Elevation of Plasma High-Density Lipoproteins Inhibits Development of Experimental Abdominal Aortic Aneurysms

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Objective—Patients with abdominal aortic aneurysms have lower concentrations of high-density lipoproteins (HDLs), leading us to investigate whether increasing plasma HDLs could influence aneurysm formation.

Methods and Results—Using the angiotensin II–induced hypercholesterolemic and the CaCl\textsubscript{2}–induced normocholesterolemic mouse model of AAA, we investigated the hypothesis that elevation of HDLs inhibits AAA. HDLs elevated before or at the time of AAA induction reduced AAA formation in both models but had no effect on early ruptures. Analysis of protein lysates from specific aortic segments demonstrated site-specific effects of HDLs on early signal transduction and cellular attrition. We found that HDLs reduced extracellular signal related kinases 1/2 activation in the suprarenal segment, while having no effect on p38 mitogen-associated protein kinase activation in any aortic segment and inhibiting c-Jun N-terminal kinase activation in all aortic segments. In addition, HDL elevation inhibited angiotensin II–induced apoptosis while inducing autophagy in the suprarenal segment of the aorta. Using Illuma gene array profiling we investigated the ability of HDL to modulate basal suprarenal aortic gene expression.

Conclusion—Increasing plasma HDLs inhibit experimental AAA formation, independent of hypercholesterolemia via reduced extracellular signal related kinases 1/2 activation and alteration of the balance of cellular attrition. HDLs modulate genes involved in matrix remodelling, cell migration, and proliferation. (Arterioscler Thromb Vasc Biol. 2012;32:2678-2686.)

Key Words: c-Jun N-terminal kinase ■ phospho–extracellular signal related kinases 1/2 ■ apoptosis ■ autophagy ■ apolipoprotein AI ■ abdominal aortic aneurysms

Abdominal aortic aneurysms (AAAs) are focal dilations of the aorta, which commonly develop in the infrarenal segment. Little is known about the mechanism of this site specificity.\textsuperscript{1} AAAs are often asymptomatic and can go undiagnosed until rupture. Prevalence is 4% to 7% in men and 1% to 2% in women depending on age and geographical location.\textsuperscript{2} Aortic diameter is the most significant risk factor for aneurysmal rupture.\textsuperscript{3} After diagnosis, growth rate is regularly monitored until the diameter reaches 5 to 5.5 cm, when the risk of rupture exceeds surgical risk. There are no pharmacological therapies for aneurysm stabilization. The demonstration that plasma concentration of high-density lipoproteins (HDLs) were lower in patients with AAA\textsuperscript{4,5} led us to investigate whether increasing the plasma concentration of HDLs would inhibit AAA development.

Mature AAAs are matrix rich, cell poor, noncompliant, vascular dilations, often lined with a remodelled thrombus, resulting from early inflammation, proteolysis, apoptosis, and oxidative stress.\textsuperscript{5,6} Involvement of these complex biological events in aneurysm pathogenesis is supported by microarray analysis of human aneurysm tissues.\textsuperscript{7} Infiltration and activation of immune cells result in modification of the proteolytic milieu, leading to elastin degradation and compensatory collagen synthesis (particularly types I and III).\textsuperscript{8,9} Further infiltration of immune cells exacerbates thrombus formation, inducing ischemia-mediated neovascularisation of the developing lesion.\textsuperscript{10} Loss of smooth muscle cell plasticity, combined with increased cell death, results in medial cellular attrition. In the later stages of aneurysm formation, there is excessive degradation of elastin and increased poorly cross-linked collagens leading to weakening of the vessel wall and eventual rupture. Little is known about the precise molecular mechanisms of rupture, which has been shown to be preceded by focal neovascularisation contributing to focal weakening of the arterial wall.\textsuperscript{11}
We used 2 well documented models. In model 1, infusion of angiotensin II (Ang II) in aged hypercholesterolemic mice generates suprarenal (SR) aneurysms within 28 days from the start of infusion, mimicking several processes described in generation of human lesions. Inflammatory changes in the vasculature result in infiltration of leukocytes within the first 3 days of Ang II infusion, followed by a detectable increase in the activity of matrix metalloproteinases within the first week. Prominent intramural hematomas are formed, after early dissection, and remodelling occurs over the following 2 weeks, resulting in formation of circumferential elastin fibers and reendothelialization at the site of dilatation.

In model 2, induction of infrarenal aortic aneurysms by application of CaCl2 to the arterial adventitia results in medial destruction and remodelling leading to dilatations within 6 weeks of injury. Analysis of CaCl2-induced aneurysm formation in mice deficient in matrix metalloproteinases-2 and -9 demonstrated that these enzymes work in concert to generate aneurysmal lesions.

HDLs are a heterogeneous family of small, dense plasma lipoprotein particles varying in size, density, lipids, proteins, apolipoproteins, and function. The dynamic remodelling is mediated through interactions with receptors and enzymes on cell membranes and other plasma lipoproteins and is concomitant with functional changes. In addition to mediating reverse cholesterol transport, HDLs have been shown to have antioxidative, anti-inflammatory, antiapoptotic, and antithrombotic properties. The ability of HDL to inhibit oxidative stress was supported in a recent study showing both mimetic peptides of apolipoprotein AI (ApoAI) and reconstituted discoidal HDLs (rHDLs) reduced NAD(P)H activation in an experimental model of nephropathy. This broad spectrum of effects provides a functional basis for their therapeutic benefit in many complex, multigenic pathologies, such as atherosclerosis and rheumatological conditions. We investigate the effect of elevation of plasma HDL concentration as a therapeutic treatment for AAA.

We show that elevation of plasma HDL concentration inhibited Ang II–induced and CaCl2–induced AAA. Inhibition was concomitant with site-specific reduction in activation of extracellular signal related kinases (ERK1/2) and modulation of cell death. HDLs also modified the transcriptional profile of the aneurysm prone site, with relative changes in specific sets of genes known to inhibit aneurysmal formation.

Materials and Methods

Animal Models

Experiments using Ang II–induced mice were conducted at St George’s, University of London, in accordance with protocols approved by the local ethics committee. AAs were induced in 6-month-old male C57Bl/6apoE−/− mice (Charles River, United Kingdom), by a 4-week infusion of Ang II (1μg/kg per minute) (Sigma-Aldrich, Dorset, United Kingdom), according to a well-established methodology. Mice numbering 94 were randomly allocated to 5 groups (Figure I in the online-only Data Supplement). All animals had access to water and normal chow (RM1/E, SDS Diets, United Kingdom) and an osmotic pump (Alzet 2004) inserted subcutaneously in the interscapulary pouch after gaseous anesthesia, at time 0. The sham operated group (SO; n=12) had pumps delivering saline and daily (SC) injections of saline, 1 week before pump insertion and 4 weeks after. The positive control group (Gp I; n=24) had pumps delivering Ang II and received saline daily (SC) injections of saline 1 week before pump insertion and 4 weeks after. The pretreatment group (Gp II; n=24) had pumps delivering Ang II and received saline daily (SC) injections of rHDLs (10 mg/kg; ApoAI) 1 week before pump insertion and 4 weeks after. The cotreatment groups had pumps delivering Ang II and were given daily saline injections (SC) of saline 1 week before pump insertion and daily injections (SC) of HDLs (10 mg/kg; ApoAI) (Gp III; n=24) or pooled total native (nHDLs) (Gp IV; n=10) from the time of pump insertion and 4 weeks after.

