Clinical and Population Studies

Cholesterol Efflux Capacity, Carotid Atherosclerosis, and Cerebrovascular Symptomatology

R.J. Doonan, A. Hafiane, C. Lai, J.P. Veinot, J. Genest, S.S. Daskalopoulou

Objective—To investigate the association of cholesterol efflux capacity with carotid atherosclerosis and cerebrovascular disease.

Approach and Results—Patients with high-grade carotid stenosis (n=154) were recruited from Vascular Surgery clinics and 9 healthy controls from the McGill University Health Network, Montreal, Canada. Cerebrovascular symptomatology history was obtained. Stenosis was assessed by carotid ultrasound. Fasting blood samples were collected and depleted of apolipoprotein B particles by polyethylene glycol precipitation from serum. Cholesterol efflux was determined by incubating apolipoprotein B–depleted serum in cAMP-stimulated J774 cells for 6 hours. Carotid specimens were classified by 2 vascular pathologists using the American Heart Association atheromatous plaque classification. Differences in efflux were assessed according to (1) stenosis, (2) American Heart Association classification, and (3) cerebrovascular symptomatology. Normalized efflux was significantly lower in patients with carotid atherosclerosis compared with controls (0.97±0.16 versus 1.5±0.46; P<0.0001). Efflux was inversely associated with stenosis; the odds ratio for 80% to 99% versus 50% to 79% stenosis of terti1 (lowest) versus tertile 3 (highest) of efflux was 3.78 (95% confidence interval, 1.18–12.06) after adjusting for age, sex, low-density lipoprotein, and high-density lipoprotein. There were significant differences in cholesterol efflux between American Heart Association fibroatheroma (Va, 0.91±0.13), mainly calcific (Vb, 0.97±0.15), and mainly fibrotic (Vc, 1.03±0.21; P=0.05). There were no significant differences in efflux according to symptomatology.

Conclusions—Cholesterol efflux capacity is inversely associated with increasing carotid stenosis and is associated with more advanced carotid plaque morphology, suggesting that cholesterol efflux capacity may be a biomarker for severity of carotid atherosclerotic burden. Whether therapies targeting high-density lipoprotein quality could be useful for stabilizing carotid atherosclerosis needs to be assessed. (Arterioscler Thromb Vasc Biol. 2014;34:921-926.)

Key Words: carotid artery diseases □ carotid stenosis

Numerous epidemiological studies have found an inverse relationship between high-density lipoprotein (HDL) cholesterol and cardiovascular events.1-3 This has led to the HDL hypothesis, which proposes that pharmaceutical intervention to increase HDL will decrease cardiovascular risk.4 However, trials such as the effects of reteorceptipib in patients at high risk for coronary events (ILLUMINATE) or a study of dalceptapib in stable coronary heart disease patients with recent acute coronary syndrome (dal-OUTCOMES) have failed to show a decrease in cardiovascular risk despite an increase in HDL.5,6 Therefore, it is now thought that the quality of HDL may be more important than the quantity. One key roles of HDL in atherosclerosis is its ability to promote reverse cholesterol transport from lipid-laden macrophages, termed cholesterol effux capacity.7 It is thought that an increased cholesterol efflux capacity of HDL indicates a better quality HDL particle.

Cholesterol efflux capacity is a potential biomarker of atherosclerotic cardiovascular disease. Indeed, cholesterol efflux capacity was shown to be inversely associated with the presence of coronary artery disease,8,9 whereas Kherra et al10 also found cholesterol efflux to be inversely associated with carotid intima-media thickness. However, these studies have not investigated the association of cholesterol efflux capacity with severity of atherosclerosis or plaque instability. Indeed, the American Heart Association (AHA) has developed a plaque classification, which ranges from type I to VI.11 Plaque types I to III are early lesions, which can be present even before the fourth decade of life. Types IV to VI are all advanced lesions, which can be clinically silent or cause symptoms. Type IV is defined as having foam cells as well as an extracellular lipid core. Both type V and VI are advanced lesions with many features that allow for further subclassification. Type V lesions can be a fibroatheroma (Va) with a lipid core and a fibrotic layer or multiple lipid cores and fibrotic layers, mainly calcific (Vb), or mainly fibrotic (Vc) with little or no lipid core. Type VI is a complicated lesion with cap rupture (Vla), hematomahemorrhage (Vlb), a thrombus (Vlc), or a combination of these features. For example, a plaque with cap rupture and a thrombus would be labeled as type VIa. The AHA plaque classification is considered to be the gold standard in the assessment

