

# Enhanced atherogenesis and altered high density lipoprotein in patients with Crohn's disease

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**Abstract** A chronic inflammatory state is a risk factor for accelerated atherogenesis. The aim of our study was to explore whether Crohn's disease (CD), characterized by recurrent inflammatory episodes, is also associated with accelerated atherogenesis. In 60 CD patients and 122 matched controls, carotid intima media thickness (IMT), a validated marker for the burden and progression of atherosclerosis, was assessed ultrasonographically. Additional subgroup analyses, including plasma levels of acute phase reactants and HDL protein profiling, were performed in 11 consecutive patients with CD in remission, 10 patients with active CD, and 15 healthy controls. Carotid IMT in patients with CD was increased compared with healthy volunteers: 0.71 (0.17) versus 0.59 (0.14) mm ( $P < 0.0001$ ), respectively. In the subgroup analysis, HDL levels in controls and patients in remission were identical [(1.45 (0.48) and 1.40 (0.46) mmol/l;  $P = 0.797$ ], whereas HDL during exacerbation was profoundly reduced: 1.02 (0.33) ( $P = 0.022$ ). HDL from patients with active CD and CD patients in remission was characterized by a reduced ability to attenuate oxidation compared with controls ( $P = 0.008$  and  $P = 0.024$  respectively). Patients with CD have increased IMT compared with matched controls, indicative of accelerated atherogenesis. The changes during CD exacerbation in terms of HDL concentration and composition imply a role for impaired HDL protection in these patients.—van Leuven, S. I., R. Hezemans, J. H. Levels, S. Snoek, P. C. Stokkers, G. K. Hovingh, J. J. P. Kastelein, E. S. Stroes, E. de Groot, and D. W. Hommes. **Enhanced atherogenesis and altered high density lipoprotein in patients with Crohn's disease.** *J. Lipid Res.* 2007. 48: 2640–2646.

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In the last decade, it has become increasingly clear that inflammation plays a pivotal role in the pathogenesis of atherosclerosis. Leukocytes can adhere to activated endothelium and transmigrate to the subendothelial space.

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Subsequently, immune cell activation leads to plaque progression and eventually plaque rupture, resulting in atherothrombotic disease (1, 2). Conversely, systemic inflammation itself has been suggested to promote the atherosclerotic process (2). Indeed, in several chronic inflammatory disorders, such as systemic lupus erythematosus (SLE) (3), rheumatoid arthritis (RA) (4), human immunodeficiency virus (5), and even periodontitis (6), systemic inflammation has been linked to enhanced atherogenesis, illustrated by an increased incidence of cardiovascular disease (CVD). Several mechanisms by which a systemic inflammatory state can accelerate the atherosclerotic process have been suggested. Cytokine-mediated damaging of the endothelium, immune cell activation, and activation of the coagulation cascade have all been implicated. In addition, inflammation can also induce changes in lipoprotein metabolism. Particularly, inflammation can decrease HDL concentrations as well as qualitatively affect HDL (7). During systemic inflammation, specific enzyme and protein components of HDL, contributing to HDL's antiatherogenic potential, are modified, thereby impeding its antiatherogenic functions, and may even render it proatherogenic (8). In several chronic inflammatory disorders, dyslipidemic changes have been linked to enhanced atherogenesis. The current exploratory study was designed to evaluate whether Crohn's disease (CD) is associated with an increased progression of the atherosclerotic process and whether inflammatory exacerbations are associated with alterations in HDL metabolism.

## MATERIALS AND METHODS

### Patients

CD patients were recruited at the outpatient inflammatory bowel disease clinic at the Academic Medical Center in Amsterdam. During study visits, disease activity was assessed

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using the Harvey Bradshaw index (HBI). The HBI is a research tool composed of clinical parameters (general well-being, abdominal pain, number of liquid stools per day, abdominal mass, and complications) used to quantify the symptoms of patients. Patients with  $HBI \geq 4$  were considered to have active CD, and patients with  $HBI < 4$ , were considered to be in remission. Blood samples were collected for C-reactive protein (CRP) and lipid profiling. CRP measurements were used for the second criteria defining active CD and CD in remission:  $CRP \geq 10$  mg/l and  $CRP < 10$  mg/l, respectively. All study patients were asked to participate in ultrasound carotid intima media thickness (IMT) measurements. Healthy controls matched for age and gender were recruited at the Department of Vascular Medicine and participated in the analysis of lipid profiles and IMT measurements. Patients gave written informed consent, and the study was approved by the local Medical Ethical Committee.

