

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2014;34:17-25; originally published online November 7, 2013;
doi: 10.1161/ATVBAHA.113.302437

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Importance of Evaluating Cell Cholesterol Influx With Efflux in Determining the Impact of Human Serum on Cholesterol Metabolism and Atherosclerosis

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Objective—Cholesterol efflux relates to cardiovascular disease but cannot predict cellular cholesterol mass changes. We asked whether influx and net flux assays provide additional insights.

Approach and Results—Adapt a bidirectional flux assay to cells where efflux has clinical correlates and examine the association of influx, efflux, and net flux to serum triglycerides (TGs). Apolipoprotein B–depleted (high-density lipoprotein-fraction) serum from individuals with unfavorable lipids (median [interquartile range]; high-density lipoprotein-cholesterol=39 [32–42], low-density lipoprotein-cholesterol=109 [97–137], TGs=258 [184–335] mg/dL; n=13) promoted greater ATP-binding cassette transporter A1–mediated [$1,2\text{-}^{3\text{H}}$] cholesterol efflux ($3.8\pm 0.3\%/4$ hour versus $1.2\pm 0.4\%/4$ hour; $P<0.0001$) from cyclic $3',5'$ -amp(CTP-amp)-treated J774 macrophages than from individuals with favorable lipids (high-density lipoprotein-cholesterol=72 [58–88], low-density lipoprotein-cholesterol=111 [97–131], TGs=65 [56–69] mg/dL; n=10). Thus, high TGs associated with more ATP-binding cassette transporter A1 acceptors. Efflux of cholesterol mass (μg free cholesterol/mg cell protein per 8 hour) to serum was also higher (7.06 ± 0.33 versus 5.83 ± 0.48 ; $P=0.04$). However, whole sera from individuals with unfavorable lipids promoted more influx (5.14 ± 0.65 versus 2.48 ± 0.85 ; $P=0.02$) and lower net release of cholesterol mass (1.93 ± 0.46 versus 3.36 ± 0.47 ; $P=0.04$). The pattern differed when mass flux was measured using apolipoprotein B–depleted serum rather than serum. Although individuals with favorable lipids tended to have greater influx than those with unfavorable lipids, efflux to apolipoprotein B–depleted serum was markedly higher (6.81 ± 0.04 versus 2.62 ± 0.14 ; $P<0.0001$), resulting in an efflux:influx ratio of ≈ 3 -fold. Thus both serum and apolipoprotein B–depleted serum from individuals with favorable lipids promoted greater net cholesterol mass release despite increased ATP-binding cassette transporter A1–mediated efflux in samples of individuals with high TGs/unfavorable lipids.

Conclusions—When considering the efficiency of serum specimens to modulate cell cholesterol content, both influx and efflux need to be measured. (*Arterioscler Thromb Vasc Biol.* 2014;34:17-25.)

Key Words: coronary artery disease ■ high-density lipoprotein-1

The ratio of cholesterol efflux (removal from peripheral cells) to cholesterol influx (uptake into peripheral cells) is critical to cellular cholesterol homeostasis in health and diseases such as atherosclerosis. Reverse cholesterol transport (RCT) is the dynamic process by which excess cholesterol is removed from peripheral tissues by extracellular acceptors and delivered to the liver for excretion. High-density lipoprotein (HDL) plays a key role in RCT as an acceptor of cellular cholesterol and promotes efflux of cholesterol from cells to lipoprotein acceptors such as HDL, considered the initial step of RCT.² Epidemiological studies have established an inverse relationship between circulating HDL-cholesterol (HDL-C) concentrations and the risk of clinical cardiovascular disease (CVD),³ an association

proposed to relate to the putative atheroprotective actions of HDL including RCT. However, a recent study revealed a disconnect between genomic loci associated with higher plasma HDL-C and the occurrence of myocardial infarction.¹ The findings of this elegant work suggest that genetic variants uniquely related to higher HDL-C may not reduce risk for myocardial infarction, whereas, as expected, variants increasing plasma low-density lipoprotein-cholesterol (LDL-C) do increase risk for myocardial infarction.¹ This study questions the proposed atheroprotective role of total HDL-C in CVD.

Assays that measure free cholesterol (FC) efflux to extracellular acceptors, such as HDL or serum, have provided useful insights into mechanisms of cholesterol transport as well as

Received on: April 22, 2013; final version accepted on: October 27, 2013.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.302437/-/DC1>.

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DOI: 10.1161/ATVBAHA.113.302437

Nonstandard Abbreviations and Acronyms	
ABCA1	ATP-binding cassette transporter A1
apoB	apolipoprotein B
cAMP	cAMP
CE	cholesteryl ester
CVD	cardiovascular disease
FC	free cholesterol
HDL	high-density lipoprotein
LDL	low-density lipoprotein
RCT	reverse cholesterol transport
TG	triglyceride
VLDL	very low density lipoprotein

the possible role of HDL-mediated efflux in atherosclerotic CVD independent of HDL-C levels.⁴ A criticism of this established approach, however, is that assays of FC efflux do not capture the influx component of total cholesterol movement and thus provide no information on changes in cellular cholesterol mass. Cholesterol flux between cells and lipoproteins is essential to maintain cell cholesterol homeostasis and overall health. Physiologically relevant acceptors, such as lipoproteins, contain both FC and cholesteryl ester and promote both efflux and influx, resulting in a bidirectional movement of cholesterol between cells and lipoproteins.⁵ FC efflux occurs via ABC (ATP binding cassette) transporters, such as ABCA1, ABCG1, and ABCG4, via the scavenger receptor class B type I and via unmediated aqueous diffusion.^{6,7} ABCA1 mediates FC efflux from macrophages to small, discoidal, lipid-free/poor pre- β -HDL, and esterification of HDL-FC by lecithin-cholesterol acyltransferase leads to mature, spherical phospholipid-rich particles that promote further efflux of FC via scavenger receptor class B type I, ABCG1, and aqueous diffusion. In turn, enzymatic hydrolysis of HDL-phospholipids from mature HDL can regenerate smaller particles with release of apolipoprotein A-I. Influx of FC and cholesteryl ester can occur by receptor-mediated selective uptake from mature HDL and by uptake of intact lipoproteins via several mechanisms.

Although there are multiple pathways of cholesterol efflux, the actual measurement of efflux is relatively simple. Efflux measures movement of FC out of cells and can be monitored by the use of fluorescent or radioactive labels that tag cellular FC pools.^{8,9} Influx involves the uptake of both FC and cholesteryl ester from lipoproteins and also occurs via multiple mechanisms such as whole particle uptake via pinocytosis, via the LDL receptor, and via the scavenger receptor class A by selective uptake of cholesteryl ester via receptors like scavenger receptor class B type I and CD36 (a member of the class B scavenger receptor family) and via aqueous diffusion of FC.^{10–13} In contrast to efflux assays, the quantification of lipoprotein-mediated cholesterol influx is more complicated. Because cholesterol in serum lipoproteins cannot be specifically tagged, its movement into cells has to be measured indirectly. Moreover, when physiological matrices, such as serum, are used, individual lipoproteins and apolipoproteins contribute to both the efflux and influx of cholesterol in a complex manner.

Here, we describe an adaptation of an established bidirectional flux assay⁵ to obtain estimates of both components of

FC movement (influx and efflux) between serum and macrophages, cells that are of direct relevance to atherosclerosis. We also report results obtained when we applied this assay to investigate how the cholesterol content of macrophages is affected by exposure to either serum or apolipoprotein B (apoB)-depleted (HDL-fraction) serum from individuals with high or low serum triglycerides (TGs). We focused specifically on individuals with high TGs and low HDL-C for the following reasons. First, recent genomic studies suggest that loci for lower HDL-C that associate with increased risk of CVD also relate to increased serum TGs, whereas loci uniquely associated with lower HDL-C have inconsistent association with CVD.^{1,14,15} Second, high serum TGs are an independent risk factor for CVD^{16,17}; however, elevated serum TGs are paradoxically associated with higher serum apolipoprotein AI (apoAI) in the pre- β -HDL-fraction, suggesting that serum of individuals with high TGs may efficiently promote FC efflux from macrophages even if HDL-C is low.¹⁸ To explore whether serum TGs could affect efflux capacity by modulating the population of HDL acceptor particles, we measured FC flux using apoB-depleted serum that eliminates any direct contribution of apoB lipoproteins to flux and allows us to better assess the effect of the HDL-fraction.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Measurement of Macrophage Bidirectional Flux of FC Mass With Serum

To measure both the efflux and influx components of bidirectional FC flux between serum and macrophages, we adapted a published method using the Fu5AH rat hepatoma cell line⁵ to the J774 macrophage model. Cells were radiolabeled as described for FC efflux assays (see Materials and Methods in the online-only Data Supplement). Influx, or cholesterol mass moving into the cell from serum, was estimated from the decrease in the average specific activity (³H-cholesterol/ μ g cholesterol per mg cell protein) of labeled cellular FC. Efflux, or cholesterol mass moving out of the cells to serum, was estimated by applying the average specific activity of FC in the cells to the cellular radioactivity released during the 8-hour incubation with acceptors. The fractional release of radioactive cellular FC was also determined. The mass of cellular FC was measured by gas liquid chromatography (GLC) using a standard method¹⁹ in extracts of cells obtained before (T0 cells) and after 8-hour incubation with serum or apoB-depleted serum. In this bidirectional flux assay, the net change in cellular FC mass can both be measured (cellular FC remaining after 8 hours) and predicted from the estimates of efflux and influx, thus serving as a convenient quality control for net flux estimation. Arbitrarily, we express net change in cellular cholesterol, net flux, as the difference between efflux and influx (efflux-influx) whereby net movement out is positive net flux and net movement in is negative net flux. In all experiments, a standard serum pool obtained from healthy donors was included, and the flux values were standardized to flux values obtained for this pool. All specimens were assayed in triplicate, and the units of flux are μ g FC/mg cell protein per 8 hour, unless otherwise indicated.

