Inflammation and its echo in atherosclerosis

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Chapter 8

Enhanced Atherogenesis And Altered High-Density Lipoprotein in Patients with Crohn's Disease

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Abstract

Background: 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.

Methods: 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.

Results: Carotid IMT in patients with CD was increased compared to 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 to controls ($p=0.008$ and $p=0.024$ respectively).

Conclusions: Patients with CD have increased IMT compared to matched controls, indicative of accelerated atherogenesis. The changes during CD exacerbation in terms of HDL concentration as well as composition imply a role for impaired HDL protection in these patients.

Introduction

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. 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 (HIV)[5] or 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 lower high-density lipoprotein (HDL) concentrations as well as qualitatively affect HDL.[7] During systemic inflammation, specific enzyme and protein components of HDL, contributing to HDL's anti-atherogenic potential, are modified thereby impeding its anti-atherogenic functions, and even may render it pro-atherogenic.[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.

Methods

Patients

CD patients were recruited at the outpatient inflammatory bowel disease (IBD) clinic at the Academic Medical Centre, Amsterdam. During study visits, disease activity was assessed 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 ≥ 4 were considered to have active CD, patients with scores <4, were considered to be in remission. Blood samples were collected for CRP and lipid profiling. CRP measurements were used for the second criteria defining active CD and CD in remission: CRP ≥ 10 mg/l and CRP < 10 mg/l respectively. All study patients were asked to participate in ultrasound carotid 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 intima-media thickness (IMT)

As is described in extenso elsewhere, IMT measurements allow for the investigation of arterial wall morphology and can describe the status as well as the present and future cardiovascular disease risk, non-invasively.[9] In summary, B-mode ultrasound cIMT measurements depict the intima-media complex of carotid arterial walls. As was shown in prospective epidemiological studies already 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 anti-hypertensive 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 -80º C and thawed once before measurement. C-reactive protein (CRP) was measured using a commercial high sensitivity-CRP reaction kit for human CRP (hsCRP, HemoIL, Lexington, MA, USA) according to manufacturer's protocol. The minimum detectable CRP concentration of the assay was 2.0 mg/l. Samples with CRP concentrations higher than 8 mg/l were diluted using CRP diluent. Serum amyloid A (SAA) analysis was carried out using a commercial available Human SAA ELISA kit from Anogen (Mississauga, Ontario, Canada) according to the manufacturer's protocol, ApolipoproteinAI (apoAI) and apoB were determined by nephelometric immunochemistry (Beckman, USA).

Lipoprotein Composition

Total cholesterol (TC) distribution amongst the lipoproteins was measured by Fast Phase Liquid Chromatography (FPLC). In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an UV-975 UV/VIS 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, Upsala Sweden) with TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Total cholesterol was determined quantitatively using 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 and SELDI-TOF MS analysis

HDL protein profiling was carried out as described previously.[11] Coating of antibodies: A 5 µL mixture containing 2.8 nM anti-apo A-I monoclonal antibodies, 3 µM ethylenediamine and 0.1 M Na_2SO_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 ˚C. Excess antibody was removed by 1 wash with distilled water and subsequently free amine-binding places were blocked by incubating the chip for 30 min at RT with 1 M Tris buffer (pH 8.0).

HDL capture: After mounting the PS-20 protein chip(s) in a 96 wells bioprocessor, 100 µL diluted plasma aliquots (1:2 diluted with TBS buffer; 50 mM Tris, pH 7.4, 150 mM NaCl) or purified HDL were applied onto single SELDI spots and were allowed to bind for 2 hours at RT on a horizontal shaker. The protein chips were washed 4 times with TBS for 10 minutes, followed by a 5 minutes TBS-Tween (0.005%) rinse unless indicated otherwise. A final wash step with Hepes solution (5 mM) was carried out to remove the excess of salt. All spots were allowed to dry and subsequently 1.2 µL sinapinic acid (10 mg/ml) in a 50/49.9/0.1 % acetonitril/H_2O/trifluoric-acid mix was applied on each spot. All chips were air dried and stored at room temperature in the dark.

