HDL phospholipid and ABCA1- mediated cholesterol efflux are reduced in patients with very high HDL-C who develop early coronary artery disease

by

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DISSERTATION

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ABSTRACT

HDL phospholipid and ABCA1- mediated cholesterol efflux are reduced in patients with very high HDL-C who develop early coronary artery disease

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Background: Plasma levels of high-density lipoprotein cholesterol (HDL-C) are strongly inversely associated with coronary artery disease (CAD), and high HDL-C is generally associated with apparent 'protection' from CAD.

Objective: We identified a number of individuals with high HDL-C levels who develop CAD, a paradoxical phenotype and hypothesized that such individuals may have HDL with altered structure and function, and compared controls with similarly high HDL-C and no coronary disease.

Methods: 55 subjects with HDL-C above the 90th percentile, early CAD, and no major known risk factors for coronary disease were identified. We selected 120 controls without CAD, each matched for race, gender, and HDL-C level.

Results: Comparison of HDL particle characteristics between cases and controls demonstrated a significant reduction in HDL phospholipid composition and cholesterol efflux capacity in cases as compared to controls.

Conclusion: Reduced cholesterol efflux capacity in cases with elevated HDL-C and CAD may explain the development of early coronary artery disease. Cholesterol efflux capacity may in fact be a better predictor of the risk of coronary disease then HDL-C levels alone. The reduction in HDL phospholipid in the cases may help account for impaired cholesterol efflux.

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PRIOR PUBLICATIONS & PRESENTATIONS

PUBLICATIONS:

A. Agarwala, J. Billheimer, A. Rodrigues, M. Risman, M. Cuchel, D. J. Rader, HDL Structure and Function in Individuals with Extreme High HDL-C Who Have Coronary Artery Disease Compared with Those Without CAD. *Arterioscler Thromb Vasc Biol*. 2013; 33: A387.

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Agarwala, A., Durakoglugil, M., Bowen, I., Herz, J. "Alternative splicing of ApoER2 is important in mediating activity- dependent synaptic plasticity." UT Southwestern 49th Annual Medical Student Research Forum, Dallas, TX, January 2011.

Agarwala, A., Bowen, I., Herz, J. "Differential AMPA and NMDA receptor activation may explain LTP changes in Apoer2 mutant mice." UT Southwestern 48th Annual Medical Student Research Forum, Dallas, TX, January 2010.

Agarwala, A., Bowen, I., Herz, J. "Balance of Apoer2 and Vldlr expression levels is a key regulator of the startle response in mice." UT Southwestern 47th Annual Medical Student Research Forum, Dallas, TX, January 2009.

Agarwala, A., Choi, H., Herz, J. "Wise Interacts with LDL Receptor-Related Protein 4." UT Southwestern 46th Annual Medical Student Research Forum, Dallas, TX, January 2008.

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A. Agarwala, E. Givens, D.K. McGuire[1], J.A. De Lemos, A.K. Rohatgi, "Rosiglitazone Increases Cholesterol Efflux Capacity in Patients with Type 2 Diabetes." Oral Presentation at the Southern Regional Meeting, New Orleans, LA, February 2014.

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CHAPTER 1

Plasma high-density lipoprotein cholesterol (HDL-C) levels are inversely correlated with coronary disease. Elevated levels of HDL-C are associated with protection against coronary disease[1]. In the 1970s, Ross et al. proposed that atherosclerosis is caused by an imbalance in the deposition and removal of cholesterol from arterial walls[2]. Miller and Miller suggested that increasing HDL-C levels may increase clearance of cholesterol from arterial walls[3]. The belief that absolute levels of HDL-C have a causal relationship to heart disease was referred to as 'the HDL cholesterol hypothesis'. Many coronary risk assessment scores use absolute levels of HDL-C as an indicator of the degree of protection against coronary disease.

Despite these findings there have been challenges to the HDL-C hypothesis. Recently, several clinical trials using agents that raise HDL-C have been prematurely terminated or have failed to show any clinical benefit. In the dal- OUTCOMES trial of the cholesterol ester transfer protein (CETP) inhibitor dalcetrapib, patients received dalcetrapib in addition to other agents that lower LDL cholesterol (LDL-C). Though a significant elevation in HDL-C levels was noted in patients treated with dalcetrapib, the trial was prematurely terminated due to futility of the study[4]. The HPS2- THRIVE trial was designed to assess cardiovascular outcomes in patients treated with ER- niacin and laropiprant, an antiflushing agent, in addition to a statin. However, HPS2- THRIVE was prematurely terminated after having missed its primary endpoint of reducing the risk of MI, stroke, or coronary revascularizations compared to statin therapy alone and showing no clinical benefit[5]. These findings prompt the question as to whether raising levels of HDL-C are

always protective against coronary artery disease.