Animals dying before termination at 4 weeks had postmortem examinations. Aneurysms were defined as lesions with a diameter of the SR aorta >50% of the normal diameter. The mean diameter of the SR aorta of the SO group was 1.24 mm; an aneurysm was defined when the SR aorta exceeded 1.86 mm in diameter. Maximal aortic diameter (mm) was estimated as an average of 5 outer-to-inner wall measurements using video micrometry, made by an investigator naive to experimental conditions (Methods and Figure II in the online-only Data Supplement). Animals were lightly sedated by (SC) injections of isofluorane:oxygen:nitrous oxide, were weighed and systolic blood pressure measured, using tail-cuff apparatus according to the manufacturer’s recommendation (Harvard Instruments, Kent, United Kingdom).

The CaCl2–induced model experiments were conducted in the University of Nebraska (W.X., T.M., B.T.B.), as previously described. All experiments were in accordance with the guidelines of the University of Nebraska Medical Centre Animal Care Committee. Mice (C57Bl/6 males, n=18) were purchased from The Jackson Laboratory (Bar Harbor, ME) and randomized to 2 groups; saline control group (saline) receiving daily (SC) saline injections and HDL group receiving daily (SC) injections of rHDL (10 mg/kg; ApoAI). Mice were pretreated for 1 week before aneurysm induction and 6 weeks after. Mice were anesthetized and underwent laparotomy at 8 to 10 weeks old. The abdominal aorta between the renal arteries and the bifurcation of the iliac arteries were isolated from retroperitoneal structures and the aorta measured in triplicate midway between the renal artery and the iliac artery bifurcation, using a video micrometer (Colorado Video, Boulder, CO). After baseline measurements, 0.25 mol/L CaCl2 was applied to the external surface of the aorta by application of a small cotton strip soaked in the solution. After 15 minutes the aorta was rinsed with 0.9% sterile saline and closed; mice were returned to their cages after recovery and 6 weeks later underwent laparotomy and dissection. Measurements were repeated at the same aortic location, by an observer naive to experimental treatments.

Lipoprotein Preparation and Analysis

rHDLs (CSL111) were supplied by CSL-Behring as a lyophilized compound reconstituted by the addition of sterile water, according to the manufacturer’s recommendations. Native HDLs were isolated as a total mixed population of particles by sequential density gradient ultracentrifugation. HDL concentration was defined by the mass of ApoAI present, and native gradient polyacrylamide gel electrophoresis was used to determine particle heterogeneity and purity (Methods in the online-only Data Supplement).

Analysis of Kinase Activation and Cell Death

Twenty-four 6-month-old male apoE−/− animals, were randomized to 3 groups: SO that received an osmotic pump delivering saline and daily (SC) injections of saline 1 week before pump implantation and up to 7 days after; GI (Ang II) that had pumps implanted delivering Ang II (1μg/kg per minute) and daily SC injections of saline 1 week before implantation and up to 7 days after; and GII that were implanted with pumps delivering Ang II (1μg/kg per minute) and a daily SC injection of rHDL (10 mg/kg; ApoAI) 1 week before pump implantation and up to 7 days after. Animals were euthanized at day 3 postimplantation for analysis of kinase activation and at 7 days postimplantation for analysis of markers of cell death. Protein lysates were prepared from 3 aortic segments (thoracic [TA], suprarenal [SR], and infrarenal/iliaic [IR/IA] regions). Abundance of activated kinases (phospho-ERK1/2, p38 mitogen-associated protein kinase, or phospho-c-Jun transduction events in cultured smooth muscle cells were determined.

HDLs Inhibit Experimental Aneurysms
N-terminal kinase (c-JNK) and markers of cell death (Caspase 9, cleaved poly-ADP ribose polymerase, or LC3II/LC3I), were measured using Western blotting with specific antibodies relative to abundance of β-tubulin or a fluorimetric assay (Caspase 3/7) according to manufacturer’s recommendations (Sigma-Aldrich, Dorset, United Kingdom; see Methods in the online-only Data Supplement).

Analysis of Plaque Burden and Neutral Lipid

Isolated hearts were harvested without perfusion, mounted in OCT, (ThermoScientific, West Sussex, United Kingdom), and cryostat sectioned (7–10 μm). Sections were discarded until the 3-valve cusps at the junction of the aorta and heart were clearly observed. Six further sections were collected and stained with Oil-Red-O and Russell-Movat’s Pentachrome (American Master Tech, CA). The extent of neutral lipid (Oil-Red-O) and atherosclerotic plaque area were calculated from digitized low-power bright-field images using the Axiomat image analysis system (Zeiss, Herts, United Kingdom) version 4.2.3.

Transcriptional Profile Analysis

Twelve 6-month-old C57Bl6/ApoE−/− male mice randomized to 2 groups, 1 receiving daily (SC) injections of saline and 1 receiving daily (SC) injections of rHDL (10 mg/kg; ApoAI). All were euthanized after 7 days and the SR aortic segment used to generate total RNA for Illumina gene array analysis using the Ref-8 mouse chip, normalized after 7 days and the SR aortic segment used to generate total RNA for Illumina gene array analysis using the Ref-8 mouse chip, version 2.0 (see the online-only Data Supplement for further details and results).

Statistics

Data are reported as mean±SD, and analyzed by 1-way ANOVA, followed by Tukey post hoc correction for multiple comparisons using GraphPad Prism version 4.0 (GraphPad Prism, CA), unless stated otherwise. Percentage incidence of aortic aneurysms was analyzed by Fisher exact test. A value of P<0.05 was considered significant.

Results

HDL-Mediated Inhibition of Experimental Aneurysm Is Unrelated to Atherosclerotic Load and Independent of Hypercholesterolemia

First, we explored the effect of delivery of HDLs in the Ang II−induced hypercholesterolemic mouse model (Figure 1). The pretreatment group receiving rHDL 1 week before in addition to during Ang II delivery (Gp II) showed complete inhibition of the ability to generate aneurysms compared with the positive control group (Gp I; 1.41±0.15 mm versus 2.29±0.17 mm; n=24; P<0.001). The cotreatment groups given either rHDL or pooled total nHDL, daily for the duration of Ang II (Gps III and IV), showed significant reduction in aeurysmal size, compared with the positive control group (Gp I), receiving Ang II alone, (1.45±0.22 mm, versus 1.42±0.20 mm; n=24; versus 2.29±0.17 mm; P<0.001; Figure 1).

HDL treatment, either pretreatment (Gp II) or cotreatment (Gps III and IV), showed no difference in the number of deaths because of rupture (Table 1). When the aneurysm and rupture data were combined, cotreatment with HDL or rHDL resulted in 37.5% or 40% incidence, whereas pretreatment resulted in a further reduction to 33% incidence. HDLs administered (SC) elevated plasma concentration of HDLs ~2-fold but had no effect on weight or systolic blood pressure between the groups (Table 2). The HDL-raising regimen used in this study was unable to significantly reduce atherosclerotic plaque burden (Figure 2A and 2B) or lipid load (Figure 2C and 2D).

