covered with amphipatic

lipids such as phospholipids and unesterified cholesterol, as well as transport proteins, referred to as apolipoproteins (69,70). The nonpolar groups of phospholipids free cholesterol and apolipoproteins are associated with the central core, whereas the polar group of the amphipathic compounds function to solubilize the lipoprotein in the hydrophilic environment of the plasma (69).

Apolipoproteins are essential components of lipoproteins. They stabilize the macromolecule, activate hydrolytic enzymes and transfer protein in the plasma in addition to being fundamental to the secretion, metabolism, and receptor-mediated uptake of lipoproteins (67,70-75). With the exception of Apo B-48 and B-100, apolipoproteins are very dynamic in their movement between different lipoproteins (68).

The transport mechanisms provided by lipoproteins accomplish several key metabolic requirements. First, dietary triglycerides, which are absorbed in the intestinal mucosa, are carried to the liver and extrahepatic tissue. Second, triglycerides stored or synthesized in the liver are delivered to peripheral tissues and either deposited for subsequent use or oxidized to provide energy. Third, dietary cholesterol is transported to the liver, and the cholesterol synthesized or stored in the liver is made available for the formation of membranes and

hormones. Finally, the excess cholesterol delivered to peripheral tissues is returned to the liver (69).

The five main classes of lipoproteins, in increasing order of density, are chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (68,69,76,77). Separation of these different classes of lipoproteins can be accomplished by ultracentrifugation in a salt solution, or by electrophoresis (68,69).

Chylomicrons, the largest lipoprotein particles, range from 800 Angstroms to 10 000 Angstroms. Their size depends on dietary triglyceride intake, since approximately 90% of the particle is comprised of triglyceride (68). Chylomicrons are the first lipoprotein to be separated by ultracentrifugation, since they are the least dense (68). Chylomicrons have a dual function in lipid metabolism. They transport dietary triglycerides to skeletal muscles and adipocytes, and also deliver dietary cholesterol to the liver (66,70). Lipid intake and specific apolipoproteins, including apo B-48, apo A-I, apo A-II and apo A-IV, provoke intestinal synthesis of chylomicrons (69,78). Following absorption, dietary fat, contained in chylomicrons traverse the basolateral membrane of enterocytes and join the circulation via the lymphatic system (69,70,72,78). Subsequently, apolipoprotein A-I and A-II is transferred

from chylomicrons to HDL particles in exchange for Apo C polypeptides and Apo E from the HDL particles to chylomicrons (68). Apo A-IV leaves the chylomicron and not only joins HDL, but also can remain unassociated in the plasma (70). It is important to note that the apolipoprotein B-48 present on the chylomicron remains in place throughout the entire metabolic process (79).

Triglycerides, which are transported in chylomicrons, are hydrolyzed by the lipolytic enzyme lipoprotein lipase in the presence of the cofactor Apo C-II (70,80). Free fatty acids, mono- and diglycerides result from the hydrolytic action of lipoprotein lipase, and can either be used by muscle cells as energy substrate or deposited in adipose cells for future use (70,80). Lipoprotein C is transferred back to HDL as chylomicrons are catabolized (66). The progressive loss of triglyceride from chylomicrons leads to the formation of chylomicron remnant particles, which contain cholesteryl ester and apolipoprotein B-48 and retain apo E (68). Apolipoprotein E on the surface of chylomicron remnants is recognized by receptors on hepatic cells and enables the removal of these particles from the circulation (66,81-83).

The liver produces VLDL particles, ranging from 300 to 800 Angstroms (68,84). Their density is between 0.94 g/ml and 1.006 g/ml (69). This lipoprotein is comprised of approximately 50% to 65% of

triglycerides (69). The major apolipoprotein associated with VLDL is apo B-100, although Apo C polypeptides and Apo E are also present (68).

The main function of VLDL particles is to transport endogenous triglycerides from the liver to extrahepatic tissues (68). As with chylomicrons, lipoprotein lipase hydrolyzes the triglycerides and makes them available to peripheral tissue (68). In the fasting state, VLDL is the most significant transporter of triglycerides (66). VLDL obtains cholesteryl esters from HDL and transfers the apo C polypeptides to HDL particles (85). The remnant VLDL particle is also referred to as intermediate-density lipoprotein (IDL) and has a density between 1.006 g/ml and 1.019 g/ml (69). IDL particles have two fates: they can be removed from circulation, mainly by the liver via the Apo E receptor-mediated pathway or they can be converted to LDL (68).

The LDL particle has a density range of 1.019 g/ml to 1.063 g/ml (69). This lipoprotein is produced in the plasma and can be regarded as the end result of VLDL catabolism (69). LDL particles are the main transporters of cholesterol and cholesteryl esters from the liver to peripheral tissue (69). Evidence provided by human fetal jejunal explant cultures and the human Caco-2 intestinal epithelial cell line, supports the hypothesis that occasionally, LDL can be synthesized in the intestine (67,86-90). The LDL of intestinal origin is enriched in triglycerides (67).

The main apolipoprotein on LDL is B-100, which assists in the receptor-mediated hepatic uptake of the LDL particle (68). In healthy subjects, nearly 75% of plasma LDL is catabolized via the receptor-mediated pathway (68).

HDL is the smallest lipoprotein, with a diameter ranging from 90 to 120 Angstroms (68). Ultacentrifugation separates HDL particles in two subclasses, HDL_2 and HDL_3, in the density ranges of 1.060 to 1.125 g/ml, and 1.125 to 1.21 g/ml respectively (69). HDL_2 contains 40% protein and 60% lipid, whereas HDL_3 is comprised of 55% protein and 45% lipid (69). The main function of the HDL particle is to transport excess cholesterol from peripheral tissue back to the liver where it can be either stored or eliminated in the form of bile acids (69). The major apolipoproteins associated with HDL are Apo A-I and Apo A-II (68).

Nascent HDL originates in the liver and intestine, with a disk-shaped structure, but as it matures in the plasma it acquires a spherical form (91). The enzyme lecithin: cholesterol acyltransferase (LCAT), which requires both Apo A-I and Apo C-I as cofactors, moves a fatty acid from lecithin to the hydroxyl group on the cholesterol molecule, resulting in the formation of lysolecithin and cholesteryl ester (66,68). Esterified cholesterol is very hydrophobic, thus it migrates to the nonpolar core of the HDL particle (66). Consequently, space is freed on

the surface of HDL that can now acquire further cholesterol molecules from other lipoproteins or from cells (66). The plasma protein cholesteryl ester transfer protein (CETP) catalyzes the movement of cholesteryl esters from HDL to VLDL, in exchange for triglycerides from the VLDL particle (83).

Oxidization and lipoproteins

Lipoproteins are susceptible to oxidation, since cholesteryl esters and phospholipids contain polyunsaturated fatty acids (92). Increased amounts of oxidized lipid products seem to be associated with the chronic intestinal inflammation seen in patients with Crohn's disease (19). In addition, studies have revealed a greater production of free radicals in inflamed intestinal and colonic cells of patients (19,58,59). Therefore, lipoproteins in patients with Crohn's disease may be very prone to lipid peroxydation since polyunsaturated fatty acids and other lipids present on lipoproteins are targets of reactive oxygen species, such as hydroxyl and hydroperoxyl radicals (23,52).

The effect of oxidation on lipoproteins has been mostly studied in the LDL particle. It has been established that chemically modified LDL, such as malonaldehyde-LDL, is no longer recognized by the Apo B and Apo E receptors in the liver and are instead taken up by scavenger

receptors on macrophages (92,93). Oxidation also alters apolipoproteins considerably, through chemical reactions between specific amino acid groups on the apolipoproteins and conjugated dienes, lipid peroxides and aldehydes (92). The result of these modifications can have substantial effects on lipid metabolism. For instance, if specific amino acids on Apo B are altered, hepatic receptors may no longer recognize the ligand and hence, the lipoprotein will either accumulate in the circulation or be internalized by alternative receptors (93). Lipoproteins are also transporters of antioxidants, for example, alpha-tocopherol, present in high amounts in the LDL particle (92). However, prior to fatty acid oxidation, levels of antioxidants decline rapidly (93). In patients with Crohn's disease antioxidant status seems to be compromised, and lipoproteins may not be adequately protected against lipid oxidation (23,62,63).

Cytokines, infection and lipoproteins

In patients with Crohn's disease bacterial agents and their products, such as lipopolysaccharide (endotoxin), as well as dietary antigens appear to traverse the intestinal mucosa with greater ease because chronic intestinal inflammation increases epithelial permeability (7,9,30). High concentrations of bacterial lipopolysaccharide can induce sepsis, fever, leukocytosis, the acute-phase response and abnormalities

in lipid metabolism (94-97). Increased plasma levels of triglycerides and VLDL, as well as a decrease in HDL concentrations have been observed in response to infection (98-104). Lipoproteins have an important function in responding to the metabolic stress related to infection, such as assisting in the removal of bacterial products from circulation and accelerating their hepatic uptake and excretion in bile (105-107). Investigators have documented that HDL and LDL have the capacity to form complexes with bacterial lipopolysaccharide, thus decreasing their systemic effects (108-110). In addition, triglyceride-rich lipoproteins such as chylomicrons and VLDL particles can also bind to bacterial lipopolysaccharide and possibly neutralize their effects (105). In vitro studies have revealed that lipoprotein-bound endotoxin can no longer stimulate immune cells, nor induce the secretion of proinflammatory cytokines, such as TNFα, IL-1 and IL-6 (111,112).

Research objectives

In view of the numerous functions of lipoproteins, we hypothesize that modifications in their chemical composition and their apolipoprotein content may adversly affect the metabolism of lipids, and consequently, play a role in the pathogenesis of Crohn's disease. To the best of our knowledge, lipid metabolism in patients with Crohn's disease has not been studied in depth. The aims of this research project are to characterize plasma lipid levels, apolipoprotein and lipoprotein levels in pediatric patients with Crohn's disease. Also, the chemical composition of lipoproteins, in terms of their triglyceride, cholesteryl ester, free cholesterol, phospholipid and protein content will be determined and expressed in terms of percentage of total lipoprotein size. Furthermore, the apolipoprotein content of the various plasma lipoproteins will be analyzed.