### Ultrasound measurements of the carotid IMT

As described elsewhere, IMT measurements allow for the investigation of arterial wall morphology and can describe the status as well as present and future CVD risk noninvasively (9). In summary, B-mode ultrasound carotid IMT measurements depict the intima media complex of carotid arterial walls. As shown in prospective epidemiological studies, a modest increase of IMT substantially increases the relative risk for myocardial infarction and stroke. IMT measurements have also shown the benefit of cholesterol-lowering and antihypertensive agents. IMT is an accepted validated surrogate marker for the status of atherosclerosis and present and future atherosclerotic disease risk (10).

### Laboratory measurements

All blood samples were immediately stored at  $-80^{\circ}\text{C}$  and thawed once before measurement. CRP was measured using a commercial high-sensitivity CRP kit for human CRP (hsCRP; Hemoll, Lexington, MA) according to the manufacturer's protocol. The minimum detectable CRP concentration of the assay was 2.0 mg/l. Samples with CRP concentrations  $> 8$  mg/l were diluted using CRP diluent. Serum amyloid A (SAA) analysis was carried out using a commercially available Human SAA ELISA kit from Anogen (Mississauga, Ontario, Canada) according to the manufacturer's protocol, and apolipoprotein A-I (apoA-I) and apoB were determined by nephelometric immunochemistry (Beckman).

### Lipoprotein composition

Total cholesterol distribution among the lipoproteins was measured by fast-phase liquid chromatography. In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and a UV-975 ultraviolet/visible light detector (Jasco, Tokyo, Japan). An extra P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line enzymatic reagent (Biomerieux, Marcy l'Etoile, France) addition at 0.1 ml/min. Plasma lipoprotein separations were performed using a Superose 6 HR 10/30 column (Pharmacia Biotech) with TBS, pH 7.4, as eluent at a flow rate of 0.31 ml/min. Total cholesterol was determined quantitatively using the PAP 250 cholesterol enzymatic method (Biomerieux, Le Fontanille, France). Computer analyses of the chromatograms for quantitative peak integration of the lipoproteins were carried out using Borwin Chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France).

### Sample preparation

HDL protein profiling was carried out as described previously (11). For coating of antibodies, a 5  $\mu\text{l}$  mixture containing 2.8 nM

anti-apoA-I monoclonal antibodies, 3  $\mu\text{M}$  ethylenediamine, and 0.1 M  $\text{Na}_2\text{SO}_4$  was added per spot of a PS-20 protein chip, and covalent binding of antibodies through primary amine-epoxide chemistry was achieved by incubating the chip in a humid chamber overnight at  $4^{\circ}\text{C}$ . Excess antibody was removed by one wash with distilled water, and subsequently, free amine binding places were blocked by incubating the chip for 30 min at room temperature with 1 M Tris buffer (pH 8.0).

For HDL capture, after mounting the PS-20 protein chip(s) in a 96-well bioprocessor, 100  $\mu\text{l}$  of diluted plasma aliquots (diluted 1:2 with TBS buffer: 50 mM Tris, pH 7.4, and 150 mM NaCl) was applied onto spot and allowed to bind for 2 h at room temperature on a horizontal shaker. The protein chips were washed four times with TBS for 10 min, followed by a 5 min TBS-Tween (0.005%) rinse unless indicated otherwise. A final wash step with HEPES solution (5 mM) was carried out to remove the excess salt. All spots were allowed to dry, and subsequently, 1.2  $\mu\text{l}$  of sinapinic acid (10 mg/ml) in a 50:49.9:0.1% acetonitrile-water-trifluoroic acid mix was applied on each spot. All chips were air-dried and stored at room temperature in the dark.