To explore the possibility that this inhibitory effect may be influenced by hypercholesterolemia, we investigated the effect of rHDL in the CaCl2-induced normocholesterolemic model (Table 3). Animals that received rHDLs daily 1 week before aneurysm induction and during the 6 weeks of aneurysm generation showed a significant reduction in percentage of animals treated for 3 days from pump implantation. On day 28 after implantation and the maximal outer-to-outer suprarenal aortic diameter (mm) was measured using computer micrometry. Ang II infusion significantly induced aneurysm formation compared with SO (2.29±0.17 mm vs 1.24±0.14 mm). Pretreatment (Gp II, Ang II/rHDL, 1.41±0.15 mm), cotreatment with HDL (Gp III, 1.45±0.22 mm), or cotreatment with nHDL (Gp IV) 1.42±0.20 mm, inhibited development of aneurysm. Data are means±SD, n as indicated, **P<0.001.

HDL-Mediated Inhibition of Aneurysm Is Concomitant With Site-Specific Differences in Signal Transduction

Because the Ang II−induced aneurysm model is associated with early inflammatory changes, we examined the effect of rHDL on activation of protein kinases known to play a fundamental role in inflammation. We analyzed the effect of rHDL elevation in protein extracts from aortic segments of animals treated for 3 days from pump implantation. On the basis that inflammatory infiltration is observed in this model within 3 to 5 days from infusion of Ang II16 and modulation of signal transduction is likely to precede this event, we decided to analyze protein extracts after 3 days
of treatment. Kinase activation resulting from the surgical stress of the pump implantation returned to baseline levels within 48 hours and by 72 hours phosphorylation was attributable to drugs delivered rather than the surgical trauma of implantation (Methods and Figure VI in the online-only Data Supplement). We found that rHDLs inhibited phosphorylation of ERK1/2 in extracts from the SR and IR/IA segments, but had no effect on extracts from TA (Figure 3A), whereas there was no significant perturbation of p38 mitogen-associated protein kinase (Figure 3B). When we examined the activation of JNK, we found that rHDLs tended to inhibit Ang II–induced c-JNK activation in all aortic segments, with a significant inhibition in the SR region (Figure 3C).

HDL-Mediated Inhibition of Aneurysm Is Concomitant With Site-Specific Differences in Cell Death

Cell death has been shown to play an important role in the development of AAA. Recently, administration of a broad-spectrum caspase inhibitor was shown to significantly reduce cell death and aneurysm formation in the Ang II–induced model.14 We explored the ability of rHDLs to influence cell death in vivo Figure 4.

Table 1. Effect of HDLs on Ang II–Induced Experimental Aneurysm

<table>
<thead>
<tr>
<th>Group</th>
<th>Pump Content</th>
<th>SC (PBS/HDLs)</th>
<th>Early Rupture wk</th>
<th>AAA Formation</th>
<th>Percentage Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>PBS</td>
<td>PBS (5)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gpl</td>
<td>Ang II</td>
<td>PBS (5)</td>
<td>24</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>GplII</td>
<td>Ang II</td>
<td>rHDL (5)</td>
<td>24</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>GplIII</td>
<td>Ang II</td>
<td>PBS/HDL (1/4)</td>
<td>24</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>GplIV</td>
<td>Ang II</td>
<td>PBS/HDL (1/4)</td>
<td>10</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

The table summarizes the clinical data showing the number of animals lost through early rupture within the 1st 2 weeks of infusion and those that generated AAA lesions by termination of the experiment. Percentage incidence (combined early rupture and aneurysm formation) was analyzed using the Fisher exact test. **P<0.01.

HDLs indicates high-density lipoproteins; rHDL, reconstituted discoidal HDL; AAA, abdominal aortic aneurysms; SO, sham operated group; Ang II, angiotensin II; Gp, group.

We measured relative protein concentration of markers of cell death, in each of the 3 aortic segments. Tissues were collected from animals after 7 days of infusion. Using a fluorometric activity assay, which detects the activity of caspase 3 and 7, we found that Ang II treatment induced ∼30% elevation of caspase 3/7 in SR extracts and a more modest elevation of caspase 3/7 activity in IR/IA extracts. In both the SR and IR/IA extracts, rHDL treatment reduced the caspase 3/7 activity to basal levels (Figure 4A). Using Western blotting, we observed that Ang II–induced caspase 9 cleavage from protein lysates of the SR segment of the aorta, reduced by treatment with HDL 3.36±0.97 versus 1.10±0.78; n=4; P=0.039. No significant effect was found in either the TA or the IR/IA segment (Figure 4B). We found that the 89-kDa–cleaved poly-ADP ribose polymerase was significantly induced in the SR region of mice treated with Ang II (0.95±0.045) and reduced to levels observed in the SO animals (SO, 0.21±0.02) by rHDL treatment (0.25±0.034), n=4; P=0.019 (Figure 4C). In addition, we measured the relative conversion of LC3I to LC3II, a marker of autophagy. Although Ang II did not influence the generation of LC3II, compared with amounts found in the SO biopsies of TA or IR/IA aorta, we found that Ang II significantly decreased LC3II conversion, compared with the SR segments from SO group (2.45±0.05 in Sham versus 0.5±0.005 in Ang II; P=0.0014). In the group treated with rHDLs, LC3II conversion was elevated =3-fold greater than the Ang II level in TA but =10-fold greater than the Ang II group in SR aorta (Figure 4D).

Elevation of Plasma Concentration of HDLs Results in Differential Expression of Genes Important in Vascular Remodelling

Transcriptional profiling of the SR aortic segment of mice after 7 daily (SC) injections of rHDL (10 mg/kg; ApoAI) compared with the SR aortic segment of mice after 7 (SC) injections of saline shows a total of 155 genes differentially expressed, 143 induced and 12 inhibited (0<0.05 after correction of multiple measures). The raw data are registered on MIAMIEExpress (HDLMusculusmuseusRef-8vs2.0, release date 2012/11/01). Quantitative real-time polymerase chain reaction validation of 6 of the top 30 induced genes have confirmed the gene array data thus far (Methods, Tables II and III in the online-only Data Supplement).

Discussion

Using 2 well-documented experimental models, we demonstrate that elevation of plasma HDLs can inhibit aneurysm formation. The inhibitory effect we achieve with rHDLs, where the sole apolipoprotein is human ApoAI, is mimicked with the use of total nHDLs, suggesting the effect may be mediated by ApoAI. We have identified 2 molecular mechanisms whereby HDLs can mediate these effects. First, we demonstrate that HDLs inhibit activation of mitogen-activated kinase, ERK1/2, and the stress activated kinase, c-JNK. Second, we demonstrate that HDLs modulate cell death via inhibition of apoptotic cell death and induction of autophagy. This differential response occurs in the SR aorta, the site specific to lesion formation in the Ang II–induced model. Most importantly, we
demonstrate the ability of HDLs to mediate vascular remodelling through modulation of vascular transcriptional profile. These data provide support for the idea that pharmacological modulation of plasma HDLs could be of therapeutic benefit for the treatment of AAA and may also add to our understanding of site specificity.

Raising plasma HDLs, either before aneurysm induction or at the same time as aneurysm induction, inhibited aneurysm formation but did not influence the incidence of early dissection/rupture. These data concur with previous studies in this model, using Fenofibrate (shown to moderately elevate plasma HDLs), also showing reduced aneurysm formation without reductions in the incidence of early rupture. The mechanism whereby elevation of HDLs is able to inhibit induction and growth of aneurysms, but not rupture, requires further investigation.

The dynamic complexity of plasma HDLs is orchestrated through interactions between lipids and proteins on cell membranes and other lipoproteins. Introduction of exogenous HDLs (either rHDLs or native total HDLs) will rapidly alter this dynamic complexity, making determination of what precise HDL subspecies is responsible for an effect a difficult task.