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Materials and Methods

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

Results

We recruited 154 patients with carotid atherosclerosis scheduled for carotid endarterectomy and 9 controls without atherosclerosis awaiting elective aortic or mitral valve replacement. Table I contains demographic and clinical characteristics of this study population. Control patients had a mean age of 56.2±17.4 years and were predominantly male (88.8%). Characteristics of subgroups (stenosis, plaque types, symptomatology) of patients with carotid atherosclerosis are presented below.

The non-normalized cholesterol efflux capacity in our patients with carotid atherosclerosis ranged from 14.4% to 92.2% and in controls from 22.9% to 35.6%. Control patients were included only as references for completeness and not intended to be part of the primary or secondary objectives. To correct for variation across plates, cholesterol efflux was normalized to a standard serum pool run on each plate. We found that subjects with carotid atherosclerosis had significantly lower normalized cholesterol efflux capacity compared with control subjects without atherosclerosis (0.97±0.15 versus 1.5±0.46; \( P=0.002 \); Figure I in the online-only Data Supplement). We found only modest correlations between normalized cholesterol efflux capacity and HDL (\( r=0.27; \ P=0.001 \)) and apolipoprotein AI (ApoAI; \( r=0.361; \ P<0.0001 \)). In linear regression analysis, age (\( \beta=0.003±0.002; \ P=0.03 \)), total cholesterol (\( \beta=0.10±0.01; \ P<0.0001 \)), triglycerides (\( \beta=0.05±0.02; \ P=0.008 \)), low-density lipoprotein (\( \beta=0.10±0.01; \ P<0.0001 \)), HDL (\( \beta=0.23±0.06; \ P<0.0001 \)), and ApoAI (\( \beta=0.34±0.06; \ P<0.0001 \)) were associated with cholesterol efflux capacity. Therefore, we have included these as covariates in the logistic regression models, except for total cholesterol and triglycerides because of colinearity. Furthermore, we tested models including triglycerides; the results were essentially unchanged and remained significant (see results below). Sex was also included because it is an established cardiovascular risk factor traditionally included in the models.

Association of Cholesterol Efflux Capacity and Carotid Artery Stenosis

There were no significant differences in patient characteristics between patients with 50% to 79% and 80% to 99% stenosis. We found an inverse association between cholesterol efflux capacity and carotid artery stenosis. There was a graded decrease in efflux capacity from controls (1.5±0.46) to the 50% to 79% stenosis group (1.06±0.15) and the 80% to 99% group (0.95±0.16; \( P<0.0001 \) for trend; Figure 1). Compared with the highest tertile of cholesterol efflux capacity, both tertile 2 (odds ratio, 2.76; 95% confidence interval, 0.99–7.66) and tertile 1 (3.06; 95% confidence interval, 1.02–9.21) had increased odds of having an 80% to 99% stenosis compared with 50% to 79% after age, sex, and low-density lipoprotein were included in the model. When triglycerides were included, the corresponding odds ratios were 3.01 (95% confidence interval, 1.07–8.48) for tertile 2 and 3.52 (95% confidence interval, 1.14–10.89) for tertile 1. These associations were robust and remained significant after adjusting for HDL or ApoAI (Table 2). The distribution of ApoAI plaque types was not significantly different according to stenosis categories (\( \chi^2=5.48; \ P=\text{not significant} \)).