SELDI-TOF analysis

Analysis was carried out using a PBS IIc protein chip reader (Ciphergen Biosystems, Fremont, CA, USA) using an automated data collection protocol within the ProteinChip Software (version 3.1). Data was 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 A-I capture. Measurement of the spectra was performed with an average of approximately 100 shots at 13 positions per SELDI spot. Calibration was done using a protein calibration chip (Ciphergen). Spectra were normalised on total ion current. Detected peaks having a signal/noise ratio > 5 were recognized as significant peaks.

HDL anti-oxidant score

The assay was performed as described previously with slight modifications[12] using historical controls. Briefly, oxidized PAPC (oxPAPC) is a pro-inflammatory phospholipid, which triggers vascular inflammation processes. The respective 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-sulphate coated magnetic beads. These beads precipitate apoB-containing lipoproteins. The cholesterol content of the HDL-containing supernatant is determined. HDL cholesterol (25uM final concentration) is added to a reaction mixture that contains oxPAPC. Adding DCF – a fluorochrome – to the reaction mixture produce a fluorescence signal dependent of the concentration of oxPAPC. Reduction of oxPAPC due to pre-incubation with HDL results in loss of fluorescence, the readout parameter of this assay. As such, HDL with an anti-oxidant score>1 is considered pro-inflammatory whereas a score <1 is considered anti-inflammatory.

Statistical analysis

SPSS statistical program 11.0.1(SPSS Inc., Chicago, USA) was used. Standard descriptive and comparative analyses were undertaken. Results are expressed as mean and standard deviations (SD). Student's *t*-test for unpaired data was used to compare CD patients with healthy volunteers. Linear regression analyses were performed to asses the relationships between CRP, SAA, HDL, ApoAI and ApoB. One-way ANOVA tests were used to investigate differences between lipid, lipoprotein and acute phase protein values. A p-value <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), but nevertheless the IMT of CD patients

(0.71(0.17) mm) was increased when compared to healthy controls (0.59(0.14) mm): p<0.001. The estimated IMT increase in CD and control subjects with age is graphically displayed in figure 1. The regression line for CD differed significantly from that of controls (p<0.05).

Table 1. Baseline Characteristics of the Study Subjects.

	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, n (%)	7 (58.3)	20 (41.7)	27 (45.0)	55 (45.1)
BMI	25.6	24.1 ± 3.9	24.4 ± 3.9	25.0 ± 8.1
HT, n(%)	1 (8.3)	4 (8.3)	5 (8.3)	7 (5.7)
DM , n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Smoking, n (%)	4 (33.3)	9 (18.8)	13 (21.7)	22 (18.0)
HBI	8.1 ± 3.2	1.3 ± 0.7	2.7 ± 4.8	NA
Total cholesterol	3.79 ± 0.89	4.73 ± 1.11	4.54 ± 1.12	5.04 ± 0.99
LDL cholesterol	2.46 ± 0.91	2.62 ± 0.97	2.59 ± 0.96	2.99 ± 0.81
HDL cholesterol	1.01 ± 0.30	1.66 ± 0.43	1.53 ± 0.48	1.47 ± 0.53
Triglycerides	0.70 ± 0.88	0.98 ± 0.54	0.92 ± 0.62	1.34 ± 1.25
hsCRP	94.5 ± 108.3	2.7 ± 2.5	21.0 ± 59.7	1.9 ± 1.7
IMT	0.62 ± 0.13	0.73 ± 0.17	0.71 ± 0.17	0.59 ± 0.14

Values are given as means ± SD. HT=hypertension, DM= Diabetes Mellitus, HBI= Harvey Bradshaw Index, LDL= Low Density Lipoprotein, HDL= High Density Lipoprotein, hsCRP= high sensitivity C-Reactive Protein, NA=not applicable. Lipid values are in mmol/L, hsCRP in mg/L, carotid IMT in mm.

