VARIABILITY OF CHOLESTEROL ACCESSIBILITY IN HUMAN RED BLOOD CELLS MEASURED USING A BACTERIAL CHOLESTEROL-BINDING TOXIN

by

RIMA SHAH CHAKRABARTI

DISSERTATION

Presented to the Faculty of the Medical School
The University of Texas Southwestern Medical Center
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

SUPPORTED BY THE SUPERVISORY COMMITTEE

Committee Chairperson's Name_____
Arun Radhakrishnan, PhD

Committee Member's Name_____
Jay Horton, MD

Committee Member's Name_____
Sandra Hofmann, MD, PhD

ABSTRACT

VARIABILITY OF CHOLESTEROL ACCESSIBILITY IN HUMAN RED BLOOD CELLS MEASURED USING A BACTERIAL CHOLESTEROL-BINDING TOXIN

RIMA SHAH CHAKRABARTI
The University of Texas Southwestern Medical Center, 2017
Supervising Professor: Helen Hobbs, M.D.

Background: The two major sources of cholesterol in humans are diet, which provides approximately 200 mg per day, and endogenous synthesis, which generates about one gram per day. To maintain cholesterol homeostasis, an equivalent amount of cholesterol must be removed from the body. Cholesterol cannot be degraded and must be transported to the liver for export into bile. This process, referred to as reverse cholesterol transport (RCT), remains poorly understood. HDL has been proposed as the primary mediator of RCT, but recent studies have revealed that HDL-deficient animals have no defect in tissue cholesterol balance. Another reservoir of cholesterol in human circulation is red blood cell (RBC) membranes, which contain an amount of cholesterol comparable to that found in lipoproteins. RBC membrane cholesterol partitions into accessible and inaccessible pools largely determined by RBC phospholipid composition.

Objective: We hypothesize that accessible cholesterol on an intact RBC membrane is measurable and is modulated by lipoproteins and other factors in plasma that participate in reverse cholesterol transport.

Methods: We describe an assay using fluorescently-tagged anthrolysin O (fALOD4), a cholesterol-binding bacterial toxin, to measure accessible cholesterol in intact human red blood cells. For this assay, RBCs from individuals are isolated, incubated with fALOD4, and the fluorescence intensity of the cells is quantified using flow cytometry.

Results: Accessible cholesterol in RBC membranes as measured by our assay is stable within individuals but varies >10-fold among individuals. Variation was unrelated to total cholesterol content, but was associated with differences in the phospholipid composition of the RBC membrane as well as with plasma levels of high density lipoprotein (HDL)-cholesterol and triglycerides (TG). Accessible cholesterol was very low in individuals with ABCA1 deficiency, whereas those with LCAT deficiency, who had similarly low HDL levels, had more modest reductions in RBC cholesterol accessibility. Plasma from various healthy individuals differentially modulated RBC cholesterol accessibility. Additionally, individuals on hemodialysis, who have unexplained increase in atherosclerotic risk, had significantly higher RBC cholesterol accessibility.

Conclusion: Our data indicate that RBC accessible cholesterol is a stable phenotype with inter-individual variability that is dependent on factors both intrinsic and extrinsic to the RBC. We speculate that variation in RBC cholesterol accessibility reflects differences in reverse cholesterol flux and that this phenotype may provide a new tool to assess cholesterol trafficking between tissues in humans.

TABLE OF CONTENTS

PRIOR PUBLICATIONS & PRESENTATIONS

PUBLICATIONS:

Chakrabarti R.S., Ingham S.A., Kozlitina J., Gay A., Cohen J.C., Radhakrishnan A., Hobbs H.H. Variability of cholesterol accessibility in human red blood cells measured using a bacterial cholesterol-binding toxin. eLife 2017: doi: 10.7554/elife.23355. PMCID: PMC5323040

PRESENTATIONS AND POSTERS:

Chakrabarti RS. A Bacterial Sensor for RBC Cholesterol Accessibility. Selected Oral Presentation at HHMI Medical Fellows Annual Meeting. HHMI headquarters, Chevy Chase MD. May 2014 & 2015.

Chakrabarti RS. Developing a Bacterial Sensor for Cholesterol Accessibility in RBCs. Oral Presentation at UTSW Department of Molecular Genetics noon seminar. UTSW. Dallas TX. May 2014 & February 2015.

Chakrabarti RS. Developing a Bacterial Sensor for Cholesterol Accessibility. Oral Presentation at McDermott Center for Human Growth and Development weekly seminar. UTSW. Dallas TX. January 2015.

Chakrabarti RS, Radhakrishnan A, Cohen JC, Hobbs HH. A Bacterial Sensor for RBC Cholesterol Accessibility. Poster Presentation at UTSW Medical Student Research Forum. UTSW. Dallas TX. January 2016.

Chakrabarti RS, Radhakrishnan A, JC Cohen, HH Hobbs. A Bacterial Sensor for RBC Cholesterol Accessibility. Poster Presentation at American Society of Clinical Investigation annual conference Chicago, IL. April 2015.

Chakrabarti RS, Radhakrishnan A, Cohen JC, Hobbs HH. A Bacterial Sensor for Cholesterol Accessibility in RBCs. Poster Presentation at HHMI Investigators Meeting at Janelia Research Campus. Ashburn, VA. January 2015.

INTRODUCTION

Cholesterol is an essential component of vertebrate cell membranes (1). Membrane cholesterol is supplied by the diet or synthesized endogenously from acetyl coenzyme A. All cells in the body except red blood cells (RBCs) synthesize cholesterol. To maintain cholesterol balance, an amount of cholesterol equivalent to the sum of what is made in cells and acquired from the diet must be removed from the body. The major pathway for cholesterol excretion in humans is via the biliary system. The proteins and processes by which cholesterol is secreted into the bile, either directly, or after conversion to bile acids, have been well characterized (2, 3). In contrast, the process by which cholesterol from peripheral tissues is delivered to the liver for excretion remains poorly defined.