### Surface-enhanced laser desorption/ionization time-of-flight analysis (SELDI-TOF)

Analysis was carried out using a PBS IIc protein chip reader (Ciphergen Biosystems, Fremont, CA) using an automated data collection protocol within the Protein-Chip Software (version 3.1). Data were collected up to 200 kDa. Laser intensity was set in a range from 190 to 220 arbitrary units, and the focus mass was set to 28 kDa specific for the anti-apoA-I capture. Measurement of the spectra was performed with an average of  $\sim 100$  shots at 13 positions per SELDI spot. Calibration was done using a protein calibration chip (Ciphergen). Spectra were normalized on total ion current. Detected peaks having a signal-to-noise ratio  $> 5$  were recognized as significant peaks.

### HDL antioxidant score

The assay was performed as described previously with slight modifications (12) using historical controls. Briefly, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) is a proinflammatory phospholipid that triggers vascular inflammation processes. The HDL OxPAPC assay measures the potential of plasma-derived HDL to reduce/inactivate previously (air) oxidized phospholipids. HDL for this assay is isolated from plasma using dextran sulfate-coated magnetic beads. These beads precipitate apoB-containing lipoproteins. The cholesterol content of the HDL-containing supernatant is determined. HDL cholesterol (25  $\mu\text{M}$  final concentration) is added to a reaction mixture that contains OxPAPC. Adding 2',7'-dichlorofluorescein, a fluorochrome, to the reaction mixture produces a fluorescent signal dependent on the concentration of OxPAPC. Reduction of OxPAPC as a result of preincubation with HDL results in a loss of fluorescence, the readout parameter of this assay. As such, HDL with an antioxidant score  $> 1$  is considered proinflammatory, whereas a score of  $< 1$  is considered anti-inflammatory.

### Statistical analysis

SPSS statistical program 11.0.1 (SPSS, Inc., Chicago, IL) was used. Standard descriptive and comparative analyses were undertaken. Results are expressed as means  $\pm$  SD. Student's *t*-test for unpaired data was used to compare CD patients with healthy volunteers. Linear regression analyses were performed to assess the relationships between CRP, SAA, HDL, apoA-I, and apoB. One-way ANOVA tests were used to investigate differences between lipid, lipoprotein, and acute-phase protein values.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Characteristics of the study groups

A total of 60 CD patients participated in this study, of which 12 suffered from active disease and 48 were in clinical remission (Table 1). In total, 122 healthy controls were included in the reference group. No differences were observed in smoking behavior or the presence of hypertension or diabetes mellitus between the three groups.

### IMT measurements

IMT measurements were performed in 60 CD patients and 122 healthy controls. Both groups exhibited a wide age range (Table 1); nevertheless, the IMT of CD patients ( $0.71 \pm 0.17$  mm) was increased compared with that of healthy controls ( $0.59 \pm 0.14$  mm) ( $P < 0.001$ ). The estimated IMT increase in CD patients and control subjects with age is graphically displayed in Fig. 1. The regression line for CD differed significantly from that of controls ( $P < 0.05$ ).

### Lipid profiles

Additional analyses were performed in the first consecutive 11 patients with CD in remission, 10 patients with active CD, and 15 healthy controls (Table 2). Mean HDL concentrations were higher in controls and patients in remission compared with patients with active CD ( $P = 0.022$  and  $P = 0.043$ , respectively). HDL concentrations did not differ between controls and patients in remission. Reduced VLDL concentrations were found in active patients compared with controls and patients in remission ( $P = 0.019$  and  $P = 0.028$ , respectively). Concentrations of LDL did not differ among the groups. With respect to apolipoproteins, controls and patients in remission had significantly higher serum apoA-I compared with CD patients with active disease (Table 2), whereas apoB levels were similar in all groups.

### Acute-phase proteins

Mean CRP concentrations were increased significantly in patients with active CD compared with patients in re-

mission and controls (Table 2). A linear regression analysis (log-transformed) showed that CRP and HDL were correlated ( $r^2 = 0.24$ ,  $P = 0.002$ ). Another acute-phase protein, SAA, was not significantly different between controls and patients in remission ( $P = 0.870$ ), whereas SAA concentrations in active CD were increased significantly compared with those in controls and patients in remission (Table 2).