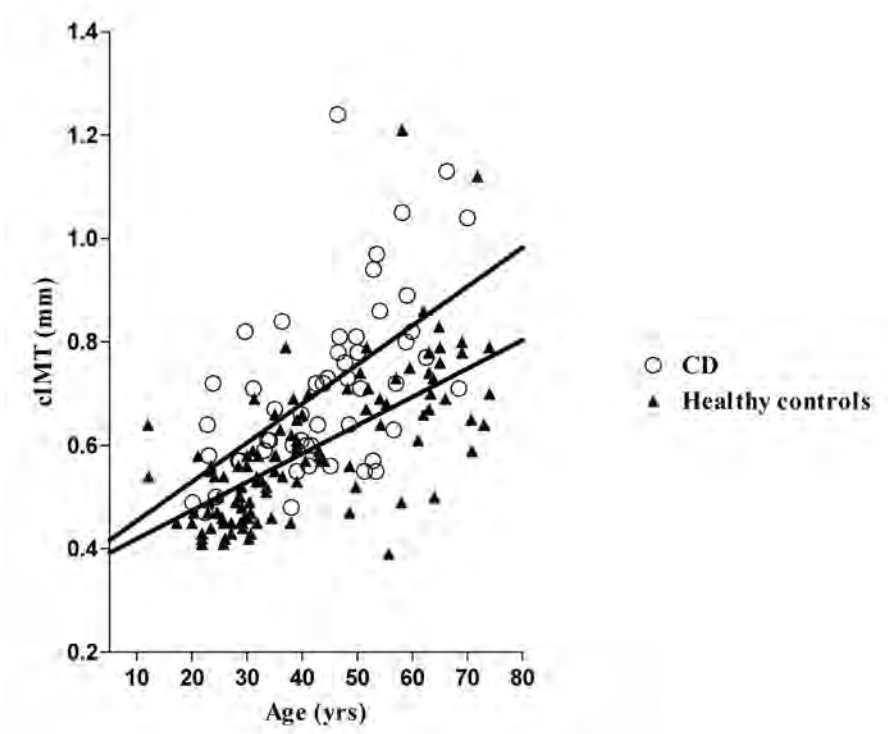

Figure 1. Carotid intima media thickness (cIMT) and age in CD patients and healthy controls.

The probability value indicates the difference in slope between the two lines.

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 to 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 to controls and patients in remission (p=0.019 and 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 apoAI compared to CD patients with active disease (table 2) whereas apoB levels were similar in all groups.

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	1.45±0.48	1.02±0.33*†	1.40±0.46
VLDL	0.37±0.20	0.18±0.13*†	0.37±0.22
LDL	2.64±0.66	2.57±0.96	2.54±0.70
CRP	0.8±0.4	108.8±113.8**††	2.6±2.7
SAA	2.6±1.4	530.8±655.3**††	25.1±61.3
ApoAI	1.48±0.27	1.11±0.27**††	1.52±0.22
ApoB	0.87±0.21	0.88±0.26	0.90±0.27

Values are presented as mean ± SD; n= number of subjects, HDL, High Density Lipoprotein; VLDL, Very Low Density lipoprotein; LDL Low Density Lipoprotein; CRP, C-reactive-protein, SAA, Serum Amyloid-A; ApoAI, ApolipoproteinAI; ApoB, ApolipoproteinB. Lipid values are in mmol/L, acute phase reactants in mg/L and apolipoprotiens in g/L. * $p<0.05$ compared to controls.† $p<0.05$ compared to patients in remission.** $p<0.005$ compared to controls.†† $p<0.005$ compared to patients in remission

Acute phase proteins

Mean CRP concentrations were increased significantly in patients with active CD when compared to patients in remission 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, $p=0.870$, while SAA concentrations in active CD were significantly elevated compared to controls and patients in remission (table 2).

