Humans with Atherosclerosis have Impaired ABCA1 Cholesterol Efflux and Enhanced HDL Oxidation by Myeloperoxidase

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Abstract

Rationale—The efflux capacity of HDL with cultured macrophages associates strongly and negatively with CAD status, indicating that impaired sterol efflux capacity might be a marker—and perhaps mediator—of atherosclerotic burden. However, the mechanisms that contribute to impaired sterol efflux capacity remain poorly understood.

Objective—To determine the relationship between myeloperoxidase-mediated oxidative damage to apoA-I, the major HDL protein, and the ability of HDL to remove cellular cholesterol by the ABCA1 pathway.

Methods and Results—We quantified both site-specific oxidation of apoA-I and HDL's ABCA1 cholesterol efflux capacity in control subjects and subjects with stable coronary artery disease or acute coronary syndrome. The CAD and ACS subjects had higher levels of chlorinated tyrosine-192 and oxidized methionine-148 than the control subjects. In contrast, plasma levels of MPO did not differ between the groups. HDL from the CAD and ACS subjects was less able to accept cholesterol from cells expressing ABCA1 than HDL from control subjects. Levels of chlorinated tyrosine and oxidized methionine associated inversely with ABCA1 efflux capacity and positively with atherosclerotic disease status. These differences remained significant after adjusting for HDL-cholesterol levels.

Conclusions—Our observations indicate that MPO may contribute to the generation of dysfunctional HDL with impaired ABCA1 efflux capacity in humans with atherosclerosis. Quantification of chlorotyrosine and oxidized methionine in circulating HDL might be useful indicators of the risk of cardiovascular disease that are independent of HDL-cholesterol.

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Keywords

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INTRODUCTION

Clinical and epidemiological studies show a robust, inverse association of high density lipoprotein cholesterol (HDL-C) levels with cardiovascular disease risk [1]. In mouse models of atherosclerosis, HDL's ability to remove cholesterol from macrophages in the artery wall is a key cardioprotective function [2, 3]. A well-established pathway involves apolipoprotein A-I (apoA-I), HDL's major protein [4]. Lipid-free apoA-I promotes cholesterol efflux from macrophages via ABCA1, a membrane-associated transporter [5, 6]. Humans lacking ABCA1 suffer from Tangier disease [7], which is characterized by low levels of HDL-C, the accumulation of cholesterol-loaded macrophage in many different tissues [8, 9]. Ablation of ABCA1 in mouse myeloid cells promotes atherosclerosis in hypercholesterolemic mice without affecting HDL-C levels [10, 11], indicating that cholesterol efflux by the ABCA1 pathway is of central importance in the cardioprotective effects of HDL. Inflammation impairs macrophage sterol efflux in animal models, rendering HDL dysfunctional [12-14]. However, the underlying mechanisms and relevance of these findings to human atherosclerosis remain unclear.

Many lines of evidence indicate that oxidative stress plays a role in the pathogenesis of cardiovascular disease [15, 16]. One important mechanism may involve damage to HDL by myeloperoxidase (MPO), a heme protein expressed at high levels by inflammatory macrophages in human atherosclerotic tissue [14, 17]. The enzyme uses hydrogen peroxide (H_2O_2) and chloride ion to produce hypochlorous acid (HOCl), which converts tyrosine (Tyr) to 3-chlorotyrosine [18-20]. It also oxidizes methionine residues [21]. When lipid-free apoA-I is oxidized by MPO in vitro, its ability to remove excess cellular cholesterol by the ABCA1 pathway is decreased [22-24]. Moreover, in vitro studies with a mutated form of apoA-I and biochemical studies with methionine sulfoxide reductase suggest that chlorination of Tyr192 in concert with oxidation of methionine (Met) impairs ABCA1 transport activity [25]. Oxidation of Met in apoA-I also impairs its ability to activate lecithin-cholesterol acyltransferase (LCAT) [26], which plays a key role in maturation of HDL. Elevated levels of chlorotyrosine have been detected in HDL isolated from coronary disease subjects [22, 24] and Tyr192 is the major chlorination site in apoA-I of HDL isolated from human atherosclerotic lesions [27]. These observations suggest that oxidation might impair human HDL's cardioprotective functions, such as cholesterol clearance from macrophages in the artery wall.

Inflammation and metabolic disorders have been proposed to convert HDL to a dysfunctional form lacking anti-atherogenic properties [12, 13, 28]. Consistent with this proposal, the ability of serum HDL to promote sterol efflux from cultured macrophages varies markedly, despite similar levels of HDL-C and apoA-I [29]. Therefore, HDL-C is not necessarily the major determinant of HDL's macrophage sterol efflux capacity. One

proposed factor is HDL's activity with the ABCA1 pathway [29]. Importantly, the efflux capacity of serum HDL with cultured macrophages associated strongly and negatively with CAD status [30], and that association was independent of HDL-C and apoA-I levels. Taken together, these observations suggest that serum HDL's capacity to promote sterol efflux from macrophages reflects its functionality, raising the possibility that this function of HDL provides insight into the relationship between HDL biology and CAD risk.

The factors that control the efflux capacity of serum HDL remain poorly understood. In the current study, we isolated HDL from control subjects and subjects with stable coronary artery disease [31] or acute coronary syndrome [32], and used tandem mass spectrometry to explore the relationship between site-specific oxidation of apoA-I and the protein's ability to promote cholesterol efflux by the ABCA1 pathway. We show that levels of apoA-I chlorinated and oxidized by MPO are elevated in CAD and ACS subjects, and that they correlate inversely with HDL's cholesterol efflux capacity. Moreover, HDL from the CAD and ACS subjects was less able to accept sterol from cells expressing ABCA1 than HDL from control subjects. Thus, MPO may help generate dysfunctional HDL in humans.

Methods

Subjects

Plasma samples were collected prospectively over 10 months from three groups of subjects at the University of Washington: (i) control subjects (n=20), (ii) stable CAD subjects (n=20), and (iii) ACS subjects (n=20). The control subjects, who were recruited by advertisement, had no clinical history of cardiovascular disease, no family history of premature CAD, and were not receiving lipid-lowering therapy. The stable CAD subjects had clinically established atherosclerotic vascular disease but had been stable for at least 3 months. CAD subjects also had at least one 50% stenotic lesion on coronary angiography or a history of myocardial infarction, percutaneous coronary intervention or coronary artery bypass grafting. All ACS subjects exhibited acute ECG changes consistent with myocardial ischemia and/or elevated troponin levels. ACS was confirmed with urgent coronary angiography; all subjects had at least one 50% stenotic lesion with ruptured plaque and/or thrombus. All subjects provided signed informed consent and all protocols were approved by the University of Washington Institutional Review Board.

Plasma levels of lipids, high-sensitivity CRP (hs-CRP) and MPO

Lipid profiles and high sensitivity CRP (hs-CRP) were determined by the Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington. All lipid analyses were standardized using the Centers for Disease Control and Prevention Reference Methods. MPO levels in plasma were measured by the Cleveland Heart Lab (Cleveland, OH).

HDL isolation from plasma

Blood was collected from overnight fasted subjects into ice-cold tubes containing EDTA (6 mM final concentration). To protect Met residues from oxidation [33], plasma was prepared immediately by centrifugation (2500 g for 15 min) and frozen at −80°C until analysis. HDL

(density 1.063-1.210 g/mL) was isolated by sequential ultracentrifugation from freshly thawed plasma [27], using buffers supplemented with 100 μM diethylenetriaminepentaacetic acid (DTPA), 100 μM butylated hydroxytoluene (BHT), and a protease inhibitor mixture (Sigma, St. Louis, MO). All studies were performed on samples that had been stored for <12 months, and freshly prepared Met (10 mM final) was added to each sample prior to work-up for MS analysis.

