Reconstituted High-Density Lipoprotein Attenuates Cholesterol Crystal–Induced Inflammatory Responses by Reducing Complement Activation

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Chronic inflammation of the arterial wall is a key element in the development of atherosclerosis, and cholesterol crystals (CC) that accumulate in plaques are associated with initiation and progression of the disease. We recently revealed a link between the complement system and CC-induced inflammatory cascades, showing that the complement system is a key trigger in CC-induced inflammation. HDL exhibits cardioprotective and anti-inflammatory properties thought to explain its inverse correlation to cardiovascular risk. In this study, we sought to determine the effect of reconstituted HDL (rHDL) on CC-induced inflammation in a human whole blood model. rHDL bound to CC and inhibited the CC-induced complement activation as measured by soluble terminal C5b-9 formation and C3c deposition on the CC surface. rHDL attenuated the amount of CC-induced complement receptor 3 (CD11b/CD18) expression on monocytes and granulocytes, as well as reactive oxygen species generation. Moreover, addition of CC to whole blood resulted in release of proinflammatory cytokines that were inhibited by rHDL. Our results support and extend the notion that CC are potent triggers of inflammation, and that rHDL may have a beneficial role in controlling the CC-induced inflammatory responses by inhibiting complement deposition on the crystals. The Journal of Immunology, 2015, 195: 000–000.

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D eposition of cholesterol-rich lipoproteins in the vessel wall is a key pathogenic step in atherosclerosis (1). The physical properties of cholesterol enable precipitation of cholesterol crystals (CC) in cholesterol-supersaturated fatty streaks, and CC are commonly found in atherosclerotic lesions at all stages of the disease (2, 3). However, the role CC play in atherogenesis has largely been unexplored, and CC was long considered as relatively inert. CC are located both extracellularly and intracellularly. Intracellular CC formation was demonstrated in vitro in lipid-laden foam cells (4, 5). Recently, Sheedy et al. (6) revealed intracellular CC formation after endocytosis of oxidized low-density lipoprotein by CD36. Phagocytosis of CC by murine or human macrophages leads to lysosomal damage, resulting in the activation of the NLRP3 inflammasome, with subsequent activation of caspase-1 and release of mature IL-1β (7, 2). Atherosclerosis is an inflammatory disease, and formation of CC may well provide a link between lipids and inflammation, the two hallmarks of the disease.

Activated complement components are present in atherosclerotic lesions, from the fatty streak to more advanced lesions (8–12), suggesting that complement in lesions may be involved in inflammatory responses associated with atherosclerosis. We recently demonstrated that the complement system controls CC-induced inflammatory cascades, showing that the complement system is a key trigger in CC-induced inflammation. HDL exhibits cardioprotective and anti-inflammatory properties thought to explain its inverse correlation to cardiovascular risk. In this study, we sought to determine the effect of reconstituted HDL (rHDL) on CC-induced inflammation in a human whole blood model. rHDL bound to CC and inhibited the CC-induced complement activation as measured by soluble terminal C5b-9 formation and C3c deposition on the CC surface. rHDL attenuated the amount of CC-induced complement receptor 3 (CD11b/CD18) expression on monocytes and granulocytes, as well as reactive oxygen species generation. Moreover, addition of CC to whole blood resulted in release of proinflammatory cytokines that were inhibited by rHDL. Our results support and extend the notion that CC are potent triggers of inflammation, and that rHDL may have a beneficial role in controlling the CC-induced inflammatory responses by inhibiting complement deposition on the crystals. The Journal of Immunology, 2015, 195: 000–000.
attributed to the ability of HDL to remove excess cholesterol from lipid-laden macrophages in a process called reverse cholesterol transport (19). In addition, HDL has antioxidant and anti-inflammatory effects (20–22). The anti-inflammatory effects of HDL on endothelium are mediated by reduction in ICAM, VCAM, and E-selectin expression, as well as increase in NO production (20, 23). The molecular mechanism by which HDL mediates its anti-inflammatory effects has been poorly understood. Recently, HDL was shown to induce expression of ATF3, a transcriptional negative regulator of several proinflammatory genes (24). The ability of HDL to increase reverse cholesterol transport and decrease inflammation has prompted clinical studies of strategies that raise these functional activities (25).

