The Ability to Promote Efflux Via ABCA1 Determines the Capacity of Serum Specimens With Similar High-Density Lipoprotein Cholesterol to Remove Cholesterol From Macrophages

Margarita de la Llera-Moya, Denise Drazul-Schrader, Bela F. Asztalos, Marina Cuchel, Daniel J. Rader, George H. Rothblat

Objective—We measured efflux from macrophages to apolipoprotein B-depleted serum from 263 specimens and found instances in which serum having similar high-density lipoprotein cholesterol (HDL-C) differed in their efflux capacity. Thus, we wanted to elucidate why efflux capacity could be independent of total HDL-C or apolipoprotein A-I (apoA-I).

Methods and Results—To understand why sera with similar HDL-C or apoA-I could differ in total efflux capacity, we assessed their ability to promote efflux via the pathways expressed in cAMP-treated J774 macrophages. Briefly, macrophages were preincubated with probucol to block ABCA1, with BLT-1 to block SR-BI, and with both inhibitors to measure residual efflux. ABCG1 efflux was measured with transfected BHK-1 cells. We used apolipoprotein B-depleted serum from specimens with similar HDL-C values at the 25th and 75th percentiles. Specimens in each group were classified as having high or low efflux based on total efflux being above or below the group average. We found that independently of HDL-C, sera with higher efflux capacity had a significant increase in ABCA1-mediated efflux, which was significantly correlated to the concentration of preβ-1 HDL. The same result was obtained when these sera were similarly analyzed based on similar apoA-I.

Conclusion—Sera with similar HDL-C or apoA-I differ in their ability to promote macrophage efflux because of differences in the concentration of preβ-1 HDL. (Arterioscler Thromb Vasc Biol. 2010;30:796-801.)

Key Words: ABCA1 • apolipoprotein A-I • cholesterol efflux • high-density lipoprotein cholesterol • macrophages

Epidemiological and interventional studies1–4 demonstrate an inverse relationship between high-density lipoprotein cholesterol (HDL-C) levels and coronary heart disease, which is an observation also supported by animal studies.5 Thus, high HDL-C levels are thought to independently reduce coronary heart disease risk. Although HDL has been shown to have both antioxidative and antiinflammatory properties,6 its beneficial antiatherogenic effect is likely attributable to its central role in reverse cholesterol transport, ie, the transport of cholesterol from peripheral tissues to the liver for excretion to reduce its accumulation in tissue cells such as vessel wall macrophages.7 Because both HDL metabolism and cholesterol transport are complex processes, it has been difficult to obtain in vivo evidence that modulating HDL levels can affect removal of cholesterol from macrophage foam cells in the vessel wall and reduce atherosclerotic lesions. Overexpression of apolipoprotein (apo) A-I in mice can reduce progression of atherosclerotic lesions,8 and infusion of apoA-I/phospholipid complexes in humans promotes lesion regression and increases fecal excretion of bile acids.9 However, results of studies in subjects with monogenic disorders of HDL metabolism10,11 and post hoc analyses of epidemiological studies raise questions regarding the mechanism underlying the association between HDL-C levels and coronary heart disease.12

We recently demonstrated that in healthy individuals having a wide range of HDL-C and apoA-I levels, the capacity of serum HDL to promote cholesterol efflux from macrophages in vitro is negatively correlated with measures of carotid intima thickness independently of HDL-C and apoA-I levels, suggesting that measures of HDL function may be additional predictors of cardiovascular risk.13 HDL exists as a heterogeneous population of particles differing in size and composition.6 In addition, efflux of cellular cholesterol is mediated by a number of pathways, including aqueous diffusion, ABCA1, ABCG1, and SR-BI, with different HDL particles best-suited to promote efflux via each of these pathways.14 Thus, the efficiency of an individual serum to accept cellular cholesterol depends on the distribution of HDL particles and the cholesterol transporters expressed in the cell being used as a cholesterol donor. Because HDL subfractions differ in their
ability to remove cholesterol from macrophages, the fact that individuals with similar HDL-C may have different distribution of HDL particles provides a rationale for the increased predictive value of measures of HDL function we observed.