Results

Correlation of Serum Triglycerides With FC Efflux From Macrophages

High serum TGs are an independent risk factor for CVD¹⁶ yet paradoxically associate with increased levels of pre- β -HDL,

an apolipoprotein A-I-containing particle that enhances macrophage FC efflux,¹⁸ a measure of HDL-function.⁴ To explore the influence of serum TGs on serum efflux capacity, we performed a retrospective analysis of the FC efflux capacity (>4 hours) of apoB-depleted serum (HDL-fraction) from a sample of individuals enrolled in the Penn HDL Inflammation and Oxidation Study (PHINOX) (N=273). This analysis of existing data showed a weak positive correlation between FC efflux to apoB-depleted (HDL-fraction) serum and serum TGs levels (Figure 1A). When the total sample

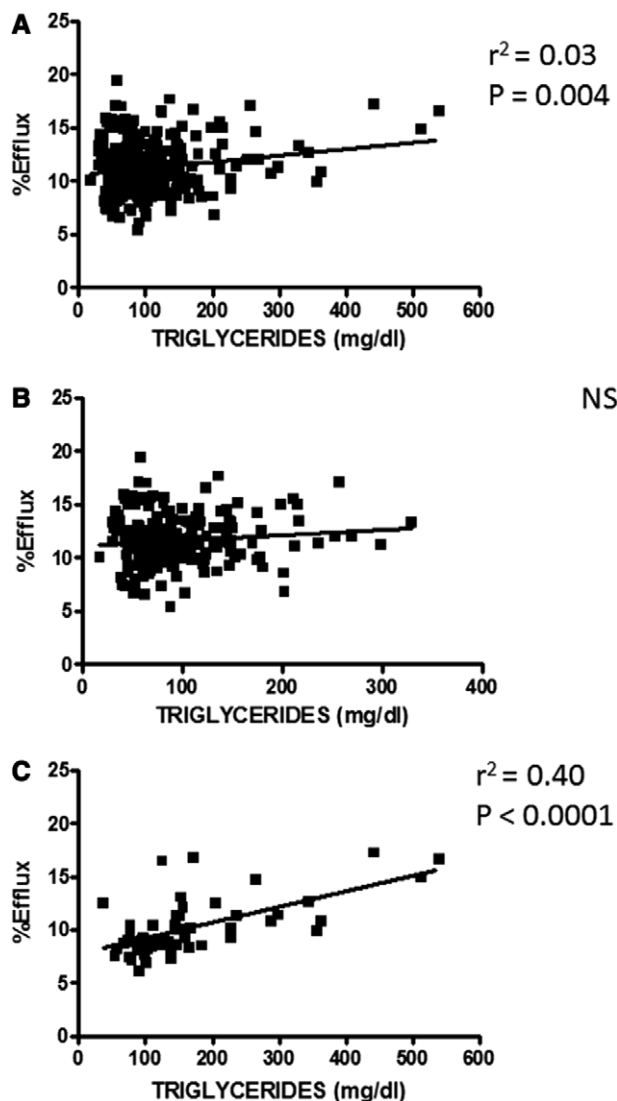


Figure 1. Retrospective analysis of J774 macrophage free cholesterol efflux in Penn HDL Inflammation and Oxidation (PHINOX) study. The PHINOX study was designed to examine inflammatory and oxidant factors related to high-density lipoprotein-cholesterol (HDL-C) and subclinical atherosclerosis in healthy adults as assessed by measures of intima-media thickness (IMT). Efflux from macrophages to the apolipoprotein B-depleted serum from these individuals showed an inverse relationship between efflux and carotid IMT. The data collected from this study were further analyzed to identify other serum factors that correlated to cholesterol efflux. Cholesterol efflux values were plotted against serum triglyceride values. **A** shows the entire population (N=273). **B** shows the specimens with HDL-C ≥ 40 mg/dL (n=215). **C** shows the specimens with HDL-C < 40 mg/dL (n=58).

was divided into individuals with HDL-C below (n=58) or above (n=215) 40 mg/dL, the cutoff point at which HDL-C is considered a risk factor for CVD,²⁰ there was no correlation between serum TGs and FC efflux in those with HDL-C > 40 mg/dL (Figure 1B). However, in the subgroup with HDL-C < 40 mg/dL, there was a significant and stronger positive correlation between FC efflux and serum TGs (Figure 1C) relative to the full sample, suggesting that TG-related factors contribute more to efflux to apoB-depleted serum when HDL-C and HDL-derived particles are reduced.

To further explore this observation and examine the specific efflux pathway underlying the association between serum TGs and FC efflux from J774 macrophages, we selected 2 groups of individuals from the Penn Diabetes Heart Study (PDHS) cohort, matched for age, sex and race, with either favorable lipids (low TG-VLDL/normal HDL-C; n=10) or unfavorable lipids (high TG-VLDL/low HDL-C; n=13; Table I in the online-only Data Supplement). Table 1 summarizes the HDL-C, LDL-C, VLDL-C, TG, and apoB composition of serum from these individuals as well as the total cholesterol, TG, and apoB values from the apoB-depleted serum. We obtained $> 98\%$ depletion of apoB, regardless of serum TG content. Recoveries of apoB and TGs in the apoB-depleted serum from individual serum specimens are given in Table II in the online-only Data Supplement. The serum LDL-C values (median [interquartile range]) for these 2 groups were the same (in low TG-VLDL/normal HDL-C participants, LDL-C=111 [97–131]; in high TG-VLDL/low HDL-C participant, LDL-C=109 [97–137]; $P=0.97$; Table 1). In addition, all PDHS subjects with unfavorable lipids except 2 of 10 with favorable lipids fulfilled criteria for definition of the metabolic syndrome,²¹ a setting of inflammation and metabolic

Table 1. Serum Lipid Profile of Selected Groups

	High TG-VLDL/Low HDL-C (n=13) Median (IQR)	Low TG-VLDL/Normal HDL-C (n=10) Median (IQR)	*P Value
Whole serum			
HDL-C	39 (32–42)	72 (58–88)	< 0.0001
VLDL-C	57 (46–68)	9 (6–13)	< 0.0001
TGs	258 (184–335)	65 (56–69)	< 0.0001
LDL-C	109 (97–137)	111 (97–131)	0.97
ApoB	108 (82–125)	81 (70–82)	0.02
ApoB-depleted serum			
Cholesterol	36 (23–27)	52 (36–58)	0.0001
TGs	22 (18–22)	15 (13–17)	0.002
ApoB	1 (0–1)	1 (1–2)	0.36

The Penn Diabetes Heart Study was designed to evaluate new risk factors related to heart disease in Type 2 diabetics and provided serum selected for high or low serum triglycerides (TGs) and very low density lipoprotein-cholesterol (VLDL-C) and low or normal high-density lipoprotein-cholesterol (HDL-C) from individuals matched for age, sex, and race. A subset was selected for the current study. The groups chosen represent individuals with similar normal low-density lipoprotein-cholesterol (LDL-C) and either a favorable (low TG-VLDL/normal HDL-C; n=10) or an unfavorable (high TG-VLDL/low HDL-C; n=13) lipid profile. ApoB indicates apolipoprotein B. Because data were not normal in distribution, lipid values (mg/dL) are presented as median and interquartile range (IQR), and statistical comparisons used the Mann-Whitney U test*.

dyslipidemia²² previously linked to changes in serum efflux capacity and HDL-function.^{23–25}

Using these sera, we measured total (Figure 2A) and ABCA1-mediated (ProbucoI-sensitive,²⁶ see Materials and Methods in online-only Data Supplement; Figure 2B) ³H-FC efflux from macrophages to apoB-depleted serum samples. Total efflux to apoB-depleted serum was equivalent in both experimental groups. However, we found that the group with the unfavorable lipid profile (high TG-VLDL/low HDL-C) had greater ABCA1-mediated FC efflux to this HDL-fraction than the group with favorable lipids (Figure 2B). This ABCA1-mediated efflux represented 47% of the total release of ³H-FC from J774+cyclic 3',5'-amp(CTP-amp) (cAMP) macrophages in the high TG-VLDL/low HDL-C group compared with 12% in the low TG-VLDL/normal HDL-C group (47±2.6%, n=13 versus 12±4.6%, n=10; P<0.0001). Although this assay measures movement of tracer FC (³H-FC) and not actual mass of FC, the observation that high TG-VLDL/low HDL-C apoB-depleted sera enhances ABCA1-mediated FC efflux from macrophages suggests an efflux driven reduction in cellular FC after incubation with the HDL-fraction of high TG-VLDL/low HDL-C sera. This conclusion, however, does not take into account the extent of FC influx, a vital component of cellular cholesterol homeostasis under normal physiological circumstances in vivo.

Characterization of the Bidirectional Flux Assay to Measure Movement of FC Mass Between J774 Macrophages and Serum

When cells are incubated with serum, the net flux of cellular cholesterol, that is, the difference between efflux and influx, can result in either net accumulation or net loss. We have shown that measures of radioactive FC efflux from either FC-normal or FC-enriched cells provide an estimate of the relative ability of serum to promote cholesterol release or efflux potential⁴ but isotopic efflux reflects the change in cellular cholesterol mass only when donor cells are enriched in FC.²⁷ To develop an assay for efflux and influx of cholesterol using J774 macrophages, we first determined the optimal conditions

in the J774 model for our bidirectional assay. Bidirectional FC flux was compared under 4 distinct conditions: FC-normal and FC-enriched J774 cells each with or without cAMP pretreatment (Figure 3A–3D). As expected, both cAMP pretreatment and FC enrichment of J774 cells (Figure 3B and 3D) increase efflux and net flux outward of cellular FC²⁷; cAMP induces greater ABCA1 efflux, whereas cellular FC enrichment also upregulates ABCA1 and reduces influx (Figure 3C and 3D). Figure I in the online-only Data Supplement further illustrates that FC enrichment increases the efflux arm of bidirectional flux whereas decreasing influx. Thus, whereas the ratio of efflux to influx in FC-enriched cells is close to 5, it approaches 1 in FC-normal cells. In addition, in this experiment (n=21), influx positively correlated with LDL-C levels in FC-normal cells ($r^2=0.104$; $P=0.010$) but not in FC-enriched cells ($r^2=0.037$, NS), suggesting that FC enrichment blunts the influence of LDL-C on cellular cholesterol levels. Table III in the online-only Data Supplement shows the interassay variability obtained when 2 different serum specimens from healthy donors as well as a standard pool were assayed in 3 separate experiments. The coefficients of variation for influx were 8% for untreated and 7.5% for cells treated with cAMP, whereas the coefficients of variation for efflux averaged 13% and 6.5% for untreated and cAMP-treated cells. Thus, we chose FC-normal cells pretreated with cAMP (Figure 3B) as the preferred model to test serum samples because of its intermediate phenotype in net flux and its superior reproducibility and sensitivity (see Figure I and Table III in the online-only Data Supplement). Furthermore, FC-normal cells may reflect dynamic interactions (both influx and efflux) of serum-derived lipoprotein particles with macrophages that are not as yet cholesterol-loaded foam cells during the initiation and progression of early atherosclerosis.