### Surface-enhanced laser desorption/ionization time-of-flight analyses

After SELDI-time-of-flight (TOF) analyses, protein spectra from HDL were obtained. Within each of the groups, all spectra showed virtually similar profiles. Comparing the fingerprints of the healthy controls and CD in remission group with those of active CD patients, statistically significant deviations were observed in relative intensity on five different markers: 3,602, 4,634, 11,695, 13,843, and 14,106  $m/z$  (Fig. 2). No statistically significant differences between the HDL fingerprints from healthy controls and patients in remission were seen.

### Antioxidant potential of HDL

The antioxidative indices of HDL of controls and patients with CD, active and in remission, are displayed in Fig. 3. The antioxidative capacity of HDL did not differ between CD patients in remission and those with active disease ( $P = 0.53$ ). HDL isolated from patients with active CD but also from CD patients in remission was characterized by a reduced ability to attenuate oxidation compared with that of controls ( $P = 0.008$  and  $P = 0.024$ , respectively).

## DISCUSSION

In the current exploratory analysis, we show that CD is associated with an acceleration of the atherosclerotic process, as illustrated by an increased carotid IMT in CD patients compared with healthy controls. In addition, CD patients were characterized during an inflammatory exacerbation by profoundly decreased levels of HDL combined with biochemical changes of the HDL particle.

TABLE 1. Baseline characteristics of the study subjects

Characteristics	Patients with Active CD (n = 12)	Patients with CD in Remission (n = 48)	Patients with CD (n = 60)	Healthy Controls (n = 122)
Age, years	34.4 ± 9.0	44.4 ± 12.9	42.4 ± 12.8	40.5 ± 16.4
Male gender, n (%)	7 (58.3)	20 (41.7)	27 (45.0)	55 (45.1)
Body mass index (kg/m <sup>2</sup> )	25.6	24.1 ± 3.9	24.4 ± 3.9	25.0 ± 8.1
Hypertension, n (%)	1 (8.3)	4 (8.3)	5 (8.3)	7 (5.7)
Diabetes mellitus, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Smoking, n (%)	4 (33.3)	9 (18.8)	13 (21.7)	22 (18.0)
Harvey Bradshaw index	8.1 ± 3.2	1.3 ± 0.7	2.7 ± 4.8	NA
Total cholesterol, mmol/l	3.79 ± 0.89	4.73 ± 1.11	4.54 ± 1.12	5.04 ± 0.99
LDL cholesterol, mmol/l	2.46 ± 0.91	2.62 ± 0.97	2.59 ± 0.96	2.99 ± 0.81
HDL cholesterol, mmol/l	1.01 ± 0.30	1.66 ± 0.43	1.53 ± 0.48	1.47 ± 0.53
Triglycerides, mmol/l	0.70 ± 0.88	0.98 ± 0.54	0.92 ± 0.62	1.34 ± 1.25
High-sensitivity CRP, mg/l	94.5 ± 108.3	2.7 ± 2.5	21.0 ± 59.7	1.9 ± 1.7
IMT, mm	0.62 ± 0.13	0.73 ± 0.17	0.71 ± 0.17	0.59 ± 0.14

Values are given as means ± SD. CD, Crohn's disease; IMT, intima media thickness.