In this study, we took advantage of the efflux data previously generated and identified subjects having similar HDL-C but significantly different total macrophage efflux. We assumed that differences in total efflux resulted from differences in the levels of functional HDL particles. We then used a published inhibitor-based assay\(^{15}\) to measure the relative contribution of different pathways to the total efflux capacity of a given serum as an indication of the relative concentration of HDL particles present. Our results show that subjects with similar HDL-C but higher total macrophage efflux capacity have significantly higher ABCA1-mediated efflux, and this efflux is associated with the level of pre\(\beta\)-1 HDL in serum. The same results were obtained with a subset of the same sera chosen to have similar apoA-I.

**Subjects and Methods**

As previously described,\(^{13}\) efflux was measured using serum aliquots specifically collected for this purpose obtained from well-characterized subjects participating in a prospective, observational study to investigate the effect of HDL-C on markers of oxidative stress and inflammation and their relationship to subclinical atherosclerosis. By design, the subjects enrolled were healthy nonsmokers with no clinically evident coronary heart disease, a broad range of HDL-C levels, and no use of drugs known to significantly affect HDL levels. The study was approved by the University of Pennsylvania Institutional Review Board and all subjects gave their informed consent for participation. Approximately equal numbers of males and females were recruited, but the experiments reported here were performed with serum from female donors because more serum aliquots were available. These were selected to have similar HDL-C by choosing sera with HDL-C within a 6% range (HDL-C ±6%), which is <7% biological variation for HDL-C and apoA-I.\(^{16}\) In addition, to confirm our results, a few critical experiments were repeated using serum from male donors also chosen to have similar HDL-C.

**Serum Lipid Parameters**

Blood was taken after a 12-hour fast, and several plasma and serum aliquots were prepared and frozen (−70°C) for future studies. EDTA plasma aliquots were used for lipid and lipoprotein analyses performed in a Centers for Disease Control-standardized lipid laboratory as previously described.\(^{17}\) The distribution of apoA-I containing HDL particles in apoB-depleted serum was measured using immunoblotting and image analysis after separation of the various particles with nondenaturing, 2-dimensional gel electrophoresis as described.\(^{18}\) To obtain particle mass, the percent distribution of HDL particles was applied to the total apoA-I concentration. Levels of pre\(\beta\)-1 HDL were also assayed using a commercial enzyme-linked immunoassay (pre\(\beta\)-1 HDL ELISA; Daiichi). We obtained a significant correlation between 2-dimensional gel and enzyme-linked immunoassay pre\(\beta\)-1 HDL values for all available values (\(r^2=0.357\); \(P=0.0069\); \(n=19\)).

**Assay of Cellular Cholesterol Efflux**

J774 cells, maintained in RPMI plus 10% fetal bovine serum and antibiotics in 5% CO\(_2\), were plated in 24 multi-well plates (70,000 cells/well) and labeled for 24 hours in the presence of ACAT inhibitor (2 \(\mu\)g/mL, CP113 818; a gift from Pfizer) using 0.5 mL/well of 2 \(\mu\)g/mL [1,2-\(^3\)H] cholesterol (Perkin Elmer) in RPMI plus 1% fetal bovine serum. To upregulate ABCA1 in J774 cells, we incubated serum from female donors because more serum aliquots were formed with serum from female donors because more serum aliquots were available. These were selected to have similar HDL-C by choosing sera with HDL-C within a 6% range (HDL-C ±6%), which is <7% biological variation for HDL-C and apoA-I.\(^{16}\) In addition, to confirm our results, a few critical experiments were repeated using serum from male donors also chosen to have similar HDL-C.