A mechanism for the centripetal trafficking of cholesterol from extrahepatic tissues to the liver, which has been termed reverse cholesterol transport, was first conceptualized by John Glomset in 1968 (4). That work, and subsequent studies, have implicated high density lipoproteins (HDL) as an essential component of this pathway (5) (***Figure 1***). In the first step of the Glomset model, HDL acquires free cholesterol from cell membranes in peripheral tissues. Free cholesterol is transferred from cells to HDL by ATP binding cassette, subfamily A, member 1 (ABCA1)(6). The free cholesterol in the HDL particle is then esterified with long-chain fatty acids by lecithin-cholesterol acyltransferase (LCAT)(4) and sequestered in a hydrophobic core below the phospholipid surface of HDL (7). This step is critical to the delivery of cholesterol to the liver; sequestration in the HDL core decreases the propensity of cholesterol to exchange with cell membranes, thus limiting the retrograde transfer of cholesterol from HDL back to tissues. The cholesteryl ester is then delivered to the liver either directly via a cell surface receptor, scavenger receptor class B member 1 (SCARB1) (8), or indirectly after transferring the cholesterol to ApoB-containing lipoproteins, which then undergo endocytosis by the low density lipoprotein receptor (LDLR) (Figure 1). Several components of the HDL-mediated reverse

cholesterol transport pathway have been supported by observations made in cultured cells and *in vivo (9).*

Despite data implicating HDL as a central component of the reverse cholesterol transport pathway, some observations in murine models of HDL deficiency are not compatible with an essential role for HDL in transport of cholesterol from peripheral tissues to liver and bile (10, 11) (Figure 1). Mice lacking ABCA1, or Apolipoprotein (Apo)A1, the major structural protein of HDL, have very low HDL levels and yet have no alterations in cholesterol turnover in peripheral tissues (10, 11).

Glomset proposed that RBCs may participate in the transport of cholesterol from tissues to the liver (4). Approximately 50% of circulating cholesterol is carried in RBC membranes and tracer studies in humans indicate that the magnitude of cholesterol flux through RBCs is comparable to the total efflux of free cholesterol from tissues (12). Cholesterol exchanges freely between RBCs, plasma, circulating lipoproteins, and cells (13) (Figure 1). More recently, Smith and colleagues showed that reducing the number of RBCs in ApoAI knockout mice, which have little to no circulating HDL, decreases transfer of radiolabeled cholesterol from macrophages to feces (14).

For RBCs to participate in reverse cholesterol transport, the cholesterol must be located in the outer leaflet of the RBC membrane and thus accessible for transfer to proteins and cells. The accessibility of cholesterol in a membrane is related to its chemical activity (15), alternatively referred to as its fugacity, which generally increases with increasing cholesterol content. At lower concentrations, cholesterol forms complexes with phospholipids, thus decreasing its activity below what would be expected in the absence of lipid-lipid interactions. As cholesterol levels increase, accessibility increases, as measured by cyclodextrin extraction (16), oxidation by cholesterol oxidase (17, 18), and by binding of cholesterol-dependent cytolysins such as perfringolysin O (PFO) or anthrolysin O (ALO) (19, 20). The increase in cholesterol accessibility with increasing cholesterol concentration in RBC membranes is

sigmoidal, rather than linear, with little change until a threshold concentration is reached (21). Once the cholesterol content of the membrane exceeds this threshold, accessibility of the membrane cholesterol increases sharply (21).

The partitioning of cholesterol between accessible and sequestered pools may allow RBCs to serve as vehicles for cholesterol transport from peripheral tissues to the liver. As a first step towards elucidating the relative contributions of the accessible and inaccessible pools of RBC cholesterol to cholesterol transport, we developed an assay using ALO, a cholesterol-binding toxin produced by *Bacillus anthracis* (22), to measure the relative amount of accessible cholesterol in RBC membranes.

Using this assay, we show that the RBC cholesterol accessibility is stable within individuals, but can vary more than ten-fold among individuals. Significant ethnic differences in RBC cholesterol accessibility were found. The variation in cholesterol accessibility was not related to the cholesterol content of the membrane, but rather to the content of other membrane lipids and to levels of circulating lipids and lipoproteins. Inactivation of key proteins in reverse cholesterol transport that are associated with major reductions in HDL-cholesterol (C) levels have different effects on RBC cholesterol accessibility. ABCA1 deficiency was associated with extremely low RBC cholesterol accessibility, whereas the lack of LCAT caused a much less dramatic reduction in activity. We find that a group of patients on hemodialysis, who have more coronary heart disease than anticipated based on known risk factors (23), have higher RBC cholesterol accessibility. Finally, we find that plasma is able change RBC cholesterol accessibility and that there is significant variability in the capacity of various individual's plasma to modulate RBC cholesterol accessibility.

EXPERIMENTAL PROCEDURES

Materials

Methyl-β-cyclodextrin (MCD) and hydroxypropyl-β-cyclodextrin (HPCD) were obtained from Cyclodextrin Technologies Development, Inc (Alachua, FL). Dithiothreitol (DTT) and pronase (Cat. No. 10165921001) were purchased from Roche (Indianapolis, IN). Phospholipase A2 (Cat. No. P9279), sphingomyelinase (SMase) (Cat. No. S8633), and Tris (2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO), and Alexa Fluor 488 C_5-maleimide, EZ-Link Sulfo-NHS-LC-Biotin, and Pierce High Sensitivity Streptavidin-HRP and SuperSignal West Pico Chemiluminescent substrate were purchased from Thermo Fisher Scientific (Waltham, MA). Newborn calf lipoprotein-deficient serum (NCLPPS) was prepared by ultracentrifugation as previously described (48). Stock solutions of cholesterol/MCD complexes were prepared at a final cholesterol concentration of 2.5 mM and a cholesterol: MCD molar ratio of 1:10 as described (49).

Buffer A contained 50 mM Tris-HCl (pH 7.5) and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP); buffer B contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM dithiothreitol (DTT); buffer C contained 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 12% (v/v) glycerol; buffer D contained phosphate buffered saline (PBS) supplemented with 1 mM EDTA and 2% (v/v) NCLPPS; buffer E contained 10 mM Tris-HCl (pH 7.5); Buffer F contains 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% (w/v) SDS, and 1 mM DTT.