Association of Cholesterol Efflux Capacity and Carotid Plaque AHA Plaque Classification

There were no significant differences in patient characteristics between patients with type V and VI plaques. However, patients with fibroatheroma (Va) plaques were more likely to be male (88.5%) compared with calcific plaques (Vb, 66.5%), and mainly fibrous (Vc, 33.3%; \( \chi^2=14.39; \ P=0.001 \)). Furthermore, patient age was significantly different (fibroatheroma=71.6±8.6 years; calcific=70.1±9.5 years; mainly fibrous=64.0±10.0 years; \( P=0.02 \) for trend). No other patient characteristics (from Table 1) were significantly different. We found no association between cholesterol efflux capacity and AHA plaque classification when we categorized patients into either AHA type V or AHA type VI (Figure II and Table I in the online-only Data Supplement). However, we found significant differences when comparing cholesterol efflux between patients with fibroatheromas (type Va, mean efflux=0.91±0.13), calcific plaques (type Vb, mean efflux=0.97±0.15), and mainly fibrous plaques (type Vc, mean efflux=1.03±0.21; \( P=0.05 \) for trend; Figure 2). In the logistic regression analysis, we also found that patients in the lower tertiles of efflux had decreased odds of having a calcific (Vb) or mainly fibrous (Vc) plaque, which further supports the notion that patients with fibroatheromas with large lipid core have lower cholesterol efflux capacity (Table 3).

Association of Cholesterol Efflux Capacity and Cerebrovascular Symptomatology

In our population, asymptomatic patients were significantly younger (65.5±6.8 years) than symptomatic patients (71.5±9.8 years; \( P<0.0001 \)). However, no other patient characteristics were significantly different. We assessed the association between cerebrovascular symptomatology (symptomatic and asymptomatic) with cholesterol efflux capacity. We found no significant difference in cholesterol efflux capacity between groups (asymptomatic, 0.99±0.15 versus symptomatic, 0.97±0.16; \( P=\text{not significant} \)) and no association in any of our logistic regression models (Figure III and Table II in the online-only Data Supplement). The distribution of fibroatheroma (Va), calcific (Vb), or mainly fibrous (Vc) plaque types was not significantly different according to cerebrovascular symptomatology (\( \chi^2=0.38; \ P=\text{not significant} \)).
plaques was not significantly different according to symptomatology ($\chi^2=1.92$; $P$ not significant). Furthermore, when we adjusted the stenosis or AHA regression models for the interaction between cholesterol efflux capacity and symptomatology, we found no significant difference in the predictive value of cholesterol efflux capacity.

### Discussion

We have investigated, for the first time, the association of cholesterol efflux capacity with carotid atherosclerosis and with cerebrovascular symptomatology. We found that cholesterol efflux was decreased in patients with carotid atherosclerosis, inversely associated with the severity of carotid stenosis, and differs between patients with different types of carotid plaques. These associations remained significant after adjustment for age, sex, low-density lipoprotein, and HDL or ApoAI. However, we did not find an association between cholesterol efflux capacity and cerebrovascular symptomatology or carotid plaque complexity (AHA plaque class V versus VI).

Previous work has found that cholesterol efflux is inversely associated with coronary artery disease.8,9,11 However, Li et al9 also found a paradoxical association of enhanced cholesterol efflux capacity with increased risk of future cardiovascular events (myocardial infarction, stroke, or death). In this study, baseline characteristics differed between cases and controls, including age and Framingham risk scores. Specifically, the control subjects in the highest efflux tertile had more cardiovascular risk factors than in other tertiles. Therefore, it is possible that the increased risk factors overcame the protective effect of increased cholesterol efflux capacity.12 However, despite this paradoxical finding, Li et al9 still found an inverse association between cholesterol efflux capacity and presence of fibroatheroma—AHA class Va (% of type V) 18.1 N/A 41.2 31.3 7.4 0.02

Calcific plaque—AHA class Vb (% of type V) 33.7 N/A 44.1 56.3 59.3 0.02

Mainly fibrous plaque—AHA class Vc (% of type V) 12.3 N/A 14.7 12.5 33.3 0.02

AHA plaque class VI, % 35.8 N/A 26.1 36.0 42.9 NS

Ever smoker, % 80.5 0 77.8 80.4 84.0 NS

CAD, % 42.2 0 34.0 43.8 46.9 NS

PAD, % 19.4 0 18.2 23.4 17.0 NS

HTN, % 85.0 66.6 89.8 84.3 80.0 NS

HTN medication, % 65.5 11.1 69.6 62.2 64.6 NS

Diabetes mellitus, % 29.2 0 30.6 21.6 34.0 NS

Diabetes mellitus medication, % 24.7 0 26.0 15.4 32.0 NS

Systolic blood pressure, mm Hg 138.1±17.3 125.4±17.1 137.3±15.3 135.8±19.9 141.0±16.6 NS