Before applying this macrophage bidirectional flux assay, additional validation was undertaken by using test incubations to promote either greater efflux or influx. Figure IIA in the online-only Data Supplement shows that when FC-enriched cells treated with cAMP were incubated with increasing concentrations of either serum or apoB-depleted serum from

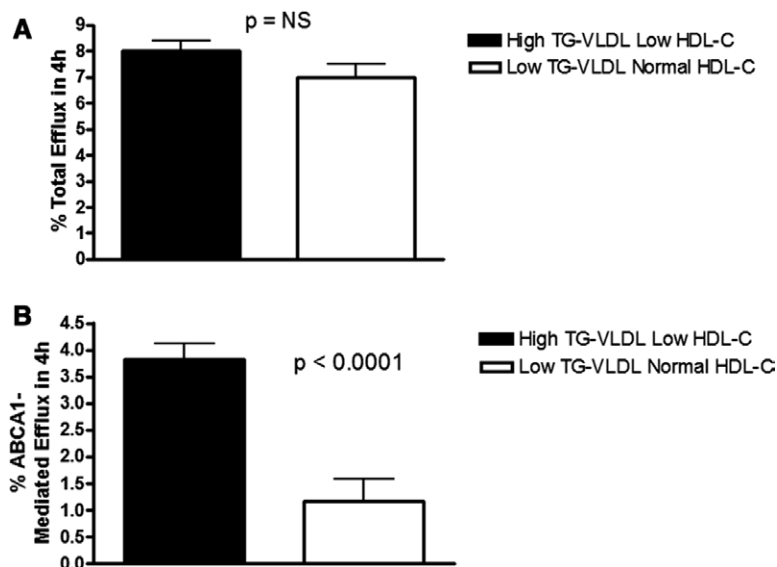


Figure 2. Total and ATP-binding cassette transporter A1 (ABCA1)-mediated free cholesterol efflux from J774 macrophages to apolipoprotein B (apoB)-depleted serum in Penn Diabetes Heart Study serum samples. [³H]-cholesterol-labeled J774+cAMP cells were incubated with apoB-depleted serum at 3.5% (equivalent to 2.5% serum dilution) for 4 hours. Total efflux was measured at 4 hours (A). ABCA1-mediated efflux from macrophages to apoB-depleted serum was measured as described in the Materials and Methods section (B). Release of [³H]-cholesterol into the medium was determined and compared with the total [³H]-cholesterol in the cell before the efflux period, and ProbucoI was used to obtain ABCA1-specific efflux. NS=not significant.

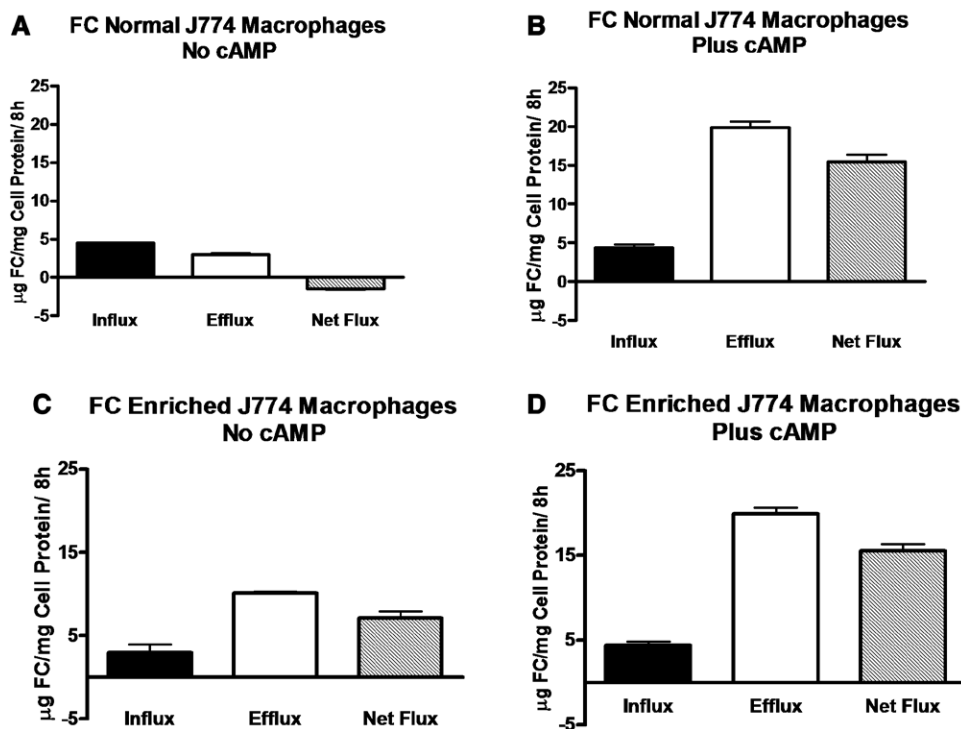


Figure 3. Bidirectional flux of free cholesterol (FC) assayed using various J774 cell models. The bidirectional flux of cholesterol between J774 cells and a pool of human serum obtained from healthy donors was measured as described in the Materials and Methods section. In **A–D**, solid bars=influx; open bars=efflux; and hatched bars=net flux (efflux–influx). **A** and **B**, Cholesterol flux between Cholesterol-normal J774 cells, not treated with cAMP (**A**) or plus cAMP (**B**) and human serum pool at 2.5%. **C** and **D**, Cholesterol flux between cholesterol-enriched J774 cells, not treated with cAMP (**C**) or plus cAMP (**D**) and human serum pool at 2.5%. Values shown are averages of triplicate measures.

healthy donors, both the efflux and net outward flux of FC (efflux-influx) increased but there was little effect on influx. Importantly, the net loss of FC calculated from the estimates of efflux and influx agreed with the change in cell FC mass directly measured by gas liquid chromatography (Figure IIB in the online-only Data Supplement). Figure 4 shows that when FC-normal J774 cells treated with cAMP were incubated with serum supplemented with acetylated low-density lipoprotein, the influx of FC significantly increased with increasing concentrations of acetylated low-density lipoprotein but efflux did not change significantly. The results shown in Figure 4 and Figure II in the online-only Data Supplement were expected but, taken together, demonstrate the reliability of our estimates of both the efflux and influx components of bidirectional flux between J774 macrophages and diluted human serum specimens.

Influence of High TG and Low HDL on Bidirectional FC Flux From Macrophages

We applied this bidirectional J774 macrophage FC flux assay to both apoB-depleted (HDL-fraction) and diluted whole

serum from PDHS individuals having favorable or unfavorable lipid profiles as described in Table 1. Even after depleting the apoB-containing lipoproteins from serum, significant FC influx occurred (Table 2; 1.32 ± 0.28 µg FC/mg cell protein per 8 hour for the unfavorable lipid group and 2.12 ± 0.45 µg FC/mg cell protein per 8 hour for the favorable lipid group). However, compared with the group with the favorable lipid profile, FC efflux to the apoB-depleted (HDL-fraction) serum from the group with unfavorable lipids was significantly reduced (2.62 ± 0.14 versus 6.81 ± 0.04 µg FC/mg cell protein per 8 hour; $P < 0.0001$). As a result, the group with the favorable lipid profile promoted a significantly higher net flux outward of FC mass from macrophages (4.65 ± 0.25 versus 1.28 ± 0.19 µg FC/mg cell protein per 8 hour; $P < 0.0001$).

Compared with individuals with a favorable lipid profile, apoB-depleted serum from individuals with unfavorable lipids promoted similar total and more ABCA1-specific efflux of ³H-FC in 4 hours (Figure 2B). However, this HDL-fraction promoted less release of actual FC mass during a longer incubation (Table 2), indicating that in some populations

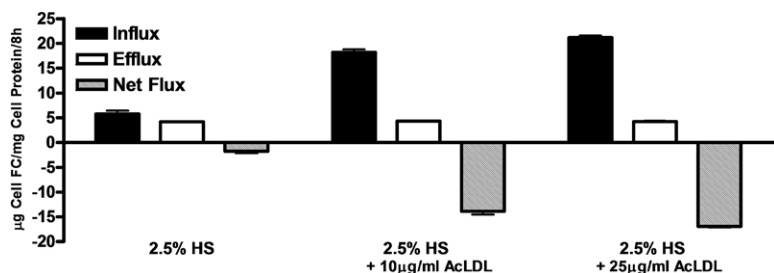


Figure 4. Validation of free cholesterol (FC) bidirectional flux estimates in J774 macrophages. Cholesterol-normal J774 cells treated with cAMP were incubated with a pool of human serum obtained from healthy donors or the same serum pool supplemented with acetylated low-density lipoprotein LDL (AcLDL) to increase cellular cholesterol mass and an acetyl-cholesterol O-acyl transferase inhibitor to prohibit formation of cholesteryl esters. The initial value for cellular cholesterol was 11.21 ± 0.049 µg/mg cell protein. The net changes in cell cholesterol as measured by gas liquid chro-

matography were 14.07 ± 3.76 and 22.57 ± 1.97 µg/mg cell protein for cells incubated with 10 and 25 µg/mL of AcLDL, respectively. The calculated net change (shown in graph) was -13.87 ± 1.18 and -16.9 ± 0.49 µg/mg cell protein for cells incubated with 10 and 25 µg/mL of AcLDL, respectively. The influx (solid bar), efflux (open bar), and net flux (defined as efflux-influx; hatched bar) of cellular cholesterol were obtained as described in the Materials and Methods section. Values shown are averages of triplicate measures.