Protein purification and labeling with fluorescent dye

A pRSETB expression vector (Life Technologies, Carlsbad, CA) that encodes domain 4 (amino acids 404-512) of anthrolysin O (ALO) (ALOD4) from *Bacillus Anthracis* (22), which contains two point mutations (S404C and C472A) and has a His_6 tag at the NH_2-terminus was used for these studies (20). Recombinant ALOD4 was purified from *E. coli* expressing ALOD4 using nickel chromatography as described (20). Typical yields from a 6L bacterial culture were ~10-15 mg.

Purified ALOD4 was concentrated to ~0.8 mg/mL (final volume 15 mL) using a 10,000 MWCO Amicon Ultra Centrifugal Filter (EMD Millipore. Billerica, MA) and mixed with 135 mL of buffer A to lower the salt concentration to a final value of 10 mM NaCl. The mixture was applied to a 1 mL HiTrap Q Sepharose High Performance column (GE Healthcare, Little Chalfont, UK) that had been pre-equilibrated in buffer B. After the column was washed with 100 column volumes of buffer A and 10 column volumes of buffer A plus 50 mM NaCl, ALOD4 was eluted with NaCl into a single 2 mL fraction using buffer A containing 500 mM NaCl. The final concentration of ALOD4 (MW, 16.2 kDa) was between 6-10 mg/mL.

Each labeling reaction contained 200 nmoles ALOD4 and 1400 nmoles Alexa Fluor 488 C_5-maleimide dye in buffer B (1 ml) with 250 mM NaCl. After incubation for 16 h at 4°C, unincorporated dye was removed by passing the sample over Ni-NTA agarose beads (1 mL) pre-equilibrated with buffer B. The column was washed with 10 column volumes of buffer B containing 50 mM imidazole. Bound proteins were eluted with buffer B containing 300 mM imidazole. Fractions were subjected to SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining and those containing protein were pooled and concentrated by anion exchange chromatography, as described above. Fluorescently labeled protein, referred to as fALOD4, was diluted to a final concentration of 30 µM in buffer C, aliquoted, flash frozen, and stored at -80°C. fALOD4 concentrations were measured using the Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientifc). Alexa Fluor 488 dye concentrations were measured with a NanoDrop instrument (Thermos Scientific, Wilmington, DE) using the extinction coefficient of Alexa Fluor 488 (ε_{495} = 76,000 M^{-1} cm^{-1}). The degree of labeling of fALOD4 by Alexa Fluor 488 ranged from 0.64-0.95.

Human subjects

The study was performed following the guidelines of the local medical ethical committee and in accordance with the declaration of Helsinki. The study protocol was approved by the

Institutional Review Board of UT Southwestern Medical Center. Written informed consent was obtained from all blood donors prior to participation in this study. Venous blood samples (7 ml) were obtained from individuals who had fasted at least 8 h. Blood samples from hemodialysis patients were obtained immediately prior to dialysis. Blood was collected in citrate-EDTA containing tubes (BD Medical Supplies, Franklin Lakes, NJ). Each study participant completed a detailed survey with information including weight, gender, BMI, socioeconomic status, medical history, and current and past medication use. Race and ethnicity were self-reported. A complete blood count [red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC) count, platelets] and measurement of plasma levels of lipids (cholesterol and triglyceride) and lipoproteins were performed by Quest Diagnostics. Blood typing for ABO and Rhesus antigen (D antigen) was performed at Carter Bloodcare in Dallas, TX for the blood samples that were obtained from that organization.

Red blood cell and plasma isolation

For isolation of the RBCs, the blood samples were maintained at 4°C after collection. Within 3 h of collection, the samples were subjected to centrifugation at 1500 x *g* for 10 min at 4°C. Plasma was transferred to a separate tube and the RBCs were resuspended in 4 mL ice-cold PBS. The RBCs in PBS were stored at 4°C until used for experiments, which were performed within 5 days. The plasma was aliquoted into 1.5 mL Eppendorf tubes and centrifuged at 2500 x *g* at room temperature for 15 minutes to remove platelets and additional RBCs. The supernatant was aliquoted into 1.5 mL Eppendorf tubes, flash frozen, and stored at -80°C until use.

Assay for fALOD4 binding to RBCs

For the first set of assays, 5 µL of RBCs (~$2x10^7$ cells) was diluted in 495 µL ice-cold PBS with gentle mixing and then subjected to centrifugation at 2500 x *g* for 2.5 min at 4°C. The pellet was resuspended in 500 µL ice-cold PBS, and the above procedure was repeated twice. The resulting pellet was resuspended in 500 µL ice-cold PBS. A total of 5 µL (~$2.5x10^5$ cells) were added to 490 µL of buffer D and 5 µL of buffer C containing the indicated amounts of fALOD4. After incubation for 3 h at 4°C on a rotator, samples were subjected to centrifugation at 2500 x *g* for 2.5 min at 4°C. The resulting supernatant was saved for analysis of released hemoglobin, and the pellet containing RBCs and bound fALOD4 was resuspended in 200 µL PBS and transferred to 12 x 75 mm round bottom polystyrene tubes (Falcon, Corning, NY) for flow cytometry analysis. For the second set of assays, fALOD4 binding assays were performed by diluting 10 µL of RBCs in 1mL ice-cold PBS with gentle mixing and then centrifuged at 2500 x g for 2.5 min at 4°C. The pellet was resuspended in 1 mL ice-cold PBS and the above washing procedure was repeated twice. A total of 10 µL of this washed dilution (~4 x 10^5 RBCs) was suspended in 485 µL ice-cold buffer D and 5 µL buffer C containing 30 µM fALOD4. The remainder of the protocol was carried out as described above.

Flow cytometry analysis of fALOD4 binding to RBCs

All data acquisition was obtained using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), equipped with an argon ion laser (488 nm) to detect Alexa Fluor 488 dye (excitation maximum: 493 nm; emission maximum: 516 nm) on RBC-bound fALOD4. Using CellQuest software (BD Biosciences), the detector for forward-scatter (FSC) was set in linear mode, and detectors for side-scatter (SSC) and FL1 (to monitor Alexa Fluor 488) were set to logarithmic mode. A total of 10,000 RBCs were analyzed for each experimental condition unless otherwise indicated. A control sample containing untreated RBCs was used to calibrate the detectors and to correct for auto-fluorescence of samples. The FSC/SSC profile of the control sample was

used to gate around RBCs with normal morphology and separate fluorescently labeled RBCS from unlabeled cells. Flow cytometry data analysis was conducted using FlowJo software version 9 (Ashland, OR). Median fluorescent intensity (MFI) of each population of fluorescently labeled RBCs was calculated and was used to quantify fALOD4 binding per RBC throughout this study.