Similar HDL but Different Efflux Capacity

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also slightly increased in the residual efflux measured in cells treated with both probucol and BLT-1. The major contribution to this efflux is aqueous diffusion, which represents $\approx 50\%$ of the total efflux in control J774 cells; however, after cAMP treatment this residual efflux may also include other inhibitor-resistant pathways such as ABCG1, which can be detected by Western blot (data not shown). Figure 1 also shows a low level of ABCA1 efflux in control J774 cells. In agreement with Favari et al.,

$$\text{this efflux is probucol-sensitive as confirmed by >90\% decrease in efflux to 20 \mu g/mL apo-A-I from both control and cAMP-treated J774 cells (Supplementary Figure II, available online at http://atvb.ahajournal.org).}$$

Because we cannot specifically measure ABCG1 efflux in cAMP J774 cells, we used transfected BHK-1 cells. In this model, ABCG1 expression is regulated by mifepristone and, as previously shown, mifepristone significantly stimulated cholesterol efflux to both HDL$_3$ and apoB-depleted serum (data not shown).

Although we previously measured the efflux capacity of apoB-depleted serum from specimens obtained from a population of healthy male and female subjects, in the current study the majority of the experiments were performed using serum from the females. There were no differences between the demographic characteristics of the entire population of female subjects (n=130) and those of subjects with similar apoA-I (apoA-I $<14.7\%$ per 4 hr) HDL-C as determined by unpaired, 2-tailed t tests. The results of this analysis are shown in Tables 1 (low HDL-C sera) and 2 (high HDL-C sera). As can be seen, the most dramatic difference we found was that specimens with high efflux capacity had significantly increased ability to promote efflux via ABCA1. We also found a significant but less pronounced increase in the ability of high efflux capacity serum to promote residual or inhibitor-resistant efflux. There were no significant differences in either SR-BI-mediated efflux from J774 cells or in ABCG1-mediated efflux from BHK-1 cells. Sera with similar apoA-I (Figure 3) can also have significantly different macrophage efflux. Thus, we selected a subset of the same specimens based on similar apoA-I (apoA-I $\pm 6\%$) and analyzed the efflux values we had obtained. Two groups were

**Table 1. Average Efflux (% per 4 Hours) for Serum Specimens From Females With HDL-C of 48±6%**

<table>
<thead>
<tr>
<th>Efflux Pathway</th>
<th>Cell Model</th>
<th>Efflux Below Mean (n=11)</th>
<th>Efflux Above Mean (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total J774</td>
<td>J774+cAMP</td>
<td>12.7±0.3</td>
<td>16.6±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Uninhibited efflux</td>
<td>J774+cAMP</td>
<td>4.8±0.2</td>
<td>5.4±0.2</td>
<td>0.011</td>
</tr>
<tr>
<td>SR-BI</td>
<td>J774+cAMP</td>
<td>0.80±0.3</td>
<td>1.2±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>ABCA1</td>
<td>J774+cAMP</td>
<td>7.6±0.3</td>
<td>10.7±0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ABCG1</td>
<td>G1-BHK</td>
<td>2.9±0.5</td>
<td>3.4±0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Efflux values of the serum apoB-depleted serum are given as mean±SEM.

Mean total J774 efflux for all sera analyzed (n=22)=$14.7\%$ per 4 hr (11.0–18.7). Average total HDL-C in each group was 48, which represents the 25th percentile for the range of HDL-C values in this set of serum specimens from all female donors (n=130).

NS indicates, not significant.

HDL-C=45–51.
formed: 1 with low apoA-I (average = 127 mg/dL; 25th percentile; range, 119–135; n = 11) and 1 with high apoA-I (average = 163 mg/dL; 75th percentile; range, 153–173; n = 10). Sera with similar low levels of apoA-I (127 mg/dL) and total macrophage efflux above the average for the group had higher ABCA1 efflux when compared to sera with total efflux below the mean for the group (9.98 ± 0.320% per 4 hours, n = 5 vs 7.72 ± 0.533% per 4 hours, n = 6; P = 0.0037). Likewise, sera with high efflux capacity and similar high apoA-I levels (163 mg/dL) also had higher ABCA1 efflux (10.74 ± 0.855% per 4 hours, n = 4 vs 7.25 ± 0.581% per 4 hours, n = 6; P = 0.0004). There were no significant differences in the efflux mediated by any other pathway. Thus, independently of HDL-C or apoA-I level, the increased efficiency of a given apoB-depleted serum to remove cholesterol from macrophages is attributable to the fact that it can more efficiently promote cholesterol efflux via ABCA1.