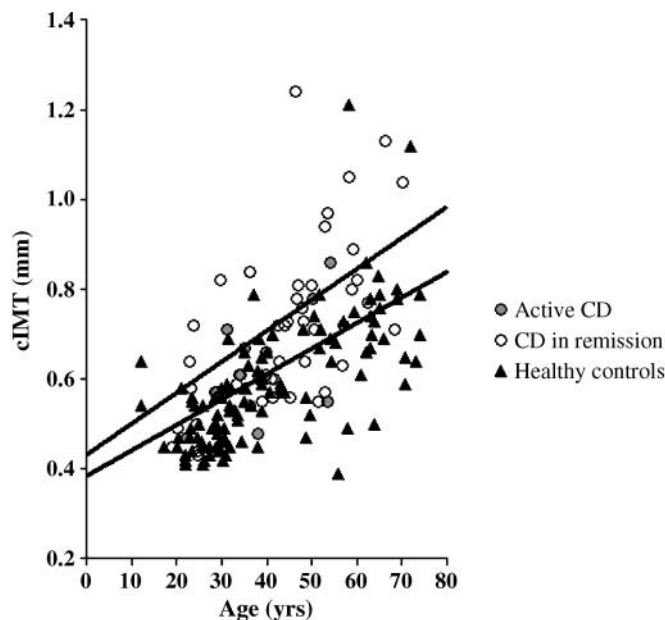


Fig. 1. Carotid intima media thickness (cIMT) and age in Crohn's disease (CD) patients and healthy controls.

These data suggest that early detection of atherosclerosis and subsequent cardiovascular prevention in patients with CD might be warranted.

#### IMT in CD

Arterial wall thickness can be measured as a continuous variable from childhood into old age and can be used in patients as well as controls. Consequently, carotid IMT measurements are used on a broad scale to assess risk in individuals with an increased cardiovascular risk, such as patients with chronic inflammatory disease such as SLE and RA and those with familial hypercholesterolemia. In the latter group, IMT measurements have also been used to evaluate the effectiveness of various therapeutic interventions. As such, IMT is now a validated surrogate marker for the assessment of atherosclerotic vascular disease (9, 10). In the current study, we also assessed the progression of the atherosclerotic process in CD by measuring IMT. Indeed, IMT was increased significantly in patients with CD compared with healthy controls. Because

of the plethora of risk factors that are of influence on IMT in CD, a CVD risk assessment based on IMT data from other populations at risk in a parallel risk assessment is premature. It must be noted, however, that the average IMT increase of 0.12 mm in CD patients and 0.59 mm in unaffected individuals is similar to the estimated IMT increase in other patient groups characterized by a proatherogenic state. This strongly implies that CD patients are at increased CVD risk. Long-term follow-up studies investigating the causality of inflammatory, dyslipidemic, and other causes of accelerated atherosclerosis in these patients, as well as clinical trials to evaluate preventive drug therapy, are warranted.

#### CVD in CD

For up to 70 years, it has been known that inflammatory bowel disease is associated with venous but also arterial thrombosis (13–21). The incidence ranges from 1.2% to 6.1% according to different studies and up to 39% in autopsy studies (22). The same risk factors that have been suggested to underlie this atherothrombotic state in CD are also risk factors for atherosclerosis, such as hyperhomocysteinemia (23), antiphospholipid antibodies (24), and a procoagulant state (25). Interestingly, atherosclerosis and CD also share a common pathway in the CD40/CD40L system (26), and although circulating activated platelets underlie a procoagulant milieu in CD (27), they have also been shown to exacerbate atherosclerosis (28). Furthermore, systemic inflammation has emerged as a causal factor for accelerated atherogenesis in inflammatory disease states such as SLE and RA (29, 30). Recently, evidence for such an association has emerged in other chronic inflammatory disorders as well. Indeed, several reports have also suggested that CD is associated with premature atherosclerosis (31–35). In addition, our findings of accelerated atherosclerosis correspond well with several other studies that showed evidence of subclinical atherosclerosis in inflammatory bowel disease by demonstrating IMT thickening (36, 37) or endothelial dysfunction (38). Although numbers on cardiovascular morbidity are lacking, cardiovascular mortality does not appear to be increased in patients with CD. However, there is significant disparity in the reported mortality rates in CD, ranging from 30% lower than expected to 70% higher than

TABLE 2. Lipid values, acute-phase proteins, and apolipoproteins of consecutive subjects in a subgroup analysis

Parameter	Controls (n = 15)	Active CD Patients (n = 10)	CD Patients in Remission (n = 11)
HDL, mmol/l	1.45 ± 0.48	1.02 ± 0.33 <sup>a,b</sup>	1.40 ± 0.46
VLDL, mmol/l	0.37 ± 0.20	0.18 ± 0.13 <sup>a,b</sup>	0.37 ± 0.22
LDL, mmol/l	2.64 ± 0.66	2.57 ± 0.96	2.54 ± 0.70
CRP, mg/l	0.8 ± 0.4	108.8 ± 113.8 <sup>c,d</sup>	2.6 ± 2.7
Serum amyloid A, mg/l	2.6 ± 1.4	530.8 ± 655.3 <sup>c,d</sup>	25.1 ± 61.3
Apolipoprotein A-I, g/l	1.48 ± 0.27	1.11 ± 0.27 <sup>c,d</sup>	1.52 ± 0.22
Apolipoprotein B, g/l	0.87 ± 0.21	0.88 ± 0.26	0.90 ± 0.27