ARTICLE: Altered Lipid Profile, Lipoprotein Composition and Oxidant/Antioxidant Status in Crohn's Disease

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ABSTRACT

Background: Growing evidence supports the role of peroxidation in the pathogenesis of the mucosal lesion in Crohn's disease (CD). The activation of inflammatory cells and release of their mediators along with excessive production of free radicals may affect circulating lipids.

Objectives and Design: The aim of this study was to examine the lipid profile, lipoprotein composition and the oxidant/antioxidant status in 22 pediatric CD subjects compared to 10 healthy controls. **Results:** Changes in plasma fatty acids consisted in an increase in saturates and monoinsaturates, and a decrease in polyinsaturates. This results in an elevation of 16:1n-7/18:2n-6 ($p<0.05$) and 20:3n-9/20:4n-6 ($p<0.04$), two established indices of essential fatty acid deficiency. Hypocholesterolemia, which is defined as total cholesterol concentration below 2.6 mmol/l was noted in CD patients, due to decreases in LDL-cholesterol (20.8%, $p<0.02$). Coincidentally, plasma apolipoprotein B (21%, $p<0.02$) and A-I (19.1%, $p<0.02$) were lower whereas plasma triglycerides (TG) were significantly increased (67%, $p<0.005$). Lipoprotein composition was altered, displaying relative TG depletion and protein enrichment in VLDL. On the other hand, IDL was characterized by an increased % of TG and protein ($p<0.005$), as well as a reduced proportion of phospholipids ($p<0.001$). Additional

abnormalities were observed in the chemical distribution of HDL_2 and HDL_3 moieties. Lipid peroxydation was documented by higher plasma malonaldehyde concentrations ($p<0.05$), accompanied by a reduction in retinol levels ($p<0.02$). **Conclusion:** Our data show that disturbances in lipid profile, lipoprotein concentration and composition, and oxidant/prooxidant status occur in CD patients.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease which may affect any region of the gastrointestinal tract. Most commonly, the distal small bowel, often including segments of the proximal large intestine as well (4,7,36). Although the etiology remains unknown, pathological factors implicated include microbial agents, immune dysfunction, genetic susceptibility, and various environmental factors (7,36). A growing body of evidence suggests that uptake of luminal antigens across the mucosal epithelium elicits an aberrant immune response, culminating in intestinal inflammation, local peroxidation and systemic circulation of inflammatory cytokines and mediators (117). These disturbances are often associated with malnutrition secondary to reduced dietary intake and malabsorption, potentially impairing essential polyunsaturated fatty acid and antioxidant status, as well as the composition of lipoprotein-lipid system (1). Of particular significance are the lipoprotein particles, which provide efficient mechanisms for moving large amounts of fat and lipid-soluble vitamins in the circulation, and contribute to the maintenance of cellular membrane structure and function. Disturbances in lipoprotein vehicles resulting from peroxidative attack, may affect their normal metabolism and subsequent distribution of both lipid and vitamin moieties to peripheral organs. Whereas inconsistent data is available in adults

(42,118-121), little information concerning these parameters has been reported in the pediatric age group, despite the fact that children and adolescents account for 25% of all new cases of CD (4). Furthermore, no thorough investigation has been carried out on lipoprotein composition in CD, characterizing lipid and apolipoprotein moieties. The aim of this study was therefore to examine fatty acid distribution, lipid profile, lipoprotein composition, as well as antioxidant levels in children with Crohn's disease.

METHODS

Subjects: Twenty-two patients with Crohn's disease were recruited from the IBD Clinic at Hospital Sainte-Justine. The diagnosis was based on confirmation of clinical, radiographic and/or endoscopic, and histological features (7). The severity of the disease was evaluated according to the Crohn's Disease Activity Index (CDAI) that includes the daily number of liquid or very soft stools, severity of abdominal pain, general well-being, extraintestinal manifestations of the disease, ability to attend school and interact socially, the presence of an abdominal mass, hematocrit, and body weight (7,152). Patients with scores of $\geq$150 are considered to have active disease. The patients' characteristics are presented in table I. Ten age and gender matched, healthy subjects served as controls.

The study protocol approved by the Ethics Committees of this institution.

Blood samples: Blood samples were collected in 1mg/ml EDTA after a 12-h overnight fast. Plasma was separated immediately by low centrifugation (2,500 rpm, 20 min) at 4 ^{0}C.

Fatty acid analysis: Fatty acids in whole plasma were assayed by an improved method described by this laboratory (123). Briefly, each sample to be analyzed underwent direct transesterification, followed by injection into an HP 5880 gas chromatograph (Hewlett Packard, Rockville, MD) using a 60-m fused capillary column coated with SP 2331.

Lipoprotein isolation: The lipoprotein fractions were isolated by discontinuous density gradient ultracentrifugation in a Beckman L5-65 preparative ultracentrifuge using a Ti-50 rotor as previously reported (128,129). Briefly, after preliminary centrifugation to remove chylomicrons (25,000 rpm, 3 min), very-low density (VLDL) , intermediate density (IDL), and low density (LDL) lipoproteins were isolated at densities of 1.006 g/ml, 1.019 g/ml, and 1.063 g/ml, respectively, running at 100,000 g for 18 h at 5^{o}C. The separation of

high density lipoprotein (HDL) subpopulations was performed at 100,00 g for 48 h at the following densities: 1,125 g/ml for HDL_2 and 1.21 g/ml for HDL_3. The lipoprotein fractions were dialized against 0.15 M NaCl, 0.001 M EDTA, pH 7.0.

Vitamin determination: Vitamins were measured according to Lepage et al (124). At the time of the assays, samples of plasma were thawed in the dark, and aliquots were processed for analysis under subdued light. Samples of 500 µl of the different specimens were mixed in tubes for 30 seconds on a vortex mixer with 500 µl internal standard (12 µl of tocopherol acetate in C_2H_5OH). After the addition of n-hexane (twice, 2.5 ml each), tubes were shaken for 10 minutes, sonicated for 3 minutes, and centrifuged for 5 minutes at 1000 g. The n-hexane layer was transferred into a tube, and the pooled organic extracts of each sample were evaporated to dryness under a gentle stream of nitrogen at 20^0C. Tubes were rapidly removed from the water bath, the residue was reconstituted with 150 µl acetonitrile/methylene chloride/methanol (70:20:10, by volume), to which 25 µg ascorbic acid in 50 µl ethanol was added, and tubes were placed in a vortex mixer for 30 seconds and sonicated for 3 minutes. Of this solution, 20 µl was injected to the high-pressure liquid chromatography system.

Table I: Clinical characteristics of the Crohn's disease group

	Mean ± SEM	Abnormal Results
Age (years)	14.3±0.6	-
Gender	11 M / 11F	-
Acute malnutrition (↓Wt/Ht %)	5M /4F	9/22 (41%)
Growth failure	1M /3F	4/22 (18%)
Hbg (n>125 g/L)	113.2±3.1	14/22 (64%)
Albumin (n<39 g/L)	34.5±1.3	13/22 (59%)
Iron (n>7 μmol/L)	7.2±1.2	10/21 (48%)
Disease active (CDAI ≥_150)	157±30*	13/22 (59%)

* Range 5-321

The chromatographic analysis was performed on a Hewlett-Packard spherical 5 μm C_{18} ODS Hypersil column (20 cm x 2.1 mm ID). A guard column of the same package preceded the main column. The reverse-phase ODS column was used for the simultaneous determination of the fat-soluble vitamins using isocratic elution with acetonitrile/chloroform/isopropanol/H_2O (79:16:3.5:2.5, vol/vol). The flow rate was 300 μl/min. The light absorption of the compounds was measured with a photodiode-array detector at wavelengths of 282 nm for tocopherol acetate, 290 nm for α-tocopherol, and 322 nm for retinol, and 446 nm for β-carotene. The amounts were calculated by using tocopheryl acetate as the internal standard. The areas under the curve of the chromatographic peaks were used in the calculations. All manipulations were carried out under subdued light to avoid photoisomerization of the compounds. All the analyses were run on a Hewlett-Packard 1090 high-pressure liquid chromatograph. The photodiode-array detector acquires chromatographic signals and spectra over the wavelength range of 190 to 600 nm.

MDA and glutathione assessment: Plasma free MDA levels were determined according to a modified method by Chirico (125). Proteins were first precipitated with 10% $NaWO_4$ solution and protein free supernatant was then reacted with an isovolume of 0.5% TBA solution

at 95^{0}C for 30 min. After cooling to room temperature, pink chromagene was extracted with n-butanol and dried over a stream of nitrogen at 37^{0}C. The dry extract was then resuspended in KH_2PO_4/methanol (70:30) mobile phase before MDA detection by high-performance liquid chromatography (HPLC) (126). Glutathione was measured by Tietze's technique (127).

Lipid and lipoprotein analysis: Plasma concentrations of total cholesterol (TC), free cholesterol (FC), and triglycerides wee measured enzymatically by a commercial kit (Boehringer Mannheim, Montreal) as reported previously (128,129). Cholesteryl esters (CE) were calculated as the difference between total and unesterified cholesterol x 1.7. Lipoprotein-protein (PR) was quantified according to Lowry et al (130) with bovine serum albumin as a standard. Phospholipids were determined by the Bartlett method (131). HDL-cholesterol was measured after precipitation of VLDL and LDL with phosphotungstic acid (128,129). Apolipoprotein composition of plasma lipoproteins was qualitatively assayed using SDS polyacrylamide gel electrophoresis. The gels were stained for 1 h with Coomassie blue and destained in 7% acetic acid. The bands of apolipoproteins were identified by comparison with the mobility of apolipoprotein standards and by standards of different molecular weights. The densitometric distribution of apolipoproteins was assayed as described previously (128,129).

Statistical analysis: All values were expressed as the mean ± SEM. Statistical differences were assessed by the Student's two-trial t-test.