The median Alexa Fluor 488 signal per RBC is hereafter referred to as fALOD4 binding per cell. fALOD4 binding values for each sample were normalized to the binding value obtained from a reference blood sample. The reference blood sample was collected from the same healthy subject and processed with the samples in each experiment.

Modulation of RBC cholesterol

RBCs were isolated from whole blood as described above. In a typical experiment, a 1:10 dilution of RBCs in 10 mL ice-cold PBS was centrifuged at 2500 x g for 5 min at 4°C. This washing procedure was repeated two times. Reaction mixtures contained 1 mL of RBCs (~$4x10^8$ cells) and either 0-2% (w/v) HPCD (to deplete RBC cholesterol) or 0-75 μM cholesterol/MCD complexes (to increase RBC cholesterol) in a final volume of 2 mL ice-cold PBS. After incubation for 1 h at 4°C on a rotator, samples were subjected to centrifugation at 2500 x *g* for 2.5 min at 4°C. The resulting supernatant was removed and saved for analysis of released hemoglobin. The RBC-containing pellets were washed three times in 1 mL of ice-cold PBS as described above. After the final wash, the pellet containing the RBCs was resuspended in 1 mL ice-cold PBS. An aliquot of the RBCs (10 μL of total, ~4 x 10^6 RBCs) was diluted in 100 μL PBS and used for fALOD4 binding assays and flow cytometry analysis as described above. The remainder of RBCs was used for cholesterol quantification (see below).

Preparation of RBC ghost membranes

RBC ghost membranes were prepared by a modified Dodge method (50). The RBC suspension was centrifuged at 2500 x g for 2.5 minutes at 4ºC. The supernatant was discarded and pellet was subjected to hemolysis by resuspension of the RBCs (990 µL) in 1 mL of hypotonic buffer E and subjecting the mixture to vigorous vortexing. The lysed RBCs were subjected to centrifugation at 13,100 x *g* for 15 min at room temperature and the supernatant was discarded. The pellet, which contained membranes from lysed RBCs and intact RBCs, was subjected to additional rounds of lysis and centrifugation until the 13,100 x *g* supernatant was clear. The pellet from the final centrifugation step, designated ghost membranes, was reconstituted in 500 µL PBS and stored at -20°C if not used immediately.

Quantification of released hemoglobin

Aliquots of 2500 x *g* supernatants (100 µL) generated during fALOD4 binding assays, RBC cholesterol modulation assays, and proteolytic cleavage assays, were transferred to 96 well clear bottom plates (Evergreen Scientific, Los Angeles, CA). Amounts of hemoglobin released from the RBCs during the assay were quantified using a microplate reader (BioTek, Winooski, VT) by measuring absorbance at 540 nm. As controls for hemolysis, an equal number of RBCs were mixed with 1% Triton-X detergent (0.5 mL final volume) or with PBS buffer alone. The samples were subjected to centrifugation (2500 x *g* for 2.5 min) and 100 µl aliquots of the supernatant were transferred to 96-well plates. Percent of hemolysis was calculated as [Hb-absorbance of sample / Hb-absorbance of positive control]*100.

Proteolytic cleavage of human RBC surface proteins

Cell surface proteins on RBC membranes were modified with biotin as described previously (51). Each reaction, in a final volume of 1 ml ice-cold PBS, contained EZ-Link™ Sulfo-NHS-LC-Biotin (0.5 mg) and 500 µl (2 x 10^7 cells) of washed RBCs. After a 30 min incubation at 4°C

on a rotator, the RBCs were washed 3 times with PBS supplemented with 100 mM glycine to remove excess biotin. The biotin-treated RBCs were resuspended in 0.5 mL ice-cold PBS. After incubation for 30 min at 4°C on a rotator, reactions were subjected to centrifugation at 2500 x *g* for 2.5 min at 4°C. The resulting supernatant was discarded and the RBC pellet was resuspended in ice-cold PBS containing 100 mM glycine to remove excess biotin. After two additional rounds of centrifugation and resuspension in PBS containing 100 mM glycine, the RBCs pellets were resuspended in 500 µL ice-cold PBS (without glycine).

RBC surface protein cleavage was carried out by pronase treatment. Pronase (0-300 µg) was added to 130 µL (~$5x10^6$ cells) of biotin-modified RBCs in a final volume of 500 µL. After a 1 h incubation at 40°C on a rotator, the samples were subjected to centrifugation at 2500 x *g* for 2.5 min at room temperature and the released hemoglobin was measured in the supernatants. RBC pellets were washed three times in PBS at room temperature and then resuspended in 130 µL PBS. An aliquot (15 µL) was used for fALOD4 binding assay and the remaining RBCs (115 µL) were subjected to centrifugation at 2500 x *g* for 2.5 min at room temperature. The pellet was resuspended in 40 µL of SDS-containing Buffer F and vortexed before being subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes and then incubated in 1.25% milk in the absence and then presence of Pierce high affinity streptavidin-HRP (0.22 µg/mL) (Thermo Fisher Scientific) to detect the biotinylated proteins. Bound streptavidin-HRP was visualized after treatment of the membranes with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) using an Odyssey FC Imaging machine (LI-COR Biosciences, Lincoln, NE).

Analysis of RBC lipids

To measure cholesterol, ghost membranes generated from ~$5x10^8$ RBCs as described above. Lipids were extracted using the Foch extraction method (52) . An aliquot of the extracted lipids (10% of total) was used to measure cholesterol by Infinity cholesterol reagent (Thermo Fisher

Scientific) or by gas chromatography (53). Another aliquot (10% of total) was used to measure choline-containing phospholipid with the Wako Phospholipids C enzymatic kit (Wako Diagnostics, Mountain View, CA). This value was divided by a calibration factor of 0.55 to estimate the total amount of RBC phospholipids. The calibration factor was derived from previous quantitative studies where it was determined that choline-containing phospholipids make up 55% of RBC phospholipids (54). Mole percentages of cholesterol were calculated by dividing moles of cholesterol by moles of total lipids (cholesterol plus phospholipids) and multiplying the result by 100. We used 389 Da for the molecular mass of cholesterol and 800 Da as an estimate for the average molecular mass of a phospholipid.