However, because rHDL is composed solely of human ApoAI as the only apolipoprotein, we are able to measure its kinetics of accumulation and clearance in the blood stream. We show that when rHDL is given as a single dose by (SC) injection, it can be detected in the blood stream of the animal within 4 hours, and the concentration peaks after 12 hours, consistent with previous reports. Using size-exclusion chromatography to analyze changes in lipoproteins over time, Chen et al have shown that within 1 hour of intravenous injection of rHDL, these nascent-like discoidal pre-β particles rapidly remodel with endogenous lipoproteins into the spherical mature HDL particle.

We have explored the mechanism of this inhibition with reference to the pleiotropic functions attributed to HDLs. The most well-defined function of HDLs is in mediating removal of cholesterol from peripheral tissues via the liver, the reverse cholesterol transport pathway. The ApoE−/− mice used in this study were at least 6 months old before implantation of the osmotic pump, and although fed normal chow, the extent of atherosclerotic burden seen in these animals was extensive, with fibro-fatty lesions throughout the aortic tree. Acute elevation of plasma HDLs, achieved in these experiments, did not confer a reduction on either lipid content or plaque area in these studies, inconsistent with observations

| Table 3. Effect of rHDL on Changes in Aortic Diameter in CaCl2-Induced Aneurysm Formation |
|---|---|---|
| Treatment | Saline | rHDL |
| Number | 9 | 9 |
| Pretreatment, μm | 478.11±28.2 | 543.78±22.4 |
| Posttreatment, μm | 726.78±34.6 | 694.67±25.7* |
| AAA develop, % | 66.6 | 0 |
| Percentage increase | 52±13 | 28±9* |
| Range of increase, μm | 35–76 | 13–45 |

Aortic diameters were measured before incubation with CaCl2 (pretreatment) and at euthanization (posttreatment). Measurements of aortic diameter are expressed as mean±SD. The percentage increase is represented as compared with pretreatment. The development of aneurysm was defined as an increase >50% relative to the original diameter of the aorta. *P<0.05, Student t test, compared with pretreatment.

AAA indicates abdominal aortic aneurysms; rHDL, reconstituted discoidal high-density lipoprotein.

Figure 2. Acute delivery of reconstituted discoidal high-density lipoprotein (rHDLs) is insufficient to reduce atherosclerotic plaque burden or the extent of neutral lipid in aortic root. On day 28, after aneurysm induction, the burden of atherosclerotic plaques was measured by quantification of area of plaque in the aortic root in serial sections stained with Russel-Movatt’s pentachrome stain (A, B), and amount of neutral lipid quantified after Oil-Red-O staining of serial sections of the aortic root (C, D). Figure shows representative photomicrographs of atherosclerosis (A) and neutral lipid (C). Histograms show plaque area (B) and neutral lipid (D) of sham operated group (SO; white bar) is not significantly different compared with the positive control group (Gp I; black bar); those animals pretreated (Gp II; fine-hatched bar); or cotreated with rHDLs (Gp III; broad-hatched bars). Data are means±SD; n=6; P=not significantly different.
reported after a 6-week treatment of low-density lipoprotein receptor–deficient mice with lipid-free human ApoAI. However, when HDLs are elevated from birth, as in ApoE−/− mice transgenically expressing human ApoAI, a significant reduction of plaque size and lipid content was observed. It may be that the short time frame of elevation of HDLs and modest increase in concentration obtained in our experiments are insufficient to effect complex changes in plaque composition. Our data support the idea that the pathological processes required for atherogenesis and aneurysm formation may have a number of divergent mechanisms.

We show that elevation of HDLs can inhibit Ang II–induced phosphorylation of c-JNK in vivo. Activation of c-JNK was previously identified as a key target in AAA repair in animal models and through gene expression profiling in human AAA. Although Yoshimura et al showed that inhibition of c-JNK activation was concomitant with inhibition and regression of AAA in this experimental model, the investigators did not explore the site specificity of this response. Our data shows that Ang II induces activation of c-JNK in all segments of the aorta, suggesting that although activation of c-JNK may be necessary, it is not specific to aneurysm formation. We show that HDLs mediate a site-specific inhibition of ERK1/2 activation, supporting the recent data where Losartan, the selective Ang II type 1 receptor blocker, inhibited the tumor growth factor-β–mediated activation of ERK1/2, by allowing continued signaling through the Ang II type 2 receptor.

Cellular attrition of the aortic wall ultimately results in a weakened, noncompliant aneurysm prone to rupture. Cellularity of the vessel wall is determined by a balance between cellular regeneration, through resident cell proliferation and stem-cell engraftment, and the processes of cell death. Cell death may also be considered a constant balance between the different forms of cell death (eg, necrosis and apoptosis and autophagy). Autophagy has been shown to be a homeostatic reparative process under normal physiological conditions. Autophagy is a multistep catabolic process whereby stable proteins and organelles are sequestered in a double membrane–bound autophagosome and degraded via lysosomes. However, in pathological conditions, where there is excessive induction of autophagy by environmental or intracellular stress, autophagy converts to a cell death pathway. The mechanisms that regulate the balance between autophagy and apoptosis, and hence, regulate cellular attrition, are largely unknown. Cell death through increased free cholesterol uptake has previously been described, and the increase of the autophagic process within the lipid-rich atherosclerotic plaques may be viewed as a defensive reparative process. Recent reports of autophagic gene expression in human AAA lesions still raises the question as to whether this forms part of the developmental process of aneurysm formation or constitutes a reactive response to redress the loss of vascular cellularity. Our study is the first to describe a site-specific imbalance between apoptosis and autophagy in this experimental aneurysm model, which may explain the localization of lesion formation.

Oxidative stress, a major inducer of cell death, has previously been shown to play an important role in the generation of AAAs. The antioxidative properties of HDLs are well documented and demonstrated to be largely a result of
the presence of paraoxonase-1, although ApoAI has also been shown to have antioxidant properties. The fact that we observe inhibition with both total nHDLs and rHDLs supports the idea that the ameliorative effect we demonstrate may be a function of ApoAI.

Studies to determine which components of HDLs are important for maintaining the antiapoptotic properties of the lipoprotein suggest that it may be attributable to the sphingosine-1-phosphate carried on the particles, whereas others have shown it can be a function of the ApoAI. Previously, we have shown that sphingosine-1-phosphate, a metabolite of ceramide induced mitogenesis, was able to suppress ceramide-mediated cell death. It is also possible that rHDL particles, used in these studies, may acquire other proteins and lipid components (eg, sphingosine-1-phosphate, which may influence their function in the Ang II–induced mouse model). Further studies are required to evaluate whether these specific changes in lipoprotein complexity play a functional role in the ability of HDLs to inhibit aneurysm formation.