HDL has a number of different functions by which it may aid against cardiovascular disease including its antioxidant and anti-inflammatory properties and its ability to participate in reverse cholesterol transport[6]. Clinical data suggest that static measurements of HDL-C do not assess its function. A study published by Khera et al. demonstrated that cholesterol efflux has an inverse association with coronary disease that is independent of HDL-C levels[7]. In this study, we sought to assess the structural and functional characteristics of HDL in a unique population of individuals with very high levels of HDL-C who develop coronary artery disease in the absence of traditional risk factors. In many instances, the development of coronary disease is earlier in life.

We posed the question of whether HDL might be "dysfunctional" in these patients and hypothesized that these individuals would have an altered structure and reduced function of their HDL as compared to healthy controls with similarly high plasma HDL-C levels. We studied one of the main functions of HDL, its ability to participate in reverse cholesterol transport, specifically cholesterol efflux capacity. Here we demonstrate in this cohort of patients with elevated plasma HDL-C levels and coronary disease, reduced function of HDL-C as measured by cholesterol efflux capacity and structural differences in the HDL particle as compared to healthy counterparts with similarly high plasma HDL-C levels.

CHAPTER 2

Study design: We identified patients that previously participated in clinical studies at the University of Pennsylvania and selected individuals with HDL-C levels above the 90th percentile with documented cardiovascular disease defined as either a history of heart attack, angioplasty, coronary artery bypass surgery, coronary calcium score above the 90th percentile, or greater than 50% stenosis on CT angiogram. We excluded subjects with plasma LDL-C level greater than 190 mg/dL, triglycerides greater than 400 mg/dL, diabetes (type I and type II), history of liver disease with LFTs greater than twice the upper limit of normal, history of kidney disease or chronic renal insufficiency, and use of medications known to significantly affect HDL-C levels, specifically including niacin doses greater than 1500 mg daily. Controls were selected based on the absence of CAD and matched to cases for race, gender, and HDL-C level within 10 mg/dL. Controls were selected to be the same age or up to 10 years older than the cases. Additional exclusion criteria for controls included history of stroke, transient ischemic attack (TIA), and history of abdominal aortic aneurysm.

Apolipoprotein measurements: Plasma concentrations of total cholesterol, HDL-C, triglycerides, and apolipoproteins were measured using blood samples obtained after an 8-hour fast using CDC-standardized methods. Measurements were performed on frozen (−80°C) EDTA plasma and serum. The Friedewald equation was used to determine the amount of LDL-C.

Radiolabeled cholesterol efflux: Cholesterol efflux capacity was measured in patient samples. J774 mouse macrophage cells were plated and labeled with 2μCi of ^{3}H cholesterol per milliliter overnight. Cells were then incubated for 6 hours in either the presence or

absence of 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP, an upregulator of ABCA1[8]. ApoB containing proteins were removed from plasma by polyethylene glycol precipitation. Efflux media containing the equivalent of 1% apolipoprotein B–depleted serum or plasma was then incubated for 2 hours at 37°C. Each patient sample was run in duplicate in both the presence and the absence of cyclic AMP. Media was collected and radioactivity determined by liquid scintillation counting after passing through a 0.22 μM filter. The quantity of radioactive cholesterol incorporated into cellular lipids was determined after isopropanol extraction[7]. Percent efflux was calculated by the formula: [cpm of ^{3}H cholesterol in the media/ (cpm of ^{3}H cholesterol in the cells + cpm of ^{3}H cholesterol in the media)] × 100. A pooled plasma control was included on each plate to which samples from patients were normalized. ATP-binding cassette transporter-1 (ABCA1)- mediated cholesterol efflux capacity was determined by subtracting the basal cholesterol efflux capacity (without cAMP) from the total cholesterol efflux capacity (with cAMP).

Lecithin-cholesterol acyltransferase (LCAT) activity: Patient plasma or serum samples were equilibrated overnight at 4°C in the presence of ^{3}H cholesterol. Samples were then incubated at 37°C for two hours. Ethanol was added to each sample to terminate the LCAT reaction. Free cholesterol and cholesterol ester fractions were subsequently separated by column chromatography. Liquid scintillation counting was used to determine number of counts in both the free cholesterol and the cholesterol ester fraction of the sample. LCAT activity (nmol/hr/ml) = (Free cholesterol concentration) x (% free cholesterol esterified)[9]. Each sample was run in duplicates to control for interassay variation, a control plasma sample was included in each assay to which all of the patient samples were normalized.