SELDI-TOF analyses

After SELDI-TOF analyses, protein spectra from HDL were obtained. Within each of the groups, patients with active CD or CD in remission and healthy controls, all spectra showed virtually similar profiles. Comparing the fingerprints of the healthy controls and CD in remission group with those of HDL from active CD patients statistically significant deviations in relative intensity on 5 different markers; 3602, 4634, 11695, 13843 and 14106 M/Z were observed (figure 2). No statistically significant differences between the HDL fingerprints from healthy controls and patients in remission were seen.

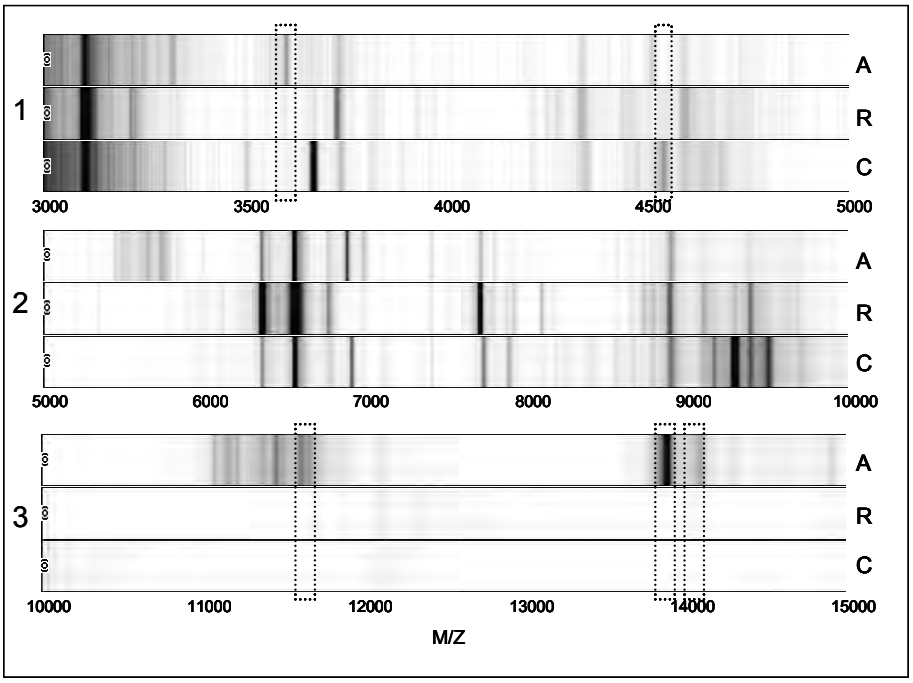

Figure 2. A representative example of HDL spectra in gelview of patients with Crohn's disease and controls respectively.

Depicted are the spectra in the M/Z mass range between 3000 –5000 Da (panel 1), 5000-10000 Da (panel 2) and 10000-15000 Da (panel 3). Within the dotted boxes the statistically significant deviations after Bonferroni correction between active Crohn (A) and remission (R) or controls (C) are seen. All spectra were normalised on total ion current.

Anti-oxidant potential of HDL

The anti-oxidative indeces of HDL of controls and patients with CD, active and in remission, are displayed in figure 3. The anti-oxidative capacity of HDL did not differ between CD patients in remission compared to 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 when compared to that of controls (p=0.008 and p=0.024 respectively).

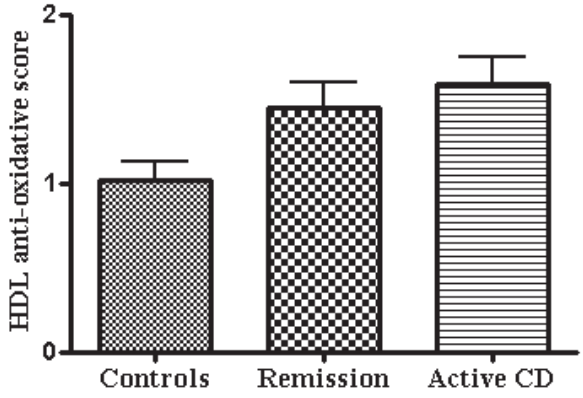

Figure 3. The anti-oxidative index of HDL isolated from controls, patients with CD in remission and patients with active CD.