Diastolic blood pressure, mm Hg 69.0±10.2 71.0±7.2 69.7±10.6 68.0±9.1 69.2±11.1 NS

Cholesterol efflux capacity 0.97±0.15 1.5±0.46 0.81±0.06 0.96±0.03 1.1±0.12 <0.0001

Total cholesterol, mmol/L 3.4 (2.9–3.9) 4.9 (4.5–5.2) 3.0 (2.7–3.4) 3.4 (2.9–3.8) 3.8 (3.3–4.4) <0.0001

LDL, mmol/L 1.7 (1.3–2.2) 2.8 (2.6–3.0) 1.5 (1.2–1.9) 1.7 (1.2–2.2) 1.9 (1.5–2.3) 0.003

HDL, mmol/L 0.9 (0.7–1.1) 1.0 (0.9–1.2) 0.87 (0.71–0.99) 0.90 (0.71–1.1) 1.0 (0.86–1.1) 0.01

Triglycerides, mmol/L 1.5 (1.1–1.8) 2.4 (1.5–2.8) 1.3 (0.97–1.7) 1.5 (1.1–1.7) 1.6 (1.1–1.9) NS

ApoAI, g/L 1.2 (1.0–1.4) 1.4 (1.3–1.5) 1.1 (1.0–1.2) 1.2 (1.0–1.4) 1.3 (1.1–1.5) <0.0001

CRP 1.5 (0.65–3.5) 2.0 (1.6–4.5) 1.6 (0.68–3.3) 1.4 (0.66–3.3) 1.4 (0.56–3.6) NS

Continuous variables are presented as mean±SD or as median (interquartile range) in the case of non-normal distribution. Categorical variables are presented as %.

AHA indicates American Heart Association; ApoAI, apolipoprotein AI; BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; HDL, high-density lipoprotein; HTN, hypertension; LDL, low-density lipoprotein; N/A, not applicable; NS, not significant; and PAD, peripheral artery disease.
of coronary artery disease on angiography at baseline. These findings underscore the need for additional research in the area.

Although previous literature reported an inverse association between carotid intima-media thickness and cholesterol efflux capacity in healthy volunteers and in individuals with ATP-binding cassette transporter A1 (ABCA1) mutations, we were interested in moving beyond preclinical carotid atherosclerosis and investigating the association between cholesterol efflux capacity and severity of carotid atherosclerosis by assessing stenosis and plaque morphology. Indeed, it has been suggested that carotid intima-media thickness is an inadequate surrogate for carotid atherosclerosis. We found a robust inverse association between cholesterol efflux capacity and carotid stenosis, a known factor for carotid plaque instability. Our data indicate that cholesterol efflux capacity may serve as a marker of atherosclerotic burden severity because we found a graded decrease in cholesterol efflux from patients with 50% to 79% to patients with 80% to 99% stenosis (Figure 1). Control subjects without atherosclerosis were included only as references for completeness.

Although we found no difference in cholesterol efflux capacity between patients with AHA class type V and AHA type VI (complicated plaque with cap rupture, thrombus, or hemorrhage), we did find that patients with fibroatheromas (Va) have lower efflux than patients with largely calcified (Vb) or mainly fibrous plaques (Vc). This suggests that increased cholesterol efflux capacity may have a protective effect against developing unstable carotid plaques because fibrous and calcified plaques are considered to be more stable than fibroatheromas. It should be noted that previous studies have assessed the expression of ABCA1 in carotid plaques. Both Liu et al found that carotid plaques had higher ABCA1 gene expression and lower protein expression compared with control arteries. Liu et al further noted that more advanced plaques (AHA plaque types III–VI) have lower ABCA1 protein expression compared with plaque types I and II, which are early lesions. Although our study contains only patients with advanced lesions, it is possible that fibroatheroma plaques express less ABCA1 than calcific or mainly fibrous plaques, whereas there is no difference in HDL, ApoAI, or C-reactive protein between patients with different AHA type V subtypes. This suggests that HDL quality rather than quantity or inflammation is associated with presence of fibroatheroma plaques. However, we cannot rule out the possibility that decreased ABCA1 protein expression in the plaque also increases the likelihood of having a fibroatheroma plaque because this remains to be investigated.