Because complement activation occurring on the surface is essential for the proinflammatory effects of CC, we investigated the effect of reconstituted HDL (rHDL) on CC-induced inflammatory responses in a human whole blood model. In this study, we show that rHDL bound directly to CC and prevented complement activation and inflammatory cytokine release in a specific manner. Furthermore, we found that high concentration of phosphatidylcholine (PC), the major phospholipid in rHDL, inhibited complement activation by CC. These data suggest an additional anti-inflammatory effect of rHDL that may be beneficial in treatment of atherosclerosis.

Materials and Methods

Reagents

Whole blood was anticoagulated with lepirudin, a recombinant form of the specific thrombin inhibitorhirudin (Refludan; Celgene). Uric acid, soybean L-α PC, and zymosan were from Sigma-Aldrich. LPS from Escherichia coli was from Invivogen (0111:B4) and Alexa Fluor Sucinimidyl Esters from Invitrogen. Human serum albumin (HSA) was from Octapharma and recombinant apolipoprotein A-I (apoA-I) from Cell Sciences. 1-Propanol for analysis EMSURE ACS was purchased from Merck Millipore. The low-density lipoprotein (LDL) was purchased from Biomedical Technologies and, according to the manufacturer, consists of 25% protein, 4–10% triglycerides, 9–11% cholesterol, 42–50% cholesterol ester, and 22–29% phospholipid. ApoB 100 constitutes 95–97% of the protein composition, and the remaining 4–5% of protein consists of smaller apoproteins (e.g., apolipoprotein E, ApoA-I, apolipoprotein M). The following Abs were used: mouse anti–HDL (IC5; Abcam), polyclonal goat anti-mouse Ig FITC (340031; BD Biosciences), mouse IgG1 (349040; BD Biosciences), anti-CD11b PE (D12, BD Biosciences), anti-CD14 FITC (MqP9; BD Biosciences), anti-CD14 PE (MqP9; BD Biosciences), polyclonal anti-C3c FITC (P0201; Dako), IgG2a conjugated to FITC (BD Biosciences), anti–C5b-9 (DIA-011; Antibodyshop), goat anti-mouse IgG directly conjugated with Alexa 488 (A1001; Invitrogen).

Preparation of crystals

Monohydrate CC were prepared as previously described (13). Utralprate cholesterol was dissolved in 1-propanol. Crystallization was performed by mixing the solution with distilled water (1:1.5). CC were then air-dried and resuspended in PBS/0.05% HSA and stored at 4˚C. This yielded CC with a size range of 1 to 2 μm. Monosodium urate (MSU) crystals were prepared as described previously (26). Uric acid was dissolved in NaOH and heated to 70˚C. The solution was continuously stirred for 24 h, resuspended in PBS/0.05% HSA, and stored at 4˚C.

Preparation of rHDL

rHDL (CSL111) with a molar ratio of apoA-I to soybean PC of 1:150 was prepared as described by Lerch at al. (27), and provided by CSL Behring. For some experiments, rHDL was prepared with cholesterol (CHDL) with a molar ratio of apoA-I/PC/cholesterol: 1:100:12. rHDL was labeled using Alexa Fluor Sucinimidyl Esters 488 according to manufacturer’s instructions. This dye should label apoA-I. A total of 20 mg/ml rHDL in 0.1M sodium bicarbonate buffer (pH 8.3) was labeled with 50 μl dye (10 mg/ml in DMSO) and incubated at room temperature (RT) for 1 h with continuous stirring. The labeled rHDL was then purified over a PD MidiTrap G-25 column (GE Healthcare), which had been equilibrated into PBS, and was run on a fast protein liquid chromatography column. Alexa 488 rHDL was able to inhibit proinflammatory cytokines.

Human whole blood experiments

CC (2 ng/ml), MSU (250 μg/ml), zymosan (10 μg/ml), PBS/BSA, or LPS (100 ng/ml) was preincubated with different concentrations of rHDL (4, 20, 100 μg/ml) or PBS for 16 h. The crystals or other stimuli were not washed free of rHDL before adding to blood. The whole blood assay was performed as described previously (28). Whole blood from healthy volunteers was collected into pyrogen-free Nunc tubes containing lepirudin anticoagulant (50 μg/ml). Stimuli and whole blood were incubated at 37˚C under constant rotation. Aliquots of blood were collected and analyzed for changes in complement receptor 3 (CR3; CD11b/CD18; detected as CD11b, 15 min), complement activation (detected as the terminal C5b-9 complement complex [TCC], 30 min), and cytokine production (6 h).