Table 2. Summary of Bidirectional Free Cholesterol Flux Values to ApoB-depleted Serum or Whole Serum

	High TG-HDL/Low HDL- C (n=13) Mean±SEM	Low TG-HDL/Normal HDL-C (n=10) Mean±SEM	*P Value
ApoB-depleted serum			
Efflux	2.62±0.14	6.81±0.04	<0.0001
Influx	1.32±0.28	2.12±0.45	0.13
Net flux	1.28±0.19	4.65±0.25	<0.0001
Whole serum			
Efflux	7.06±0.33	5.83±0.48	0.04
Influx	5.14±0.65	2.48±0.85	0.02
Net flux	1.93±0.46	3.36±0.47	0.04

Flux values were obtained as described in the Materials and Methods section and represent free cholesterol (FC) flux between FC-normal J774+ cAMP-treated cells and serum from 2 groups defined by their lipoprotein profile as described in Table 1. ApoB indicates apolipoprotein B; HDL, high-density lipoprotein; and TGs, triglycerides. ApoB-depleted serum was used at 3.5%, equivalent to the 2.5% whole serum dilution used. All values represent average of triplicate determinations. Because flux data ($\mu\text{g FC/mg cell protein per 8 h}$) were normal in distribution, values are presented as mean±SEM, and statistical comparisons used the *Student *t* test.

measurement of tracer cholesterol will not accurately predict cholesterol mass movement over time.

The results are more complex, yet striking, when whole serum (not depleted of apoB) was used. Serum from the group with the unfavorable lipid profile promoted greater FC efflux from cAMP-treated J774 macrophages than serum from individuals with the favorable lipid profile (Table 2). However, influx from serum of the group with unfavorable lipids was almost twice that obtained with the group having favorable profiles; thus net outward FC flux to serum for the group with unfavorable lipids was significantly lower than that for the group with favorable lipids (1.93±0.46 versus 3.36±0.47 $\mu\text{g FC/mg cell protein per 8 hour}$, respectively, $P<0.04$). These results underscore the importance of measuring both components of bidirectional flux, not just FC efflux, in determining the overall impact of specific lipoproteins and serum specimens on macrophage FC flux and cholesterol homeostasis. Although high serum TG-VLDL associates with increased efflux of isotopic FC via ABCA1, our estimates of bidirectional flux suggest that with time this serum profile would promote accumulation of cellular cholesterol mass, consistent with the observed association of increased TGs with atherosclerosis.

Discussion

Cholesterol represents a striking paradox; it gives plasma membranes the physical characteristics necessary to maintain cellular integrity and life and yet, in excess, it results in atherosclerosis CVD, the leading killer in the Western world.²⁸ The process by which excess cholesterol is removed from cells by extracellular acceptors such as HDL is called efflux. Indeed, efflux of cholesterol from peripheral cells is thought to be the first step in RCT, the process by which excess cholesterol is removed from the body.²⁹ Because the health of an organism is intimately linked to cellular cholesterol levels, the process

of cholesterol efflux has garnered much attention. In fact, the capacity of apoB-depleted human serum samples to promote cholesterol efflux from J774 cells was recently shown to be an inverse correlate of carotid intima-media thickness and coronary artery disease independent of HDL-C levels.⁴

Cellular cholesterol movement, however, is bidirectional. Cholesterol not only effluxes out of cells, but it is also moves into cells (influx). As mentioned above, efflux and influx occur through various regulated processes as well as by aqueous diffusion. The balance of cholesterol in a cell is not only dependent on how much cholesterol goes out, or efflux, but also how much comes in, or influx. The difference between efflux and influx yields the total or net flux of cholesterol, which we have defined as positive (if there is net efflux, ie, efflux > influx) or negative (if there is net influx, ie, influx > efflux). Ideally, cholesterol influx as well as efflux should be considered when studying the capacity of serum or lipoproteins to regulate cellular cholesterol homeostasis.

A retrospective analysis of efflux to apoB-depleted serum (HDL-rich fraction) obtained from PHINOX study individuals, a study that showed that ¹H-FC efflux from macrophages inversely correlated with carotid atherosclerosis and coronary artery disease,⁴ indicated that there was a modest positive correlation between increasing serum TG levels and the capacity of an individual's serum to promote cholesterol efflux. At face-value, this result seems counterintuitive because high TGs are typically associated with higher cardiovascular risk. We analyzed the data further by dividing this population into individuals with low HDL-C and those with normal HDL-C. Interestingly, there was no correlation between efflux and TGs in the individuals with normal HDL-C levels. The correlation seen in the whole population seems to be driven by the more pronounced correlation between efflux and TGs in individuals with the low HDL-C, perhaps suggesting efflux efficiency is not simply a function of HDL-concentration. In these individuals, factors related to the metabolism of increased TG-rich lipoproteins may result in a population of acceptors and HDL skewed toward particles that are efficient ligands for cholesterol transporters.

Next, we studied an independent sample from PDHS with 2 groups of individuals specifically selected for either favorable lipid profile (low TG-VLDL/normal HDL-C) or unfavorable profile (high TG-VLDL/low HDL-C). Interestingly, apoB-depleted serum from the group with the unfavorable lipid profile had greater ABCA1-mediated ¹H-FC efflux than the group with the favorable lipid profile (Figure 2). This result is consistent with a previous report that individuals with elevated TG-VLDL levels have increased levels of pre- β -HDL.^{18,30} Despite the positive association of serum TGs with apparently atheroprotective ABCA1-mediated efflux, both high serum TGs and, notably more recently, high serum pre- β levels are positively correlated with increased atherosclerotic CVD in epidemiological studies.^{3,31–34} Thus, it seems that macrophage ¹H-FC efflux data cannot be interpreted in isolation presumably because it neglects to account for macrophage FC influx, a vital component of cellular cholesterol homeostasis and likely disease mechanism for atherogenic lipoproteins. We have not addressed the specific biological mechanisms that lead to the presence of particles with increased efflux capacity

in patients with high TGs but note that the PDHS participants with unfavorable lipids also had the metabolic syndrome. Thus, abnormalities described in this syndrome, including insulin resistance, chronic inflammation,^{22,35} increased phospholipid transfer protein activity,^{23,30} and altered lipase activity, as we have described,²⁴ may contribute to altered serum efflux capacity in these individuals.

Past experiments have focused on measuring efflux of cholesterol using radioisotopes to trace cholesterol movement out of cells. Although these studies have yielded valuable information on the mechanism(s) of cholesterol efflux, it is noteworthy that often cholesterol efflux values do not predict the level of cholesterol in the cell. Previously, we have described an assay for measuring bidirectional cholesterol flux in an Fu5AH rat hepatoma cell line.⁵ With this assay, cholesterol efflux as well as cholesterol influx can be measured, thereby obtaining a more complete estimate of the cholesterol status of the cell. Because macrophages are relevant to the process of atherosclerosis, we adapted this assay to J774 macrophages, a model with established clinical relevance,⁴ and used it to study serum from selected human samples.

The assay measures cholesterol flux by using radiolabeled cholesterol to detect cholesterol movement. Cholesterol influx is measured by changes in specific activity of radiolabeled cholesterol and cholesterol mass measurements. As well, efflux of FC mass can be estimated from specific activity of cellular FC and the radioactivity released. After adapting the assay for use with macrophages, we measured the interassay variation of the bidirectional flux assay and obtained values $\leq 10\%$, especially with cAMP-treated cells (Table III in the online-only Data Supplement). We validated the assay by varying serum composition, cholesterol status of the cell, and by using cells stimulated with or without cAMP (which upregulates ABCA1; Figures 3 and 4). We chose cAMP-treated, FC-normal cells because when tested this model demonstrated an intermediate phenotype relative to FC flux and less variability (Figure I and Table III in the online-only Data Supplement). In addition, we have shown that cAMP-treated, FC-normal J774 cells express all the known pathways that promote cholesterol flux between cells and cholesterol acceptors in serum. Furthermore, they provide a model of peripheral cells³⁶ poised to maintain cholesterol homeostasis or accumulate cholesterol and potentially become foam cells on long-term exposure to an unfavorable lipoprotein profile. Although the efflux of radioactive FC to either apoB-depleted or diluted serum was significantly correlated to the efflux of FC mass released to the same specimens, we found that in some instances efflux does not actually predict the net movement of cholesterol because of a large and variable influx component (Figure 2, Table 2, and Figure 4).

When the bidirectional flux assay was applied to serum of individuals specifically selected for either high TG-VLDL/low HDL-C (unfavorable) or low TG-VLDL/normal HDL-C (favorable) lipid profiles (Table 1), we found that individuals with unfavorable lipids had greater efflux of FC mass to serum than those with favorable lipids (Table 2). Conversely, in apoB-depleted serum, individuals with an unfavorable lipid profile had decreased efflux of FC mass (Table 2), indicating that in these individuals, efflux to apoB-containing lipoproteins contributes to the release of FC mass. However, the

elevated apoB-containing lipoproteins in individuals with unfavorable lipids also contributed to marked FC influx (Table 2). On the contrary, in individuals with favorable lipids, the apoB lipoproteins did not contribute much to FC flux. Thus, in spite of the increased ABCA1-mediated efflux to the HDL-fraction from individuals with unfavorable lipids and the contribution of their apoB lipoproteins to efflux, when cells are incubated with this serum, efflux does not balance the increased apoB-mediated FC influx. In contrast, efflux of FC mass to apoB-depleted serum and serum from individuals with favorable lipids was similar.