To measure RBC phospholipids, the ghost membranes were prepared as described above and lipids were extracted using a modified Bigh-Dyer extraction (1:1:1 mixture of dichloromethane: methanol: PBS) (Bligh EG, Dyer WJ 1959). In short, membranes were suspended in a volume of 3 mL PBS and added to an 8 mL, 13x100 mm glass culture tube (Fisherbrand, Pittsburgh, PA) containing 3 mL methanol that was immersed in a water bath sonicator (Branson model # B2510MT, Danbury, CT) set a maximum speed. After removal of the tube from the sonicator, a total of 3 mL of dichloromethane (DCM) was added to the tube. The samples were capped (Fisherbrand black phenolic Teflon coated screw cap), vortexed, and centrifuged at 2,500 rpm for 5 min at room temperature. The bottom phase, which contained the lipids, was transferred to a fresh glass culture tube. An additional 4 mL DCM was added to the original tube, which was again capped, vortexed and centrifuged at 2,500 rpm for 2.5 min at room temperature. The bottom phase was combined with the material from the initial phase separation (total volume of approximately 5 mL). The samples were placed at 42ºC under a gentle stream of nitrogen gas until the volume was approximately 0.5 mL. The samples were transferred to 1 mL glass culture tubes, which were again placed at 42ºC under a gentle stream of nitrogen gas until all solvent had evaporated. The glass tubes containing the dried lipids were

immediately capped, flash frozen with liquid nitrogen, placed on dry ice, and stored at -80ºC until they were shipped on dry ice to the Kansas Lipid Core for detailed analysis of polar lipids.

Phospholipase A2 (PLA2) and sphingomyelinase (SMase) treatment of RBCs

Lyophilized phospholipase A2 (PLA2) (1 mg) (Sigma Aldrich, St. Louis, MO) was suspended in 1 mL PBS. A 1:10 dilution of RBCs were isolated and washed as described above. Ringers buffer containing divalent cations (Mg2+ and Ca2+), which are required for PLA2 activity (Yu BZ 1993*), was prepared as described previously (Dinkla S 2012*) . A total of 300 µL washed RBCs ($1.6x10^8$ RBCs) and 0- 40 U/mL of PLA2 was suspended in Ringers buffer (active PLA2) or divalent ion free PBS buffer (inactive PLA2) to a final volume of 1 mL and incubated for 1 h at room temperature. RBCs were then washed three times with 1 mL ice-cold PBS buffer. fALOD4 binding assays were performed on a 3 µL aliquot (~$2x10^5$ RBCs) of PLA2 treated cells as described above.

A similar experiment was performed using neutral sphingomyelinase from Staphylococcus aureus (SMase) (0.195 mL in 0.25 M PBS containing 50% glycerol, pH 7.5) (Sigma Aldrich, St. Louis, MO). Neutral SMase, like PLA2, requires divalent cations for activity (Ago H 2006*). Therefore, an aliquot of SMase was diluted 100 fold in Ringers lactate (300 µl total volume) for the active enzyme conditions and a separate aliquot was diluted 100 fold in divalent ion free PBS buffer for the enzyme inactive control. A total of 300 µL washed RBCs ($1.6x10^8$ RBCs) and 0- 20 milliunits/mL of SMase was suspended in Ringers buffer (active SMase) or PBS buffer (inactive SMase) to a final volume of 1 mL and incubated for 1 h at 37°C on a rotator. RBCs were then washed three times with 1 mL ice-cold PBS buffer. fALOD4 binding assays were performed on a 3 µL aliquot (~$2x10^5$ RBCs) of SMase treated cells as described above.

Plasma activity assay

Fifteen microliters ($6x10^7$) RBCs were diluted in 1.5 mL ice cold PBS and were washed 3 times as described for the fALOD4 binding assay. The final pellet was resuspended in 1.5 mL ice-cold PBS. Aliquots of plasma, stored as described above, were thawed on ice and gently vortexed prior to use. A total of 30 μL of washed RBCs were added to 420 μL PBS and 50 μL of thawed plasma. After incubation for 3 h at 37°C on a rotator, RBCs were washed three times with 1 mL ice-cold PBS buffer. Resuspend final pellet in 20μL PBS. fALOD4 binding assays were performed on a 10 μL aliquot (~$2x10^5$ RBCs) of plasma treated cells as described above.

Statistical methods

Spearman's correlation and associated P values were calculated using the software package Prism 6 (GraphPad, La Jolla, CA). Differences between groups were compared using two-tailed T-tests. Data are reported as means ± SEM. P values of <0.05 were considered statistically significant.

RESULTS

Measurement of RBC Cholesterol Accessibility Using Cholesterol Binding Domain of ALO

As described previously, a recombinant protein consisting of the cholesterol-binding domain (D4) of ALO was purified from *E. coli* and its sole engineered cysteine was covalently modified with a maleimide-linked fluorescent dye (Alexa Fluor 488) (20). This reagent is referred to hereafter as fALOD4 (Figure 2*A*). An aliquot of $2.5x10^5$ RBCs was incubated with increasing amounts of fALOD4 for 3 h prior to analysis by flow cytometry. The forward and side light scatter patterns and the median fluorescence per RBC are shown in Figure 2B. Dose-response and time course experiments were performed to determine the initial conditions for the assay (Figure 2*C*). The extent of fALOD4 binding per cell increased linearly with increasing concentrations of fALOD4 (right panel). Even at the highest concentrations of fALOD4, binding did not saturate. A value within the linear range of fALOD4 binding (250 nM) was used to perform the time-course experiment (right panel). At this concentration, binding approached saturation after 3 h and this incubation time was chosen for subsequent assays. Subjecting RBCs to these assay conditions did not result in significant hemolysis (<5%).