RESULTS

Plasma lipids, lipoproteins and apolipoproteins

Several alterations were noted in plasma lipids, lipoproteins and apolipoproteins among the Crohn's disease compared with control group (Fig. 1). Triglyceride levels were higher whereas total cholesterol concentrations were lower. The latter was characterized by a significant decrease in LDL-cholesterol, with only a slight trend towards a decrease in HDL-cholesterol. As anticipated in view of these findings, apo A-I and apo B levels were reduced in the Crohn's disease patients. Using semiquantitative agarose gel electrophoresis, diminished intensity was particularly noted in the β-lipoprotein fraction (data not shown).

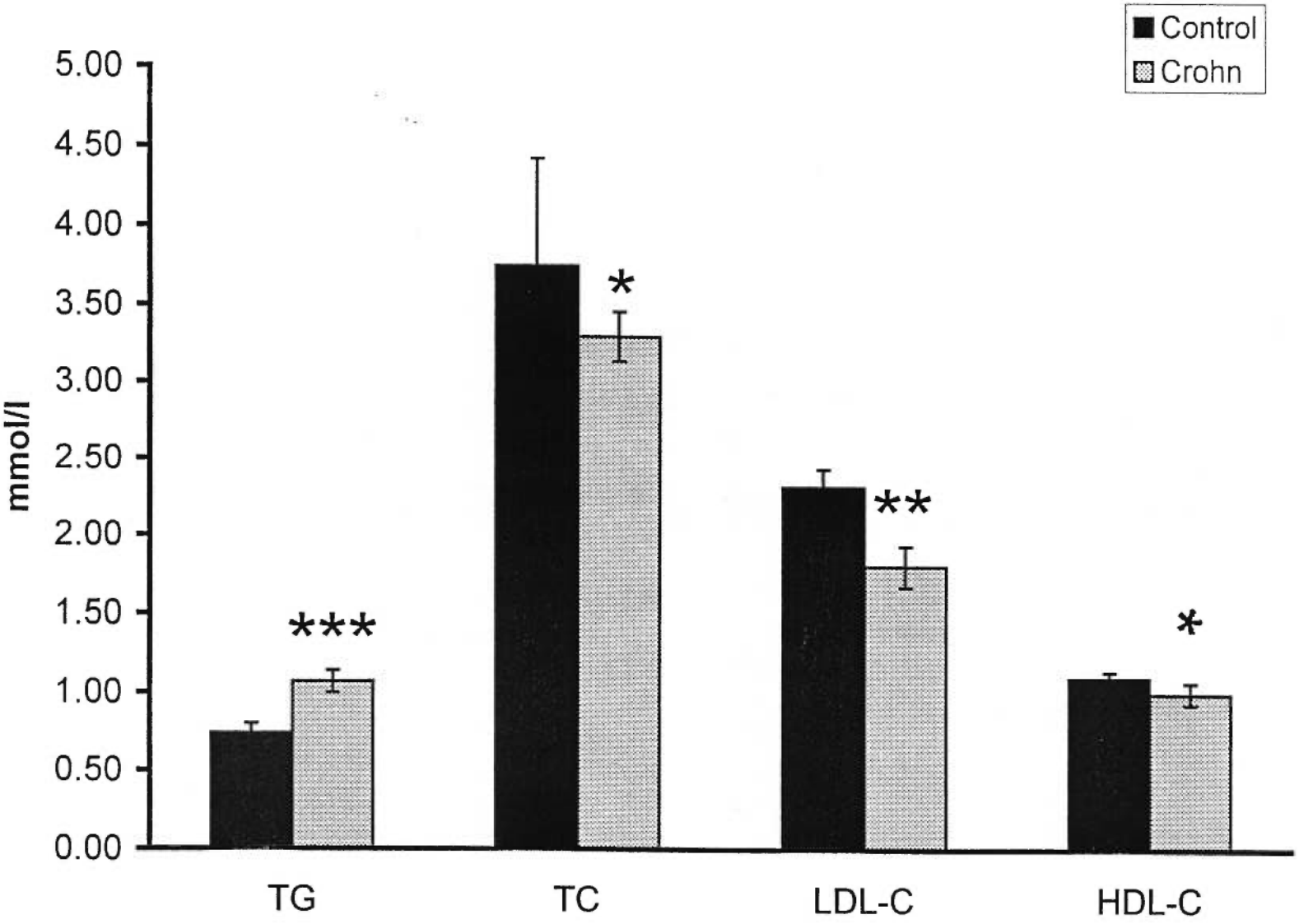

Figure 1. Plasma lipids, lipoprotein cholesterol and apolipoproteins in pediatric Crohn's disease. The biochemical determinations were performed in fasting plasma of control and CD subjects. Values of TG, TC, LDL-C, HDL-C, apo A-I and apo B. Data represent means ± SEM for 10 controls and 22 patients with CD.
*p<0.01, **p<0.02, ***p<0.005.

Fatty acid profile

The Crohn's disease patients' plasma fatty acid profiles (table II) also showed several significant abnormalities compared with age-matched controls. Plasma linoleic acid, an essential fatty acid, was lower in the Crohn's disease group. On the other hand, the proportion of monoinsaturates showed higher values in CD patients. The overall changes in the various species and ratios of fatty acids relevant for the assessment of EFA-deficiency are summarized in table III. Both total n-3 and n-6 polyunsaturated were decreased, whereas saturates and monoinsaturates (n-7 and n-9) were increased. The ratios of 20:3n-9 to 20:4n-6 and 16:1n-7 to 18:2n-6, established indices for essential fatty acid status, were higher in CD patients than in controls, confirming deficiency .

Lipoprotein composition

The lipoprotein composition of the CD patients and healthy patients is summarized in table IV. The VLDL fraction was poorer in triglycerides and relatively protein-enriched. In IDL, the percentage of triglycerides and protein were higher, whereas the proportion of phospholipids was lower. No significant changes were noticed in the LDL fraction. The two HDL subpopulations were also found to have

lipoprotein disturbances in the Crohn's disease patients. Triglycerides, free cholesterol and cholesteryl ester were increased, while phospholipids and protein were decreased in HDL_2. The marked alterations in HDL_3 consisted in a reduction of cholesteryl ester, concomitant with a rise in phospholipids. As expected from these changes in lipoprotein components, particle size was affected, as inferred from the mass ratio of core (TG+CE) to surface constituents (FC+PR+PL). The calculated value of these ratios indicated that VLDL, LDL and HDL_3 were smaller, and IDL and HDL_2 larger.

Apolipoprotein distribition in lipoprotein fractions is illustrated in Fig. 2. The gradient SDS-gel electrophoresis (4-15%) showed no differences in VLDL-apoproteins, except for the apo E bands. There was also no consistent difference between the apo B patterns of LDL. Apo A-I and apo A-II in HDL fractions, determined by 15% SDS-PAGE, are shown in Fig.3. Apo A-I.apo A-II ratios were higher in the HDL_2 and HDL_3 fractions for the Crohn's disease patients.

Oxidant/Antioxidant status:

In order to assess the antioxdant status of Crohn's disease, their plasma retinol, β-carotene, vitamin E and γ-tocopherol levels were determined (Fig. 3). Only retinol concentrations were observed to be

significantly reduced. Glutathione, a key endogenous soluble antioxidant, was increased compared to healthy controls (Fig. 4). Increased oxidative stress was observed in the Crohn's disease group, as evidenced by a 70% increase of MDA level relative to controls (Fig. 4).

Effect of disease acitivity on nutritional status:

The 22 Crohn's disease patients were separated into those with active versus quiescent disease based on their CDAI scores (Table V). Although many differences were observed between the two groups in terms of their nutritional status, they did not reach statistical significance. Furthermore, both glutathione and MDA levels showed a trend towards higher values, without attaining statistical significance.

Table II: Plasma fatty acid profile

Fatty acid	Controls	CD	P
14:0	0.98±0.06	0.93±0.09	NS
15:0	0.27±0.02	0.25±0.01	NS
16:0	21.99±0.33	23.20±0.32	0.01
17:0	0.33±0.01	0.32±0.01	NS
18:0	7.46±0.20	7.35±0.21	NS
20:0	0.25±0.01	0.30±0.01	0.036
22:0	0.57±0.04	0.58±0.02	0.030
24:00	0.53±0.03	0.42±0.02	0.003
18:3n-3	0.72±0.05	0.61±0.04	NS
20:5n-3	0.49±0.05	0.42±0.03	NS
22:5n-3	0.56±0.04	0.49±0.03	NS
22:6n-3	1.61±0.09	1.37±0.10	NS
18:2n-6	29.66±0.83	25.45±0.84	0.001
18:3n-6	0.40±0.04	0.35+0.02	NS
20:2n-6	0.28±0.01	0.22±0.01	0.001
20:3n-6	1.57±0.06	1.55±0.08	NS
20:4n-6	6.86±0.27	6.71±0.30	NS
22:4n-6	0.24±0.01	0.24±0.01	NS
16:1n-7	1.65±0.12	2.02±0.15	0.081
18:1n-7	1.54±0.03	1.69±0.06	0.067
18:1n-9	19.97±0.47	22.79±0.74	0.006
20:1n-9	0.16±0.01	0.17±0.01	NS
20:3n-9	0.11±0.01	0.14: ±0.01	NS
24:1n-9	0.88±0.03	0.84±0.05	NS

Results are expressed as the mol % of total fatty acids present.
All data represent mean values ± SEM

Table III: Fatty acid families, EFA deficient ratios and desaturase index activity

Fatty acid	Controls	CD	P
Saturated (%)	32.50±0.35	33.52±0.35	0.057
MUFA (%)	24.99±0.60	28.91±0.87	0.002
PUFA (%)	42.50±0.72	37.57±0.99	0.001
PUFA/Saturated	1.31±0.03	1.12±0.03	0.001
Total n-3 (%)	3.38±0.14	2.90±0.15	0.031
Total n-6 (%)	39.01±0.77	34.53±0.93	0.001
Total n-7 (%)	3.19±0.15	3.72±0.18	0.041
Total n-9 (%)	20.77±0.54	23.95±0.75	0.003
16: 1n-7/18:2n-6	0.058±0.006	0.085±0.010	0.051
20: 3n-9/20:4n-6	0.016±0.001	0.022±0.022	0.036