Because the differences in efflux shown in Tables 1 and 2 were not related to serum HDL-C, we used 2-dimensional gel electrophoresis to measure pre-β-1 HDL levels.14 Figure 4 shows that there is a significant correlation between ABCA1 efflux and the level of pre-β-1 HDL in all the specimens analyzed using 2-dimensional gels (n = 29). The $r^2$ value ($r^2 = 0.425$) obtained suggests that 43% of the variability in ABCA1 efflux can be explained by the level of pre-β-1 HDL in these sera. There were also significant associations between ABCA1 efflux and the pre-β-1 HDL levels in sera having similar low or high HDL-C (low HDL-C: $r^2 = 0.220$, "Table 2. Average Efflux (% per 4 Hours) for Serum Specimens From Females With HDL-C of 73±6%"

<table>
<thead>
<tr>
<th>Efflux Pathway</th>
<th>Cell Model</th>
<th>Efflux Below HDL Mean ± SEM</th>
<th>Efflux Above HDL Mean ± SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total J774</td>
<td>J774 + cAMP</td>
<td>11.7 ± 0.4</td>
<td>15.0 ± 0.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Uninhibited</td>
<td>J774 + cAMP</td>
<td>4.6 ± 0.2</td>
<td>5.7 ± 0.3</td>
<td>0.0102</td>
</tr>
<tr>
<td>SR-BI</td>
<td>J774 + cAMP</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>ABCA1</td>
<td>J774 + cAMP</td>
<td>6.2 ± 0.5</td>
<td>8.5 ± 0.5</td>
<td>0.0072</td>
</tr>
<tr>
<td>ABCG1</td>
<td>G1-BHK</td>
<td>2.7 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Efflux values of the serum apoB-depleted serum are given as mean ± SEM. Mean total J774 efflux for all sera analyzed (n = 18) = 13.4% per 4 hr (10.2–17.6). Average total HDL-C in each group was 73, which represents the 75th percentile for the range of HDL-C values in this set of serum specimens from all female donors (n = 130).

HDL-C = 69–77.

Figure 3. Efflux from cAMP-treated J774 cells to serum pairs with similar apoA-I. Total cholesterol efflux from cAMP-treated J774 macrophages was measured as in Figure 2. There were significant differences in efflux (P < 0.05) between all pairs with the same or similar apoA-I as determined by unpaired, 2-tailed t tests.

Figure 4. Correlation of ABCA1 efflux to the concentration of pre-β-1 HDL in serum with similar high or low HDL-C. ABCA1 efflux from cAMP J774 cells was the probucol-sensitive efflux measured after 4-hour incubation with 2.8% apoB-depleted serum from specimens with similar HDL-C (HDL-C ± 6%) at either the 25th percentile (open circles, HDL-C of 48; range, 45–51; n = 22) or the 75th percentile (open triangles, HDL-C of 73; range, 69–77; n = 18). The specific contribution of ABCA1 to cholesterol efflux from cAMP-treated J774 macrophages was significantly associated with the serum level of pre-β-1 HDL ($r^2 = 0.425$; $P = 0.0002$; n = 29) measured by 2-dimensional gels. All procedures performed as in Methods.

P = 0.05, n = 18; high HDL-C: $r^2 = 0.535$, $P = 0.011$, n = 11. Although we did not have pre-β-1 HDL values for all the sera chosen on the basis of similar apoA-I, the ABCA1 efflux mediated by all the specimens in this smaller subset was also significantly associated with the serum pre-β-1 HDL levels measured ($r^2 = 0.357$, $P = 0.040$, n = 12).

Although we had focused these studies on the apoB-depleted serum from female donors, confirmatory experiments were performed with apoB-depleted serum from healthy males who participated in the same clinical study and have a similar demographic profile (data not shown). We found that whether the specimens from male donors had similar low HDL-C (HDL-C = 38 mg/dL ± 6%; n = 14) or similar high HDL-C (HDL-C = 63 mg/dL ± 6%; n = 16), sera with higher capacity to promote efflux from J774 macrophages had significantly higher ABCA1 efflux (Supplementary Tables II and III).