It is important that we found that cholesterol efflux capacity is inversely associated with plaque instability but not symptomatology. Symptomatology is an inadequate surrogate for plaque instability because asymptomatic patients may have unstable plaques that have simply not yet ruptured. Indeed, there was no difference in the distribution of AHA plaque types between symptomatic and asymptomatic patients. Therefore, had we only assessed symptomatology and not plaque morphology, we would have missed important information about this relationship. There is the possibility that we simply did not have enough power to detect a difference between asymptomatic and symptomatic patients. However, there were no previous studies on which to base sample size calculations. Indeed, with a small difference of only 2% (0.99±0.15 in asymptomatic patients versus 0.97±0.16 in symptomatic patients), we would need 5934 patients to detect a statistically significant difference. However, because cholesterol efflux capacity was found to be associated with stenosis and plaque morphology,

### Table 2. Association of Cholesterol Efflux Capacity and Carotid Artery Stenosis

<table>
<thead>
<tr>
<th>Efflux Tertile</th>
<th>No. of Patients</th>
<th>Univariate</th>
<th>Odds Ratio for 80%–99% Versus 50%–79% Stenosis (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertile 1 (0.66–0.89)</td>
<td>51</td>
<td>3.75 (1.33–10.54)</td>
<td>3.06 (1.02–9.21) 3.78 (1.18–12.06) 3.91 (1.18–12.92)</td>
</tr>
<tr>
<td>Tertile 2 (0.90–1.02)</td>
<td>51</td>
<td>2.95 (1.14–7.64)</td>
<td>2.76 (0.99–7.66) 2.98 (1.05–8.44) 3.00 (1.06–8.51)</td>
</tr>
<tr>
<td>Tertile 3 (1.03–1.51)</td>
<td>52</td>
<td>Reference</td>
<td>Reference Reference Reference</td>
</tr>
</tbody>
</table>

*Patients in the lowest tertile of cholesterol efflux compared with patients in the highest tertile have increased odds of having an 80% to 99% stenosis plaque instead of a 50% to 79% plaque. ApoAI indicates apolipoprotein A1; CI, confidence interval; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.*
both known factors that affect plaque instability, it is possible that efflux contributes indirectly to developing cerebrovascular symptomatology by affecting plaque instability.

Because APOA1 is the rate-limiting step in reverse cholesterol transport,4 it has been suggested that variations in pre-β HDL between patients may explain observed differences in cholesterol efflux capacity.22 However, the HDL particle is heterogeneous in charge, size, and protein composition1,4; these differences are also likely to explain, at least in part, differences in cholesterol efflux between patients. It is important also to consider the effect of covariates on cholesterol efflux. We have included traditional cardiovascular risk factors such as age, sex, and low-density lipoprotein in our models as well as HDL or ApoA1 because these are known to affect cholesterol efflux. We cannot exclude the possibility that medications that alter lipids, such as statins, affect cholesterol efflux as well. However, statins have been previously shown to not significantly affect cholesterol efflux,4 statin use did not differ between groups in our study, and was not associated with cholesterol efflux capacity when assessed by linear regression.

Our study has limitations, including the cross-sectional design. We only recruited patients scheduled to undergo carotid endarterectomy. However, this was inevitable because we needed to collect carotid surgical specimens. It would be important in the future to establish causation by following patients with low-grade carotid stenosis to determine whether cholesterol efflux capacity is associated with progression of stenosis and development of cerebrovascular symptomatology and also to determine whether cholesterol efflux capacity can be used as a biomarker. The efflux technique captures only information of the ability of HDL to accept cholesterol from macrophages and not other steps in reverse cholesterol transport, such as cholesterol uptake into the liver.

**Conclusion**

We assessed for the first time the association between cholesterol efflux capacity and carotid atherosclerosis and cerebrovascular symptomatology. We found cholesterol efflux capacity was inversely associated with carotid stenosis and was associated with carotid plaque morphology but not with symptomatology. It remains to be investigated whether cholesterol efflux capacity could be a biomarker for severity of carotid atherosclerosis and whether therapies targeting HDL quality could be useful for stabilizing carotid atherosclerosis.

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**Disclosures**

None.