X±SEM

Table IV: Chemical Composition of lipoproteins

Lipoprotein	Composition							
	TG	CE	FC	PL	PR	TG/PL	CE/PR	TG+CE/FC+PL+PR
VLDL (1.006 g/ml)								
CD	49.28±1.2	9.73 ±1.01	6.13±0.43	18.47±0.64	16.24±1.24	2.68±0.12	0.68±0.10	1.44±0.07
Controls	55.9±0.9	10.7±1.12	5.6±0.3	16.7±0.3	10.0±0.6	3.34±0.05	1.07±0.03	2.06±0.23
P	0.001	NS	NS	NS	0.001	0.001	0.005	0.05
IDL (1.029 g/ml)								
CD	31.02±2.1	17.59±2.13	8.51±2.13	19.44±2.21	23.44±1.57	1.98±0.33	0.79±0.11	1.02±0.11
Controls	20.9±1.3	22.4±1.9	7.5±0.8	31.3±3.1	17.9±0.4	0.66±0.10	1.25±0.04	0.76±0.07
P	0.005	NS	NS	0.01	0.005	0.005	0.005	NS
LDL (1.063 g/ml)								
CD	6.54±0.42	35.76±1.08	10.81±0.63	22.03±1.03	24.08±1.37	0.30±0.02	1.50±0.09	0.74±0.03
Controls	6.2±1.0	39.1±1.5	10.3±0.4	20.8±1.9	23.5±0.6	0.30±0.03	1.66±0.03	0.83±0.05
P	NS	NS	NS	NS	NS	NS	NS	NS
HDL_2 (1.125 g/ml)								
CD	6.91±0.57	25.08±0.92	7.4±0.38	23.44±1.57	36.03±0.73	0.30±0.03	0.70±0.13	0.47±0.02
Controls	3.5±0.2	21.8±0.3	5.7±0.3	31.0±0.5	38.0±1.22	0.11±0.01	0.57±0.01	0.34±0.01
P	0.001	0.005	0.005	0.001	0.05	0.001	0.001	0.001
HDL_3 (1.21 g/ml)								
CD	3.82±0.33	16.06±0.67	3.39±0.67	29.2±0.98	47.49±1.54	0.13±0.03	0.35±0.07	0.25±0.03
Controls	3.2±0.2	20.2±0.5	2.8±0.1	24.0±0.3	49.9±0.7	0.13±0.01	0.40±0.02	0.30±0.01
P	NS	0.001	NS	0.001	NS	NS	NS	NS

Data are means ± SEM (mmol/L)

TG, triglyceride; CE, cholesteryl ester; FC, Free cholesterol; PL, phospholipid; PR, protein; NS, non significant

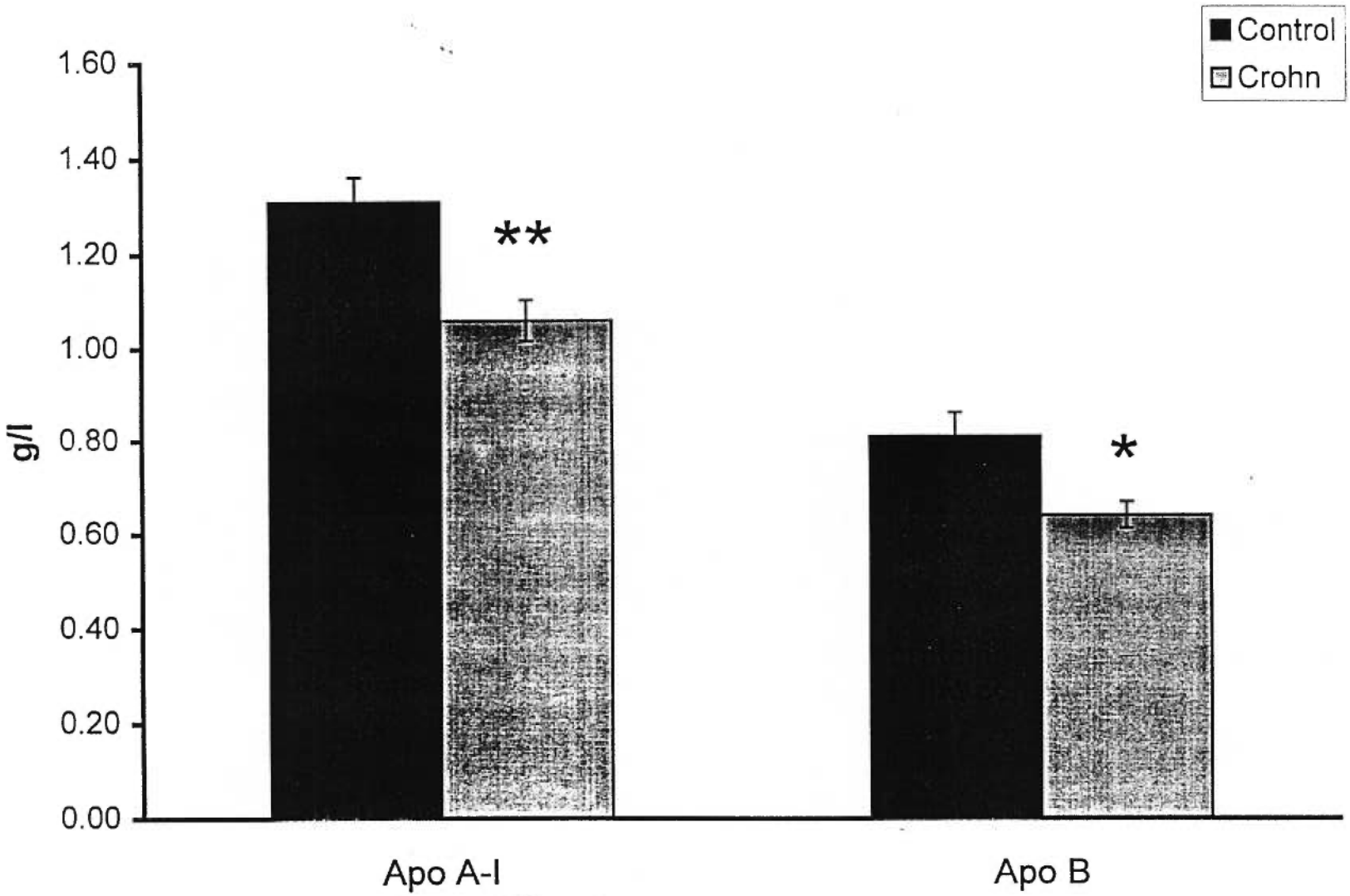

Figure 2. **Plasma concentrations of apolipoproteins A-I and B (g/l) in patients with Crohn's disease and in the control group (*p<0.01 vs controls, **p<0.001 vs controls).**

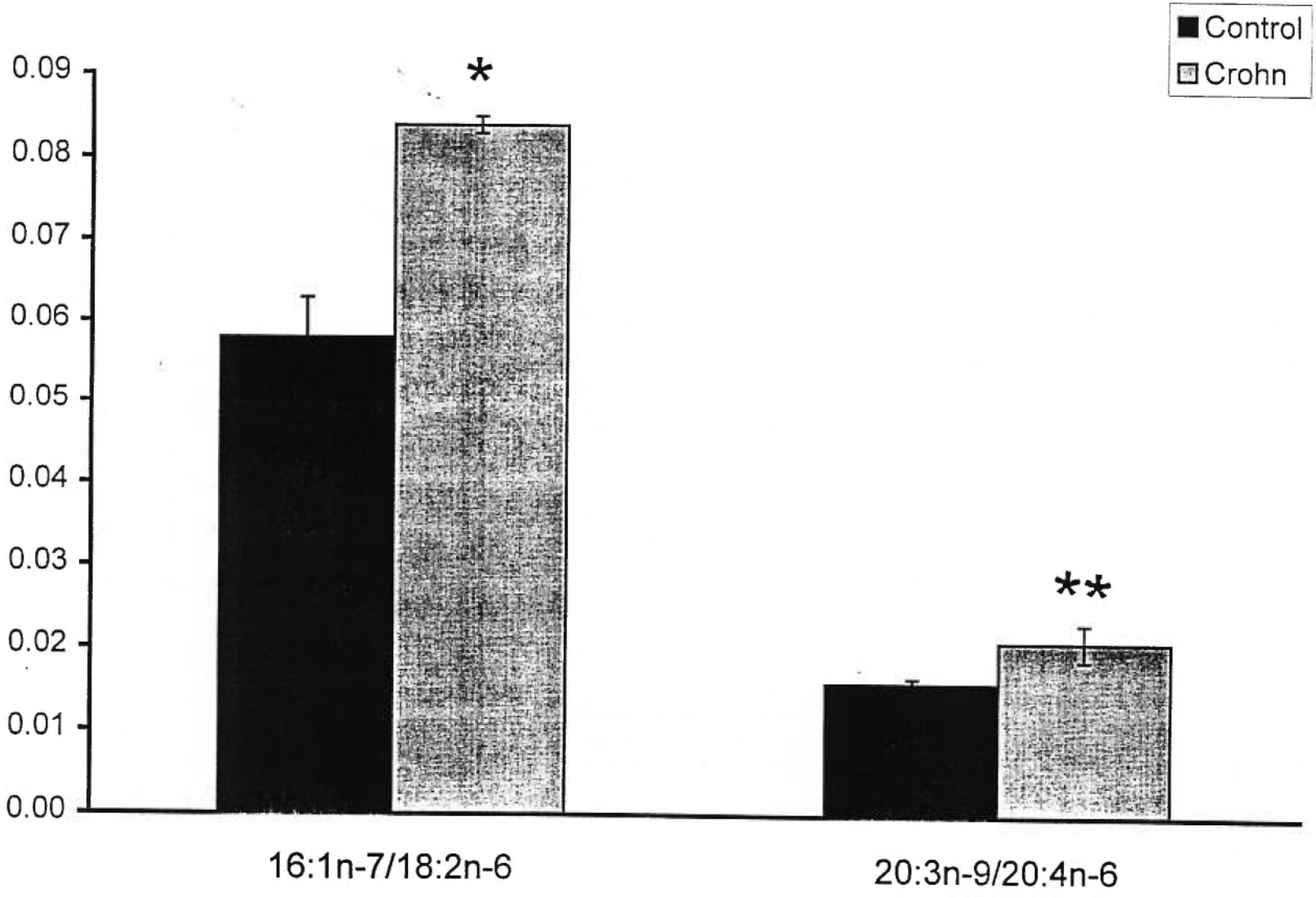

Figure 3. Plasma vitamin status in pediatric CD. The fasting CD patients and controls was analyzed for retinol, β-carotene, α-tocopherol and γ-tocopherol as described in Methods. Values were means ± SEM for 10 healthy control and 22 subjects with CD
* $p<0.02$.