To better-link high efflux capacity to increased ABCA1 and pre-β-1 HDL, we calculated the average pre-β-1 HDL concentration for all sera with high vs low efflux capacity. As expected, high efflux sera had higher pre-β-1 HDL. However, the average values were not significantly different (high = 25 + 3.5 mg/dL, n = 15 vs low = 19 + 2.6 mg/dL, n = 14), possibly because of the low number of sera in each group. To increase the statistical power of this analysis, we obtained a commercially available enzyme-linked immunosassay to establish pre-β-1 HDL values measured in specimens with high vs low ABCA1 efflux ($high = 27 ± 1.7 ng/mL$, n = 33 vs low = 22 ± 1.7 ng/mL, n = 29; $P = 0.017$).

Discussion

Studies have shown that measures of HDL subclasses in different populations, such as participants in the VA-HIT study,22 postmenopausal women,23 and Finnish families with low HDL,24 are better predictors of coronary artery disease.

![Image](https://via.placeholder.com/150)
HDL subfractions, especially pre-

types of HDL have different capacities to promote cellular

The experiments reported in this article were performed with apoB-depleted serum from female donors; however, comparable results were obtained in a small study using apoB-depleted serum from healthy male donors who participated in the same clinical study and have a similar demographic profile. Thus, for specimens with similar low or high HDL-C, a higher capacity to promote efflux from J774 macrophages was attributable to significantly higher ABCA1 efflux (Supplementary Tables II and III).

Conclusion

In summary, our experiments show for the first time to our knowledge that the apoB-depleted serum from individuals with similar levels of HDL-C or apoA-I may have different capacities to remove cholesterol from macrophages. In our cell model, specimens from subjects with either similar HDL-C or similar apo A-I that have higher efflux capacity can better promote efflux via ABCA1, and this is related, in
large part, to the serum concentration of pre-β-1 HDL. These novel results add to the increasing evidence that measures of HDL function, such as its efflux capacity, can be useful when assessing an individual’s coronary heart disease risk.

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Disclosures

None.

References

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<table>
<thead>
<tr>
<th>Parameter</th>
<th>All Subjects (n=130)</th>
<th>Subjects with Low HDL-C (n=22)</th>
<th>Subjects with High HDL-C (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>55±8</td>
<td>54±7</td>
<td>54±7</td>
</tr>
<tr>
<td>Race (%AA/W)</td>
<td>23/75</td>
<td>32/68</td>
<td>33/61</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.5±5.3</td>
<td>29.4±5.5</td>
<td>28.8±5.6</td>
</tr>
<tr>
<td>Syst BP (mm Hg)</td>
<td>122±15</td>
<td>122±12</td>
<td>121±17</td>
</tr>
<tr>
<td>Diast BP (mm Hg)</td>
<td>74±9</td>
<td>76±8</td>
<td>76±12</td>
</tr>
<tr>
<td>HDL-C</td>
<td>63±17</td>
<td>48</td>
<td>73</td>
</tr>
</tbody>
</table>

SD, Standard Deviation; yrs, years; AA, African American; W, White; BMI, body mass index; Syst BP, systolic blood pressure; Diast BP, Diastolic blood pressure; HDL-C, High density lipoprotein cholesterol.
Supplementary Table II. Average Efflux (% per 4h) For Serum Specimens From Males With HDL-C=38±6% (HDL C=36 to 40)