Measurements of cytokines and chemokines

For the analysis of cytokines and chemokines, plasma was collected after 6-h stimulation as described earlier. The cytokine and chemokine levels were analyzed using Bio-Plex Human cytokine 6-Plex Panel (Bio-Rad Laboratories, Hercules, CA) containing the following analytes: IL-1β, IL-6, IL-8 (IL-8 or CXCL8), MCP-1, MIP-1α, and TNF. The analysis was performed according to the manufacturer’s protocol.

Complement measurements

CC (1 mg/ml) or PBS/BSA was preincubated with different concentrations (1, 4, 20, 100 μg/ml) of rHDL, CHDL, apoA-I, LDL, and of PC (0.1, 0.4, 2, and 10 mg/ml), or PBS for 16 h. LDL and rHDL are given in micrograms or milligrams of protein. The crystals were not washed free of rHDL, CHDL, apoA-I, LDL, or PC before adding to plasma. Thereafter, plasma from whole blood was incubated with stimuli for 30 min at 37˚C, and the reaction was stopped by adding EDTA (10 mM final concentration). Complement activation in the fluid phase was measured by ELISA for TCC as described in detail elsewhere (29). Shortly, the assay is based on a monoclonal capture Ab specific for a neopterin epitope expressed in C9 after assembly of the TCC, but hidden in the native C9 molecule, thus reflecting the degree of activation. Thus, only the activated component is detected. Complement deposition on CC was detected by the same mAb reacting with TCC, and C3 deposition was detected using a polyclonal anti-C3c.

Binding studies

CC were preincubated with unlabeled HDL (100 μg/ml), LDL (100 μg/ml), or PBS for 60 min at RT. Competitive binding of Alexa 488–labeled rHDL (10 μg/ml), in the absence or presence of unlabeled HDL or LDL, was determined after 16 h of incubation. To investigate binding of CC to native HDL, we incubated plasma with CC for 16 h. After incubation with plasma, the CC were washed twice and resuspended in a solution of PBS/BSA, and stained for native HDL binding using mouse mAb to human HDL (10 μg/ml) or control mouse IgG1. Monoclonal anti-HDL reacts with HDL2, HDL3, and apoA-I. Goat anti-mouse Ig (FITC) was used as a secondary Ab.

Measurements of CC dissolution

To investigate whether rHDL or PC dissolved CC, we incubated a solution of 1 mg/ml CC with rHDL (100 μg/ml), PC liposomes (0.1, 2 mg/ml), PBS, or 50% ethanol for 22 h at 37˚C under constant rotation. Twenty thousand CC events were acquired for each sample. CC were first visualized by forward scatter versus side scatter, and a gate was drawn around the CC cloud at time 0 h and used as a reference. The decrease of percentage in this gate shows how much CC that are dissolved in the different samples.

Caspase-1 detection

CC were preincubated with rHDL or PBS for 16 h. Caspase-1 detection (FAM FLICA in vitro Caspase detection kit; Immunochemia Technol-ogies) was performed following the manufacturer’s recommendations with minor revision. Whole blood was stimulated for 4 h and incubated for 2 h with FLICA probes for caspase-1 detection. Blood was stained with anti–CD14-PE (5 μg/ml) before lysis with FACS lysing solution (BD Biosciences).

CD11b and reactive oxygen species detection

CD11b was detected in whole blood fixed with 1% paraformaldehyde for 4 min at 37˚C and stained for 15 min with anti–CD11b-PE (5 μg/ml) and anti–CD14-FITC (5 μg/ml). Reactive oxygen species (ROS) was detected
using the oxidative Burst Test [PHAGOBURST] kit; BD Biosciences) following the manufacturer’s protocol with some modification. Whole blood was stimulated for 10 min, after which DHR 123 substrate was added for 10 min. After lysis and fixation for 20 min at RT, samples were washed and stained with anti-CD14-PE (5 μg/ml) for 15 min at RT.