RBC Cholesterol Accessibility was Nonlinearly Related to RBC Cholesterol Content

Next, we assessed the effect of the cholesterol content of the RBCs on fALOD4 binding. RBCs were incubated with either hydroxypropyl-β-cyclodextrin (HPCD) or cholesterol/methyl-β-cyclodextrin (MCD) to reduce or increase the cholesterol content of RBC membranes, respectively (Figure 2D, left panel). No fALOD4 binding was observed when the cholesterol content of the RBCs was reduced below ~46 mole%. Above this apparent threshold, the binding increased sharply. When the data were plotted on a log-linear scale the relationship between cholesterol content of the membranes and fALOD4 binding was linear (Figure 2D, inset, left panel). In cells incubated with increasing concentrations of HPCD, a small reduction in cholesterol content of the cell was associated with a marked reduction in fALOD4 binding

(Figure 2D, right panel). Such sigmoidal dependences on cholesterol concentration have been observed previously for the binding of cholesterol-binding toxins like ALO to model liposomes (thresholds vary from 20 mole% to 50 mole% depending on the phospholipid) (20, 24), to plasma membranes (PM) of human fibroblasts (threshold ~40 mole%)(25), and to purified ER membranes from cultured CHO-K1 cells (threshold ~5 mole%) (26).

RBC Cholesterol Accessibility Varied Between Individuals in a Reproducible and Stable Manner

We then measured fALOD4 binding to RBCs isolated from unrelated healthy individuals using flow cytometry. Independent measurements were made on different aliquots of RBCs from the same individual's sample. The data were expressed relative to a reference sample that was used in all assays and the results were expressed as normalized relative fluorescence units (nRFU). The nRFUs varied over a 10-fold range (from 0.2 to 2.6) between samples from different individuals but the correlation between the two measurements on the same sample was very high (R^2 = 0.94) (Figure 3A). The intra-assay coefficient of variation for the assay was 10.7 ± 5.3%.

To determine if fALOD4 binding is stable over time in individuals, blood was drawn from five subjects on three separate days over a 25-day interval (Figure 3B). The intra-individual variation in fALOD4 binding per cell was 8.8 ± 4.2% (range: 3.6%-12.8%). Thus, there is low intra-individual but high inter-individual variation in fALOD4 binding. Representative data showing the distributions of fALOD4 binding to RBCs for an individual with low (top), medium (middle), and high (bottom) levels of fALOD4 RBC binding are shown in ***Figure 3A***. Although the distribution of cells by size is similar in the three samples (left), the distributions of fluorescence intensity differ among these individuals (right). Since the distribution of fluorescence signal within individual samples is skewed, the median rather than mean fluorescence intensity is used as a measure of fALOD4 binding per cell in the studies to follow.

We next tested whether variations in fALOD4 binding arose because the fALOD4 probe was present in limiting amounts. For this experiment, we used RBCs from three individuals with relatively low, medium, and high fALOD4 binding per cell (***Figure 3B***). We incubated increasing amounts of RBCs with a fixed concentration of fALOD4 (250 nM). When 10^4– 10^6 RBCs were used, fALOD4 binding values clearly separated into the low, medium, and high ranges. At higher RBC concentrations, fALOD4 binding per cell decreased in all cases and resolution among high, medium and low binding was lost. Therefore, in all subsequent assays, 2.5 x 10^5 RBCs/assay were used to ensure that the amount of fALOD4 was not limiting.

To examine the relationship between fALOD4 binding and the cholesterol content of the RBC membranes, we prepared duplicate aliquots of RBCs from 27 individuals. One aliquot was used to determine fALOD4 binding. Cholesterol was measured in lipid extracts from RBC ghost membranes prepared from the second aliquot. Surprisingly, no relationship was found between the cholesterol content of the RBC membranes and fALOD4 binding (***Figure 3D***). Thus, fALOD4 binding does not simply reflect the cholesterol content of the RBC membrane.

Significant Ethnic Differences in RBC Cholesterol Accessibility

We assayed RBCs from 364 men and women of White (European-American), Black (African-American) and Hispanic (all self-assigned) ancestry who had blood drawn either at a local blood bank or as part of a convenience sample of healthy individuals in Dallas. A control sample was included in each assay and was used to normalize measurements. Since relative binding differed among the three ethnic groups, we plotted the data for each group separately. In all three groups, the distribution of fALOD4 binding to RBCs was positively skewed and varied over a > 10-fold range among the study participants (***Figure 4A***). Median fALOD4 binding was lowest in Whites (0.77 nRFU), highest in Blacks (1.18 nRFU) and intermediate in Hispanics (0.86 nRFU) ($P<0.0001$). A summary of the demographic and clinical variables in the sample is provided in Table 1. The ethnic breakdown was 50% White, 27% Black and 23% Hispanic. The

Hispanics in the samples were significantly younger than the other two groups. The hematocrits, red blood cell counts, and plasma cholesterol levels were similar among the three groups. Several differences in the other measured parameters were seen.

RBCs were collected from a second group of 164 men and women of White (European-American), Black (African-American) and Hispanic (all self-assigned) ancestry and fALOD4 measurements were made by a separate technician. A different control sample was used to normalize these measurements. As in the first group, median fALOD4 binding was lowest in Whites (0.93 nRFU), highest in Blacks (1.65 nRFU) and intermediate in Hispanics (1.28 nRFU) ($P<0.0001$). Demographic and clinical variables of group 2 are provided in Table 3. The ethnic breakdown was 24% White, 27% Black and 49% Hispanic. No significant differences in other parameters were observed between group 1 and group 2.

A multivariate analysis was performed to determine which factors listed in tables 1 and 3 contributed most significantly to differences in fALOD4 binding. In both populations, the differences in RBC cholesterol accessibility did not correlate with age (Tables 2 and 4). Males had slightly higher fALOD4 binding than females ($p=0.01$) in group 1. In both groups, ethnicity accounted for the largest fraction of variation in fALOD4 binding, accounting for 14.6% of the variance in group 1 and 38.53% of the variance in group 2.