Phospholipid transfer protein (PLTP) activity: PLTP activity was measured using the Kamiya Biomedical PLTP activity assay (Cat. No. KT-206).

Nuclear Magnetic Resonance: Particle size was measured by NMR spectroscopy using the LipoProfile-3 algorithm at LipoScience, Inc. (Raleigh, NC). VLDL, LDL, IDL, and HDL subclasses of different size were quantified from the amplitudes of their spectroscopically distinct lipid methyl group NMR signals. Large HDL particle subclass diameters range from 9.4 nm to 14 nm, medium HDL particle subclass diameters range from 8.2 nm to 9.4 nm, and small HDL particle subclass diameters range from 7.3 nm to 8.2 nm.

Statistical Analysis: Data was analyzed using a logistic regression model in which data was looked at without statistical adjustment.

CHAPTER 3

Characteristics of the 55 cases and 120 controls are shown in table 1. Mean age at participation was 64 ± 11 for the cases and 69 ± 12 for the controls with 36.4% and 40% of them being female respectively. The mean age of onset of coronary disease was approximated to be around age 60 in female cases and 61 in male cases. For subjects whose age of onset of coronary disease was not available, the age at participation was substituted, thus the mean age of onset may actually be lower.

Plasma lipid values for the cases and controls are depicted in table 2. There is no difference in the mean HDL-C between the cases and controls (86 ± 21 mg/dL vs. 86 ± 20 mg/dL) as cases and controls were matched for HDL-C level. LDL-C is significantly lower in the cases as compared to the controls (97 ± 38 mg/dL vs.125 ± 33 mg/dL) as are total cholesterol (201 ± 47 mg/dL vs. 228 ± 37 mg/dL) and ApoB (77 ± 21 mg/dL vs. 89 ± 19 mg/dL). These differences can be attributed to treatment of the cases with statin therapy. Median Lpa values for cases and controls are 22.5 mg/dL and 13.7 mg/dL respectively. No difference was observed in ApoA1 or ApoA2 levels between cases and controls (195 ± 42 mg/dL vs. 194 ± 40 mg/dL and 43 ± 11 mg/dL vs. 40 ± 15 mg/dL respectively). No difference was observed in ApoC3 levels (15 ± 5 mg/dL vs. 13 ± 5 mg/dL); however a reduction in ApoE was noted in the cases (5 ± 2 mg/dL vs. 6 ± 2 mg/dL).

A comparison of the composition of HDL between cases and controls is shown in table 3. Although HDL-C did not change, HDL phospholipid concentrations were reduced by approximately 15% in cases as compared to controls (92 ± 37 mg/dL vs. 109 ± 43 mg/dL, p value 0.0095). Total phospholipid content was also reduced in cases (253 ± 55 mg/dL vs. 274

± 52 mg/dL, p value 0.017). No change was observed in the non-HDL phospholipid (162 ± 48 mg/dL vs. 165 ± 57 mg/dL) in cases and controls, suggesting that the difference in total phospholipid is attributed to the HDL fraction of the phospholipid. HDL triglyceride levels were elevated in cases as compared to controls (13 ± 9 mg/dL vs. 11 ± 4 mg/dL).

HDL particle number and size remain unchanged between cases and controls:

No difference was observed in total HDL particle number (40 ± 8 μmol/L vs. 39 ± 8 μmol/L, p value 0.46) or in the number of large HDL particles (12 ± 5 μmol/L vs. 13 ± 4 μmol/L, p value 0.87), medium HDL particles (11 ± 6 μmol/L vs. 11 ± 7 μmol/L, p value 0.68), and small HDL particles (17 ± 6 μmol/L vs. 16 ± 7 μmol/L, p value 0.55) between cases and controls (table 3).

Total cholesterol efflux and ABCA1- mediated cholesterol efflux is reduced in subjects with high HDL-C and CAD:

A functional analysis of HDL was also determined. The cholesterol efflux capacity of HDL from patient plasma was determined (table 4). Total cholesterol efflux is significantly reduced in cases (1.96 ± 0.39 %efflux/ 2hr/ 1% plasma vs. 2.11 ± 0.43 %efflux/ 2hr/ 1% plasma, p value 0.040). Similarly, a significant reduction in ABCA1- mediated cholesterol efflux capacity (defined as efflux + cAMP minus –cAMP) was noted in the cases (0.60 ± 0.24 %efflux/ 2hr/ 1% plasma vs. 0.7 1± 0.32 %efflux/ 2hr/ 1% plasma, p value 0.033). There is a reduction in total efflux to HDL-C ratio in cases (0.023 ± 0.005 %efflux/ 2hr/ 1% plasma/ mg/dL HDL-C vs. 0.025 ± 0.006 %efflux/ 2hr/ 1% plasma/ mg/dL HDL-C, p value 0.029).