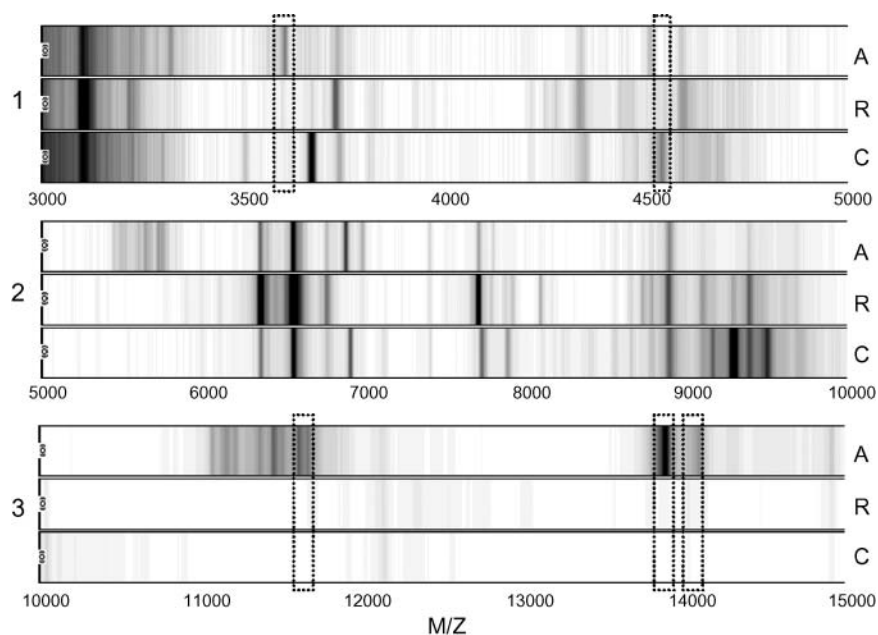
Values are given as means ± SD.

<sup>a</sup>  $P < 0.05$  compared with controls.

<sup>b</sup>  $P < 0.05$  compared with patients in remission.

<sup>c</sup>  $P < 0.005$  compared with controls.

<sup>d</sup>  $P < 0.005$  compared with patients in remission.



**Fig. 2.** A representative example of HDL spectra in Gelview of patients with CD and controls. Depicted are the spectra in the  $m/z$  mass range between 3,000 and 5,000 Da (panel 1), 5,000 and 10,000 Da (panel 2), and 10,000 and 15,000 Da (panel 3). Within the dotted boxes, the statistically significant deviations after Bonferroni correction among active CD patients (A), CD patients in remission (R), and controls (C) are seen. All spectra were normalized on total ion current.

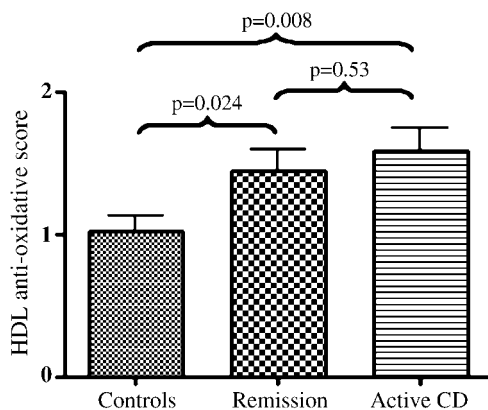
expected (39). Most of the patients in these cohorts were identified retrospectively and were diagnosed before improved medical treatment became available. In a more recent study, the mortality risk of CD patients was increased significantly, and a standardized mortality ratio for cardiovascular mortality was 1.49 (40). Long-term follow-up studies are required to resolve these issues.