To mediate the profound inhibitory changes we have shown, HDLs must alter expression of a broad spectrum of genes important in aneurysm pathogenesis. We show that of the 155 genes that are significantly modulated by increasing plasma concentration of rHDLs, 26% of the top 30 upregulated genes are involved in the synthesis, cross-linking, and degradation of extracellular matrix, known to be essential to the pathogenesis of abdominal aneurysm: Carboxypeptidase X1; Serpins A3n; tissue inhibitor of metalloproteinase-1; Prolyl Hydroxylase-β; Lysyl Oxidase; Elastin; Collagens (type 1, 5,14, and 16 α1); Osteonectin; Nidogen; Laminin B1. Twenty-five percent of the top 30 upregulated genes are involved in cell cycle progression and processing of growth factors, which also have a role in vascular remodelling: Cyclin B1; Cyclin C20; Protein Regulator of Cytokinesis; Cell Cycle Associated Protein 3. Of the top 12 downregulated genes, the membrane attack complex inhibitory factor CD59a has been previously reported to have a role in aneurysm formation and other genes (eg, Sortilin-1, very-low-densiy lipoprotein receptor, and Peroxisomal Biogenesis Factor) may play more of a role in modulating the changes in lipoprotein metabolism/complexity. Thus, HDL-mediated modulation of transcriptional profile may explain the ability of pretreatment with rHDL to confer additional protection against Ang II–induced aneurysm formation we have observed.

The list of potential candidate targets for aneurysm therapy is continually increasing, as differential gene expression reveals changes in expression concomitant with aneurysm formation, and investigators explore function through genetic modification. However, further investigation of the kinetics of expression, cell specificity, and functional activity of each of these genes in a variety of models is essential to fully understand their precise role in aneurysm pathobiology. For example, whereas SerpinA3n appears important in aneurysm formation, increasing circulating concentrations of Serpin A3n can inhibit early rupture of Ang II–induced aneurysms but transgenic overexpression of
the gene did not inhibit CaCl₂-induced aneurysm formation.58 Interestingly, one of the latest new candidate genes, the angio-
poietin-like protein 2, a member of the large family of secreted angiopoietins, shown to accelerate CaCl₂-induced aneurysm for-
formation59 has also been shown to reduce plasma concentrations of HDLs through interaction with lipoprotein lipases.60

These studies are proof of the concept that raising plasma concentration of HDLs has potential to reduce AAA. It is a
timely report, coming at a point when HDL-raising strategies are gaining momentum as a therapeutic regimen for cardiovas-
cular diseases. Taken together, our findings support the idea that raising HDLs may provide a viable therapeutic strategy for aneurysm treatment which warrants further investigation.

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Disclosures
None.

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Elevation of Plasma High-Density Lipoproteins Inhibits Development of Experimental Abdominal Aortic Aneurysms

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Supplement Methods

Design of Ang II-induced aneurysm experiments

Ninety four six month old ApoE deficient mice were randomised to five groups (see Figure I). Animals viable at day 28, following pump implantation, were sacrificed humanely and weighed. Blood was collected by cardiac puncture to measure lipid profiles and hearts were collected and blocked without perfusion, to estimate plaque load and neutral lipid. The supra-renal aortae were exposed following removal of retroperitoneal structures in order to measure outer-to-outer wall diameter using video micrometry.

Measurement of aneurysm diameter

Both aneurysm models used in this study employ video micrometry to measure the external aortic diameter. The Ang II-induced studies measure the aortic diameter of the supra-renal aorta between the last intercostal artery and the renal artery, and the CaCl$_2$-induced model measures the infra-renal aorta mid-way between the renal artery and the iliac bifurcation. Whilst the Ang II-induced model only measures the aortic diameter at termination of the experiment, investigators are able to measure the infra-renal aorta at the beginning and at termination in the CaCl$_2$-induced model.

Measurements are made once the retroperitoneal structures have been removed to reveal the outer wall of the vessels. An average estimate is made by an investigator blinded to the treatment regimen of each animal. Figure II below illustrates an example of the supra-renal aortic measurement on day 28 following a sham operated animal (SO, top panel) animal and an animal that has received Ang II ($1\mu$g/kg/min) for the duration of the experiment (Ang II, bottom panel). In sham operated animals five measurements are made at random sites across the supra-renal region, and in the Ang II animal with a large aneurysm, the maximal dilation is located, and five sequential measures are taken at this site. The figure II shows the mean ± SD of these particular animals.

Isolation and characterisation of HDLs.

(i) Reconstituted discoidal high-density lipoprotein (rHDL)
Reconstituted HDLs were prepared from plasma derived human ApoAI and soybean phosphatidylcholine by cholate dialysis$^1$ and provided as a lyophilised compound by CSL-Behring. Reconstitution, according to the manufacturers recommendations, results in a solution of discoidal particles with an average protein: phospholipid ratio of 1:150.

(ii) Total native high-density lipoproteins (nHDLs)
To isolate sufficient native HDLs to ensure that the animals received a comparable complexity of particles, we commenced preparation with 400mls of pooled human plasma
from eight normal healthy volunteers. Since each mouse receives 10mg/kg/day (as a measure of Apo AI mass) for 28 days, and with a batch of n=10 and an average mouse weight of 25g, a minimum amount required, to ensure provision for the entire experimental group, was 100 mg nHDL as determined by the mass of ApoAI.

Briefly: Batches of 50 mls of whole blood from each of 8 normal healthy volunteers (with an average HDL concentration of 1mg/ml) were collected into EDTA (1.5mg/ml) and plasma collected following centrifugation at 3000rpm at 4°C. Native HDLs in the density range of 1.07 – 1.21 g/ml, were isolated by sequential density gradient ultracentrifugation as previously reported\(^2\). Resulting preparations of HDLs were dialysed against four changes of phosphate buffered saline before filter sterilising. Aliquots of the pooled material were stored at -80°C and thawed as required. Storage at low temperature ensured that the process of oxidation was not progressive.

**Sequential density gradient ultracentrifugation for isolation of total native HDL**

Plasma, containing 0.01% EDTA (w/v), to minimise auto-oxidation, was adjusted to a density of 1.07, using KBr and centrifuged for 24 hours at 70K in 70 Ti, using the formula:

\[
\text{Mass KBr (g)} = \text{Vi} \times (\text{pf} - \text{pi})/(1 - \text{V}_5) \times \text{pf}
\]

Where Vi - initial volume of plasma; pf - desired density; pi - initial density and V\(_5\) - partial specific volume of KBr at 5°C (at density 1.07 V\(_5\) is 0.2943 and at density 1.21 V\(_5\) is 0.3503). Solubilisation of KBr in plasma was facilitated by stirring at 4°C, on a bed of wet ice, protected from the light (to minimise oxidation).

Following ultracentrifugation for 24 hours at 70k in 70 Ti, the top fraction was discarded (containing mainly LDL and VLDL) and the subnatant adjusted to a density of 1.21 g/ml by addition of solid KBr, on wet ice in the dark. Once in solution, the fraction was further centrifuged at 70 K in 70 Ti for 24 hours.

The top fraction, containing HDL was harvested, re-adjusted to a density 1.21 g/ml and centrifuged at 100k overnight in 100.4 Ti. The top of the tubes were harvested and pooled, and the material dialysed against four changes of PBS (1L containing 0.01% EDTA) and filter sterilised through a 0.2 \(\mu\)m acrodisc ™ (Gelman, UK) for analysis.

**Measurement of oxidation products**

[i] HPLC. Oxidation products (cholesteryl arachidonate and cholesteryl lineate - rapid products) and 7-ketocholesterol and cholesteryl linoleate hydroxide (slower products) were assessed by HPLC according to previously reported method of HPLC\(^3\). 7-ketocholesteryl standard was purchased from SigmaAldrich, Dorset, UK.

We were unable to detect oxidation products and thus the abundance of oxidation product was estimated to be < 20 pmole/injection, the lowest limit of detection.
[ii] Monocyte adhesion – bioassay. Since detection of oxidation products were below the level of detection using the HPLC protocol, ie., < 20 pmoles/injection, we used stimulation of monocyte adhesion to endothelial cells as an appropriate bioassay for oxidative products.