Discussion

In the current exploratory analysis we show that CD is associated with acceleration of the atherosclerotic process as illustrated by an increased carotid IMT in CD patients compared to 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. These data suggest that early detection of atherosclerosis and subsequent cardiovascular prevention in patients with CD might be warranted.

Intima Media Thickness in Crohn's Disease

Arterial wall thickness can be measured as continuous variable from childhood into old age and can be used in patients as well as in controls. Consequently, carotid IMT measurements are used on a broad scale to assess risk in patients with an increased cardiovascular risk such as patients with chronic inflammatory disease such as SLE and RA but also familial hypercholesterolemia (FH). In the latter group IMT measurements have also been used to evaluate the effectiveness of various therapeutic interventions. As such, IMT is now an validated surrogate marker for the assessment

of atherosclerotic vascular disease.[9,10] In the current study we also assessed progression of the atherosclerotic process in CD by measuring IMT. Indeed, IMT was significantly increased in patients with CD when compared to healthy controls. Due to the plethora of risk factors that are of influence on IMT in CD, a CVD risk assessment based on IMT data of other populations at risk in parallel risk assessment is premature. It must be noted however, that the on average IMT increase of 0.12 mm in CD patients on top of 0.59 mm in the unaffected is of similar order to the estimated IMT increase in other patients groups characterized by a pro-atherogenic state. This strongly implies 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 therefore warranted.

Cardiovascular disease in Crohn's Disease

For up to seventy 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 underly 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 while 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 which showed evidence of subclinical atherosclerosis in IBD 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 reported mortality rates in Crohn's disease ranging from 30% lower than expected to 70% higher than 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, mortality risk of CD patients was significantly increased 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 Crohn's Disease

The outcome of the atherosclerotic process is determined by the balance between pro- and anti-atherogenic stimuli. HDL is amongst 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 transport (RCT) 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 anti-atherogenic potential. Firstly, 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 recruitment of leukocytes to the subendothelial space.[43] Oxidative modification of lipoproteins plays a pivotal role in atherogenesis. HDL has potent anti-oxidant 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. Thirdly, HDL has several antithrombotic properties which are of interest since 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 pro-atherogenic state in CD, particularly during exacerbation of the disease. With regard to the effects of HDL beyond its role in RCT, 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 line with this, 'dysfunctional' HDL particles have been demonstrated in patients with overt cardiovascular disease.[45] Interestingly, HDL from patients with active CD, without cardiovascular disease, was characterized by a higher anti-oxidative score compared to healthy volunteers, indicating an attenuated anti-atherogenic potential.[45] The fact that there was still a higher anti-oxidative score of HDL in CD patients in remission compared to controls may imply that even in remission the low-grade inflammatory state has an impact on HDL quality in spite of normalization of HDL levels. These findings bear close resemblance to those reported in patients with SLE and RA, in whom pro-inflammatory changes in HDL have been observed.[46] In line, Navab et al. have elegantly demonstrated that during acute phase reactions the concentration of 'protective' proteins on the HDL particle actually decreases significantly.[47] Overall, our findings imply that during CD exacerbation, loss of HDLs' atheroprotective effects, on top of the drop 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-DOF 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 ms is not a direct approach for identification of proteins, the 11695 marker can be identified as SAA. This has already been confirmed in previous reports by using SELDI TOF combined with MALDI TOF ms for 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 has recently been shown that pro-inflammatory cytokines such as TNF-α and IL-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 apoAI in the HDL particle which renders them less 'protective'.[54,55]

In conclusion, these data suggest 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 anti-atherogenic mediator during these exacerbations might contribute to the progression of atherogenesis. The present findings call for consideration of the implementation of cardiovascular disease detection and prevention in patients with CD.

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