Table V: **Comparison of clinical and nutritional parameters in pediatric Crohn's disease patients with active and inactive disease**

	Active Disease	**Disease Remission**
Sex	7M/6F	4M/4F
CDAI	263.7±15.7*	50.4±17.7
Weight (kg)	42.97±3.39	39.51±3.33
Height(cm)	155.81±4.24	149.56±4.21
Albumin(g/L)	32.85±1.53	37.12±1.98
Iron	6.44±1.01	8.76±2.97
Hemoglobin(g/L)	112.69±4.18	114.12±4.63
Platelet count(xIO^9/L)	455.31±48.74	388.50±51.47
Sedimentation rate (mm/h)	38.08±4.54	31.75±6.02
Triglycerides (mmol/L)	1.04±0.10	1.13±0.10
Total cholesterol (mmol/L)	3.23±0.23	3.39±0.22
LDL- cholesterol (mmol/L)	1.83±0.17	1.84±0.21
HDL- cholesterol (mmol/L)	0.93±0.12	1.04±0.08
Apo A-I (g/L)	0.99+0.06	1.16±0.05
Apo B- (g/L)	0.61±0.04	0.60±0.06
Lp(a) (g/L)	0.97±0.22	0.63±0.19
Apo E	3/3 (n=11),4/3 (n=2)	3/3 (n=8)
MDA (mmol/L)	449.14±333.06	76.00±17.50
Glutathione (mmol/L)	36.93±6.05	19.45±2.72

*p<0.001

Liposoluble vitamins and MDA

In order to assess the antioxidant status of patients with CD, the levels of retinol, β-carotene, vitamin E and γ-tocopherol were determined (Fig. 4 and 5). As illustrated in Figure 4, only retinol concentrations were found significantly reduced in CD patients. Examination of glutathione, an endogenous soluble antioxidant, revealed increased glutathione levels compared to healthy controls (Fig. 6). Besides, analysis of plasma samples from patients with CD for the presence of peroxidation index displayed a 232% increase of MDA relatively to controls (Fig. 6).

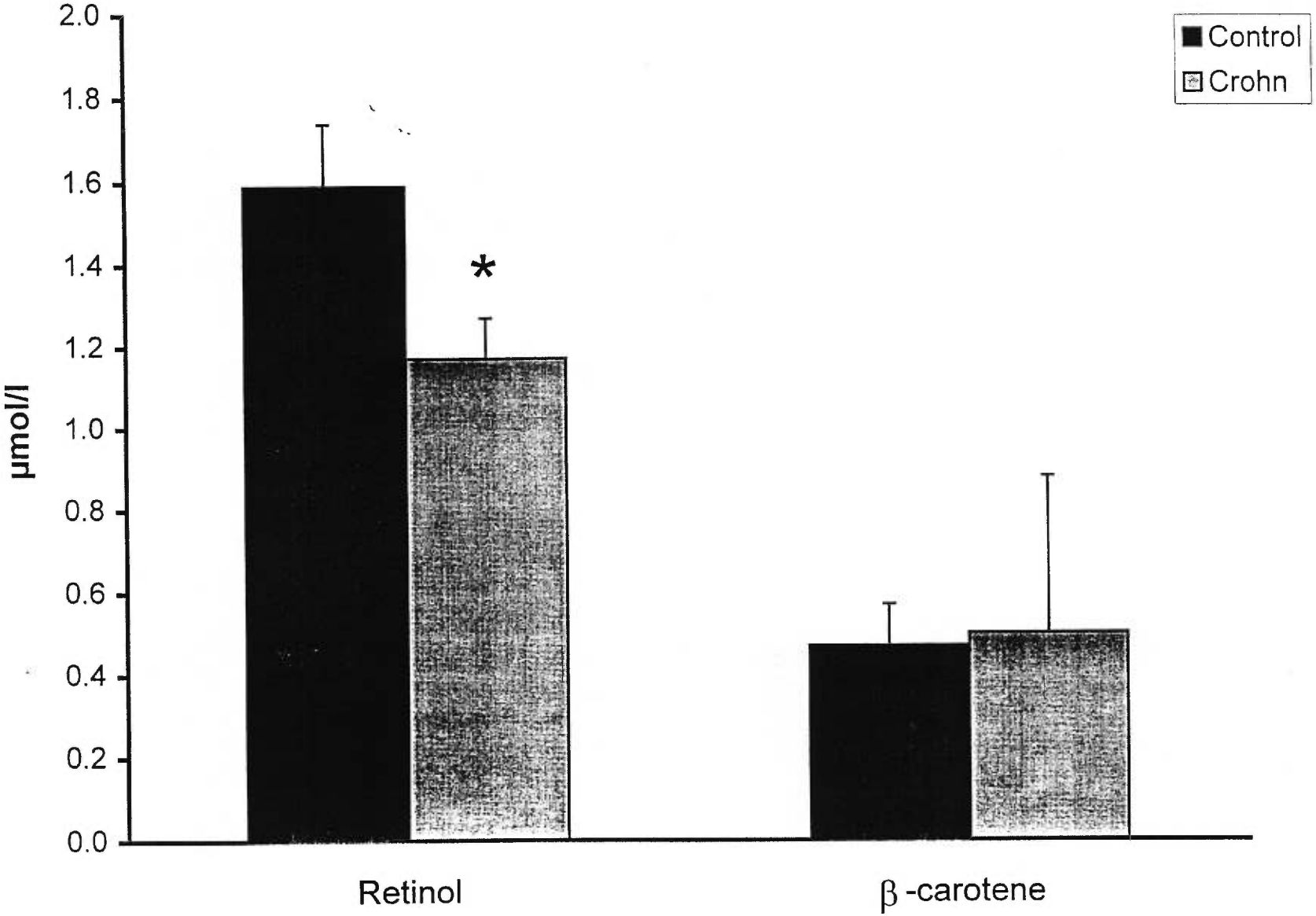

Figure 4. Oxidant/antioxidant status in pediatric CD. Malonaldehyde (MDA_and glutathione (GSH) levels were measured in the plasma of patients with CD and age-matched controls. Values are means ± SEM for 10 controls and 22 individuals with CD.
* $p<0.05$.

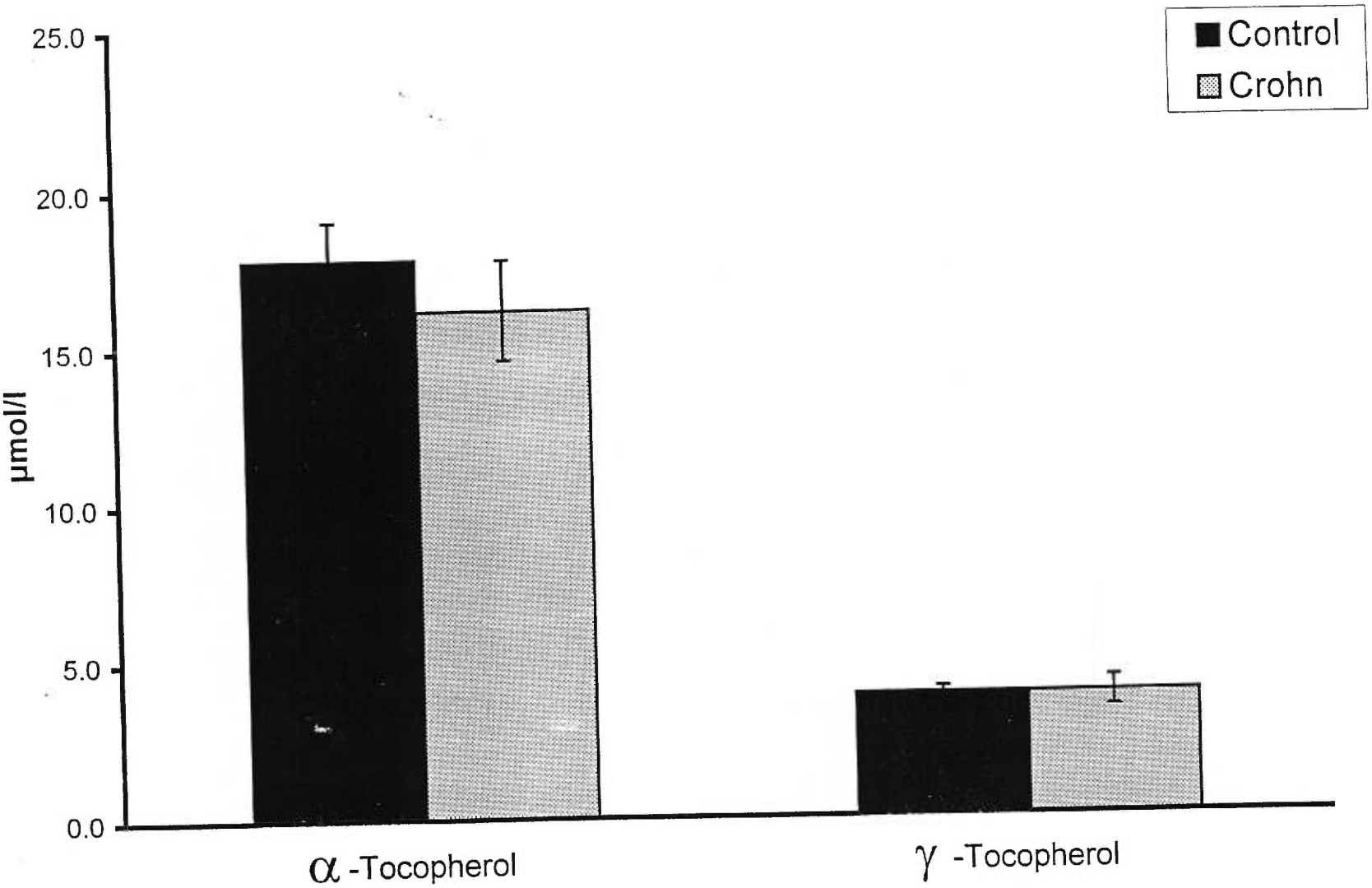

Figure 5. Plasma concentrations of α-tocopherol and γ-tocopherol (µmol/l) in patients with Crohn's disease and in the control group .