Isotope-labeled apoA-I

[^{15}N]apoA-I was prepared by growing bacteria that stably express human apoA-I in minimal medium supplemented with [^{15}N]ammonium chloride [34].

Mass spectrometric (MS) analysis with selected reaction monitoring (SRM)

The protein concentration of HDL was determined using the Lowry assay (BioRad), with albumin as the standard. Following the addition of freshly prepared Met (10 mM final concentration), proteins were reduced with dithiothreitol and alkylated with iodoacetamide [27]. Following digestion of apoA-I with sequencing grade modified trypsin (Promega) or endoproteinase Glu-C (from Staphylococcus aureus V8, Roche Applied Science), site-specific oxidation of apoA-I was quantified by SRM, a quantitative and sensitive MS/MS technique for detecting peptides and their post-translational modifications [27, 35], in peptide digests of HDL with a nano-LC-MS/MS as described [27]. Additional details are provided in Supplemental Materials.

Ex vivo oxidation of HDL

Preliminary studies demonstrated that Tyr chlorination was negligible in recombinant apoA-I and reconstituted HDL (rHDL) analyzed by tandem MS, indicating that sample work-up and MS analysis did not contribute significantly to *ex vivo* oxidation. However, when rHDL disks made from apoA-I and phospholipid containing linoleic acid (which is highly susceptible to oxidation) were isolated by ultracentrifugation, oxidation of Met86 and Met112 in apoA-I increased significantly (Supplemental Fig. I). In contrast, there was no evidence of increased oxidation of Met148 (Supplemental Fig. I). Moreover, there was no additional oxidation of Met148 in ^{15}N-apoA-I when rHDL (made from ^{15}N-apoA-I) was incubated in plasma and then isolated by ultracentrifugation (Supplemental Fig. II). Addition to plasma of azide (10 mM), a potent inhibitor of MPO, did not affect Met oxidation or Tyr chlorination of rHDL or HDL (Supplemental Fig. II, III). These data suggest that lipid oxidation products, but not plasma MPO or other heme proteins, can contribute to *ex vivo* oxidation of Met86 and Met112 – but not Met148 – as previously described [36].

ABCA1 efflux capacity of serum HDL and in vitro oxidized HDL

Serum was derived from plasma by adding calcium [29]. Polyethylene glycol was then used to precipitate lipoproteins containing apolipoprotein B, and the supernatant was centrifuged to generate serum HDL. ABCA1-specific sterol efflux to serum HDL was quantified using baby hamster kidney (BHK) cells expressing mifepristone-inducible human ABCA1 [30, 37]. HDL isolated by ultracentrifugation was oxidized with HOCl and reduced with pilB as described [25, 26]. ABCA1-specific efflux capacity of oxidized and reduced HDL was

measured as previously described [25, 37]. Additional details are provided in Supplemental Materials.

Statistical analysis

Continuous variables are presented as means and SDs and categorical variables as frequencies and percentages. Because levels of chloroTyr192 and Met(O)148 exhibited a non-normal distribution in our study population, we used logarithmic transformation in all analyses. Linear regression analysis with continuous variables used Pearson's coefficient. Multiple logistic regression was used to estimate the association between 3-chloroTyr192, Met(O)148, cholesterol efflux capacity, and cardiovascular disease status after adjustment for HDL-C levels. Odds ratios are reported for 1-SD change for continuous variables. Significant P values were $\alpha < 0.05$ on two-tailed analysis. Statistical analyses were performed with SPSS (Windows version 19, Chicago, IL) or OriginPro (version 8.6, Origin Lab, Northampton, MA).

RESULTS

The subjects' clinical characteristics and lipid values are shown in Table 1. Analysis with one-way ANOVA indicated a significant difference in HDL-C levels among the groups ($P=0.0003$). Further analysis with Fisher's least significance difference [LSD] test demonstrated that the stable CAD (42±11 mg/dL, n=20) and ACS groups (47±17 mg/dL, n=20) had significantly lower HDL-C levels ($P=0.0001$ and $P=0.003$, respectively) than the healthy control group (61±16 mg/dL, n=20). LDL-C levels were similar in the control and ACS groups but lower in the CAD group ($P=0.009$, Fisher's LSD test, CAD vs. control), likely because all CAD subjects were on statin therapy. Plasma levels of MPO were similar in the control and CAD groups, but higher in the ACS group. However, the differences in MPO levels between the groups were not significant by one-way ANOVA. In contrast, the ACS and CAD groups had significantly higher levels of hs-CRP than the control group ($P=0.02$ and $P<0.0001$, respectively, Fisher's LSD test).

Stable CAD and ACS subjects have elevated levels of chlorinated and oxidized apoA-I

To quantify oxidation sites in HDL isolated by ultracentrifugation from human plasma of the control, CAD and ACS subjects, we supplemented the HDL with [^{15}N]apoA-I chlorinated with reagent HOCl before digesting the proteins with trypsin or Glu-C. Levels of 3-chlorotyrosine and Met(O) were quantified by tandem mass spectrometry with SRM and isotope dilution [27, 35]. This sensitive and quantitative approach detected all seven peptides that contain Tyr, all three peptides that contain Met, and the corresponding chlorinated and oxidized products.

Chlorinated peptides were readily detectable in the proteolytic digests of apoA-I prepared from HDL isolated from human plasma (Fig. 1A). Moreover, we identified Tyr192 as the major chlorination site in apoA-I of HDL isolated from both the diseased and control subjects ($P<0.0001$ vs. Tyr-18, Student's t-test), as previously demonstrated for HDL isolated from human atherosclerotic lesions [27]. The average level (mean±SD) of 3-chloroTyr192 in all the subjects was 62±26 μmol/mol Tyr. Tyr18 was the second major site

of chlorination, with an average level of 29±11 μmol/mol Tyr. Lower levels of 3-chlorotyrosine (<10 μmol/mol Tyr) were detected at the other five Tyr residues.

To determine whether the healthy and diseased groups had different levels of 3-chlorotyrosine in the apoA-I from their plasma HDL, we calculated the overall levels of Tyr chlorination for all seven Tyr residues. The total level of protein-bound 3-chlorotyrosine in the control group was 14±2.5 μmol/mol Tyr (n=20). In the CAD (n=20) and ACS (n=20) groups, it was 17±3.9 μmol/mol Tyr and 20±8.2 μmol/mol Tyr, respectively. Analysis by one-way ANOVA indicated total chloroTyr levels differed among the groups (*P*=0.009). Total levels of 3-chlorotyrosine in apoA-I of HDL from the CAD or ACS subjects were significantly higher than in the healthy control subjects (*P*=0.04 and *P*=0.002, Fisher's LSD test). Levels of 3-chlorotyrosine ranged from 10 to 19 μmol/mol Tyr in the control group, 11 to 24 μmol/mol Tyr in the CAD group, and 10 to 41 μmol/mol Tyr in the ACS group.