**Flow-cytometric analysis**

Data acquisition of CD11b expression and caspase-1 on leukocytes, and the binding analysis were performed on a BD FACSCanto II (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Ashland, OR).

**Statistical analysis**

Results from a minimum of six volunteers were pooled and analyzed using GraphPad Prism version 5.04 (GraphPad Software) for evaluation of significance, with \( p < 0.05 \) considered as statistically significant. Column figures are presented as means ± SEM. Data in Figs. 1 and 4–6 were analyzed using Wilcoxon matched-pair signed rank test.

**Study approval**

Approval no. 2009/2245 was received from the Regional Committee for Medical and Health Research Ethics in Central Norway (REC Central), The Norwegian Ministry of Education and Research. Written, informed consent was obtained from all participants.

**Results**

**rHDL inhibits CC-induced complement activation (TCC) and CD11b expression in whole blood**

Protective properties of HDL in animal models of cardiovascular diseases are thought to be a result of several beneficial functions, such as mediating cholesterol efflux, reducing oxidative stress, and protecting endothelial integrity (30). We have previously demonstrated that the inflammatory cytokine response to CC is strongly dependent on complement activation (13). We therefore investigated the effect of rHDL on CC-induced inflammatory responses. To do so, we first preincubated CC with rHDL and incubated with whole blood for 30 min. CC induced a strong TCC response that was dose-dependently reduced (\( p < 0.001 \) for 100 μg/ml rHDL) upon preincubation with rHDL (Fig. 1A). When whole blood was incubated with CC for 15 min, both monocytes and granulocytes displayed a rapid increase in CR3 expression (detected as CD11b), which was significantly (\( p < 0.01 \) for 100 μg/ml rHDL) reduced by preincubation of CC with rHDL (Fig. 1B, 1C). The inhibitory effect of rHDL on TCC and CD11b was restricted to CC because no effect was seen on MSU crystals and zymosan controls (Fig. 1). These data demonstrate that rHDL reduces the ability of CC to initiate complement activation and subsequent up-regulation of the CR3.

**rHDL binds to CC and inhibits C3c and TCC deposition on CC surface**

Earlier reports suggested that direct interactions between HDL and CC resulted in solubilization of CC (31, 32). To verify whether the reduced complement activation by CC could be caused by dissolution of CC by rHDL, we incubated CC with rHDL, ethanol, or PBS. Whereas ethanol dissolved nearly 100% of the CC, a minimal change was observed in CC incubated with rHDL (Fig. 2). We next examined whether the inhibitory effect of rHDL on CC-induced TCC and CD11b was due to rHDL binding to CC. We conducted competitive binding studies where CC were incubated with unlabeled rHDL or PBS for 60 min and stained by Alexa 488 HDL for 16 h. As shown in Fig. 3A, rHDL bound strongly to CC, and the binding to Alexa 488HDL was reduced upon preincubation with unlabeled rHDL. By using a mAb detecting apoA-I, HDL2, and HDL3, we showed that native HDL also bound to CC surface in human plasma (Fig. 3B). We next investigated whether rHDL reduced opsonization of CC. In the presence of human plasma, a considerable deposition of C3c and TCC on the CC surface was detected. Preincubation of CC with rHDL inhibited C3c and TCC deposition on CC substantially (Fig. 3C, 3D), suggesting that the inhibition is a consequence of rHDL interfering with CC, and thus diminishing opsonization and the complement activating potential of CC.
Comparison of LDL and components of rHDL on the ability to reduce CC-induced complement activation