**References**


Table 3. Association of Cholesterol Efflux Capacity and AHA Plaque Classification

<table>
<thead>
<tr>
<th>Efflux Tertile</th>
<th>No. of Patients</th>
<th>Odds Ratio for Calcific Plaque vs Fibroatheroma (95% CI)</th>
<th>Odds Ratio for AHA Class Mainly Fibrous Plaque vs Fibroatheroma (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertile 1</td>
<td>33</td>
<td>0.13 (0.66–0.88)</td>
<td>0.07 (0.01–0.50)</td>
</tr>
<tr>
<td>Tertile 2</td>
<td>33</td>
<td>0.22 (0.89–1.00)</td>
<td>0.08 (0.01–0.68)</td>
</tr>
<tr>
<td>Tertile 3</td>
<td>33</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
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</tbody>
</table>

Patients in the lowest tertile of cholesterol efflux compared with patients in the highest tertile have decreased odds of having a type calcific plaque (Vb) or mainly fibrous plaque (Vc) plaque instead of a fibroatheroma (Va). AHA indicates American Heart Association; ApoAI, apolipoprotein AI; CI, confidence interval; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.


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**Significance**

Recently, the high-density lipoprotein (HDL) hypothesis has been called into question, and it is now thought that HDL quality may be more important than HDL quantity. We have shown for the first time that HDL’s cholesterol efflux capacity from macrophages is a predictor of carotid stenosis and carotid plaque instability. It remains to be investigated in the future whether cholesterol efflux capacity is a useful biomarker for severity of carotid atherosclerosis and whether HDL quality could be used as a target in the future to treat carotid atherosclerosis.
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Methods

Patients and Clinical Variables
Consecutive patients with high-grade carotid stenosis (n=154) scheduled for carotid endarterectomy were identified through our collaborating vascular surgeons and recruited from the Vascular Surgery pre-operative clinics from the McGill University Health Network in Montreal, Canada. All patients underwent pre-operative screening including physical examination. Patients were excluded if they had a previous intervention on their carotid artery (restenosis). Patients with symptomatic disease were screened and excluded if they had non-carotid related sources of cerebrovascular disease, such as a cardio-embolic source or aortic arch disease. Healthy control patients (n=9) awaiting elective aortic or mitral valve replacement or repair were recruited through a collaborating cardiologist. Control patients were included only as references for completeness and not intended to be part of the primary or secondary objectives. Controls had no coronary artery disease or carotid plaque as documented by angiography and carotid ultrasound, respectively. Plaque was defined as a focal structure encroaching into the arterial lumen of at least 0.5mm or 50% of the surrounding intima-media thickness value, or demonstrates a thickness >1.5mm as measured from the media-adventitia interface to the intima-lumen interface. These healthy control patients were used as a comparison group to assess if cholesterol efflux capacity is significantly different between patients with and without carotid atherosclerosis.

Patient demographics and clinical information containing detailed symptomatology, past medical history including neurological history, and medications were obtained from 1) patient interview, 2) a detailed patient questionnaire, and 3) patient medical records. Using sources from pre-operative clinics, height and weight were measured to calculate body mass index, and brachial blood pressure (HEM-705CP, Omron Corp.) was measured according to guidelines.

Ultrasound Examinations
Using a Philips IU22 (Andover, United States) ultrasound machine and a linear 9-3 MHz probe, bilateral carotid examinations were performed by the same, experienced vascular ultrasonographer. Doppler signals were acquired from each arterial segment (common carotid, internal carotid, and external carotid) to obtain velocities and calculate degree of stenosis according to the North American Carotid Endarterectomy Trial (NASCET) criteria.

Surgical Samples
Surgical carotid plaque specimens were collected from the operating room; they were immediately fixed in 10% formalin for 24 hours and decalcified (Surgipath Decalcifier I, Leica Microsystems, Richmond, United States) for 24-96 hours, depending on the extent of calcification. Plaques were dissected into 3-4 mm transverse segments and processed for paraffin embedding.