Our bidirectional flux data suggest that in cases where the serum lipoprotein profile is skewed, efflux to whole serum or apoB-depleted serum should not be interpreted in isolation. Indeed, cholesterol influx from whole serum with high TG-VLDL/low HDL-C is more than twice that from serum of the group with favorable lipids. When influx is considered, individuals with a favorable lipid profile have 43% higher net outward flux of cholesterol compared with those individuals with an unfavorable lipid profile. Interestingly, our data also indicate that there is measurable influx of cholesterol mass from the apoB-depleted fraction of serum (Table 2), possibly via uptake from HDL but the precise mechanism remains to be determined. This observation further underscores that, even in the absence of apoB lipoproteins, one needs to account for cholesterol influx when determining net cholesterol movement and atherogenic potential of human serum samples because cholesterol flux between cells and cholesterol-containing lipoproteins (even HDL) is bidirectional and depends on the total lipoprotein profile, not just on HDL-concentration.

In large clinical studies, we have previously found that J774 macrophage cholesterol efflux is an independent risk factor for atherosclerotic CVD.⁴ Recently, Li et al³⁷ confirmed these findings and demonstrated that cAMP-treated RAW 264.7 macrophage efflux of labeled free cholesterol to the apoB-depleted (HDL-fraction) serum was indeed associated with lower levels of prevalent coronary atherosclerosis in cohorts with coronary artery disease. Remarkably, however, they observed that this efflux to the HDL-fraction (presumed to have a significant ABCA1 contribution because cells were treated with cAMP) predicted increased rates of incident CVD events in a 3-year follow-up of coronary artery disease patients (although this observation was made on a relatively small number of incident events), whereas HDL-C, apolipoprotein A-I, and apolipoprotein A-II levels predicted lower event rates. This article suggests that efflux to the HDL-fraction may not be a straightforward bioassay of HDL-function. The conflicting data for prevalent versus incident CVD underscore that macrophage cholesterol homeostasis in vivo as it relates to atherosclerosis and HDL-function is complex and that assays of efflux alone may not capture this complexity. Although our studies do not directly address the issues raised by Li et al,³⁷ our work is novel in that it examines cholesterol influx as well as efflux, thus exploring how the bidirectional cholesterol flux between individual serum specimens and a macrophage model can affect cellular cholesterol homeostasis.

The work we present here indicates that, although apoB lipoproteins are considered atherogenic by virtue of their role in promoting cholesterol influx, they affect the efflux component

of flux even when absent from the incubation likely by affecting the distribution of HDL-particles and cholesterol acceptors. Thus, additional functional assays, such as bidirectional flux, are required to fully understand the impact of serum and its apoB-depleted HDL-fraction on CVD.³⁷ When considering specific populations such as individuals with high TG-VLDL/low HDL-C, efflux of isotopic FC from macrophages to serum does not predict the net flux of cholesterol or the actual cholesterol content of cells. In addition, our study suggests that although serum with increased pre- β -HDL may be efficient at promoting the early release of ³H-FC, small particles seem to be less efficient at promoting sustained efflux of FC mass. Therefore, macrophage efflux capacity, when considered in isolation, may not always predict reduced risk of CVD or provide an accurate measure of the atherogenic or atheroprotective potential of serum samples.

In future studies, it is important to define whether measures of bidirectional flux provide value beyond efflux capacity alone in CVD risk prediction. It is possible that the apoB lipoproteins in serum account fully for the influx component of our bidirectional assay. However, this requires additional study. Whether measures of influx provide incremental value in our understanding of CVD beyond plasma levels of apoB lipoproteins and assays of HDL efflux capacity is an open question. Large clinical studies are required to compare efflux capacity with bidirectional flux parameters across the full spectrum of serum lipid profiles and to determine their relationship with measures of atherosclerosis and the occurrence of clinical CVD.

Sources of Funding

The studies described in this article were supported by the National Institutes of Health grant HL22633 (to G.H. Rothblat, G.L. Weibel, and M.d.l. Llera-Moya). M.P. Reilly is supported by K24-HL-107643.

Disclosures

None.

References

- Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a Mendelian randomisation study. *Lancet*. 2012;380:572–580.
- Rader DJ, Alexander ET, Weibel GL, Billheimer J, Rothblat GH. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J Lipid Res*. 2009;50 Suppl:S189–S194.
- Castelli WP. Epidemiology of coronary heart disease: the Framingham study. *Am J Med*. 1984;76(2A):4–12.
- Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med*. 2011;364:127–135.
- Zimetti F, Weibel GK, Duong M, Rothblat GH. Measurement of cholesterol bidirectional flux between cells and lipoproteins. *J Lipid Res*. 2006;47:605–613.
- Baldán A, Bojanic DD, Edwards PA. The ABCs of sterol transport. *J Lipid Res*. 2009;50 Suppl:S80–S85.
- Kellner-Weibel G, de la Llera-Moya M. Update on HDL receptors and cellular cholesterol transport. *Curr Atheroscler Rep*. 2011;13:233–241.
- Rothblat GH, de la Llera-Moya M, Favari E, Yancey PG, Kellner-Weibel G. Cellular cholesterol flux studies: methodological considerations. *Atherosclerosis*. 2002;163:1–8.
- Sankaranarayanan S, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Asztalos BF, Bittman R, Rothblat GH. A sensitive assay for ABCA1-mediated cholesterol efflux using BODIPY-cholesterol. *J Lipid Res*. 2011;52:2332–2340.
- Kruth HS. Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native low-density lipoprotein particles. *Curr Opin Lipidol*. 2011;22:386–393.
- Thuahnai ST, Lund-Katz S, Dhanasekaran P, de la Llera-Moya M, Connelly MA, Williams DL, Rothblat GH, Phillips MC. Sr-bi-mediated cholesteryl ester selective uptake and efflux of unesterified cholesterol: Influence of HDL size and structure. *J Biol Chem*. 2004;279:12448–12455.
- Go GW, Mani A. Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. *Yale J Biol Med*. 2012;85:19–28.
- de Winther MP, van Dijk KW, Havekes LM, Hofker MH. Macrophage scavenger receptor class A: A multifunctional receptor in atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2000;20:290–297.
- Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature*. 2010;466:707–713.
- Dastani Z, Hivert MF, Timpson N, et al. Novel loci for adiponectin levels and their influence on type 2 diabetes and metabolic traits: A multi-ethnic meta-analysis of 45,891 individuals. *PLoS Genet*. 2012;8:E10002607.
- Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, Goldberg AC, Howard WJ, Jacobson MS, Kris-Etherton PM, Lennie TA. Triglycerides and cardiovascular disease. *Circulation*. 2011;123:2292–2333.
- Talayero BG, Sacks FM. The role of triglycerides in atherosclerosis. *Curr Cardiol Rep*. 2011;13:544–552.
- Attia N, Ramaharo A, Paul JL, Cambillau M, Beaune P, Grynberg A, Simon A, Fournier N. Enhanced removal of cholesterol from macrophage foam cells to serum from type IV hypertriglyceridemic subjects. *Atherosclerosis*. 2008;198:49–56.
- Klansek JJ, Yancey P, St Clair RW, Fischer RT, Johnson WJ, Glick JM. Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters. *J Lipid Res*. 1995;36:2261–2266.
- Assmann G, Gotto AM Jr. HDL cholesterol and protective factors in atherosclerosis. *Circulation*. 2004;109(23 Suppl 1):III8–III14.
- Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr, Spertus JA, Costa F; American Heart Association; National Heart, Lung, and Blood Institute. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation*. 2005;112:2735–2752.
- Reilly MP, Rader DJ. The metabolic syndrome: more than the sum of its parts? *Circulation*. 2003;108:1546–1551.
- Ji J, Watts GF, Johnson AG, Chan DC, Ooi EM, Rye KA, Serone AP, Barrett PH. High-density lipoprotein (HDL) transport in the metabolic syndrome: application of a new model for HDL particle kinetics. *J Clin Endocrinol Metab*. 2006;91:973–979.
- Badellino KO, Wolfe ML, Reilly MP, Rader DJ. Endothelial lipase concentrations are increased in metabolic syndrome and associated with coronary atherosclerosis. *PLoS Med*. 2006;3:e22.
- Nestel P, Hoang A, Sviridov D, Straznicki N. Cholesterol efflux from macrophages is influenced differentially by plasmas from overweight insulin-sensitive and -resistant subjects. *Int J Obes (Lond)*. 2012;36:407–413.
- Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probuocol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler Thromb Vasc Biol*. 2004;24:2345–2350.
- Sankaranarayanan S, de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Weibel GL, Rothblat GH. Importance of macrophage cholesterol content on the flux of cholesterol mass. *J Lipid Res*. 2010;51:3243–3249.
- CDC. National center for health statistics 2010
- Rothblat GH, Phillips MC. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr Opin Lipidol*. 2010;21:229–238.
- Dullaart RP, Groen AK, Dallinga-Thie GM, de Vries R, Sluiter WJ, van Tol A. Fibroblast cholesterol efflux to plasma from metabolic syndrome subjects is not defective despite low high-density lipoprotein cholesterol. *Eur J Endocrinol*. 2008;158:53–60.
- Asztalos BF, Collins D, Cupples LA, Demissie S, Horvath KV, Bloomfield HE, Robins SJ, Schaefer EJ. Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial. *Arterioscler Thromb Vasc Biol*. 2005;25:2185–2191.
- Kane JP, Malloy MJ. Prebeta-1 HDL and coronary heart disease. *Curr Opin Lipidol*. 2012;23:367–371.
- Guey LT, Pullinger CR, Ishida BY, O'Connor PM, Zellner C, Francone OL, Laramie JM, Naya-Vigne JM, Siradze KA, Deedwania P, Redberg

- RF, Frost PH, Seymour AB, Kane JP, Malloy MJ. Relation of increased prebeta-1 high-density lipoprotein levels to risk of coronary heart disease. *Am J Cardiol.* 2011;108:360–366.
34. de Vries R PF, van Tol A, Dullaart RP. Carotid intima media thickness is related positively to plasma preBeta high density lipoproteins in nondiabetic subjects. *Clin Chim. Acta.* 2012;413:473–477.
35. Reilly MP, Rohatgi A, McMahon K, Wolfe ML, Pinto SC, Rhodes T, Girman C, Rader DJ. Plasma cytokines, metabolic syndrome, and atherosclerosis in humans. *J Investig Med.* 2007;55:26–35.
36. de la Llera-Moya M, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar HDL-C to remove cholesterol from macrophages. *Arterioscler. Thromb. Vasc. Biol.* 2010;30:796–801
37. Li XM, Tang WH, Mosior MK, Huang Y, Wu Y, Matter W, Gao V, Schmitt D, Didonato JA, Fisher EA, Smith JD, Hazen SL. Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks. *Arterioscler Thromb Vasc Biol.* 2013;33:1696–1705.