CAD and ACS subjects have elevated levels of chlorinated Tyr192 and oxidized Met148

Because Tyr192 was the major chlorination site in apoA-I of HDL isolated from human plasma, we determined whether CAD or ACS subjects had higher levels of that chlorinated residue (Fig. 1B). The average level of 3-chloroTyr192 in the control group was 50±8.5 μmol/mol Tyr. After log transformation of 3-chloroTyr192 values, levels differed significantly among the groups (*P*=0.01, one-way ANOVA). Levels were significantly higher (Fisher's LSD test) in the CAD subjects (64±20 μmol/mol Tyr, *P*=0.02) and ACS subjects (71±36 μmol/mol Tyr, *P*=0.004) than in the control subjects. However, the difference between the CAD and ACS levels was not significant.

We quantified levels of oxidation of all 3 Met residues in apoA-I (Supplemental Fig. IV). However, we focused on Met(O)148 because oxidation of that residue strongly associates with impaired ABCA1-mediated cholesterol efflux activity and reduced ability to activate LCAT [25, 26]. Importantly, preliminary studies indicated that Met148 was resistant to *ex vivo* oxidation (Methods and Supplemental Fig. I, II). In the control group, the average level of Met(O)148 was 75±13 mmol/mol Met (Fig. 1C). After log transformation of Met(O)148 values, levels were significantly higher in the CAD subjects (120±69 mmol/mol Met, *P*=0.002) and ACS subjects (110±62 mmol/mol Met, *P*=0.02) than in the control subjects.

Studies of MPO-deficient mice strongly suggest that MPO is the only generator of 3-chlorotyrosine during acute inflammation [38]. In contrast, other oxidative pathways can produce methionine sulfoxide [21]. To determine whether MPO might be an important pathway for promoting methionine oxidation in humans, we assessed the relationship between 3-chloroTyr192 levels and Met(O)148 levels (Fig. 1D) in all of the subjects (n=60). Linear regression analysis demonstrated a strong correlation (Pearson's r=0.57; *P*<0.0001) The levels of 3-chloroTyr192 also correlated with levels of Met(O)86 (r=0.52, *P*<0.0001, Supplemental Fig. IV D) and Met(O)112 (r=0.58, *P*<0.0001, Supplemental Fig. IV E). Our observations suggest that MPO contributes to Met oxidation in vivo in human apoA-I.

We also assessed the relationship between plasma MPO levels and levels of 3-chloroTyr192 and oxidized Met residues in HDL (Supplemental Fig. V). Linear regression analysis revealed that MPO levels were modestly correlated with levels of 3-chloroTyr192 (r=0.34,

P=0.008) but failed to correlate with levels of Met(O)148 (r= 0.24, *P*=0.06). Supplementation of plasma with 10 mM azide, a potent inhibitor of MPO, had no effect on Met oxidation or Tyr chlorination (Supplemental Material, Fig. II, III). These observations suggest that MPO in plasma has little influence on HDL oxidation.

Correlations of 3-chloroTyr192 and Met(O)148 with traditional CAD risk factors

We examined the relationship between 3-chloroTyr192 levels in HDL and traditional CAD risk factors in this cohort of control and high-risk patients (Fig. 2). Levels of 3-chloroTyr192 did not associate with sex or hypertension; they also failed to correlate with total cholesterol, non-HDL-C, age, or triglycerides (data not shown). However, they were significantly related to smoking status (53.4 mol/mol vs. 70.6 mol/mol, non-smoker vs. smoker, *P*=0.007, two-tailed Student's t-test) and diabetes (56.4 mol/mol vs. 83.2 mol/mol, non-diabetic vs. diabetic subjects, *P*=0.001). Levels of 3-chloroTyr192 correlated inversely with HDL-C levels (r=-0.27; *P*=0.03, Fig. 2A) but not with LDL-C levels (r=-0.13; *P*=0.31, Fig. 2B). Levels of 3-chloroTyr192 also correlated modestly with levels of hs-CRP (r=0.38; *P*=0.003, Fig. 2C).

We next examined the relationship between Met(O)148 levels and traditional CAD risk factors. Sex, age, hypertension, smoking status, total cholesterol, non-HDL-C, and triglyceride did not associate with levels of Met(O)148. However, Met(O)148 associated significantly with diabetes (92 mmol/mol Met vs. 138 mmol/mol Met, non-diabetic subjects vs. diabetic subjects, *P*=0.01). Unlike 3-chloroTyr192, Met(O)148 was not correlated with HDL-C (r=-0.23; *P*=0.08, Fig. 2D) but inversely correlated with LDL-C (r=-0.27; *P*=0.04, Fig. 2E). The correlation between Met(O)148 and hs-CRP was not significant (r=0.08; *P*=0.49, Fig. 2F).

Cholesterol efflux capacity of serum HDL with the ABCA1 pathway is impaired in CAD and ACS subjects

We assessed the capacity of serum HDL to promote cholesterol efflux by the ABCA1 pathway with BHK cells expressing or not expressing mifepristone-inducible human ABCA1. Analysis by one-way ANOVA indicated that serum HDL efflux capacity differed among the groups (*P*=0.0004). Serum HDL of CAD and ACS subjects had significantly less cholesterol efflux capacity (10.8% and 10.0% sterol efflux, *P*=0.02 and *P*<0.0001, Fisher's LSD test) than that of control subjects (11.8% sterol efflux) when cells expressing ABCA1 were cholesterol donors (Fig. 3A). There was a trend towards greater impairment of sterol efflux from ABCA1 expressing cells to serum HDL of ACS subjects than CAD subjects (*P*=0.06).

Levels of 3-chloroTyr192 and Met(O)148 inversely correlate with serum HDL's ABCA1 cholesterol efflux capacity

We investigated the relationship between levels of oxidized residues in apoA-I and the cholesterol efflux capacity of serum HDL, using cells that expressed ABCA1. Linear regression analysis of all the subjects (n=60) revealed an inverse correlation with levels of both 3-chloroTyr192 (r=−0.33, *P*=0.01, Fig. 3B) and Met(O)148 (r=−0.32, *P*=0.01, Fig. 3C).

The cholesterol efflux capacity of serum HDL also inversely correlated with levels of Met(O)86 and Met(O)112 (Supplemental Fig. VI).

To investigate the potential contribution of Met oxidation in vivo to impaired sterol efflux, we exposed isolated HDL to low levels of HOCl. When 29% of Met148 of apoA-I was converted to Met(O)148, we observed ~30% loss of cholesterol efflux by the ABCA1 pathway (Fig. 4). Met(O)148 was almost completely reduced back to native Met when oxidized HDL was incubated with pilB, a methionine sulfoxide reductase that reduces both epimers of Met(O) [39]. Reduction of Met(O) to Met completely restored the efflux capacity of HOCl-oxidized HDL (Fig. 4). The efflux capacity of control HDL, which contained ~9% Met(O)148, was also increased by incubation with pilB. Collectively, these observations provide strong evidence that the levels of Met(O) observed in HDL isolated from coronary artery disease subjects are likely high enough to contribute to impaired sterol efflux by the ABCA1 pathway.

Serum HDL's cholesterol efflux capability with macrophages associates with both HDL-C levels [30] and the functionality of the HDL particles [40]. We therefore determined the relationship between HDL-C levels and the ABCA1 activity of serum HDL for all of the subjects (n=60), using linear regression analysis (Fig. 5A). Efflux capacity correlated with HDL-C levels (P=0.02) and accounted for approximately the same variance in the data (r=0.31) as was observed for apoA-I chlorination (r=−0.33). Thus, the impaired cholesterol efflux in the CAD and ACS groups appears partially due to lower HDL-C levels. After we controlled for HDL-C level, logistic regression analysis revealed that the inverse correlations between 3-chloroTyr192 or Met(O)148 with efflux capacity remained significant (r=−0.27, P=0.04 and r=−0.27, P=0.04, respectively). Therefore, modification of apoA-I by MPO correlated with impaired HDL cholesterol efflux capacity via the ABCA1 pathway, even after adjusting for HDL-C levels. In contrast, we observed no correlation of the efflux capacity of serum HDL with plasma levels of MPO (r=-0.07, P=0.60, Fig. 3D).