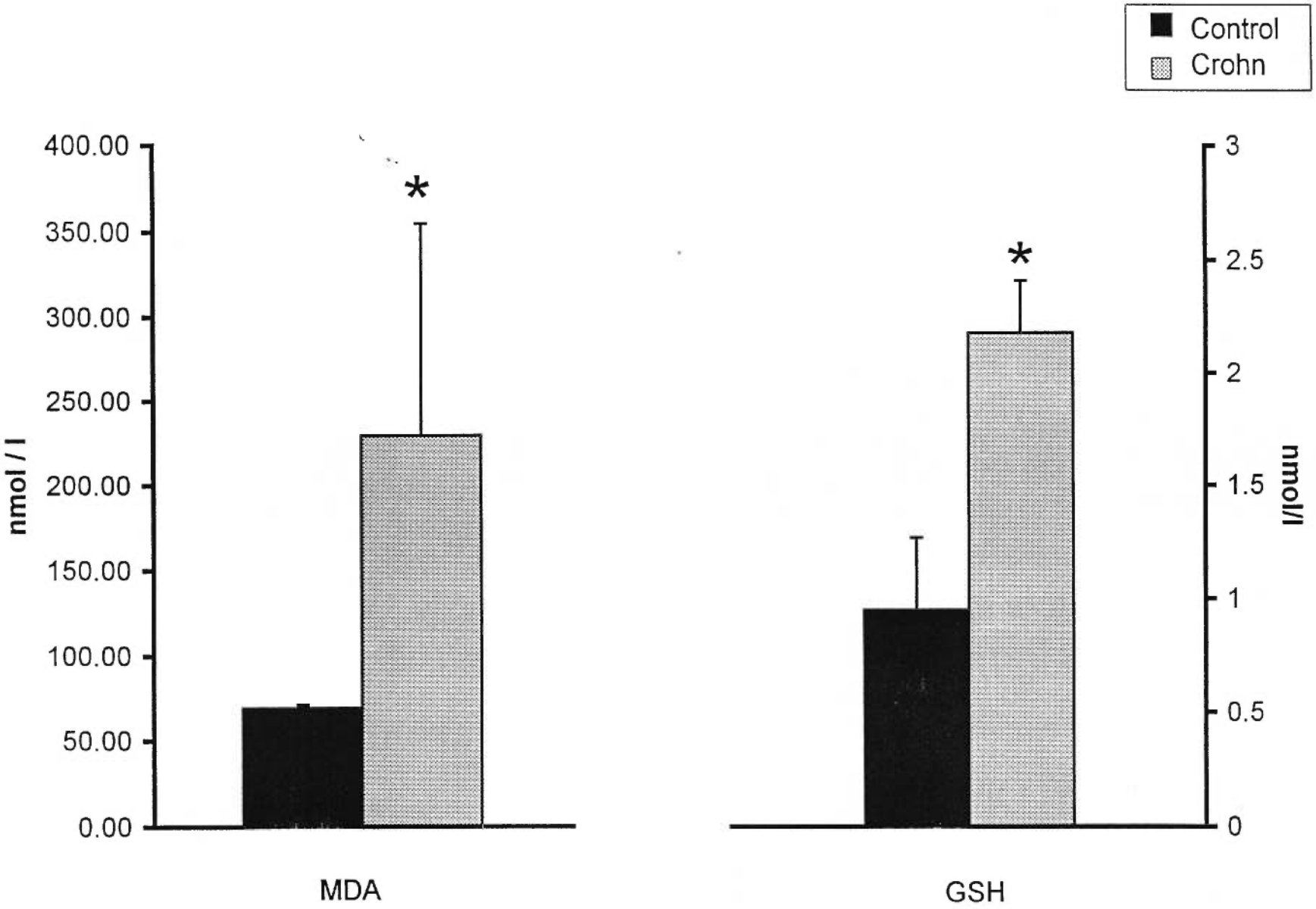

Figure 6. Plasma concentration of malonaldehyde and glutathione (nmol/l) in patients with Crohn's disease and in the control group (*p<0.05 vs controls).

DISCUSSION

The present study identified various alterations in lipid profile, lipoprotein composition and oxidant/antioxidant status in patients with CD, compared to age-matched healthy controls. Abnormal fatty acid pattern characterized the CD patients who displayed a decreased percentage of n-3 and n-6 families and increased proportion of n-7 and n-9 species. The changes in plasma fatty acids induced mainly an increment in the two indices of EFA deficiency 16:1n-7/18:2n-6 and 20:3n-9/20:4n-6. Our data documented abnormally low plasma levels of cholesterol, LDL-cholesterol and apo B with a concomitant rise in triglycerides. Finally, altered oxidant/antioxidant status was demonstrated by decreased plasma retinol, increased glutathione, and the high levels of plasma MDA. The latter finding is consistent with abnormal lipid peroxidation in the circulation.

PUFA play an active role in cell membrane function and eicosanoid synthesis (132,133). Depletion in PUFA in IBD patients is predictable , given their high demand for intestinal tissue repair and their heightened conversion into eicosanoids (134), key mediators of inflammation. However, contradictory findings were previously reported concerning long chain PUFA in CD. Decreased, normal and increased

values of n-3 and n-6 FA were observed (14,42,119). In this study, employing technological refinements of gas-liquid chromatography such as direct transesterification and separation of FA by a 60-m capillary column (123), we detected abnormal PUFA levels in pediatric patients with Crohn's disease. The decline in n-3 and n-6 families, concomitant with the increment of n-7 and n-9 FA let to high ratios of 16:1n-7/18:2n-6 and 20:3n-9/20:4n-6, two indices of essential fatty acid deficiency. Although the magnitude was not extreme as we had reported in cystic fibrosis (125,126), our data demonstrate that Crohn's disease patients are vulnerable to EFA deficiency. Contributing risk factors can include fat malabsorption, inadequate nutritional intake and hypermetabolism. The inconsistent results in the various studies reported to date may be due to methodological differences, the activity or extent of the disease, as well as the patients' variable intake. The evaluation of EFA status in patients with Crohn's disease may be clinically useful, since therapeutic trials have shown beneficial effects of n-3 fatty acids (137).

Crohn's disease is characterized by inflammatory cell infiltrates in the mucosal lesions. The excessive local production of soluble mediators from activated monocytes and polymorphonuclear leukocytes has been implicated in mediating tissue injury (138). Important among the mediators are oxygen free radicals. The chronic gut inflammation promotes an imbalance between pro-oxidant and antioxidant

mechanisms at the tissue level (3)., and may even compromise circulating antioxidant concentrations. In this respect, the plasma levels of ascorbic acid, vitamin E and β-carotene in CD compared to control adult subjectc, despite multivitamin supplementation (63,139). However, increased plasma antioxidant concentrations has been reported in children with CD (121). We did not observe alterations in vitamin E, β-carotene and γ-tocopherol levels. Retinol concentrations were significantly decreased, consistent with other reports in adults with Crohn's disease (63,64). The differences in circulating antioxidants in between reports to date may be due to the degree of inflammation, medication and supplement use, enteric losses, altered mobilization from inflamed mucosa, malabsorption and decreased nutrient intake. In view of the risk for physiologic sequelae associated with vitamin A deficiency , special attention should be devoted to retinol status in Crohn's disease.

Glutathione is a very important intracellular antioxidant. This tripeptide helps detoxify free radicals, peroxides and electrophilic compounds of endogenous and exogenous origin (141,142). Interestingly, increased glutathione content in red blood cell of CD patients was observed in our CD patients, in keeping with the recent study of Hoffenberg et al (42). Additional investigation is needed to

verify whether the high values of glutathione constitute an adaptation response to the increased oxidative stress. We have recently obtained similar results in animals exposed to iron-induced lipid peroxidation (126). These high levels of glutathione may prevent oxidation of α-tocopherol oxidation (143) and preserve ascorbic acid concentrations (144).

Free radicals are known to occur as natural by-products under physiologic conditions. However, their over-production has been implcated in the pathogenesis of gut inflammation and intestinal injury in IBD (3,138). Oxyradical-induced cytotoxicity gives rise to lipid peroxidation by reacting with PUFA in cellular membranes, resulting in MDA formation. Crohn's disease patients exhibited a significant increase in circulating MDA in the present study. It is unclear as to whether the excessive MDA was generated in the patients' blood or was produced by the inflammed intestine and translocated into the circulation. Its presence in the circulation may explain the noted reduction in essential fatty acids and the increased glutathione formation as a means to prevent oxidative damage. Our previous studies in various disorders such as cystic fibrosis clearly established a relationship between malnutrition, malabsorption and essential fatty acid deficiency on the one hand, and defective lipid profile and lipoprotein

composition on the other (128,136,145-147). Moreover, pro-inflammatory cytokines have the potential to adversely affect lipoprotein metabolism (148). All these conditions are encountered in Crohn's disease. Nevertheless, our data failed to demonstrate an effect of disease acitivity on these parameters, due to the relatively small number of patients in the subgroups with active and quiescent disease. Further studies are required, analyzing these parameters serially in a cohort of Crohn's disease patients, over time.