Briefly – a spontaneously transformed human endothelial cell line (C11STH) was grown to confluence in 24 well plates and following an overnight incubation with lipids, the monocytic cell line (THP1, 4.5 x 10⁴/well) was added for 20 minutes at 37°C. Monocyte adhesion was measured, in comparison with the level obtained through cytokine-induction of the endothelium, by counting the number of cells remaining in five high-power fields after washing off the non-adherent cells three times in phosphate buffered saline (PBS) at 4°C (see Figure III).

**Endothelial cell culture**

C11STH cells were grown in Medium M199 with Earle’s salts, supplemented with 20% foetal calf serum, 1mmol/L sodium pyruvate, 20 mmol/L L-HEPES, 2 mMol/L glutamine, 100 u/ml penicillin, 100µg/ml streptomycin, 50µg/ml Heparin (monoparin) 50 µg/ml ECGF (Sigma, Dorset, UK) at 37°C in 5% CO₂. All reagents are from Gibco (Renfrewshire, UK), unless otherwise stated.

**THP-1 cell culture**

THP-1 cells purchased from ATCC, (ATCC TIB-202) were grown in RPMI 1640 medium containing 10% foetal calf serum, 100 U/ml penicillin and 100µg/ml streptomycin at 37°C in 5% CO₂.

**Adhesion protocol**

Endothelial cells were grown to confluence, then washed in serum low medium (2%) and treated overnight with activators (lipids or cytokines). THP1 cells were added (4.5 x 10⁴ cells /24 well dish) for 20 mins at 37°C. The assays were then washed three times in PBS, by filling the well with PBS and flicking off the washing medium. The degree of monocyte adhesion was measured as the cell numbers in five random high-power fields per well, in triplicate.

**Preparation of oxidised lipoproteins**

oxLDL was prepared by the addition of 7.5 mmol/L CuSO₄ for 6 hours at 37°C. The reaction was stopped by addition of 0.01 mmol/L EDTA and the product dialyzed in four changes of PBS and 0.01% EDTA at 4°C before passing through a 0.2µm acrodisc™ (Gelman Sciences, UK).

**Measurement of apolipoproteins.**

The concentrations of apoA-I and apoB in the preparations of native HDLs were determined using an ELISA, according to the manufacturer’s recommendations (MabTech Inc., Antibes, France). 
Native gradient gel electrophoresis of HDL.

The major lipoprotein subclasses in the total native HDLs were visualised using non-denaturing gradient polyacrylamide gel electrophoresis, followed by western blotting using a goat-anti human ApoAI polyclonal antiserum. A concave non-denaturing 3-35% polyacrylamide gel was prepared using the Biorad gel former (Gel Former 495, Biorad, UK) in an apparatus (Biorad Protean II XL™) using a 16 x 20 cm x 3mm format. Once assembled into the running tank, the wells were gently rinsed out with fresh running buffer (taking care not to distort the well) and pre-run for 60 mins at 50V. Total native sequential density gradient purified HDL (nHDL) samples were loaded in the continuous buffering system (Tris borate pH 8.3) containing 10% sucrose and bromophenol blue. Samples were run for a total of 3000Vhrs (15V for 15 mins, then 70V for 20 mins then 125V for 24 hours). Particle sizes were calculated relative to migration of standards of known Stoke’s hydrated diameter (Catalase (11.3 nm), Aldolase (7.3 nm), Ovalbumin (3.5 nm), Chymotrypsinogen A (2.6 nm)). Native gradient gels were transferred to PVDF membrane and western blotted using a goat-anti-human ApoAI polyclonal antibodies (Millipore, Herts, UK).

Following electrophoresis, gel was blotted onto a PVDF membrane (Millipore, Herts., UK) at 20V overnight at 4°C. Following transfer, the membrane was fixed with 10% acetic acid for 15 mins then air dried for a further 15 mins. Blocking was achieved by incubating the membrane in 50mM TrisCl pH 8.0, 80mM NaCl, 2 mM CaCl₂ containing 5% nonfat dry milk and 0.2% Nonidet P40 for 1 hour at room temperature. After which, the membrane was incubated in 1:2000 dilution of goat anti-human Apo-Al antisera (IgG) (Millipore, Herts., UK) for 1 hour at room temperature, then washed and incubated at 1:3000 dilution of HRP-conjugated donkey anti-goat IgG for a further hour. The membrane was finally washed in Tris-Saline MgCl₂ pH 9.6 before incubation with Pierce Super Signal™ West Pico Chemiluminescence substrate. Chemiluminescence was visualised using an Odyssey Fc Imager (Li-Cor Biosciences, GmbH). Particle size was defined by comparison to profiling standards of known Stoke’s Radius (Figure IV).

Establishing that the subcutaneous delivery of rHDL (CSL-111) raises circulatory levels of plasma HDLs.

Reconstituted HDLs (rHDLs), containing human apolipoprotein Al (ApoAI) as the sole protein, and soybean phosphatidylcholine as the sole phospholipids in a molar ratio of 1:153, were provided by CSL-Behring AG (Product Code CSL-111, Bern, Switzerland), and prepared from human plasma as previously described. This lyophilised material was reconstituted with sterile water according to manufacturer’s recommendations, aliquoted and stored at -80°C.

To establish the rate of entry into, and clearance of rHDLs from, the circulation in six month old ApoE deficient mice, we gave a single injection (10mg/kg ApoAI) to a group of 24 animals. The animals (n=4 per time point) were sacrificed at time 0, and 4, 8, 12, 24 and 48
hours after delivery of rHDLs. Pooled whole blood was collected by cardiac puncture into Potassium EDTA (1.5 mg/ml) and plasma was harvested following centrifugation at 3000 rpm for 20 mins at 4°C. The concentration of human ApoAI (the sole apolipoprotein in rHDLs) in murine plasma was measured using an ELISA kit (MabTech, Antibes, France) for human ApoAI, according to the manufacturer’s recommendation. The antibodies in this ELISA did not cross-react with mouse ApoAI.

**Tissue collection preparation and western blotting.**

**Collection of tissues:** Termination of the experiments occurred 4 weeks following insertion of the osmotic pumps (Pre-treatment and co-treatment study), and at 6 weeks following aneurysm generation (Therapeutic regression study). Animals in which aortic segments were collected for analysis of protein kinase activation were terminated 3 days following pump implantation and those analysed for cell death were terminated 1 week after implantation of the pump. All animals were fasted for 6 hours before termination, during which time MRI scanning was performed for regression studies (see supplemental methods). Following heavy surgical anesthesia (fentanyl/fluanisone/midazolam (1:1:2) Hypnorm™:Hypnovel™:water) (10ml/kg (i.p)) (VetPharma Ltd., Sussex, UK), whole blood was collected by cardiac puncture into pediatric collection pots containing 1.5 mg/ml K$_2$EDTA. Animals were perfused with ice-cold phosphate buffered saline (PBS), containing protease inhibitors, via a cannula inserted into the left ventricle of the heart. Infusion was maintained at 100 mmHg, using a Harvard pump. Perfusion was continued for 15 minutes, following which the aortic tree was dissected, photographed and dimensions measured using computer assisted micrometry (Ziess, Herts, UK). The thoracic abdominal aorta (TA) was excised from bottom of the descending aorta to the second pair of intercostal arteries, the supra-renal (SR) abdominal aorta was excised between the second pair of intercostals to just below the right renal artery, and the infra-renal and iliac artery (IR/IA) excised from below right renal artery to just below the iliac bifurcation. Tissue segments were stored in liquid nitrogen (for protein extraction), until required for analysis.