Significance

Because cholesterol flux between cells and lipoproteins is bidirectional, measures of efflux do not capture the influx component of flux and cannot predict the direction of changes in cellular cholesterol mass. We have adapted an assay previously used to measure bidirectional cholesterol (free cholesterol, FC) flux from Fu5AH hepatoma cells for use in the J774 macrophage model, where FC efflux has been related to clinical atherosclerosis. We used this assay system to explore whether estimates of FC influx and bidirectional flux might provide additional insights beyond FC efflux into the role of FC flux in cardiovascular disease (CVD). Application of the bidirectional cholesterol flux assay using our macrophage model provides measures of the net change in cellular cholesterol mass after exposure to acceptors, such as serum and HDL, as well as estimates of efflux and influx, thus adding insights into serum components, disease states, and therapies that either prevent or promote deposition of cholesterol in a cell model that is relevant to atherosclerosis.

Supplemental Material

Importance of Evaluating Cell Cholesterol Influx with Efflux in Determining the Impact of Human Serum on Cholesterol Metabolism and Atherosclerosis

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Supplemental Material

Rationale for cell model used. Although *in vivo* the pathology of atherosclerosis is largely characterized by the presence of foam cells in the vessel wall, the initiation of lesions likely reflects dynamic interactions between serum-derived lipoprotein particles and cells such as macrophages that are not yet loaded with cholesterol, where cellular cholesterol content is kept in check by mechanisms that promote FC efflux and influx. J774+cAMP macrophages, the cell model chosen for these studies, has been shown to have an important clinical correlate¹ and we believe that the adaptation of an assay that allows us to obtain estimates of influx and net change in cellular cholesterol is an important extension of previous work.

Figure 3 in the text shows estimates of bidirectional flux using four cell models: FC normal and FC-enriched J774 cells \pm cAMP pre-treatment. As expected, both cAMP pre-treatment and FC enrichment of J774 cells increased both efflux and net outward flux of cellular FC increasing the sensitivity of these measurements. However, since enrichment with FC blunted influx (3C, 3D vs. 3A, 3B) and cAMP treatment decreased assay variability when measuring efflux (Supplementary Table III), we chose to use FC-normal cells pre-treated with cAMP as the preferred model to test serum specimens with our assay. Supplementary Fig I further documents the effects of FC enrichment. This figure summarizes flux data from cholesterol “normal” and “enriched” J774+cAMP cells to the same serum specimens from healthy adults (n=21) and clearly shows that when cells are enriched in cholesterol the efflux arm of bidirectional flux is disproportionately increased and LDL-C has no influence on the rate of FC influx (Influx vs. LDL-C: FC-Normal, p=0.01; FC-Enriched p= 0.172; N=21). We believe that using cholesterol-normal cells, where both arms of bidirectional flux are well-represented (E/I=0.63), will better allow us to identify which arm of the pathway is producing the change in cellular cholesterol mass observed upon incubation with a specific serum specimen. An important aspect of our assay will be to understand which serum components are likely to promote accumulation of cellular cholesterol upon prolonged exposure. In addition, using cholesterol-normal cells results in more reproducible initial cell cholesterol content as it is not easy to obtain reproducible cholesterol enrichment from experiment to experiment because not all batches of AcLDL enrich cells to the same extent.

Rationale for serum concentration used. We chose to use 2.5% whole serum in these experiments because, when using cholesterol-normal cells, 2.5% serum allowed us to assure changes in cellular FC during an 8h incubation that could be detected and measured accurately. For this reason, 2.5% was also the serum concentration used in the published assay with cholesterol-normal Fu5AH cells². At lower serum concentrations, the changes in cellular mass obtained in 8 hours in cholesterol-normal cells are difficult to reproduce and we were concerned that incubations longer than 8h could lead to cellular toxicity with some test sera. In addition, we have experimental results showing that both total and ABCA1-dependent efflux of isotopic cholesterol is in the linear range when 2.5% serum is

used (unpublished results). Initial experiments using FC-normal cells showed that, when compared to 2.5%, increasing the serum concentration to 5% and 10% increased both efflux and influx to a similar extent (about 3-fold). The experiment shown in Supplementary Figure II was done using FC-enriched cells where efflux is increased. This experiment is presented to further validate our assay by demonstrating that both arms of FC flux are dependent on the serum concentration. In addition, as also shown in Figure 4, the data validates our estimate of influx by showing that net flux values calculated as the difference between efflux and influx coincide with gas liquid chromatography measures of the actual FC remaining in the cells after incubation with serum.

Effect of Probucol on Bidirectional Flux. To validate the use of Probucol to block ABCA1 function, bidirectional flux was assayed after a 2h pre-treatment with Probucol as described in Methods. Supplementary Table IV shows that Probucol has a marked effect on the efflux arm of bidirectional flux and minimal or no effect on influx.

Depletion of ApoB. ApoB-depleted (HDL-fraction) serum was prepared from aliquots of all PDHS sera used and apoA-I, apoB and triglycerides in both whole serum and apoB-depleted serum were analyzed as described in Methods. As expected, an average of over 98% of apoA-I (not shown) but less than 2% of apoB was recovered in the apoB-depleted serum. The recoveries of apoB and TG in individual specimens are given in Supplementary Table II.

Supplementary Table I. Demographic, Clinical and Biochemical variables for PDHS participants

Variable	High TG-VLDL /Low HDL-C (n=13)	Low TG-VLDL /Normal HDL-C (n=10)	P Value
	Median (IQR) or N (%)	Median (IQR) or N (%)	
Age (years)	58 (50-67)	60 (55-67)	NS
Male, N (%)	3 (23.1%)	4 (40%)	
Race			
Caucasian, N (%)	7 (53.9%)	6 (60%)	
African-American, N (%)	6 (46.1%)	4 (40%)	
BMI (kg/m ²)	31.9 (30.6-37.6)	28.5 (23.9-30.1)	<0.05
Waist circumference (cm)	104.1 (96.5-111.8)	90.2 (81.3-100.3)	<0.05
Right SBP (mm/Hg)	136 (132-155)	126.5 (116-146)	NS
Right DBP (mm/Hg)	80 (77-84)	73 (68-81)	NS
Total cholesterol (mg/dl)	207 (191-228)	198.5 (179-210)	NS
Triglycerides (mg/dl)	230 (178-331)	65 (54-69)	<0.05
VLDL-Cholesterol (mg/dl)	54 (45-63)	8.5 (6-13)	<0.05
HDL-Cholesterol (mg/dl)	39 (34-42)	72 (53-91)	<0.05
LDL-Cholesterol (mg/dl)	110 (104-143)	111 (95-133)	NS
ApoB (mg/dl)	108 (96-119)	81 (70-82) (*n=9)	<0.05
ApoA-I (mg/dl)	122 (113-129)	150 (119-172)	NS
ApoA-II (mg/dl)	32 (31-33)	27 (26-39)	NS
HbA1c (%)	7.3 (6.9-7.7)	6.5 (6-7)	NS
Duration of diabetes (years)	5 (1.5-8) (*n=12)	5 (4-7)	NS
Diabetes medication			
Metformin, N (%)	8 (61.5)	5 (50)	
Sulfonylurea, N (%)	4 (30.8)	2 (20)	
Thiazolidinediones, N (%)	2 (15.4)	2 (20)	
Exanetide, N (%)	2 (15.4)	0 (0)	
Meglitinides, N (%)	1 (7.7)	1 (10)	
Smoking history			
Never smoked, N (%)	3 (23.1)	3 (30)	
Past smoker, N (%)	5 (38.5)	7 (70)	
Current smoker, N (%)	5 (38.5)	0 (0)	

IQR = interquartile range

*data were available for subset "n" participants

Supplementary Table II. Recovery of Triglyceride and Apolipoprotein B (ApoB) in ApoB-depleted Serum. ApoB-depleted serum was prepared and assayed for residual apoB and triglyceride as described in Methods. Recoveries were calculated as percent of whole serum values. ApoB was efficiently depleted regardless of serum triglyceride concentration. Values are reported as mg/dl. ND=not determined.