In conclusion, this study attracts attention to substantial abnormalities in the concentration of plasma lipids, MDA and antioxidants as well as in fatty acid and lipoprotein composition in pediatric patients with Crohn's disease. Further investigation is required in order to elucidate the mechanisms involved in the hope of improving the current management of Crohn's disease patients.

Acknowledgements

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DISCUSSION

Inflammatory bowel disease, which is the general term employed for chronic, idiopathic inflammatory disorders involving the gastrointestinal tract, includes both ulcerative colitis and Crohn's disease. Ulcerative colitis is localized to the mucosa of the colon, whereas the inflammatory process in Crohn's disease is discontinuous and transmural, affecting any part of the gastrointestinal tract (4). The incidence of Crohn's disease in adults has been progressively increasing in Western countries, a trend also noted in the pediatric population (5-7). Clinically, recurrent periods of relapses and remissions characterize Crohn's disease. In addition to primary gastrointestinal symptoms, systemic (fever, anorexia) and extraintestinal manifestations (arthritis, uveitis, erythema nodosum, etc) are frequently encountered.

While the etiology of Crohn's disease remains unknown, several features of this disease have indicated that certain important factors need to be considered. The increased incidence of Crohn's disease in certain ethnic groups, familial cases and its association with certain hereditary diseases such as cystic fibrosis suggests a genetic predisposition (7,9). Genetic "susceptibility" markers have been identified on chromosomes 3, 7, 12 and 16 (25-29). Many infectious agents such as the measles virus and certain dietary factors such as a

high intake of omega-6 polyunsaturated fatty acids, low omega-3 polyunsaturated fatty acids, high animal protein and a lack of fresh fruits and fiber have been postulated to be involved in Crohn's disease. However the mechanisms involved need further clarification (31-37).

A key role for the immune system in the etiology of Crohn's disease is based on several lines of evidence. First, extraintestinal manifestations may accompany the illness. Second, immunosuppressant drugs such as glucocorticoids, azathioprine and cyclosporine are usually associated with relief of symptoms. Third, immune mediators such as the proinflammatory cytokines IL-1, IL-6 and TNF-α are found in elevated levels in the intestinal mucosa of individuals with active Crohn's disease (17,41).

As seen in Table 1, our patients were on average 14.3 years old and there was an equal distribution of males and females. We observed that 41% of our patients suffered from acute malnutrition and 18% had growth failure. Acute malnutrition in patients with Crohn's disease may be a consequence of the chronic intestinal inflammation which decreases the absorptive surface, the overgrowth of bacteria, surgical treatment such as bowel resection and iatrogenic causes, for instance,

corticosteroid therapy (7). However, the most important cause is inadequate intake compared to energy needs.

Disturbances is the intestinal immune system are believed to play a key role in the pathogenesis of Crohn's disease. Oxidant-mediated tissue injury, particularly via metabolites and pro-inflammatory cytokines, has been shown to have detrimental effects on epithelial cells (42,57). As the integrity of the mucosal membrane is compromised, absorption of nutrients is decreased and protection against foreign antigens is ineffective. Furthermore, elevated pro-inflammatory cytokine levels and eicosanoid concentrations induce the structural and functional damage to cellular membranes by decreasing their fluidity and electrochemical potential, enhancing their permeability to hydrogen and other ions, disrupting enzyme activity and causing membrane fragmentation. The intestinal barrier is rendered unable to prevent the entry of microbial agents, dietary antigens and other harmful agents, which can further aggravate the underlying tissue injury. Under normal circumstances, protective substances such as antioxidants inactivate reactive oxygen species and prevent excessive cytokine production. However, sustained and excessive formation of free radical species in Crohn's disease can overwhelm these defenses, exacerbating the damage to the intestinal mucosa (23,60).

In this research project we measured plasma lipid levels, lipoprotein composition, apolipoprotein content and antioxidant/oxidant status in pediatric patients with Crohn's disease, compared to a healthy control group. Oxidative stress in the Crohn's disease group was increased in the present study as seen by a decreased plasma retinol level as well as an increased plasma concentration of malonaldehyde and gluthatione (Figures 4 and 6). Plasma membrane phospholipids are rich in polyunsaturated fatty acids. When exposed to an environment rich in free radicals, these highly reactive species can disrupt the double bonds of PUFA chains. The lipoperoxyl free radicals formed can attack neighboring PUFA residues, thereby initiating a chain of free radical reactions, and amplifying the damage to membrane structures. The lipoperoxyl free radicals produced by the chain reactions continue to release more reactive species as well as breakdown compounds such as malonaldehyde (52). Malonaldehyde is a three-carbon dialdehyde that is produced during autoxidation of polyunsaturated acids. In chronic inflammatory disorders such as Crohn's disease, lipid peroxydation takes place, leading to an increase in malonaldehyde, a marker for lipid peroxydation (54). Furthermore, in our study and as reported by Hoffenburg et al. (42), plasma concentrations of glutathione were found to be increased compared to the control group (Figure 6). Glutathione is the substrate of the important intracellular enzyme glutathione peroxydase, which converts toxic lipid peroxides into less reactive

compounds. The increased levels of glutathione observed in our pediatric population may be a result of a high rate of enzyme production in an attempt to control the quantity of free radicals.

Evidence for the pro-inflammatory imbalance is also supported by a decrease in the plasma concentration of retinol in patients with Crohn's disease, as seen in Figure 4. These results are in concordance with those of Fernandez-Banãres et al. (63). Intake of vitamin A occurs mainly as retinyl esters, which are hydrolyzed to retinol in the intestinal lumen during digestion. Retinol is then freely absorbed into the intestinal mucosa. Retinol is reesterified primarily with palmitic acid in the intestinal mucosa before being incorporated into chylomicrons, which are then carried to the circulation and to the liver (151). Therefore, the low levels of retinol may be a consequence of inadequate intake of vitamin A or a component of malnutrition. However, there is insufficient evidence that indicates that diets of patients with Crohn's disease are deficient in vitamin A. Also, retinol levels were reported as being decreased in patients with Crohn's disease with respect to the control group despite the fact that not all the patients in our study suffered from malnutrition, in whom we would expect decreased retinol binding protein concentration (Table 1). Vitamin A, which is stored in the liver, is transported to peripheral tissues by retinol binding protein (151). If oxidative stress structurally modified this transport protein, retinol would no longer reach its target areas. However, studies on such a

modification to retinol binding protein in patients with Crohn's disease are lacking. The observed level of vitamin A could also be a consequence of intestinal malabsorption of retinol secondary to a decrease of absorptive surface of the intestinal mucosa. Additional studies on the absorptive process of retinol in the presence of chronic intestinal inflammation would thus be beneficial in furthering our understanding of the consequences of Crohn's disease on the absorption, transport and metabolism of vitamin A. Interestingly, the plasma concentration of β-carotene, which has the highest provitamin A activity of all carotenoids, did not differ in patients with Crohn's disease from those in the control group (Figure 4). As with retinyl esters, β-carotene is converted to retinol in the intestinal mucosa followed by reesterification to form mainly retinyl palmitate, which is incorporated into chylomicrons (151).

We noted no difference in the plasma concentrations of α-tocopherol and γ-tocopherol between patients with Crohn's disease and the control group (Figure 5). Fernandez-Banãres et al. reported significantly lower plasma levels of vitamin E and β-carotene in CD patients whereas Hoffenburg et al. observed an increase in α-tocopherol (42,139). The discrepancy in results between various studies may reflect differences in disease activity, drug therapy, extent of intestinal

mucosal damage, degree of malabsorption and dietary intake. Taking into account the considerable role of free radicals in the pathogenesis of Crohn's disease, further investigations of antioxidant status in these patients would also be warranted.

Analysis of plasma lipid levels in patients with Crohn's disease revealed several differences as compared to the control group (Figure 1). Triglyceride levels were significantly higher. This could result from an increased mobilization of triglycerides from its storage site, the liver, as a source of metabolic energy required to fulfill an energy deficit during periods of relapse. Also, to compensate for inadequate dietary intake or malabsorption of lipids, there may be an increased mobilization of triglycerides. Endogenous triglycerides are mainly transported from the liver to peripheral tissue by the lipoprotein VLDL. Triglyceride enrichment was observed in IDL, a VLDL-remnant particle in our Crohn's disease group,. Triglyceride enrichment in HDL_2, but not in VLDL particles was also noted. This suggests that following their mobilization, the transport of excess triglycerides back to the liver may be inefficient in patients with Crohn's disease, leading to an accumulation of triglycerides in peripheral tissues.

Second, plasma levels of cholesterol were significantly lower in patients with Crohn's disease as compared to the control group (Figure

1). LDL primarily transports cholesterol to peripheral tissues, while excess cholesterol is returned to the liver by HDL. Membrane cholesterol replacement occurs due to the normal turnover of lipids in the plasma membrane. However, in the presence of chronic inflammation and free radical attack cell membranes, this repair process may be accelerated. We observed a decrease in plasma levels of LDL-cholesterol and HDL-cholesterol, suggesting that the absorption of exogenous cholesterol may be inadequate either due to poor nutritional status, or as a result of decreased intestinal absorptive surface. Also, synthesis of endogenous cholesterol may be impaired in patients with Crohn's disease. Alternatively, cholesterol lost in bile acids due to an inefficient reabsorption in the diseased ileum may exceed the body's ability for their synthesis. Paralleling the changes in HDL-cholesterol and LDL-cholesterol was a reduction in the plasma concentration of apolipoproteins A-I and B. These results are not surprising, since apo B and apo A are predominantly found in LDL and HDL particles, respectively (68).