Histology
The area of maximum stenosis or the visible culprit lesion area (in symptomatic patients, if different from the area of maximum stenosis) were used for histological analysis. Two 4µm sections were stained with hematoxylin and eosin. Two vascular pathologists (CL and JV) independently examined the sections and classified them according to Stary’s American Heart Association (AHA) plaque classification. The AHA plaque classification system ranges from type I, an initial lesion, to type VI, a lesion with a hematoma, hemorrhage, or thrombus. Types IV-VI are advanced lesions and types V and VI have distinguishable features, which allow for further subdivision. Indeed, type V can be subclassified into type Va-fibroatheroma with a large lipid core, Vb-calcified plaque, or Vc-mainly fibrous plaque. Type VI can be subclassified into
VIA-disrupted cap, VIB-hematoma or hemorrhage, or VIC-thrombosis (or a combination of these features). In case of disagreements a consensus was reached.

**Blood samples and tests**

Blood was collected by venipuncture and allowed to clot for 30 minutes. Serum was separated (2000g, 4°C for 20min) and frozen at -80°C in 0.5 ml aliquots. Assays for total cholesterol, HDL, and triglycerides were performed enzymatically on an autoanalyzer (Cobas Mira, Roche). Low-density lipoprotein (LDL) cholesterol was calculated with the Friedwald formula. Apolipoprotein (Apo) AI concentration was measured by nephelometry (Behring Nephelometer 100 Analyzer). All measurements were performed in the McGill University Health Centre central biochemistry labs.

**Cholesterol Efflux Capacity Measurements**

Cholesterol efflux assays were performed using apoB-depleted serum. ApoB depleted serum was obtained by polyethylene glycol (PEG, MW8000, Sigma, Oakville, Canada) precipitation 20% 40:100 plasma (v/v). A serum standard pool was obtained from 6 healthy volunteers (3 men and 3 women) and stored at -80°C until use. This serum pool was run on each plate to control for inter-assay variation and allow for efflux normalization.

Efflux assays were performed as previously described. J774 cells (ATCC TIB-67, Cedarlane, Burlington, Canada) were maintained in RPMI with 10% fetal bovine serum (FBS) supplemented with 100U/ml penicillin and 100U/ml streptomycin (Penicillin-Streptomycin, Invitrogen), 5% CO₂. Cells were distributed (150,000 cells/well) in 24 well plates. In the presence of 2ug/ml acyl-coenzyme A acyltransferase inhibitor (Sandoz, Sigma, Oakville, Canada) cells were labelled for 24h with 2 µCi/ml [3][H]-cholesterol (0.5ml.well, RPMI/1% FBS, 0.3mM 8-(4-Chlorophenylthio)-cyclic AMP (cpt-cAMP, Sigma, Oakville, Canada) in RPMI (0.2% bovine serum albumin) was used to upregulate ATP binding cassette transporter (ABC) A1 for 18-20h. [3][H]-cholesterol loaded cells were incubated with cholesterol 2.8% ApoB depleted serum (Figure 1 shows dose response curve) diluted in MEM-HEPES (0.5ml/well) for 6 hours (Figure 2 shows time response curve) for the efflux assays. Cholesterol efflux capacity was calculated as: percent cholesterol efflux = [3][H]-cpm_{medium}/[3][H]-cpm_{medium} + [3][H]-cpm_{cells} × 100%. Liquid scintillation counting (Perkin Elimer, Montreal, Canada) was used to quantify the [3][H]-cholesterol in the medium and cells. Each patient sample was run in triplicate. The coefficient of inter-variability of 5.83%. To correct for inter-assay variation across plates the pooled serum standard was included on each plate and values were normalized to this control pool (patient efflux/control pool efflux).