We also examined the relationship between ABCA1 cholesterol efflux capacity and traditional risk factors for atherosclerosis in our subjects (Fig. 5). Cholesterol efflux correlated weakly with HDL-C (r=0.31, P=0.02, Fig. 5A), consistent with the observation that lipid-free or poorly lipidated apolipoproteins are the major ligands for ABCA1 [6]. Efflux capacity also correlated with LDL-C (r=0.26, P=0.04, Fig. 5B) but adjusting for HDL-C levels attenuated that relationship (r=0.24, P=0.07). Cholesterol efflux did not correlate with triglyceride level (r=0.17, P=0.19, Fig. 5C). However, after we controlled for HDL-C levels, that relationship became significant (r=0.49, P=0.0001). Efflux did not associate with gender or hypertension. Efflux did not correlate with age or total cholesterol. However, it related significantly to diabetes (11.2% vs. 10.0%, non-diabetic vs. diabetic, P=0.01, two-tailed Student's t-test). Efflux also associated with smoking status (11.3% vs. 10.6%, non-smoker vs. smoker, P=0.047). The efflux capacity of serum HDL also correlated with hs-CRP levels (r=-0.35, P=0.006, Fig. 5D).

3-ChloroTyr192, Met(O)148, and efflux capacity associate with cardiovascular disease status

We investigated the relationships among apoA-I oxidation, ABCA1 efflux capacity, traditional cardiovascular risk factors, and cardiovascular disease (CVD) status (Fig. 6). To increase power, and because the levels of oxidized amino acids and sterol efflux capacity of HDL were similar in CAD and ACS subjects, we included both CAD and ACS subjects in the CVD group.

Compared with control subjects, CVD subjects had significantly lower levels of HDL-C (mean values for case and control subjects, 45 mg/dL and 61 mg/dL, respectively; P = 0.0003). Cholesterol efflux capacity of serum HDL was significantly lower in case group compared with control group (mean values for case and control subjects, 10.4% and 11.8%, respectively; P = 0.001). Increased efflux capacity by the ABCA1 pathway associated with a decreased risk of CVD (odds ratio [OR] per 1-SD change, 0.30; 95% confidence interval (CI), 0.14-0.66; P=0.003). This association remained robust after adjusting for HDL-C levels (adjusted OR, 0.33; 95% CI, 0.15-0.76; P=0.009). Elevated levels of 3-chloroTyr192 and Met(O)148 of apoA-I in HDL were also highly significantly associated with CVD status (ORs 4.1 and 7.3, P=0.008 and 0.006, respectively). In contrast, plasma levels of MPO and LDL-C were not associated with CVD status (Fig. 6).

DISCUSSION

Understanding the pathways that impair sterol efflux by HDL may lead to new diagnostic and therapeutic approaches to atherosclerosis. In this study, we found that levels of 3-chloroTyr192 and Met(O)148 were significantly higher in apoA-I of HDL isolated from CAD or ACS subjects than in HDL from apparently healthy control subjects. We also found that serum HDL of CAD and ACS subjects was significantly less able to promote cellular sterol efflux by the ABCA1 pathway. There was a strong inverse association between ABCA1 efflux capacity with CVD status, and this association persisted after adjustment for HDL-C. Levels of 3-chloroTyr192 and Met(O)148 positively associated with CVD status. Moreover, serum HDL's ability to promote sterol efflux by the ABCA1 pathway correlated inversely with levels of 3-chloroTyr192 and Met(O)148. Because chlorination of Tyr192 in concert with oxidation of Met residues in vitro impairs the ABCA1 activity of apoA-I [23, 25], and 3-chlorotyrosine is a characteristic chemical fingerprint of MPO [38], our observations suggest that MPO contributes to the generation of dysfunctional HDL with impaired ABCA1 efflux capacity in humans with atherosclerotic vascular disease.

A key issue is whether the levels of oxidized apoA-I we detected in apoA-I of HDL of CAD and ACS subjects were high enough to potentially reduce HDL's sterol efflux capacity. When HDL oxidized in vitro with HOCl contained levels of Met(O) similar to those observed in vivo, its ability to promote sterol efflux by the ABCA1 pathway was significantly impaired. Importantly, the ability of oxidized HDL to promote sterol efflux by the ABCA1 pathway was completely restored when Met[O] was converted to Met by methionine sulfoxide reductase. In contrast, the levels of 3-chlorotyrosine we detected in HDL were low and unlikely to contribute directly to impaired sterol efflux capacity by the ABCA1 pathway. Collectively, these observations provide strong evidence that the levels of

Met(O) observed in HDL isolated from coronary artery disease subjects likely impair sterol efflux. In future studies, it will be important to determine if other modifications of apoA-I, such as lipid adduction [32] and tryptophan oxidation [41], contribute to impaired sterol efflux by the ABCA1 pathway.

Although methionine can be oxidized by lipid hydroperoxides in HDL as well as other pathways [42, 43], the correlation between levels of Met(O)148 and levels of 3-chloroTyr192 (a unique product of MPO [38]) indicate that MPO likely contributes to oxidation of methionine residues in vivo. In contrast to our observations with the ABCA1 pathway and LCAT activation [25, 26], oxidation of Met residues in apoA-I does not impair sterol efflux from human monocyte-derived macrophages [36]. However, the contribution of the ABCA1 pathway to efflux in these cells is uncertain, and the clinical relevance of these observations is unclear, because there is no evidence that impaired efflux with human macrophages associates with CVD status. In a secondary analysis, we also found that levels of 3-chloroTyr192 and Met(O)148 associated with smoking and diabetes status. Indeed, increased levels of oxidized Met residues in apoA-I of isolated HDL were observed in a study of type 1 diabetic subjects [44]. In future studies, it will be of interest to determine if smoking and diabetes—two major risk factors for CAD—associate with impaired sterol efflux capacity and HDL modification by MPO.

There was a strong trend (P=0.06) towards greater impairment of serum HDL's efflux capacity with the ABCA1 pathway in ACS subjects than in CAD subjects. However, the two groups contained similar levels of 3-chlorotyrosine and Met(O) in their HDLs. These observations suggest that additional factors impair sterol efflux in ACS subjects. One important contributor may be acute inflammation [12-14, 45], a prominent component of unstable angina and acute myocardial infarction [45]. Consistent with this suggestion, we observed a significant, inverse correlation between hs-CRP levels and the sterol efflux capacity of serum HDL in our cohort. Acute inflammation alters the concentration of HDL-associated enzymes and remodels the human HDL proteome [46], and animal models and human studies provide strong support for the hypothesis that inflammation converts HDL into a dysfunctional form [12-14, 28, 47]. Cholesterol efflux to HDL may be one important target because lipopolysaccharide, a potent inducer of acute inflammation, impairs cholesterol efflux from macrophages in mice [13].