Lipoproteins have a high content of polyunsaturated fatty acids and contain apolipoproteins; both of these constituents are highly susceptible to oxidation, which may alter their metabolic functions. We therefore thoroughly studied the chemical composition of lipoproteins in patients with Crohn's disease as compared to the control group. Several

differences became apparent as shown in table IV. VLDL particles in patients with Crohn's disease contained lower amounts of triglycerides and proteins, which consequently decreased the mass ratio of core to surface constituents. These changes could be the result of the chemical modifications to polyunsaturated fatty acids and apolipoproteins that are present in VLDL particles. Triglycerides are hydrolyzed from VLDL by the enzyme lipoprotein lipase in the circulation. There is also an exchange of triglycerides and phospholipids for cholesteryl esters from VLDL to HDL particles. The decrease in triglyceride content in VLDL particles may be a physiologic response to the high concentration of triglycerides in the circulation. In addition, the exchange between VLDL and HDL particles could be abnormal. Oxidative modifications to apolipoproteins may account for the altered protein content in VLDL particles.

IDL particles of patients with Crohn's disease were enriched with triglycerides and proteins but were deficient in phospholipids (Table IV). Therefore, IDL particles, which are VLDL-remnant particles, should be triglyceride depleted and richer in cholesteryl ester. The differences in the chemical composition of both VLDL and VLDL-remnant particles may be a consequence of oxidative changes to the many polyunsaturated fatty acids that are present in VLDL particles. Therefore, the variations observed could reflect different nutritional and

physiologic states between patients with Crohn's disease and healthy controls.

LDL particles, whose main function is to transport cholesterol and cholesteryl esters from the liver to peripheral tissues, did not differ in patients with Crohn's disease as compared to the control group. Therefore, the decreased plasma concentrations of total cholesterol, LDL-cholesterol and HDL-cholesterol seen in individuals with Crohn's disease is not likely due to a disturbance in cholesterol delivery to peripheral tissues, but rather, to other mechanisms discussed above.

HDL_3 particles in patients with Crohn's disease had a higher content of phospholipids and a decreased amount of cholesteryl esters. The acquisition of triglycerides and phospholipids from VLDL particles may be abnormal since we also observed a deficiency of triglycerides in VLDL, although phospholipid content also differed in Crohn's disease patients as compared to the control group. HDL_2 particles in the plasma of patients with Crohn's disease contained an elevated percentage of triglyceride, cholesterol ester and free cholesterol but a decreased percentage of phospholipids and proteins. Also, the mass ratio of core to surface constituents was elevated as compared to the control group. The transfer of triglycerides from VLDL to HDL_2 particles may exceed the movement of cholesteryl ester from HDL_2 to VLDL, whereas the

transfer of phospholipids from VLDL to HDL_2 particles may be deficient. An important factor to consider is the quantity and chemical composition of apolipoproteins, since they not only solubilize lipoproteins and maintain their structural integrity, but are also essential in directing lipoprotein metabolism of . Apo A-I and B were investigated in this research project. However, further studies on the effects of oxidation on apolipoprotein structure and function, as well as their interaction with specific receptors would assist in providing additional information on lipoprotein diturbances in patients with Crohn's disease.

The fatty acid profile of patients with Crohn's disease revealed several significant differences from the control group (Tables II and III). Saturated and monounsaturated fatty acids has a higher percentage of total fatty acids, whereas polyunsaturated fatty acids were decreased in patients with CD in comparison to the control group. Consequently, the ratio PUFA/saturated fatty acids was lower in patients with Crohn's disease. Polyunsaturated fatty acids play a substantial physiological role in determining the fluidity of membrane lipid bilayers, thereby modulating protein conformation and altering membrane signal transduction processes (16). Fatty acid synthesis in mammalians generates only saturated and monounsaturated fatty acids of the n-9 series since they lack the ability to desaturate fatty acids in the n-6 and n-3 position of the fatty acid chain. Polyunsaturated fatty acids are the

precursors of eicosanoids, which play a significant role in mediating inflammation (2,16). Therefore, the lower percentage of polyunsaturated fatty acids may result from an elevated consumption related to increased synthesis of prostaglandins and leukotrienes. Furthermore, the decrease in membrane fluidity due to a reduction in polyunsaturated fatty acids may have serious consequences on cell function and could potentially play a considerable role in the pathogenesis of Crohn's disease. The health benefits and risks of monounsaturated fatty acids cannot be generalized to all monounsaturated fatty acids, but certainly its elevation in comparison to the control group suggests a physiological or metabolic alteration in lipid metabolism. Saturated fatty acids and their contribution to atherogenesis have been extensively studied. Although the patients in our study did not have elevated plasma cholesterol levels, an important factor in atherogenesis, lipid peroxydation is elevated, as shown by the increase in MDA. Therefore, high amounts of saturated fatty acids could further deteriorate the health status of patients with CD.

As shown on table III, several variances were noted between the different fatty acid series studies. The percentage of total n-3 fatty acids was lower than in the control group, whereas total n-6, n-7 and n-9 fatty acids were significantly higher in CD patients. A competitive interaction exists between fatty acids of the n-3, n-6, n-7 and n-9 series, since the

same desaturase enzymes are involved. Plasma fatty acid profiles generally reflect dietary intake. Therefore, differences between fatty acid profile of Crohn's disease patients and the control group could be a direct consequence of nutrient intake variations. Investigators such as Shoda et al. have reported an epidemiological association between an increase in the incidence of Crohn's disease in Japan with the increased intake of n-6 fatty acids combined with a decline of n-3 fatty acid consumption (33). The variation in fatty acid profile may therefore be implicated in the underlying pathological processes in Crohn's disease. As noted above, fatty acids of the n-6 series are the precursors of eicosanoids, important mediators of inflammatory vasodilation, vascular permeability, aggregation and adhesion of inflammatory cells,, as well as abdominal pain and fever (2,4). In contrast, prostaglandins and leukotrienes from n-3 polyunsaturated fatty acids not only decrease the production of pro-inflammatory eicosanoids originating from n-6 PUFA but are also active vasodilators, weak inducers of inflammation and weak chemotactic agents (16). Therefore, the n-6/n-3 fatty acid imbalance seen in patients with Crohn's disease may further aggravate the chronic inflammatory process, a key element in the pathogenesis of the illness. In addition, the two well-established indices of fatty acid deficiency, 16:1n-7/18:2n-6 and 20:3n-9/20:4n-6 were both significantly higher in patients with Crohn's disease in comparison to the control group. Although there was no clinical manifestation of the classical

symptoms of essential fatty acid deficiency, inadequacy of essential fatty acids is known to alter immune function, most probably through modifying membrane phospholipid fatty acid composition, which will in turn alter membrane function (118).

CONCLUSION

In summary , in this research project we investigated in depth the lipid metabolism and the pro-oxidant/antioxidant status of 22 patients with Crohn's disease as well as in 10 healthy control subjects. Alterations in the lipid profile, lipoprotein composition and fatty acid profile of CD patients revealed disturbances in their lipid metabolism. Furthermore, the elevation in the plasma concentration of malonaldehyde and glutathione un addition to the decrease in the level retinol suggested that antioxidant defenses in CD patients are jeopardized. The possible role of immunomodulation with n-3 fatty acid supplements, either by nutritional or pharmacological therapies may be beneficial. However, the pro-oxidant/antioxidant imbalance suggested by our results suggest that additional studies are warranted. Future challenges for clinicians and scientists lie in further elucidating the impact of oxidative stress on the relationship between antioxidants and lipid metabolism, so that novel nutritional therapeutic strategies aimed at modifying the course of the disease may be developed.

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Annexe A. Crohn's Disease Activity Index (CDAI)

	Date (Day/Month/Year)	Sum	Factor	subtotal
	1 2 3 4 5 6 7			
# liquid or very soft stools			X 2 =	
Abdominal pain rating *			X 5 =	
General well-being[θ]			X 7 =	
# of infirm days[Ω]			X 5 =	
Symptoms or findings present during the past week (√ all that apply):	□arthritis or arthralgia □skin or mouth lesions □iritis or uveitis □anal fissures, fistula or perianal abscess □other external fistula □fever > 100^{0}F during the week		X 20 =	
Abdominal mass (√ one)	□ 0=No □ 2=Questionable □ 5=Definite		X 10	
Hematocrit[σ]	normal – actual value		X 6 =	
	100x[1-(body wgt/ideal body wgt]		X 1 =	
	Total the above subtotal columns to give CDAI			

* 0 = none 1 = mild 2 = moderate 3 = severe

[θ] 0 = generally well 1 = slightly indisposed 2 = poor 3 = very poor 4 = terrible

[Ω] Infirm days = child unable to attend school or unable to participate in normal activities as a result of the disease

Annexe B. Maladie de Crohn: relevé des symptômes

Inscrire le chiffre correspondant selon votre évaluation personnelle.

DATE							
Combien de selles liquides ou très molles avez-vous eu aujourd'hui?							
Avez-vous ressenti une douleur abdominale aujourd'hui? 0 = aucune 1 = légère 2 = modérée 3 = intense							
Comment vous sentez-vous aujourd'hui? 0 = généralement bien 1 = plus ou moins bien 2 = mal 3 = très mal 4 = affreusement mal							
Avez-vous dû manquer l'école ou des activités normales aujourd'hui en raison de la maladie de Crohn?							

Relevé de symptômes préparé par: Lise Bouthillier, dt.p.
Décembre 1996.

Annexe C. Nomenclature of major fatty acids

Abbreviation	Common name
14:0	Myristic acid
16:0	Palmitic acid
16:1(n-7)	Palmitoleic acid
18:0	Stearic acid
18:1(n-7)	Vaccenic acid
18:1(n-9)	Oleic acid
18:2(n-6)	Linoelaidic acid
18:3(n-3)	α-linolenic acid
18:3(n-6)	γ-linolenic acid
20:0	Arachidic acid
20:1(n-9)	Gondoic acid
20:3(n-6)	Dihomo-γ-linolenic acid
20:3(n-9)	Mead acid
20:4(n-6)	Arachidonic acid
20:5(n-3)	Timnodonic acid
22:0	Behenic acid
22:4(n-6)	Adrenic acid
22:5(n-3)	Docosapentaenoic acid
22:6(n-3)	Clupanodonic acid
24:0	Tetrasanoic acid
24:1(n-9)	Nervonic acid