### HDL and CD

The outcome of the atherosclerotic process is determined by the balance between proatherogenic and antiatherogenic stimuli. HDL is among the most powerful endogenous mediators in atheroprotection, which is illustrated by the strong inverse relationship between HDL levels and the incidence of CVD (41, 42). Via the reverse cholesterol trans-

port pathway, HDL can transport cholesterol from peripheral tissues, such as the arterial wall, back to the liver. Several additional properties of HDL contribute to its antiatherogenic potential. First, HDL exerts various anti-inflammatory effects and can reduce vascular inflammation in atherogenesis. For instance, HDL reduces the endothelial expression of adhesion molecules and chemokines, thereby reducing the recruitment of leukocytes to the subendothelial space (43). Oxidative modification of lipoproteins plays a pivotal role in atherogenesis. HDL has potent antioxidant properties and can reduce oxidative stress by a transport mechanism that binds oxidant molecules and carries anti-oxidative enzymes as well (43). Consistent with inflammation, there is increased oxidative stress in CD patients, resulting in increased lipid peroxidation (44). This will stimulate atherogenesis in patients with CD but can be neutralized by HDL. Third, HDL has several antithrombotic properties that are of interest because CD is associated with a procoagulant state, illustrated by an increased risk of venous thromboembolism. Thus, distinct changes in HDL are likely to contribute to the proatherogenic state in CD, particularly during exacerbation of the disease.


With regard to the effects of HDL beyond its role in reverse cholesterol transport, it has been advocated that changes in HDL's functional characteristics may provide information on its vasculoprotective effects over and beyond merely focusing on HDL levels. In agreement with this, "dysfunctional" HDL particles have been demonstrated in patients with overt CVD (45). Interestingly, HDL from patients with active CD, without CVD, was characterized by a higher anti-oxidative score compared with healthy volunteers, indicating an attenuated antiatherogenic potential



**Fig. 3.** The antioxidative index of HDL isolated from controls, patients with CD in remission, and patients with active CD.

(45). The fact that there was still a higher antioxidative score of HDL in CD patients in remission compared with controls may imply that even in remission the low-grade inflammatory state has an impact on HDL quality, in spite of the normalization of HDL levels. These findings bear close resemblance to those reported in patients with SLE and RA, in whom proinflammatory changes in HDL have been observed (46). In agreement, Navab et al. (47) have elegantly demonstrated that during acute-phase reactions the concentration of “protective” proteins on the HDL particle actually decreases significantly. Overall, our findings imply that during CD exacerbation, the loss of HDL’s atheroprotective effects, combined with the decrease in HDL levels, may contribute to accelerated atherogenesis.

Simultaneously, HDL protein profiling has emerged as a promising tool to unravel the biochemical composition of HDL (11, 48, 49), with which functional characteristics are intertwined. In addition to showing diminished levels of HDL during an inflammatory exacerbation of CD, using SELDI-TOF analysis we were able to show alterations in HDL composition. Interestingly, it has been suggested that HDL during an inflammatory episode can lose its protective properties and can even enhance atherogenesis (8). In the present study, we observed changes in the biochemical composition of the HDL particle. Even though SELDI-TOF mass spectrometry is not a direct approach for the identification of proteins, the 11,695 marker can be identified as SAA. This has already been confirmed in previous reports using SELDI-TOF combined with MALDI-TOF mass spectrometry for the identification of this specific marker (50–52). The acute-phase response in patients with active CD, characterized by increased SAA levels, thus seems to underlie an increased presence of SAA within the HDL particle. In fact, it was recently shown that proinflammatory cytokines such as tumor necrosis factor- $\alpha$  and interleukin-6, which are increased in patients with CD, induce SAA expression in hepatocytes (53). It is already known that SAA has the capacity to replace apoA-I in the HDL particle, which renders them less protective (54, 55).

In conclusion, these data suggest that atherogenesis is enhanced in patients with CD. Changes in HDL concentration as well as in HDL’s compositional and functional characteristics during exacerbations of CD imply that attenuation of this antiatherogenic mediator during these exacerbations might contribute to the progression of atherogenesis. The present findings call for consideration of the implementation of CVD detection and prevention in patients with CD. 

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