Our detection of higher levels of 3-chloroTyr192 and Met(O)148 in plasma HDL from CAD and ACS subjects might seem surprising, given that blood is richly endowed with antioxidants that scavenge HOCl. Moreover, levels of the oxidized residues were similar in the two groups, suggesting that acute inflammation is unlikely to promote oxidation of circulating HDL. Thus, the oxidized HDL in plasma might be generated elsewhere, perhaps in a microenvironment that is depleted of antioxidants but enriched in macrophages, MPO and HOCl [17]. Many lines of evidence indicate that atherosclerotic lesions provide such a milieu [17]. One intriguing possibility is that HDL is oxidized in atherosclerotic lesions (or other inflamed tissues rich in leukocytes [45]), resulting in a feedback loop in which reduced sterol efflux due to damage to HDL prompts macrophages to become foam cells, accelerating atherosclerosis. In turn, these events promote additional HDL oxidation and the appearance of MPO-oxidized HDL in blood. Regardless of whether it originates in blood or

lesions, however, 3-chloroTyr and Met(O) in circulating HDL might be a useful indicator of the risk of cardiovascular disease and the efficacy of antioxidant interventions.

The levels of chloroTyr192 and Met(O)148 were similar in ACS and stable CAD patient, raising the possibility that the ACS subjects had elevated levels of the two oxidized amino acids *before* they developed clinical symptoms. In future studies, it will be of great interest to determine if levels of MPO-oxidized HDL can identify apparently healthy subjects who are at increased risk of CAD, and whether HDL oxidation is enhanced in conditions that predispose humans to clinically significant atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

ABCA1	ATP-binding cassette transporter A1
ACS	acute coronary syndrome
apoA-I	apolipoprotein A-I
BHK	baby hamster kidney
BHT	butylated hydroxytoluene
CAD	coronary artery disease
CVD	cardiovascular disease
DTPA	diethylenetriaminepentaacetic acid
H_2O_2	HDL, high density lipoprotein
rHDL	reconstituted HDL
HDL-C	high density lipoprotein cholesterol
HOCl	hypochlorous acid
hs-CRP	high-sensitivity C-reactive protein
LC	liquid chromatography
LCAT	lecithin-cholesterol acyltransferase
LDL-C	low density lipoprotein cholesterol
LSD	Fisher's least significance difference
Met(O)	methionine sulfoxide
MPO	myeloperoxidase
MS	mass spectrometry
MS/MS	tandem MS
OR	odds ratio
PEG	Polyethylene glycol
SRM	selected reaction monitoring

Novelty and Significance

What Is Known?

- The ability of HDL to accept cholesterol from macrophages is negatively associated with cardiovascular disease status.

- HDL receives cholesterol from ABCA1, a membrane protein that exports the sterol from macrophages.

- HDL can be damaged by myeloperoxidase, which oxidizes methionine residues and chlorinates Tyr192 of apolipoprotein A-I (apoA-I) in HDL impairing the ability of HDL to accept cholesterol from the ABCA1 pathway.

What New Information Does This Article Contribute?

- Subjects with stable coronary artery disease (CAD) or acute coronary syndrome (ACS) have elevated levels of oxidized HDL.

- Levels of chlorinated Tyr192 and oxidized Met148 in apoA-I strongly associate with cardiovascular disease status and the degree to which HDL is unable to accept cholesterol from cells via the ABCA1 pathway.

- Because chlorinated Tyr192 and oxidized Met148 are produced by myeloperoxidase, the enzyme may help generate dysfunctional HDL in humans.

The ability of HDL to remove cholesterol from artery wall macrophages is one of its key cardioprotective functions. The sterol efflux capacity of HDL shows a robust negative association with CAD status, suggesting that loss of efflux capacity contributes to HDL dysfunction. However, the underlying mechanisms remain poorly understood. We found that subjects with CAD or ACS have higher levels of oxidized and chlorinated HDL than control subjects, and that the oxidation pattern—chlorinated Tyr192 and oxidized Met148—matches that produced by myeloperoxidase. HDL from the CAD and ACS subjects was also less efficient in promoting sterol efflux by the ABCA1 pathway. Converting oxidized methionine residues of HDL back to methionine improved sterol efflux capacity in vitro. Importantly, the levels of oxidized and chlorinated HDL were inversely correlated with ABCA1 efflux capacity and positively associated with atherosclerotic status. These observations indicate that dysfunctional HDL with impaired sterol efflux capacity could be generated by myeloperoxidase. Thus, inhibiting HDL oxidation by myeloperoxidase in vivo might be a new approach for treating atherosclerosis.
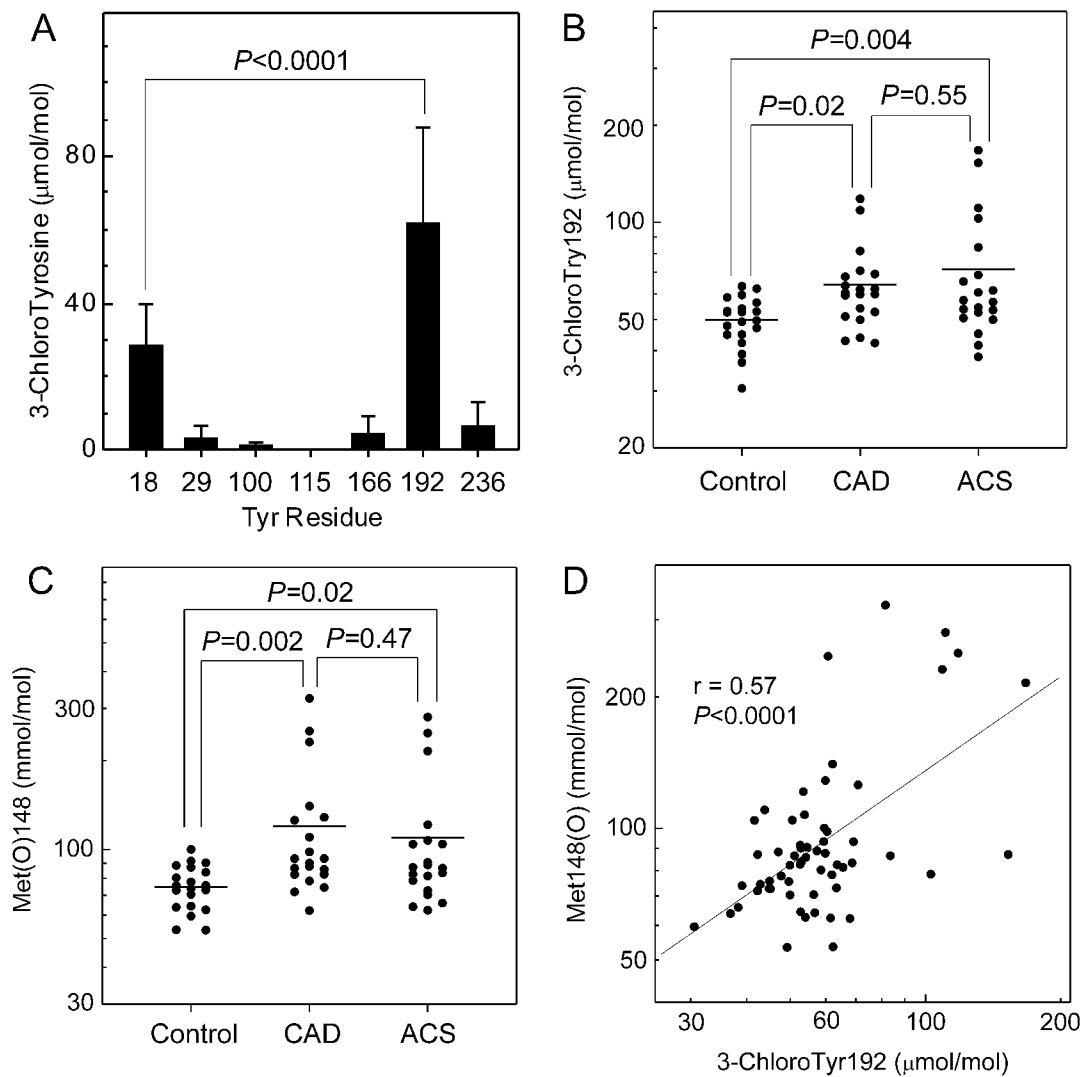
Figure 1. Site-specific tyrosine chlorination and methionine oxidation of apoA-I in control subjects, CAD subjects, and ACS subjects