In both groups, no significant association was found between fALOD4 binding and RBC indices, including hematocrit, hemoglobin concentration, RBC count, mean corpuscular hemoglobin content (MCHC), mean corpuscular volume (MCV) or RBC distribution width (RDW) (***Figure 6***). Thus, the differences in RBC cholesterol accessibility levels did not appear to be related to the gross morphology or hemoglobin content of the RBC.

fALOD4 Binding was Inversely Related to Plasma Triglyceride Levels and Directly Related to Plasma HDL-C Levels

We also examined the relationship between fALOD4 binding and plasma lipid and lipoprotein levels. Binding to RBCs was not related significantly to plasma cholesterol levels and correlated only modestly with plasma low density lipoprotein-cholesterol (LDL-C) levels (***Figure 7A***). fALOD4 binding was inversely related to plasma triglyceride levels and directly related to plasma HDL-C levels (***Figure 7A***). In a multivariate analysis of factors including demographic factors as well as plasma lipid and lipoprotein levels, only plasma triglycerides ($p = 0.0002$) and total cholesterol levels ($p = 0.026$) contributed significantly to the variance of fALOD4 binding and accounted for 21.6% of the variance (Table 2).

fALOD4 Binding was Reduced in Patients with Tangier Disease but not in Patients with LCAT Deficiency

To elucidate the molecular basis of the relationship between fAOLD4 binding and HDL-C levels, we measured fALOD4 binding of RBCs of patients with Tangier disease (patients described in ref. (6)), who have no functional ABCA1, and in patients with LCAT deficiency (***Figure 7B*** and Table 5). Blood was collected from a proband with Tangier disease, an affected sibling, two children, the proband's spouse as well as the affected sibling's family. Additionally, blood was collected from a proband with LCAT deficiency, an affected sibling, two parents, and the patient's spouse. For each proband, assays were performed on blood samples obtained about 6 months apart. The fALOD4 binding to RBCs from the Tangier disease proband and the affected sibling were lower than any of the 528 other individuals assayed. RBC fALOD4 binding of the proband's children and nephews, who are all obligate heterozygotes, was in between that of the affected and unaffected parents. Both the proband and affected sibling with LCAT deficiency also had relatively low fALOD4 binding, although not as low as that of the individuals with Tangier disease. The parents of the LCAT deficient patients also had relatively low fALOD4 binding.

Relationship Between fALOD4 Binding and RBC Membrane Proteins

To determine if membrane proteins contribute to the differences in RBC cholesterol accessibility, we labeled the membrane proteins with biotin and then treated the RBCs with pronase (***Figure 8A***). Western blotting was performed using streptavidin-HRP to assess the effect of pronase treatment on biotin labeled surface membrane proteins. The surface protein content of the RBC membranes fell progressively with increasing amounts of pronase. Hemolysis measurements show that the integrity of the RBC membranes was not significantly affected by the pronase treatment, suggesting only surface proteins were affected. At the lowest concentration of pronase (0.02 μg/mL), fALOD4 binding increased by ~25% (***Figure 8A***). Higher concentrations of pronase did not further increase fALOD4 binding despite further decreases in membrane protein content. Based on these results, we concluded that the protein composition of the RBC membrane is not a major determinant of fALOD4 binding, although we cannot exclude the possibility that a protein(s) that is resistant to pronase treatment contributes to the inter-individual differences in fALOD4 binding.

We also examined the relationship between fALOD4 binding and ABO blood groups in participants that were recruited at a blood donation facility (***Figure 8B*** and Table 6). These study subjects were White. No significant differences were apparent between individuals with blood types A, B, and O (***Figure 8B***), whereas individuals who were Rhesus antigen negative (Rh-) had significantly lower fALOD4 binding than did individuals who were Rh+. The relationship between blood type and accessible cholesterol binding will need to be confirmed in an independent sample and examined in other ethnic groups.

fALOD4 Binding Increases with Treatment of RBCs with Phospholipase A2 (PLA2) and Sphingomyelinase (SMase)

To determine if the phospholipid composition of the RBC membrane contributes to fALOD4 binding, we treated RBCs with PLA2, which hydrolyzes the ester linkage at the sn-2 position of

phospholipids, or with SMase, which hydrolyzes the ester linkage between the sn-1 carbon and phosphate group of sphingomyelins (SM). fALOD4 binding increased progressively when the RBCs were incubated with increasing amounts of PLA2 (***Figure 9A***) as well as with increasing amounts of SMase (***Figure 9B***), which is consistent with previous observations that SMase treatment increases cholesterol accessibility to perfringolysin O in fibroblasts (25), MCD extraction of cholesterol from erythrocytes (27) and increases plasma membrane to endoplasmic reticulum trafficking of cholesterol in fibroblasts (28-30).

fALOD4 Binding is Related to the Phospholipid Composition of the RBC Membrane

Next, we examined the lipid composition of the RBC membranes in a subset of individuals of group 2 as described earlier. Specifically, we looked at 41 Black and 82 Hispanic individuals whose RBC ghost membrane extracts had been prepared and stored at -80°C after blood collection. The demographics and the distribution of fALOD4 binding of this group are shown in Table 3. Lipids were extracted from RBC ghosts and then the major glycerophospholipids were quantitated by LC-MS/MS at the Kansas Lipidomics Research Center. The overall lipid composition of the RBC membranes (Table 7) was like what has been reported previously (31). Differences in levels of several of the RBC lipids were observed between Blacks and Hispanics, however in a multivariable model adjusted for age, gender, and ethnicity, only differences in lysophosphatidylethanolamine (LPE), SM, and phosphatidic acid levels were associated with differences in fALOD4 binding. These lipids accounted for 8.55% (LPE), 0.01% (SM), and 3.62% (phosphatidic acid) of variation in fALOD4 binding, respectively (Table 8). When taken together, the RBC lipids accounted for 17.6% of the inter-individual variation in fALOD4 binding.

Patients on Hemodialysis Have Increased fALOD4 Binding

Patients on hemodialysis have accelerated atherosclerosis that is not accounted for by known risk factors (23). To determine whether RBC cholesterol activity is altered in these individuals,

we measured fALOD4 binding to RBCs from 50 patients attending a hemodialysis clinic and in 48 ethnicity-matched controls (Table 9). fALOD4 binding was significantly higher in RBCs from dialysis patients, irrespective of ethnicity (***Figure 10A***). In eight individuals, fALOD4 binding was measured in RBCs prepared from blood samples obtained before and after a single dialysis treatment. fALOD4 binding was similar in the paired samples (***Figure 10B***). Therefore, the increase in fALOD4 binding observed in dialysis patients was not an acute result of dialysis *per se*.