Group	Subject	Whole Serum			ApoB-depleted Serum			
		VLDL-C	TG	ApoB	TG	% recovery	ApoB	% recovery
High TG-VLDL /Low HDL-C								
	1	46	168	89	18	10.7	1	1.1
	2	76	527	96	13	2.5	2	2.1
	3	68	455	108	18	4.0	1	0.9
	4	57	335	80	25	7.5	1	1.3
	5	63	395	114	28	7.1	0	0.0
	6	71	331	125	19	5.7	0	0.0
	7	54	310	137	17	5.5	1	0.7
	8	57	230	77	23	10.0	2	2.6
	9	38	134	82	19	14.2	1	1.2
	10	54	184	148	22	12.0	0	0.0
	11	42	178	129	22	12.4	2	1.5
	12	45	206	102	22	10.7	0	0.0
	13	68	258	119	22	8.5	1	0.8
Low TG-VLDL /Normal HDL-C								
	1	6	64	108	19	29.7	1	0.9
	2	7	49	61	15	30.6	2	3.3
	3	13	86	70	19	22.1	0	0.0
	4	10	66	93	15	22.7	1	1.1
	5	14	69	65	15	21.7	0	0.0
	6	14	73	ND	10	13.7	2	ND
	7	2	54	82	12	22.2	1	1.2
	8	7	62	91	16	25.8	1	1.1
	9	11	50	70	10	20.0	6	8.6
	10	3	69	81	17	24.6	2	2.5

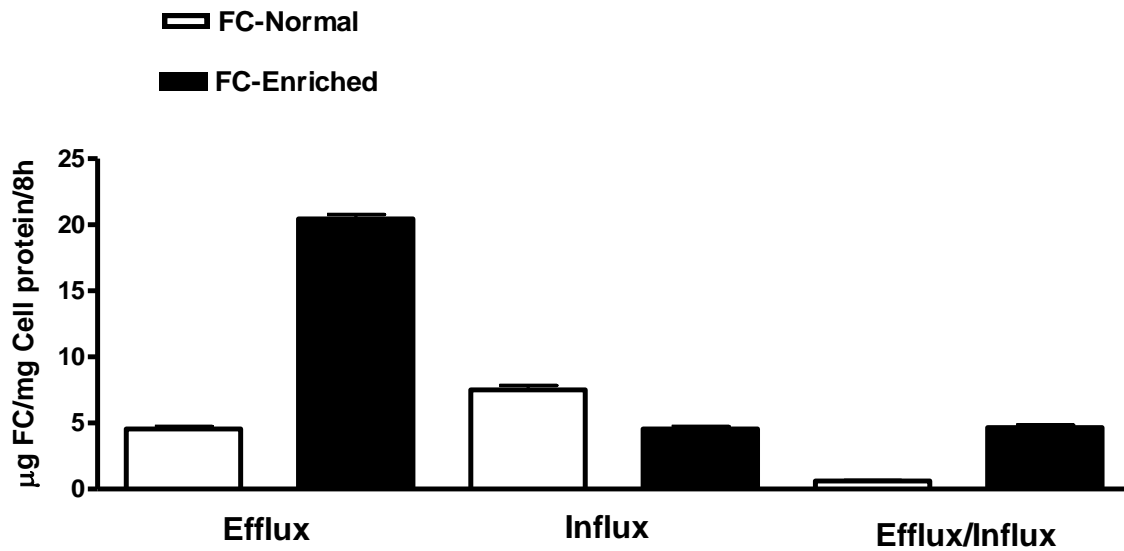
Supplementary Table III. Inter-assay Variability for Estimates of Bidirectional Flux of FC Mass Between J774 Macrophages and Whole Human Serum. Flux values ($\mu\text{g FC/mg cell protein/8h}$) were obtained as described in methods and represent the means \pm standard deviation (SD) of 3 separate experiments using J774 macrophages \pm cAMP incubated with a pool of human serum or 2 different human serum specimens. All serum was obtained from healthy donors and was used at 2.5% dilution. The coefficient of variation was calculated from the standard deviation of the means. SD = standard deviation. cv = coefficient of variation.

	Influx No cAMP	Efflux No cAMP	Influx With cAMP	Efflux With cAMP
	<i>Mean\pmSD (cv)</i>	<i>Mean\pmSD (cv)</i>	<i>Mean\pmSD (cv)</i>	<i>Mean\pmSD (cv)</i>
Pool	4.32 \pm 0.67 (0.15)	15.06 \pm 1.82 (0.12)	3.98 \pm 0.16 (0.04)	23.81 \pm 2.29 (0.10)
Donor #1	4.43 \pm 0.27 (0.06)	15.82 \pm 2.15 (0.14)	4.47 \pm 0.56 (0.13)	26.55 \pm 2.08 (0.08)
Donor #2	4.09 \pm 0.13 (0.03)	15.03 \pm 1.98 (0.13)	4.64 \pm 0.28 (0.06)	25.33 \pm 0.55 (0.02)

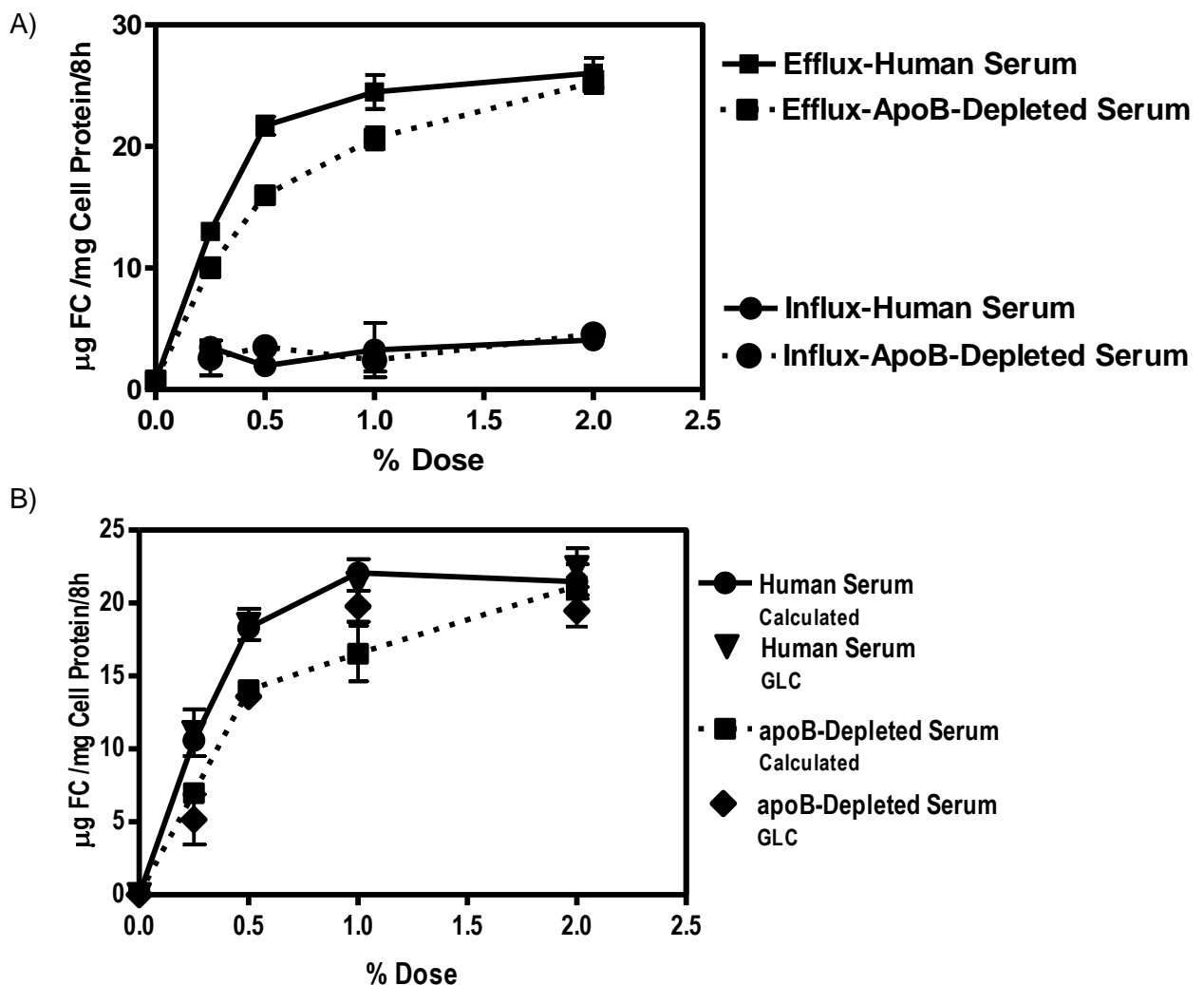
Supplementary Table IV. Effect of Probucol Treatment on Bidirectional Flux. J774+cAMP cells were treated with Probucol for 2 hours prior to bidirectional flux assay using a whole serum pool at 2.5% or apoB-depleted serum at 3.5%. Values are mean \pm SEM, N=3.

	Efflux			Influx		
	$\mu\text{g FC / mg Cell Protein/6h}$			$\mu\text{g FC / mg Cell Protein/6h}$		
	No Probucol	Plus Probucol	Probucol Sensitive Flux	No Probucol	Plus Probucol	Probucol Sensitive Flux
Experiment 1						
2.5% Human Serum Pool	4.32 \pm 0.06	2.28 \pm 0.02	2.04=47%	2.44 \pm 0.06	2.17 \pm 0.13	0.27=11%
Experiment 2						
2.5% Human Serum Pool	3.71 \pm 0.05	2.02 \pm 0.05	1.69=46%	2.49 \pm 0.14	2.33 \pm 0.06	0.16=6%
3.5% ApoB-depleted Serum	2.96 \pm 0.09	1.45 \pm 0.03	1.51=51%	1.35 \pm 0.05	1.50 \pm 0.06	NA

Supplementary Figure I. Bidirectional Flux of Cholesterol from free cholesterol (FC)-normal and FC-enriched J774+cAMP Cells to the Same Human Serum Specimens. FC flux between J774+cAMP cells and 21 whole serum specimens from healthy donors was measured. Cells were enriched in FC by incubation with AcLDL. In FC-normal cells, but not in FC-enriched cells, LDL-C correlated positively with influx and negatively with net efflux. Open bars indicate flux parameters for FC-normal cells; initial FC=11.8 $\mu\text{g FC/mg cell protein}$. Closed bars indicate flux parameters for FC-enriched cells, initial FC=46.6 $\mu\text{g FC/mg cell protein}$.



Supplementary Figure II. Effect of Serum Concentration on Free Cholesterol Bidirectional Flux. FC-enriched J774 cells treated with cAMP were incubated with increasing concentrations of either a pool of whole serum from healthy donors or apoB-depleted serum from the same pool, prepared as described in methods. Free cholesterol (FC) flux was measured as described in Methods. **Panel A** shows the dependence of both efflux (squares) and influx (circles) on the concentration of whole serum (solid line) and apoB-depleted serum (dotted line). **Panel B** shows the dependence of net flux on concentration of whole serum (solid line) and apoB-depleted serum (dotted line). Net flux is defined as the difference between efflux and influx and positive values indicate net outward movement of cellular cholesterol during the incubation. The circles and squares represent values obtained by subtracting the calculated estimates of efflux from calculated estimates of influx. The inverted triangles and diamonds represent cellular FC values obtained by GLC (see methods) and were obtained by subtracting the cellular cholesterol remaining after an 8h incubation from the cellular FC measured in duplicate cells not exposed to serum.