We first compared the effect of rHDL to LDL, a lipoprotein that has not been shown to have anti-inflammatory properties. The concentration range for HDL and LDL in blood normally refers to the cholesterol content of the lipoprotein particles. The rHDL contains no cholesterol, and thus the concentration is given as micrograms/milliliter of protein. In this experiment, LDL was used as a lipoprotein control for rHDL and its concentration is also given as micrograms/milliliter of protein. rHDL inhibition of TCC was concentration dependent, with the greatest inhibition attained at 100 μg/ml concentration of rHDL (p < 0.001; Fig. 4A). In contrast with rHDL, LDL did not inhibit CC-induced TCC formation. This result was in line with the low binding activity of LDL to CC (Fig. 4B). This finding indicates that complement inhibition by rHDL is specific for this lipoprotein particle. CHDL also inhibited CC-induced TCC production; however, this effect was only significant at the highest CHDL concentration (p < 0.01; Fig. 4C). rHDL used in this study was prepared with apoA-I and PC at a ratio of PC/apoA-I of 150:1, and we thus sought to identify whether any of the rHDL components were able to regulate TCC production in response to CC. Treatment with recombinant apoA-I had no inhibitory effect on CC-induced TCC (Fig. 4C). Addition of PC reduced CC-induced complement activation; however, a dose of at least 2 mg/ml was needed to obtain significant inhibition (p < 0.01; Fig. 4C). Thus, at least 50-fold excess of PC compared with rHDL was needed to obtain comparable inhibition of CC-induced TCC production. In contrast with rHDL, pre-incubation of 1 mg/ml CC with 2 mg/ml PC liposomes dissolved 84% of the crystals (measured by flow cytometry). This indicates that high concentrations of PC can solubilize CC, which, in turn, may cause the observed inhibition of the CC-initiated TCC production.

rHDL attenuates CC-induced cytokines and chemokines in whole blood

Having observed a strong inhibition of rHDL on CC-induced complement activation, we next assessed whether rHDL also reduced cytokine and chemokine responses initiated by CC. The ability of CC to induce cytokine production in whole blood is to a large extent dependent on complement activation (13). Addition of CC to whole blood resulted in strong induction of proinflammatory cytokines (IL-1β, TNF, and IL-6) and chemokines (IL-8, MIP-1α, and MCP-1). However, once exposed to rHDL, CC-induced cytokine and chemokine response were significantly (p < 0.01) reduced (Fig. 5). The same inhibitory profile was observed for all CC-induced cytokines and chemokines measured. For IL-1β, TNF, and MIP-1α, the inhibition with rHDL was >60%, whereas the inhibition was >50% for IL-6 and IL-8. Based on our previous findings that CC leads to complement-dependent ROS production and caspase-1 activation, we next examined whether rHDL would have a regulatory effect on CC-induced inflammasome activation (13). Indeed, the CC-induced ROS production and caspase-1 activation by monocytes was significantly (p < 0.01 for 100 μg/ml rHDL) reduced by exposure of CC to rHDL (Fig. 6).

Discussion

Previously considered as inert particles, crystalline cholesterol formed in atherosclerotic lesions is emerging as an endogenous initiator of inflammation. Recent studies implicate CC-induced inflammasome activation as one of the forces driving atherosclerosis development and progression (2, 7, 33). We have recently
shown a link between CC-induced inflammasome activation and the complement system activation (13, 34). In this study, we report that rHDL composed of lipoprotein apoA-I with PC as the sole phospholipid binds to CC and impairs the ability of CC to activate complement, and consequently attenuates the inflammatory responses induced by CC.

We used a whole blood model of inflammation to investigate the influence of rHDL on inflammatory responses to CC. The model is unique in exploring the role of the complement system in inflammation because it specifically blocks thrombin to inhibit coagulation but preserves all other biological systems intact to mutually interact, in contrast with the traditionally used anticoagulants like EDTA, citrate, and heparin. Thus, it allows interactions of cellular and fluid-phase mediators by using an anticoagulant that does not affect either complement activation or other inflammatory systems like the cytokine network (28). Our data demonstrate that CC-induced complement activation, revealed by high amounts of soluble TCC, was dose dependently inhibited by rHDL. The effect of rHDL was apparent at 100 μg/ml concentration where rHDL inhibited TCC production by ~70%. We found that the rHDL effect on TCC was specific to CC because no inhibition was observed on MSU crystals. Earlier in vitro studies showed that HDL, apoA-I, and apoA-II inhibited complement-mediated lysis of erythrocytes (35). Recent analysis of the HDL proteome revealed that multiple complement components and complement regulatory proteins reside in HDL (36), raising the possibility of other protective roles of HDL on cells exposed to activated components of complement.