(**A**) Chlorination of Tyr residue in apoA-I for all subjects. (**B**) Levels of chloroTyr192 in control subjects, CAD subjects, and ACS subjects. (**C**) Levels of Met(O)148 in control subjects, CAD subjects, and ACS subjects. (**D**) Correlation of Tyr chlorination and Met oxidation in all subjects. HDL was isolated by ultracentrifugation from freshly prepared plasma of CAD, ACS, or healthy subjects. Following the addition of oxidized [^{15}N]apoA-I as internal standard, HDL was subjected to proteolytic digestion. Peptide digests were analyzed by tandem MS with SRM. Values for chloroTyr192 and Met(O)148 were logarithmically transformed to achieve a normal distribution.
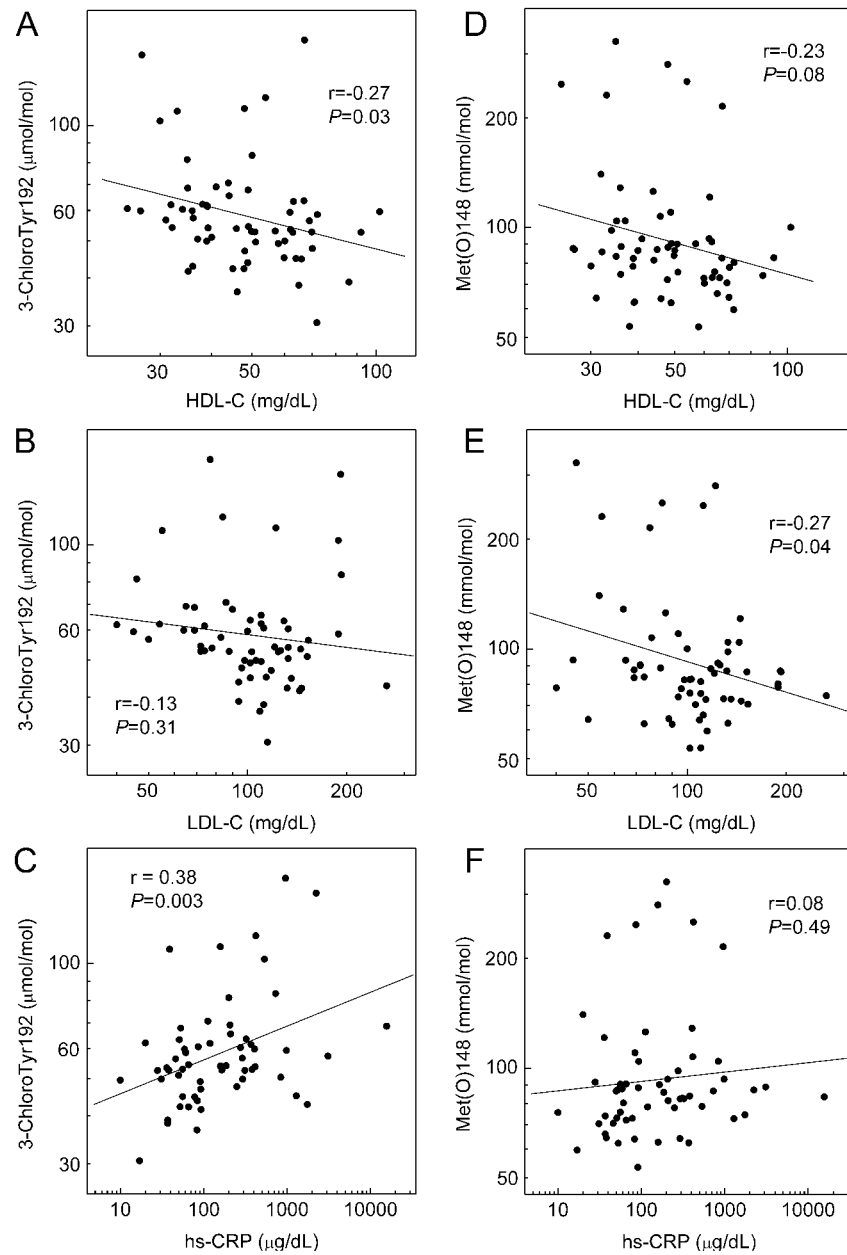
Figure 2. Correlation of 3-chloroTyr192 and Met(O)148 with cardiovascular risk factors
Levels of 3-chloroTyr192 and Met(O)148 were quantified as described in the legends of Fig. 1. Relationships between 3-chloroTyr192 or Met(O)148 and HDL-C (**A, D**), LDL-C (**B, E**), and hs-CRP (**C, F**) were linear regression analyses using Pearson's correlation coefficient. Where indicated, values were logarithmically transformed.
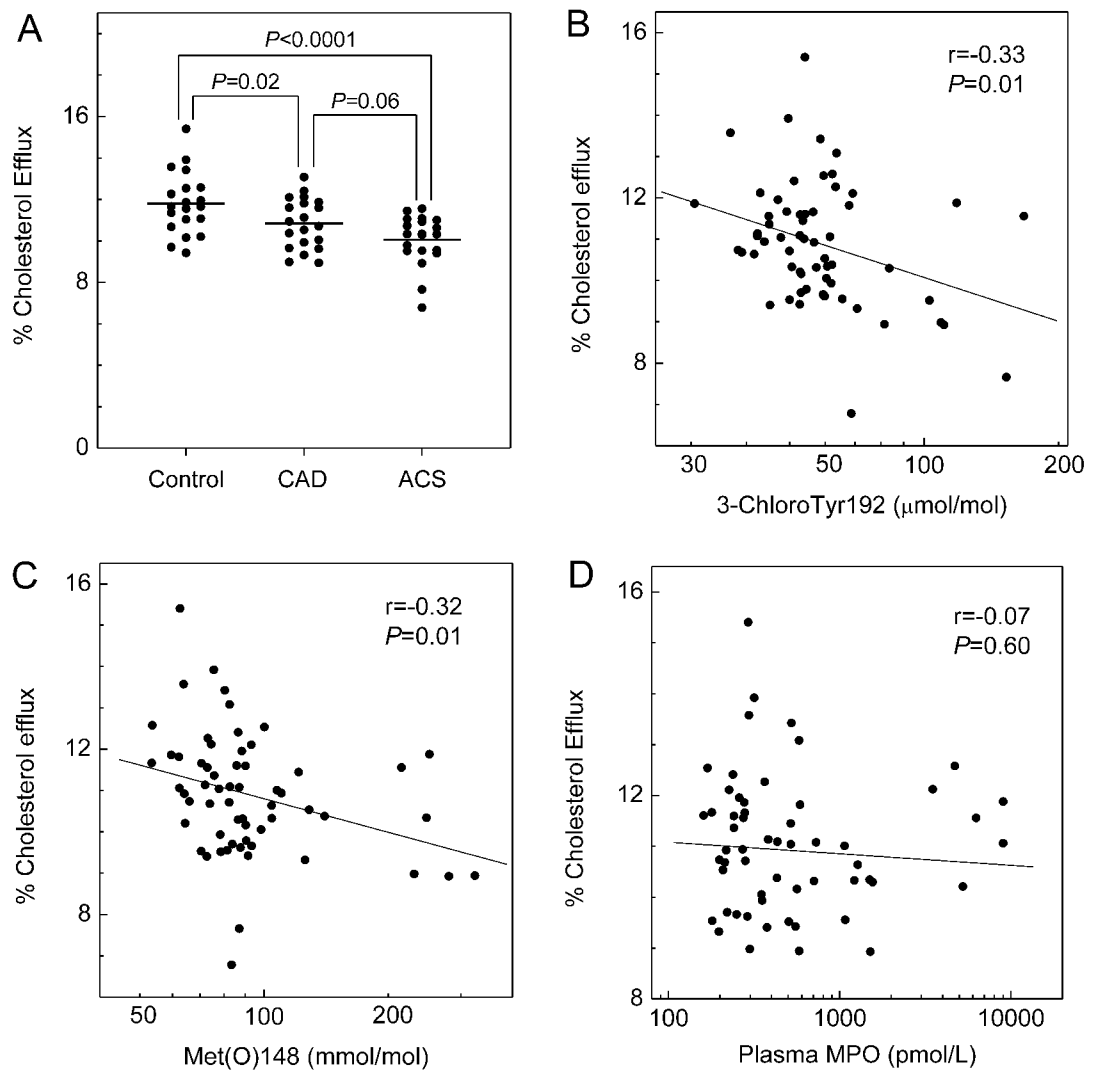
Figure 3. Cholesterol efflux capacity of serum HDL in control subjects, CAD subjects, and ACS subjects