References

1. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *New England Journal of Medicine*. 2011;364:127-135
2. Zimetti F, Weibel GL, Duong MN, Rothblat GH. Measurement of cholesterol bidirectional flux between cells and lipoproteins. *Journal of Lipid Research*. 2006;47:605-613

Materials and Methods

Materials

Tissue culture plasticware was purchased from Corning (Corning, NY) and Thermo Fisher (Rochester, NY). RPMI 1640 and phosphate-buffered saline (PBS) were purchased from Mediatech Cellgro (Manassas, VA). MEM buffered with 10mM HEPES (pH 7.4) was prepared in the laboratory from MEM purchased from BioWhittaker (Walkersville, MD) and HEPES purchased from Fisher Scientific (Newark, DE). Fetal bovine serum (FBS), 8-(parachlorophenylthio) cyclic-3',5'-AMP (CTP-cAMP, cAMP), calf serum (CS), gentamicin, trypsin EDTA, polyethylene glycol (PEG) 6000, Probucol and the ACAT inhibitor Sandoz 58-035 were all purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents were purchased from VWR (Radnor, PA). BSA was purchased from Millipore (Kankakee, IL) and [1,2-³H]-cholesterol was purchased from Perkin Elmer Analytical Sciences (Waltham, MA).

Clinical Studies

We used serum samples from two different studies enrolled at the University of Pennsylvania. The Penn HDL Inflammation and Oxidation study (PHINOX) has been described in detail previously.^{1, 2} Briefly, PHINOX was designed to examine inflammatory and oxidant factors related to HDL-C and subclinical atherosclerosis in healthy adults, as assessed by ultrasound measurement of carotid artery intimal media thickness (IMT). Cholesterol efflux from J774 macrophages to the apoB-depleted serum from these individuals showed an inverse relationship of serum efflux capacity and carotid IMT¹. Here, we utilize existing data in PHINOX to explore the relationship of serum TGs to serum efflux capacity in the J774 model. The Penn Diabetes Heart Study (PDHS) has also been described in detail elsewhere³⁻⁵. Briefly, PDHS was designed to evaluate novel genetic and biochemical risk factors for atherosclerosis in Type 2 diabetic patients who were free of clinical cardiovascular disease and kidney dysfunction at enrolment. We designed a nested sub-study in PDHS to examine the relationship of extremes of serum TG / HDL-C with serum bidirectional flux capacity from macrophages as well as with our established measure of serum efflux capacity. From PDHS individuals, free from medical therapy with statins and insulin, two groups matched for age, gender and race (Supplementary Table I), were selected for a “favorable” serum lipid profile (low TG / normal HDL-C; HDL-C=72 (58-88), LDL-C=111 (97-131), TG=65 (56-69) median (IQR) mg/dl; n=10) or an “unfavorable” profile (high TG / low HDL-C; HDL-C=39 (32-42), LDL-C=109 (97-137), TG=258 (184-335) median (IQR) mg/dl; n=13), both groups having similar normal LDL-C levels (Table 1 & Supplementary Table I). Bidirectional flux as well established efflux assays were measured in whole serum and apoB-depleted (HDL-fraction) serum samples of these two groups of PDHS participants.

Biochemical assays

At the end of experiments, the cell monolayers were washed with PBS and cell lipid was extracted using 2-propanol containing cholesteryl methyl ether (CME) (Sigma, St. Louis, MO) as an internal standard for gas liquid chromatography (GLC). A fraction of the extracted lipid was used to measure total cholesterol radioactivity incorporated into cellular lipids using scintillation counting. The remaining lipid was prepared for GLC to measure FC mass as previously described.⁶ Cell protein was measured by a modified Lowry assay⁷.

Serum lipids and lipoproteins were measured in PHINOX and PDHS enzymatically (WAKO Diagnostics, Richmond, VA) on a Hitachi 912 analyzer (Roche Diagnostics, Basel, Switzerland) as previously described⁴. Removal of apolipoprotein B (apoB) containing lipoproteins from whole serum was done as we described; PEG treatment removes over 97% of apoB while retaining over 90% of the apolipoproteins A-I and A-II⁸. Briefly, the whole serum samples were combined with a solution containing 20% PEG in 200mM glycine, pH 7.4, at a ratio of 100 parts serum to 40 parts PEG solution. The mixture was incubated at room temperature for 20 minutes and spun at 4°C at 10,000 rpm for 30 minutes. The supernatant containing the HDL lipoprotein fraction was removed and diluted in MEM-HEPES at the desired concentration.

FC Enriched Macrophages

J774 cells were plated in 24-well plates (150,000 cells/well). To enrich the cells with cholesterol (FC), 50 µg/ml AcLDL was added to labeling medium, which was prepared as follows: 2µCi/ml ³H-FC in RPMI-1640 supplemented with gentamycin and 2 µg/ml ACAT inhibitor. The cells were incubated with the supplemented labeling medium for 24h. Supplementation with AcLDL increases the cellular cholesterol content by approximately 3-fold.

Measurement of macrophage FC efflux to serum

FC efflux from macrophages was measured using a standard assay¹. J774 macrophages were maintained in RPMI 1640 medium supplemented with 10% FBS and 0.5% gentamicin and then plated in growth medium in 24-well plates at a density of 150,000cells/well and incubated for 24h. The cells were labeled for 24h with 2µCi/ml [1,2-³H] cholesterol in medium supplemented with 5% FBS and 2µg/ml ACAT inhibitor to prevent the presence of radio-labeled CE pools that complicate the determination of the release of labeled cholesterol or the estimate of the specific activity of cell cholesterol. After labeling, the cells were washed 2x with MEM HEPES and equilibrated for 18h in RPMI 1640 medium containing 0.2% BSA and 2µg/ml ACAT inhibitor as well as 0.3mM cAMP (cyclic-3',5'-amp (CTP-cAMP); to upregulate ABCA1). After the equilibration/upregulation period, a triplicate set of cells was harvested prior to the

incubation with cholesterol acceptors (time zero, T0) to provide a baseline value for total cholesterol content (radioactivity and mass). T0 cell lipids were extracted and measured as described below. The remaining cells were washed with HEPES-buffered MEM, 0.5ml of this same medium containing acceptor was added to triplicate wells and they were incubated for 4 or 8h at 37°C. 0.03mM cAMP (a tenth of the concentration used to upregulate) was added to the efflux medium along with ACAT inhibitor to prevent cholesterol esterification. After incubation with cells, the media were collected, filtered and an aliquot was removed for scintillation counting to determine the amount of [1,2-³H] cholesterol released into the medium. The radioactivity released into the incubation medium was expressed as a fraction of the total [1,2-³H] free cholesterol in T0 cells. These values were corrected for the small amount of radioactivity released in the absence of an acceptor.

ABCA1-specific FC efflux was determined by calculating the difference in efflux in the absence and presence of Probucol in 8-(parachlorophenylthio) cyclic-3', 5'-amp, cAMP treated cells as previously described⁹. Briefly, Probucol was dissolved in 100% DMSO to form a 10 mM stock solution. The stock solution was diluted to a 20 mM Probucol (an inhibitor of ABCA1-mediated efflux)⁹, 0.2% BSA MEM HEPES solution. Equivalent amount of DMSO was added to all incubations. Cells were plated and labeled with [1,2-³H] cholesterol as described above. Cell monolayers were incubated for 2h in 0.2% BSA containing medium alone or with 20mM Probucol before efflux of macrophage FC to whole serum or apoB-depleted serum was measured. ABCA1-mediated efflux was calculated by subtracting the efflux value obtained from the Probucol treated cells from the efflux value obtained from the untreated cells.

Statistical analysis

All values were presented as means \pm standard deviations (SD) or standard errors of means (SEM) for data with normal distribution or as median and interquartile range (IRQ) for non-normal data. Statistical significance was determined by unpaired *t* tests (for data with normal distribution) or Mann Whitney U test (for non-normal data). Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using GraphPad Prism (San Diego, CA) software.

References

1. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *New England Journal of Medicine*. 2011;364:127-135
2. Heffron SP, Parastatidis I, Cuchel M, Wolfe ML, Tadesse MG, Mohler ER, Ischiropoulos H, Rader DJ, Reilly MP. Inflammation induces fibrinogen nitration in experimental human endotoxemia. *Free Rad Biol Med*. 2009;47:1140-1146
3. Martin SS, Qasim AN, Wolfe M, St Clair C, Schwartz S, Iqbal N, Schutta M, Bagheri R, Mehta NN, Rader DJ, Reilly MP. Comparison of high-density lipoprotein cholesterol to apolipoprotein A-I and A-II to predict coronary calcium and the effect of insulin resistance. *Am J Cardiol*. 2011;107:393-398
4. Martin SS, Qasim AN, Mehta NN, Wolfe M, Terembula K, Schwartz S, Iqbal N, Schutta M, Bagheri R, Reilly MP. Apolipoprotein B but not LDL cholesterol is associated with coronary artery calcification in type 2 diabetic whites. *Diabetes*. 2009;58:1887-1892
5. Qasim A, Mehta NN, Tadesse MG, Wolfe ML, Rhodes T, Girman C, Reilly MP. Adipokines, insulin resistance, and coronary artery calcification. *J Am Coll Cardiol*. 2008;53:231-236
6. Klanssek J, Yancey P, St.Clair RW, Fischer RT, Johnson WJ, Glick JM. Cholesterol quantitation by GLC: Artifactual formation of short-chain steryl esters. *J.Lipid Res*. 1995;36:2261-2266
7. Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal.Biochem*. 1978;87:206-210
8. Asztalos BF, de La Llera-Moya M, Dallal GE, Horvath KV, Schaefer EJ, Rothblat G. Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux. *J. Lipid Res*. 2005;46:2246-2253
9. Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probucol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler.Thromb.and Vascular Biol*. 2004;24:2345-2350