The cholesterol-loaded version of rHDL, which converts rHDL to a diffusional cholesterol donor, inhibited CC-induced TCC formation only at the highest concentration of CHDL. Thus, the inhibitory effect of rHDL can also occur when the particle is loaded with cholesterol. We found that apoA-I had no effect on CC-induced TCC formation. A previous study has shown that PC liposomes can dissolve CC in tissue fragments isolated from human aortic plaques (37). Our data demonstrate that a 50-fold excess of PC was needed to mediate a similar inhibitory effect as rHDL on the CC-initiated TCC production. At concentrations where PC liposomes inhibited CC-induced complement activation, a marked PC-mediated dissolution effect of CC was observed. Thus, it is possible that the effect of PC on CC solubility may explain its inhibitory effect on complement activation. PC is likely to bind to the CC surface, because it dissolves the crystal. Previous reports have shown that native HDL can solubilize CC (31, 32); however,
in these studies, >30 times higher HDL compared with rHDL was needed to see this effect. Moreover, native HDL needed to be enriched in PC to dissolve CC (37). We suggest that PC mediates the binding of rHDL to the CC surface, and thereby has a role in the rHDL-mediated inhibition of TCC production.

PC is the most abundant phospholipid in HDL (38, 39). The HDL isolated from plasma of different human subjects varies in their inhibitory activity. Some have reported that the anti-inflammatory effect of HDL does not depend on the particle size or the composition of apolipoproteins (40), but rather depends on the composition of the phospholipids (41). A number of studies have demonstrated an anti-inflammatory potential of PC in various conditions such as acute arthritis (42), ischemia (43), and oxidative stress (44). The amount of PC in LDL is lower than in HDL (45). Moreover, the PC in the LDL particle is tightly packed and difficult to extract, whereas that in HDL is much more loosely associated (46), thereby accessible for binding to CC. This may explain why rHDL binds stronger to CC compared with LDL.

Complement activation induced by CC results in opsonization and increased expression of the integrin phagocytosis receptor CR3 (CD11b/CD18) on monocytes and granulocytes by a C5a-dependent mechanism (13). A recent study confirmed the importance of C5a in the inflammatory responses also to MSU crystals (47). In this article, we found that rHDL bound firmly to CC and reduced complement deposition on the CC surface with subsequent downregulation of CR3 expression. One explanation for this effect may be that rHDL reduces CC-induced C5a production. Native HDL has previously been shown to inhibit expression of CR3 on PMA-activated monocytes and granulocytes (48, 49). Thus, HDL may inhibit CR3 expression through several mechanisms.

In this article, we demonstrate an inhibitory effect of rHDL on the secretion of a number of proinflammatory cytokines in response to CC activation in whole blood. Many of these cytokines have been implicated in the development of atherosclerosis (50). Internalization of CC by monocytes is complement dependent and the

FIGURE 5. rHDL attenuates CC-induced cytokines and chemokines in whole blood. (A–F) Stimuli CC, PBS, or LPS were preincubated with different concentrations of rHDL (4, 20, 100 μg/ml) or PBS for 16 h before activation of human whole blood for 6 h. Cytokines and chemokines were quantified in plasma by multiplex analysis. T0 represents the start of the experiment. Dataset on the left of the dividing line (T0, PBS and CC) is plotted on the left y-axis, and dataset on the right of the dividing line (LPS) is plotted on the right y-axis. Mean values ± SEM are shown as column graphs (n = 12). *p < 0.05, **p < 0.01, ***p < 0.001 for CC + rHDL versus CC + PBS.

FIGURE 6. Effect of rHDL on CC-induced ROS and caspase-1 on monocytes. Whole blood was incubated with CC preincubated with rHDL (4, 20, 100 μg/ml) or PBS for 16 h. (A) ROS production is shown as a percentage (%) of dihydrorhodamine (DHR) 123+ monocytes. (B) Activation of caspase-1 was detected as percentage of FLICA+ monocytes in whole blood. Mean values ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 for CC + rHDL versus CC + PBS.
CC-induced inflammation is critical in the initiation and progression of atherosclerotic plaques in a more beneficial direction. Given that the balance between anti-inflammatory and proinflammatory actions is important, studies (56, 57) have shown a novel function of rHDL that reduces the expression of ROS and caspase-1 on monocytes in vitro. flammasome activation by CC. In this study, rHDL significantly inhibited LPS-induced secretion of TNF, IL-6, and other cytokines. The conditions in our whole blood model macrophage foam cells. The anti-inflammatory properties of HDL.

**Disclosures**

S.D.W. is a paid employee at CSL Behring. The other authors have no financial conflicts of interest.

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