No significant change was observed in the total efflux to ApoA1 ratio between cases and controls (0.010 ± 0.002 %efflux/ 2hr/ 1% plasma/ mg/dL ApoA1 vs. 0.011 ± 0.004 %efflux/ 2hr/ 1% plasma/ mg/dL ApoA1).

Given the observed difference between cases and controls in HDL phospholipid and cholesterol efflux capacity, we investigated the hypothesis that there may be a causal relationship between HDL PL and cholesterol efflux capacity. We compared total and ABCA1 mediated cholesterol efflux per HDL PL in cases and controls and found that the previously observed individual differences were no longer significant (0.02 ± 0.007 %efflux/ 2hr/ 1% plasma per mg/dL HDL PL vs. 0.02 ± 0.008 %efflux/ 2hr/ 1% plasma per mg/dL HDL PL, p value 0.06 and 0.008 ± 0.004 %efflux/ 2hr/ 1% plasma per mg/dL HDL PL vs. 0.007 ± 0.073 %efflux/ 2hr/ 1% plasma per mg/dL HDL PL, p value 0.77 respectively).

LCAT and PLTP activity remain unchanged

Additional functions of HDL include the esterification of plasma cholesterol by LCAT and the transfer of plasma phospholipids by PLTP. No difference was observed between cases and controls in either LCAT activity (153.73 ± 55.23 nmol esterified/ hr/ mL vs.169.28 ± 56.13 nmol esterified/ hr/ mL, p value 0.30) or PLTP activity (0.49 ± 0.18 nmol/mL/min vs. 0.51 ± 0.20 nmol/mL/min, p value 0.46) as depicted in table 4.

CHAPTER 4

Due to recent clinical data with niacin[5,10] and CETP inhibitors[11,12] suggesting that absolute levels of HDL-C may not consistently correlate with the extent of cardioprotection, we approached our study by looking at one of the important functions of HDL, cholesterol efflux capacity. Cholesterol efflux is the first step of the reverse cholesterol pathway that can be assessed separately from the rest of the pathway by a method developed by Rader et al[7]. In this study we observed a significant reduction in cholesterol efflux capacity by the radiolabeled method in the subjects with HDL cholesterol levels above the 90th percentile and coronary disease. Taken together with the reduction of efflux per HDL particle suggests that the cases may have dysfunctional HDL as compared to the controls. These findings may be, in part, the reason for development of coronary disease in our study population. Several differences were observed between cases and controls in HDL particle composition. The reduction in ApoE and HDL phospholipid as well as the increase in HDL triglyceride may contribute to the reduced atheroprotection seen in the cases. These findings were particularly interesting given that there was no difference in HDL-C, ApoA1, or ApoA2 levels. Piperi et al. have shown an association between HDL phospholipid and the development of coronary disease[13]. Additional analyses of cholesterol efflux capacity per mg/dL of HDL PL no longer showed a difference, suggesting that the two parameters are interrelated. Taken together, the reduction in HDL phospholipid levels and the increase in HDL triglyceride levels may play a causative role in the reduced cholesterol efflux capacity of HDL, thereby aiding the progression of coronary disease.

A significant difference was observed in LDL-C and ApoB levels between the cases

and controls. This difference was attributed to the fact that the cases had known coronary disease and were treated with LDL-C lowering therapy, namely statins.

The reduction in total phospholipid and HDL phospholipid in the cases led us to hypothesize that the cases would have smaller HDL particles as compared to controls. However, there was no difference in HDL particle size as measured by nuclear magnetic resonance (NMR). Potential limitations include the utility of NMR for measuring HDL particle size as our population was anomalous due to the high HDL levels. Additionally, samples were run on NMR without precipitation of ApoB containing lipoproteins. Thus it is possible that some of the largest HDL particles may have come out with the small LDL fraction. In addition to HDL particle size, we hypothesized that PLTP activity would be higher in the cases, however we found no significant differences between the two groups. The etiology and effect of the reduction in HDL phospholipid in subjects with coronary artery disease remains to be determined.