**Preparation of protein lysates:** Aortic segments were ground to a fine powder in liquid nitrogen then sonicated in ice-cold PBS and lysed at 4°C in lysis buffer, and analysed by western blotting. (20 mmol.L$^{-1}$ sodium HEPES, pH 7.4; 1mmol.L$^{-1}$ EDTA; 2mmol.L$^{-1}$ MgCl$_2$; 10 µg.ml$^{-1}$ PMSF; 2 µg.ml$^{-1}$ leupeptin; 2 µg.ml$^{-1}$ aprotinin), followed by centrifugation (14,000 g x 15 min), boiling (5 min at 100°C). Total cell lysates (10 – 20 µg protein) were separated on an SDS-PAGE (10 – 12 % running gel, 4% stacking gel). Protein was then transferred to PVDF membrane (Millipore, Herts, UK), using the rapid room temperature Trans-blot Turbo Transfer System™ (Biorad, UK). Blocking was performed overnight at 4°C in Tris-buffered saline (TBS) plus 5% non-fat dry milk. Blots were incubated with affinity purified primary antibodies (1:1000) (Table 1), overnight at 4°C in TBS with 0.05% Tween-20, 5% non-fat dried milk solid followed by incubation with HRP-conjugated species appropriate secondary
antibody (1:2000) (New England Biolabs, Herts, UK) for 45 minutes. Immunodetection was accomplished using chemiluminescence (Super Signal-HRP, Pierce Chemical Corp.). Relative abundance was calculated as a ratio of test protein compared with β-tubulin following scanning the specific bands using an Odyssey Fc Imager (Li-Cor Biosciences, GmbH).

**Establishing the time course of surgical stress on activation of MAPKs**

Twelve, six month old ApoE deficient mice were randomised to three groups. All received surgical implantation of an osmotic pump delivering only saline, according to the method described. Groups of four mice were sacrificed at 6, 18 and 72 hours following implantation, and the aortic segments harvested at 4°C following physiological perfusion with saline containing protein inhibitors and processed for protein lysates to analyse phospho-JNK, phospho-p38MAPK and phospho-ERK1/2 activation by western blotting.

**Micro-array analysis of gene expression.**

*Total RNA isolation and quality control.* Total RNA was isolated from the supra-renal aortic region between the last intercostals artery and the left renal artery. Tissue biopsies were ground to a fine powder in liquid nitrogen and total RNA extracted using RNeasy™ Fibrous Tissue RNA extraction kit (Qiagen, UK). The integrity and concentration of the extracted total RNA were tested using RNA 60000 Nano labChip™ kit and Agilent 2100 Bioanalyser (Agilent Technologies, UK). Features of the successful total RNA were RIN value >9.0 and observation of two ribosomal peaks (18s and 28s) in an approximate stoichiometry 1:2.

*Illumina™ Gene Array analysis.* Briefly, total RNA (1µg/sample) was reverse transcribed followed by a single in vitro transcription amplification to incorporate biotin-labelled nucleotides. The labelled transcripts were hybridized according to the manufacturers recommendation and stained with streptavidin-Cy3, according to manufacturers recommendations. The chip (Mouse Ref 8 vs 2, containing 25,697 transcripts and variants, Illumina, Essex, UK) were scanned with an Illumina Bead Array Reader to measure signal intensity to the labelled target genes.

**Bioinformatics.** Data analysis was completed at Cambridge Genomic Services, Cambridge, UK. Raw data was analysed with the microarray software (Bean Studio Gene Exp v 3.4.0) for quality control, background analysis and normalisation with rank invariant algorithm. Genes showing differential gene expression were identified by filtering, using the p value >0.05 as the sole criteria for selection. *Entrez Gene,* available in the NCBI homepage (www.ncbi.nlm.nih.gov/gene), was used to identify all known gene ontology and the processes associated with the products of those genes. Benjamini-Hochberg multiple testing correction was applied to normalised data.
Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). QRT-PCR was carried out using an Mx4000 Multiplex Quantitative PCR System (Stratagene, UK), using Assays on Demand (Applied Biosystems, Cheshire, UK), according to manufacturers recommendations (see Table 4). The PCR cycle started with an initial 10 min denaturation step at 95°C, followed by 40 cycles of shuttle heating at 95°C for 15 s and 60°C for 1 min. The associated Mx4000 software was used to analyze the data and determine the threshold count \( \text{C}_t \). The specimens were normalized to the endogeneous reference controls, transferrin receptor and GAPDH. For each sample, \( \text{C}_t \) \text{target gene} and \( \text{C}_t \) \text{Average controls} were determined, and \( \Delta \text{C}_t = \text{C}_t \text{target gene} - \text{C}_t \text{Average transferrin receptor, GAPDH} \). The relative quantification of target gene normalised to the average of Transferrin receptor and GAPDH was determined by calculating \( 2^{-\Delta\Delta\text{C}_t} \).

For the \( 2^{-\Delta\Delta\text{C}_t} \) calculation to be valid, preliminary experiments were performed to verify that the efficiencies of target gene amplification and the efficiency of control gene amplification to be approximately equal. To achieve this, standard curves of template dilution and \( \text{C}_t \) were obtained for each target gene and an average of transferrin receptor mRNA and GAPDH mRNA. In this study, the values of the slope of log amount total RNA vs \( \text{C}_t \) was sufficiently small (\( < 0.1 \) or \( > -0.1 \)) for efficiencies of amplification of target genes and transferrin receptor and GAPDH to be considered approximately equal.

Supplemental Results

Whilst endothelium treated with cytokines (TNFα, 10ng/ml) and oxidised LDL (100µg/ml) are able to support a two fold induction on monocyte adhesion, neither native or reconstituted HDL induce monocyte adhesion significantly above the basal level (Figure III), suggesting a lack of significant oxidation products in the HDLs in agreement with the HPLC findings.

The total native HDLs isolated gave two major alpha migrating populations which identified with sizes consistent with HDL2b and HDL3a. Although their was no ApoB, suggesting little LDL contamination, other species of HDLs were apparent as minor components of the preparation as was evidenced by the background staining with antiApoAI antibody on western blotting.

A single dose (10 mg/kg, ApoAI) reaches a maximal plasma concentration within 12 hours following subcutaneous (s.c) injection, and is largely cleared within 48 hours (Figure V). These kinetics were consistent with those reported previously, using subcutaneous injections of 500 µg of human ApoAI every other day for a period of six weeks. Daily injections with native total HDL or rHDLs resulted in no adverse effects over the maximal time of our experiment.
Surgical implantation causes significant activation of all three MAPKs examines at 18 hours in both the thoracic (TA) and supra-renal (SR) region of the aorta (Figure VI). However, by 72 hours the level of activation of JNK, p38MAPK and ERK1/2 has returned to that measured at 6 hours post implantation (basal).