**Statistics**

Categorical variables are presented as percentages and continuous variables are presented as mean ± standard deviation for normally distributed variables or median and interquartile range for non-normally distributed variables. The primary objectives were to evaluate the association of cholesterol efflux capacity, with carotid stenosis and carotid plaque instability (Type V vs. VI or Va vs. Vb vs. Vc). The secondary objective was to evaluate the association of cholesterol efflux capacity and with cerebrovascular symptomatology. Independent sample t-test or one-way ANOVA were used to assess differences in cholesterol efflux capacity between patient groups based on stenosis, plaque histological classifications, or cerebrovascular symptomatology. Non-parametric tests (Mann-Whitney or Kruskal Wallis) were used to compare distributions between healthy control subjects and subjects with carotid atherosclerosis. Logistic regression was used to estimate the association of cholesterol efflux capacity (divided by tertile) and carotid artery stenosis category (50-79% or 80-99%), AHA
plaque classification (V or VI) and AHA type V subclassification (Va, Vb, or Vc), and symptomatology (symptomatic or asymptomatic). Odds ratios (OR) are presented with 95% confidence intervals (CI) are presented after adjustment for age, sex, and LDL. HDL or ApoA1 were added into additional models. Chi square or independent sample t-test were performed to evaluate distributions of AHA classifications according to degree of stenosis and presence or absence of symptomatology as well clinical characteristics according to cholesterol efflux tertile and between different patient groups (stenosis, AHA plaque type, and symptomatology). Spearman's correlation coefficients were calculated between cholesterol efflux capacity, HDL, and ApoA1. Statistics were performed in SPSS version 20 (IBM, Armonk, Unites states).

**Ethics**
This study was approved by the McGill University Ethics Review Board. All patients provided written informed consent.
Figure 1. Dose Response Curve of Increased Serum Values on Cholesterol Efflux Over 6 Hours
Figure 2. Effect of Incubation Time on Cholesterol Efflux from J774 cell line.
References


6. 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 high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler Thromb Vasc Biol. 2010: 30: 796-801.

Supplemental Figure I. Cholesterol Efflux Capacity in Controls and Patients with Carotid Atherosclerosis.

Results presented as mean normalized cholesterol efflux. Error bars represent standard deviation. Analysis performed by Mann Whitney test.
Supplemental Figure II. Cholesterol Efflux Capacity According to American Heart Association Plaque Classification.

Results presented as mean normalized cholesterol efflux. Error bars represent standard deviation. Analysis performed by independent t-test.
Results presented as mean normalized cholesterol efflux. Error bars represent standard deviation. Analysis performed by independent t-test.
### Supplemental Table I. Association of Cholesterol Efflux Capacity and American Heart Association Plaque Type.

<table>
<thead>
<tr>
<th>Efflux Tertile</th>
<th>No. of Patients</th>
<th>Odds Ratio for AHA VI vs. V (95% CI)</th>
<th>Univariate</th>
<th>Age, sex, LDL</th>
<th>Age, sex, LDL + HDL</th>
<th>Age, sex, LDL + ApoAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tertile 1</td>
<td>51</td>
<td>0.47 (0.19-1.12)</td>
<td>0.49 (0.20-1.23)</td>
<td>0.41 (0.16-1.07)</td>
<td>0.42 (0.16-1.12)</td>
<td></td>
</tr>
<tr>
<td>(0.66-0.89)</td>
<td></td>
<td>0.75 (0.33-1.68)</td>
<td>0.80 (0.34-1.85)</td>
<td>0.76 (0.32-1.78)</td>
<td>0.76 (0.32-1.79)</td>
<td></td>
</tr>
<tr>
<td>Tertile 2</td>
<td>51</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>(0.90-1.02)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tertile 3</td>
<td>52</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>(1.03-1.51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| P for trend    | NS             | NS                                  | NS         | NS            | NS                 | NS                    |

LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, apolipoprotein. Patients in the lowest tertile of cholesterol efflux compared to patients in the highest tertile had no difference in the odds of having a type VI plaque compared to type V.
Supplemental Table II. Association of Cholesterol Efflux Capacity and Cerebrovascular Symptomatology.

<table>
<thead>
<tr>
<th>Efflux Tertile</th>
<th>No. of Patients</th>
<th>Odds Ratio for Symptomatic vs. Asymptomatic Patients (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Univariate</td>
</tr>
<tr>
<td>Tertile 1 (0.66-0.89)</td>
<td>51</td>
<td>1.26 (0.51-3.09)</td>
</tr>
<tr>
<td>Tertile 2 (0.90-1.02)</td>
<td>51</td>
<td>0.80 (0.34-1.86)</td>
</tr>
<tr>
<td>Tertile 3 (1.03-1.51)</td>
<td>52</td>
<td>Reference</td>
</tr>
<tr>
<td>P for trend</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo, apolipoprotein. Patients in the lowest tertile of cholesterol efflux compared to patients in the highest tertile had no difference in the odds of having cerebrovascular symptoms.