Serum HDL was obtained by polyethylene glycol (PEG) precipitation of serum derived from plasma. Cholesterol efflux from serum HDL to ABCA1-expressing BHK cells (**A**) was measured as described in Methods. Correlations between 3-chloroTyr192 (**B**), Met(O)148 (**C**), or MPO levels in plasma (**D**) and ABCA1 efflux capacity were determined by linear regression analysis using Pearson's correlation coefficient.
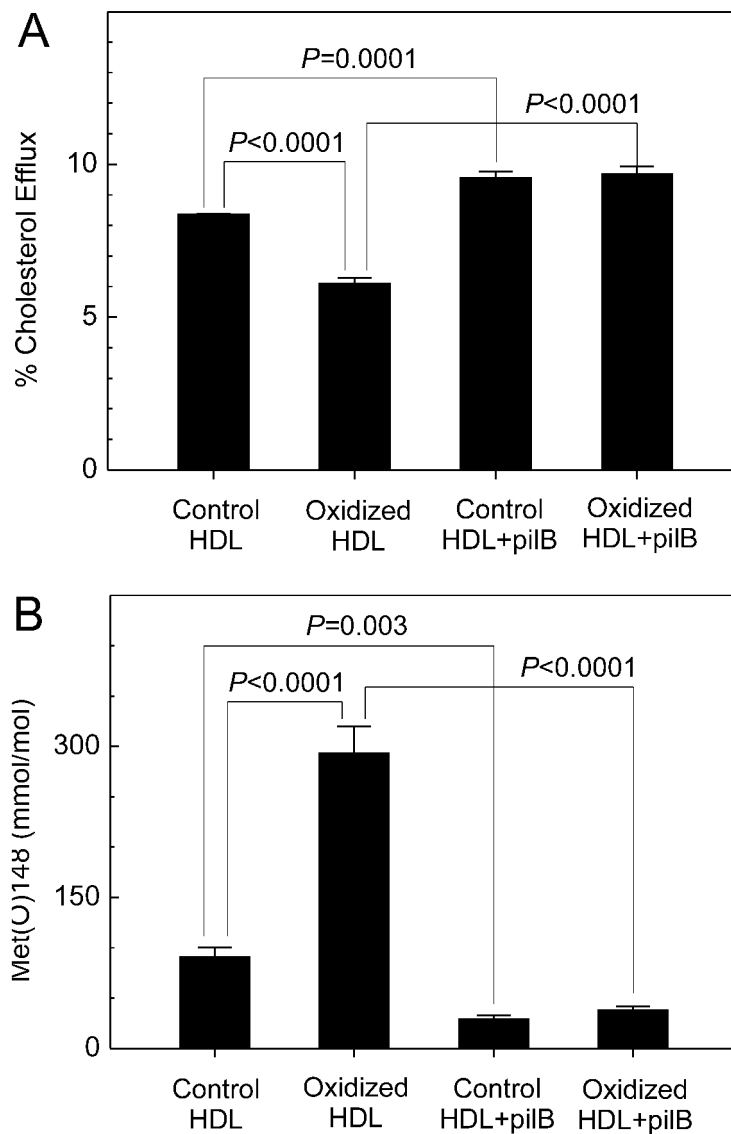
Figure 4. Methionine oxidation and cholesterol efflux capacity of HDL exposed to HOCl in vitro
(A) Cholesterol efflux capacity of HDL exposed to HOCl and reduced by pilB; (B) Levels
of Met(O)148 in HDL exposed to HOCl and reduced by pilB. HDL (1 mg/mL) was oxidized
by HOCl (180 μM) at 37°C for 1 h. Where indicated, HDL (0.5 mg/mL) was incubated
subsequently with the methionine sulfoxide reductase pilB (4:1, HDL/pilB, w/w) at 37°C for
2 h. Cholesterol efflux from ABCA1-expressing BHK cells to control HDL, HOCl-oxidized
HDL, and pilB-reduced HDL was measured as described in Methods. Levels of Met(O)148
were quantified by tandem MS with SRM. Results are means ± SDs of three determinations
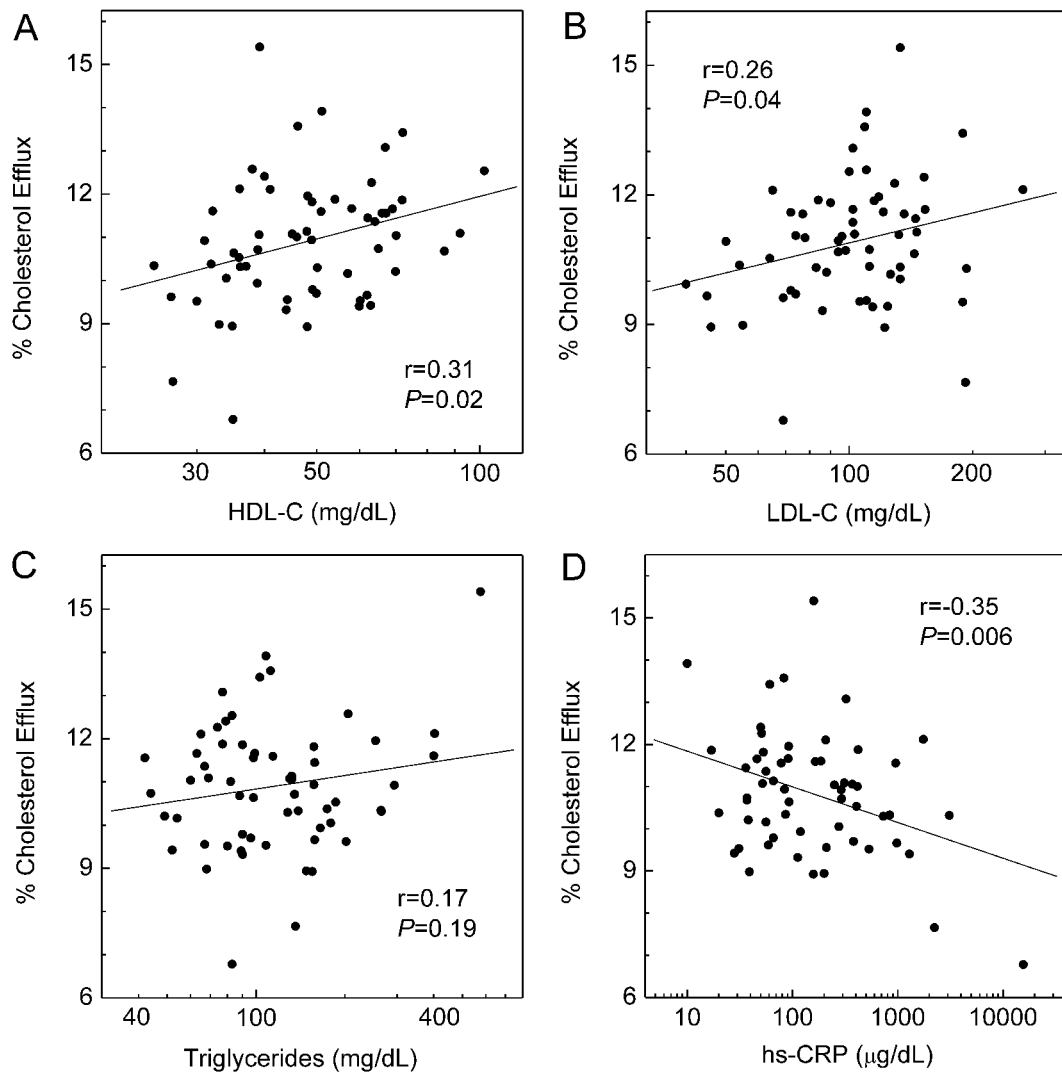and are representative of two independent experiments.