**Supplemental Conclusion.**

The native HDLs isolated from normal healthy volunteers showed little evidence of oxidation or inflammatory potential. The measurement of activation of MAPKs from aortic segments harvested at 72 hours, following implantation of an osmotic pump, is unlikely to be confounded by activation due to surgical stress.
Supplemental Figures

Figure I

Time (wks) from pump insertion at time zero (0):

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Figure II

SO

1209.54 μm
1245.21 μm
1193.34 μm
1106.92 μm
1201.63 μm 1191.33 ± 51.16

Ang II

2389.92 μm
2417.29 μm
2404.44 μm
2381.80 μm
2352.19 μm  2369.13 ± 24.73
Figure III

![Bar chart showing cell counts](image)

Figure IV.

![Stoke's Diameter chart](image)
Figure V.

Figure VI.

[a] [b] [c]
Supplemental Figure Legends

Figure I Schematic diagram of experimental design in Ang II-induced aneurysm model.

Ninety-four mice were randomly allocated to five groups. All animals had an osmotic pump inserted subcutaneously, at time zero. Sham operated group (SO, \(n=12\)) had pumps delivering saline and daily injections (s.c) of saline one week prior to pump insertion and four weeks following.

Group I, the positive control (Gp I; \(n=24\)), had pumps delivering Ang II and received daily subcutaneous (s.c) injections of saline for one week prior to pump insertion and four weeks following.

Group II, the pre-treatment group (Gp II; \(n=24\)) had pumps delivering Ang II and received daily (s.c) injections of reconstituted HDLs (rHDLs) (10mg/kg, ApoAI) one week prior to pump insertion and four weeks following.

Groups III, the rHDL co-treatment group (Gp III; \(n=24\)) had pumps delivering Ang II and received daily injections of saline (s.c) one week before pump insertion and rHDL (10mg/kg, ApoAI) four weeks following.

Group IV, the nHDL co-treatment group (Gp IV; \(n=10\)) had pumps delivering Ang II and received daily injections of saline (s.c) one week before pump insertion, and nHDL (10mg/kg, ApoAI) four weeks following.

Figure II Examples of measurement of supra-renal aortic aneurysm using video micrometry

An average estimate of each aortic diameter is made by drawing a cursor from outer wall to outer wall, once the artery has been exposed, at five random sites on aortae which are not affected (top panel, Sham Operated (SO)), and five times at the site of maximal dilatation in animals which clearly have an aneurysm (bottom panel, Ang II (AngII)).

Figure III Effect of lipoproteins on monocyte adhesion to endothelium

Confluent endothelial cell cultures were incubated overnight with culture medium (2% foetal calf serum) with the addition of nothing (Nil), TNF\(\alpha\) (10ng/ml), native HDL (nHDL, 1mg/ml), reconstituted discoidal HDL (rHDL, 1mg/ml), oxidised LDL (oxLDL, 100\(\mu\)g/ml), native LDL (LDL, 100\(\mu\)g/ml). THP-1 monocytic cells (4.5 x 10^4 cells/well) were added for 20 mins at 37\(^{\circ}\)C, and the non-adherent cells washed off with three changes of PBS. Cells attached were counted in 5 random high-power fields per well and treatments were analysed in triplicate. Data are mean ± SD, \(n=3\), \(p^*=<0.01\)
**Figure IV** Western blot of human ApoAI following native gradient gel electrophoresis of total human HDLs.

There was no evidence of contamination of LDLs in the native HDL preparations, as apoB was undetected in the HDL preparations. Evidence of oxidation products were below the level of detection using HPLC, and nHDLs did not support monocyte adhesion to activated endothelium. All preparations of native HDLs contained two major populations: one with particles of Stoke’s diameter 10.45 nm (HDL2b) and one with particles of diameter 8.6 nm (HDL3a).

**Figure V** Pharmacokinetics of Human ApoAI following a single subcutaneous dose of rHDL in ApoE deficient mice.

Graph showing the rate of plasma clearance of a single dose of rHDL (10 mg/kg ApoAI), given s.c. in six month old ApoE deficient mice. Twenty four animals were given a single dose and four animals sacrificed at 0, 4, 8, 12, 24 and 48 hours after injection. Human ApoAI, the sole protein in rHDL, was measured using by ELISA. Data are mean ± SD, n=4.

**Figure VI** Activation of MAPK as a result of surgical implantation of the osmotic pump is resolved within 72 hours.

Histogram showing aortic concentration of phosphorylated MAPK [a] Phospho-JNK, [b] Phospho ERK and [c] Phospho-p38MAPK, estimated using western blotting relative to the abundance of total kinase, in aortic segments (Thoracic [TA] and Supra-renal [SR]) following sham pump implantation over time. White bars are samples taken 6 hours after implantation, hatched bars are taken at 18 hours after implantation and the black bars are samples taken at 72 hours following implantation. Data are mean ± SD, n=4, p as indicated.
**Supplemental Tables**

*Table I. Antibodies used in this study.*

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MAb – monoclonal antibody, PC – polyclonal antibody
### Table II. rHDL-mediated differentially upregulated genes (top 28)

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<td>Carboxypeptidase X1</td>
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<td>Sushi-repeat-containing PX</td>
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<td>CAPG</td>
<td>Capping protein gelsolin-like</td>
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<td>COL1A1</td>
<td>Collagen 1alpha1</td>
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<td>TIMP1</td>
<td>Tissue inhibitor metalloproteinase1</td>
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<td>P4HB</td>
<td>Prolyl 4 hydroxylase beta</td>
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<td>ERP29</td>
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<td>GRB10</td>
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<td>Sushi-Von Willebrand-Pentraxin gene</td>
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<td>CCNB1</td>
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<td>CXCL16</td>
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<td>TCF19</td>
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<td>IGFBP4</td>
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<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
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<td>1.793</td>
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<td>CDC20</td>
<td>Cyclin C20</td>
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<td>LOX</td>
<td>Lysyl Oxidase</td>
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<td>FK506 binding protein 11</td>
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<td>NID1</td>
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### Table III. rHDL-mediated differentially downregulated genes

<table>
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<th>Gene Abbreviation</th>
<th>Full Name</th>
<th>Corrected Fold Change (Illumina™)</th>
<th>P value</th>
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<td>IL17RE</td>
<td>Interleukin 17 receptorE</td>
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<tr>
<td>CD59a</td>
<td>Cluster of Differentiation 59a</td>
<td>-1.524</td>
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<tr>
<td>IVD</td>
<td>Iso-valeryl CoA dehydrogenase</td>
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<td>IGFBP5</td>
<td>IGF binding protein 5</td>
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<td>SORT1</td>
<td>Sortilin 1</td>
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<td>VLDLR</td>
<td>VLDL Receptor</td>
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<td>LRTM1</td>
<td>Leucine rich rpt transmembrane 1</td>
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<td>PEX</td>
<td>Peroxisome assembly factor 2</td>
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<td>ATG-12</td>
<td>Autophagy-related protein 12</td>
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### Table IV. Assays on Demand ™ probe sets used in QRT-PCR

<table>
<thead>
<tr>
<th>Full Name of Gene</th>
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<tr>
<td>SerpinA3n</td>
<td>Mm00776439-m1</td>
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<td>Elastin</td>
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<td>Mm00441818-m1</td>
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<td>Prolyl4Hydroxylaseβ</td>
<td>Mm01243188-m1</td>
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<tr>
<td>Lysyl Oxidase</td>
<td>Mm00495386-m1</td>
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</tbody>
</table>
References:


2. Rye KA, Garrety KH, Barter PJ. Preparation and characterization of spheroidal, reconstituted high-density lipoproteins with apolipoprotein A-I only or with apolipoprotein A-I and A-II. *Biochim Biophys Acta.* 1993;1167:316-325.