<table>
<thead>
<tr>
<th>Efflux Pathway</th>
<th>Cell Model</th>
<th>Efflux Below Mean (n=7)</th>
<th>Efflux Above Mean (n=7)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>J774+ c-AMP</td>
<td>9.16±0.316</td>
<td>13.30±0.512</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Uninhibited Efflux</td>
<td>J774+ c-AMP</td>
<td>3.91±0.137</td>
<td>4.81±0.257</td>
<td>0.009</td>
</tr>
<tr>
<td>SR-BI</td>
<td>J774+ c-AMP</td>
<td>0.48±0.100</td>
<td>0.32±0.156</td>
<td>NS</td>
</tr>
<tr>
<td>ABCA-1</td>
<td>J774+ c-AMP</td>
<td>4.79±0.325</td>
<td>7.71±0.436</td>
<td>0.0002</td>
</tr>
<tr>
<td>ABCG-1</td>
<td>G1-BHK</td>
<td>4.10±0.335</td>
<td>4.23±0.417</td>
<td>NS</td>
</tr>
</tbody>
</table>

Efflux values to the serum HDL fraction are given as Mean ± SEM. Mean total J774 efflux for all sera (n=14) was 11.23%/4h (7.66-15.57). Average total HDL-C in each group was 38 which represents the 25th percentile for the range of HDL-C values in this set of serum specimens from male donors (n=138)
Efflux values to the serum HDL fraction are given as Mean ± SEM. Average total J774 efflux for all sera (n=16) = 14.46 (9.90-20.08). Average total HDL-C in each group was 62 which represents the 75th percentile for the range of HDL-C values in this set of serum specimens from male donors (n=138).

**Supplementary Table III. Average Efflux (% per 4h) For Serum Specimens From Males With HDL-C = 62±6% (HDL C=59 to 66)**

<table>
<thead>
<tr>
<th>Efflux Pathway</th>
<th>Cell Model</th>
<th>Efflux Below Mean (n=8)</th>
<th>Efflux Above Mean (n=8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>J774+ c-AMP</td>
<td>12.24±0.527</td>
<td>16.68±0.886</td>
<td>0.0002</td>
</tr>
<tr>
<td>Ubinhibited Efflux</td>
<td>J774+ c-AMP</td>
<td>3.83±0.345</td>
<td>5.50±0.303</td>
<td>0.003</td>
</tr>
<tr>
<td>SR-BI</td>
<td>J774+ c-AMP</td>
<td>1.08±0.254</td>
<td>1.70±0.367</td>
<td>NS</td>
</tr>
<tr>
<td>ABCA-1</td>
<td>J774+ c-AMP</td>
<td>6.85±0.417</td>
<td>10.30±0.539</td>
<td>0.0002</td>
</tr>
<tr>
<td>ABCG-1</td>
<td>G1-BHK</td>
<td>2.50±0.407</td>
<td>2.04±0.244</td>
<td>NS</td>
</tr>
</tbody>
</table>

Efflux values to the serum HDL fraction are given as Mean ± SEM. Average total J774 efflux for all sera (n=16) = 14.46 (9.90-20.08). Average total HDL-C in each group was 62 which represents the 75th percentile for the range of HDL-C values in this set of serum specimens from male donors (n=138).
Supplementary Figure I. Dependence of Efflux on the Concentration of Apo B-depleted Serum. The dependence of receptor–mediated efflux on the dose of apo B-depleted serum was measured as described in methods. The following cells were used: J774 Cells ±c-AMP (ABCA1), A1-BHK-1 Cells ± Mifepristone (ABCA1), Fu5AH Cells (SR-BI) and G1-BHK-1 Cells ± Mifepristone (ABCG1)
Supplementary Figure II

Supplementary Figure II. Probucol Sensitivity of Efflux to apo A-I. Efflux from J774 + c-AMP macrophages to 20µg/ml was measured before and after 2h pretreatment with 20µM Probucol as described in methods. Data shown is the average of 2 experiments.
Supplementary Figure III

Supplementary Figure III. Correlation Between Cholesterol Efflux and HDL Parameters. Cholesterol efflux from AMP treated J774 macrophages to the isolated HDL fraction obtained from serum specimens from 127 female donors was correlated to HDL parameters measured in serum. Panel A shows a significant correlation between efflux (%/4h) and HDL-C (r²=0.355, p<0.001, n=127). Panel B shows a significant correlation between efflux (%/4h) and apo A-I (r²=0.339, p<0.001, n=130).