Figure 5. Correlation of ABCA1 cholesterol efflux capacity with cardiovascular risk factors
Serum ABCA1 cholesterol efflux was measured as described in the legend of Fig. 3. Correlations between serum efflux and HDL-C (**A**), LDL-C (**B**), triglyceride (**C**), and hs-CRP (**D**) were determined by linear regression analysis using Pearson's correlation coefficient. Where indicated, values were logarithmically transformed.
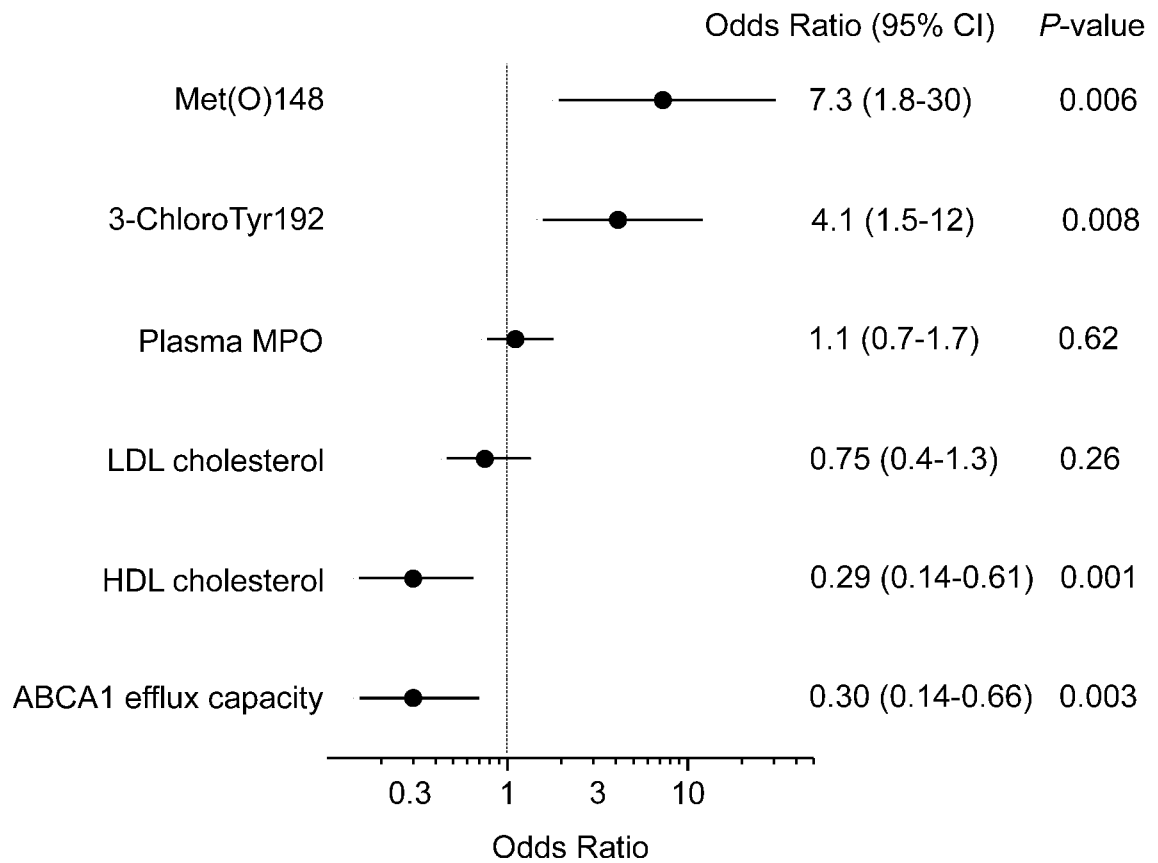
Figure 6. Odds ratios for cardiovascular disease status for 3-chloroTyr192, Met(O)148, plasma MPO, HDL-C, LDL-C and ABCA1 sterol efflux capacity
Cardiovascular disease included both ACS and CAD subjects (n=40). Bars represent 95%
confidence intervals for each odds ratio. Odds ratios are per 1-SD increase.

Table 1

Clinical Characteristics of Study Subjects

	Control (n=20)	Stable CAD (n=20)	ACS (n=20)
Age (years)	56 ± 8	64 ± 11[*]	63 ± 12
Male (%)	15 (75%)	14 (70%)	12 (60%)
Hypertensive patients (%)	2 (10%)	15 (75%) [***]	10 (50%) [***]
Diabetic patients (%)	0 (0%)	3 (15%)	9 (45%) [***]
Smokers (%)	4 (20%)	13 (65%) [***]	12 (60%) [**]
Total cholesterol (mg/dL)	203 ± 35	N/A	186 ± 41
HDL cholesterol (mg/dL)	61 ± 16	42 ± 11[***]	47 ± 17 [***]
LDL cholesterol (mg/dL)	117 ± 25	94 ± 52	114 ± 42
Triglycerides (mg/dL)	123 ± 118	158 ± 93	126 ± 72
MPO (pmol/L)	809 ± 1,431	920 ± 2,033	1610 ± 2,267
hs-CRP (µg/dL)	88 ± 92	290 ± 406 [*]	1,369 ± 3,442 [***]

Plus-minus values are means and SDs. MPO and hs-CRP values were log transformed before one-way ANOVA analysis. NA, not available.

[*], [**], [***] P-value less than 0.05, 0.01 or 0.001, respectively, versus control subjects by Fisher's least significant differences test (after one-way ANOVA with $P<0.05$).