Our findings suggest that impaired cholesterol efflux capacity may contribute to the development of coronary artery disease in a subset of patients with very high HDL-C levels. A major limitation of this study is that the baseline characteristics of these subjects prior to the onset of coronary disease. To minimize this problem, we excluded any subjects on significant doses of HDL-raising medications. We selected controls that were either the same age or older than the cases. The lack of prospective data also makes it uncertain whether the reduction in cholesterol efflux capacity is a cause or consequence of the cardiac event. Another limitation in our study is the assessment of only one of the major functions of HDL. HDL is a heterogeneous particle that has additional antioxidant and anti-inflammatory

functions, which when all considered together, may give a more comprehensive picture of HDL functionality.

In conclusion, these findings demonstrate the heterogeneity in the structure and function of the HDL particle and suggest that these differences may impact on the development of coronary disease. Findings from this study may impact the clinical management of patients with high plasma levels of HDL-C who may be lulled into a false sense of security by using traditional cardiovascular risk assessment methods.

LIST OF TABLES

Table 1: Basic Demographics

	Cases	Controls	P value
N	55	120	-
Age (mean ± SD)	64 ± 11	69 ± 12	0.07
Female (%)	36.4	40	0.65
African American (%)	3.6	6.1	0.95

Table 2: Plasma Lipid Values

	Cases	Controls	P value
Triglycerides	80 ± 34	85 ± 38	0.34
Total Cholesterol	201 ± 47	228 ± 37††	0.0003
HDL-C	86 ± 21	86 ± 20	0.97
LDL-C	97 ± 38	125 ± 33††	1.6 e-5
ApoB	77 ± 21	89 ± 19††	0.0007
Lp(a)	22.5*	13.7*	0.1
ApoA1	195 ± 42	194 ± 40	0.91
ApoA2	43 ± 11	40 ± 15	0.40
ApoC3	15 ± 5	13 ± 5	0.89
ApoE	5 ± 2	6 ± 2†	0.046
Total phospholipid	253 ± 55	274 ± 52†	0.017

Lipid and apolipoprotein parameters are mg/dl.

† p <0.05 compared to cases, †† p <0.001

* Data reported as median

Table 3: HDL composition

	Cases	Controls	P value
HDL-C	86 ± 21	86 ± 20	0.97
HDL Phospholipid	92 ± 37	109 ± 43†	0.0095
Non HDL Phospholipid	162 ± 48	165 ± 57	0.48
HDL Triglyceride	13 ± 9	11 ± 4†	0.049
Total HDL Particle number	40. ± 8	39 ± 8	0.46
Large HDL Particle number	12 ± 5	13 ± 4	0.87
Medium HDL Particle number	11 ± 6	11 ± 7	0.68
Small HDL Particle number	17 ± 6	16 ± 7	0.55

Lipid and apolipoprotein parameters are mg/dL. HDL particle number is reported in μmol/L

† p <0.05 compared to cases, †† p <0.001

Table 4: Functional Properties of HDL

	Cases	Controls	P value
Total efflux	1.96 ± 0.39	2.11 ± 0.43†	0.040
ABCA1- mediated	0.60 ± 0.24	0.71 ± 0.32†	0.033
Total efflux/ HDL-C	0.023 ± 0.005	0.025 ± 0.006†	0.029
Total efflux/ ApoA1	0.010 ± 0.002	0.011 ± 0.004	0.20
Total efflux/ HDL PL	0.02 ± 0.007	0.02 ± 0.008	0.06

ABCA1 efflux/ HDL PL	0.008 ± 0.004	0.007 ± 0.073	0.77
Total efflux/ HDL TG	0.18 ± 0.08	0.20 ± 0.07	0.12
ABCA1 efflux/ HDL TG	0.05 ± 0.03	0.07 ± 0.04[†]	0.02
LCAT activity	153 ± 55	169 ± 56	0.30
PLTP activity	0.49 ± 0.18	0.51 ± 0.20	0.46

Efflux activity given as %efflux/ 2hr/ 1% plasma, LCAT activity is given in nmol esterified/ hr/ mL. PLTP activity is given in nmol/mL/min.

[†] $p < 0.05$ compared to cases

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VITAE

Anandita Agarwala (June 21st 1987-present) is a 4th year medical student at the University of Texas Southwestern Medical Center at Dallas. She completed her undergraduate studies at The University of Texas at Austin where she received a B.S. in Biology and a B.A. in French. During her medical school training, Anandita took a year off to participate in the Doris Duke Clinical Research Fellowship at the University of Pennsylvania where she worked with Dr. Daniel J. Rader. Her work for her thesis was completed in Dr. Rader's laboratory. She will be starting her internal medicine residency in July. After completing residency, Anandita will pursue a cardiology fellowship and would like to have a career in academic medicine.

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