Plasma activity of individuals correlates with RBC fALOD4 Binding

To determine if factors in plasma directly affect the amount of fALOD4 binding of RBCs, we incubated RBCs from a control individual with plasma isolated from several different individuals. Specifically, we tested plasma collected from a subset of individuals from group 2 (Table 3) whose plasma had been stored at -80°C after blood collection. We defined plasma activity as the degree to which an individual's plasma changed RBC fALOD4 binding of the control individual; it was calculated as shown in ***Figure 11A***. Control RBCs were incubated with plasma from 40 Whites, 25 Hispanics, and 40 Blacks. In this population, plasma activity was highest in Whites, lowest in Blacks and intermediate in Hispanics. These ethnicity-related differences in plasma activity are inverse to the differences observed in RBC fALOD4 binding (***Figure 11B***). When plasma activity is plotted against fALOD4 binding, there is a significant inverse correlation observed in the White group (***Figure 11C***). No significant correlation between plasma activity and fALOD4 binding was observed in the Hispanic or Black groups.

TABLES

Table 1. Characteristics of 364 healthy subjects (Group 1)

Characteristic	N	All (N=364)	White (N=182)	Black (N=98)	Hispanic (N=84)	P-value
Age, years	364	40.9 ± 16.5	42.2 ± 17.7	44.9 ± 12	33.4 ± 16.2	3.5E-06
Male, N (%)	364	136 (37.4)	91 (50)	19 (19.4)	26 (31)	1.1E-06
Female, N (%)	364	228 (62.6)	91 (50)	79 (80.6)	58 (69)	1.1E-06
Hct (%)	345	41.1 ± 5.6	41.4 ± 6.1	40.6 ± 5.5	40.8 ± 4.5	0.27
Hb (g/dL)	344	13.2 ± 1.9	13.5 ± 1.9	12.9 ± 1.8	13 ± 2.0	0.98
RBC count (106/uL)	345	4.6 ± 0.6	4.7 ± 0.6	4.6 ± 0.6	4.6 ± 0.5	0.065
MCHC	344	32.3 ± 0.8	31.8 ± 0.7	32.6 ± 0.8	32.2 ± 0.8	7.5E-10
MCV (fL)	345	87.9 ± 8.3	88.3 ± 8.8	87.2 ± 9.5	88.0 ± 5.3	0.23
RDW	345	14.4 ± 1.8	14.7 ± 1.7	14.2 ± 1.9	14.3 ± 1.7	0.11
Total cholesterol (mg/dL)	363	178.4 ± 36.8	180.3 ± 37.3	179.6 ± 34.6	173.1 ± 38.0	0.47
HDL-chol (mg/dL)	345	51.9 ± 14.4	50.3 ± 14.6	57 ± 14	49.1 ± 13.2	0.012
LDL-chol (mg/dL)	341	101.4 ± 35.4	99.5 ± 32.2	102.9 ± 31.6	103.3 ± 44.6	0.53
TG (mg/dL)	345	76 (105-164)	129 (85-184)	83 (67-117)	106 (85-159)	2.8E-07

Data are mean ± SD or median (25^{th} – 75^{th} percentile), unless otherwise indicated. Blood samples were obtained from healthy subjects after fasting for at least 8 hours. Plasma lipids and lipoproteins and RBC indices were measured by Quest diagnostics as described in the *Methods.* *C*ontinuous variables were compared between groups using ANOVA adjusted for age and gender where appropriate and categorical variables were compared using chi-square tests. Variables with non-normal distributions were inverse-normally transformed before analysis.

Table 2. Factors associated with fALOD4 binding in 345 healthy subjects (Group 1)

Factor	Beta	SE	P-value	Partial R^2 (%)
Age	0.017	0.023	0.46	0.16
Male Gender	0.119	0.046	0.010	1.93
Ethnicity:				
White	Reference	-	-	14.63
Black	0.403	0.053	2.8E-13	
Hispanic	0.130	0.053	1.6E-02	
TG	-0.089	0.024	0.00021	3.99
Total cholesterol	0.053	0.024	0.026	1.45
Total				**21.6**

Beta coefficients are in SD units. For quantitative variables (age, lipids and CBCs), betas are given per 1 SD change in the predictor. Partial R^2 indicates the proportion of variance in fALOD4 binding explained by a given predictor after adjusting for other factors.

Table 3. Characteristics of 164 healthy subjects (Group 2)

Characteristic	All (N=164)	White (N=39)	Black (N=44)	Hispanic (N=81)
Age	38 ± 0.9	39 ± 2	42 ± 2	35 ± 1
Male (%)	30	41	18	32
Female (%)	70	59	82	68
Hb (g/dL)	13.7 ± 0.1	14 ± 0.3	13.2 ± 0.2	13.7 ± 0.2
Hct (%)	41.1 ± 0.3	42.0 ± 0.6	40.0 ± 0.6	41.3 ± 0.5
RBC count (10^6/uL)	4.7 ± 0.03	4.7 ± 0.07	4.6 ± 0.07	4.7 ± 0.05
MCV (fL)	88 ± 0.7	87.3 ± 2.2	87.3 ± 0.8	87.7 ± 0.7
MCHC (g/dL)	33.1 ± 0.2	33.2 ± 0.6	32.9 ± 0.1	33.2 ± 0.1
RDW (%)	14.3 ± 0.1	13.7 ± 0.2	14.6 ± 0.2	14.3 ± 0.2
Total cholesterol (mg/dL)	183 ± 2.9	179 ± 6.2	186 ± 5.8	184 ± 3.9
HDL-chol (mg/dL)	54. ± 1.2	60 ± 3	59 ± 2.1	49 ± 1
LDL-chol (mg/dL)	105 ± 2.5	100 ± 5.5	109 ± 5.4	103 ± 3
TG (mg/dL)	125 ± 7.2	104 ± 8.2	90 ± 6.8	156 ± 12.7

Data are mean ± SD or median (25^{th} – 75^{th} percentile), unless otherwise indicated. Blood samples were obtained from healthy subjects after fasting for at least 8 hours. Plasma lipids and lipoproteins and RBC indices were measured by Quest diagnostics as described in the *Methods. C*ontinuous variables were compared between groups using ANOVA adjusted for age and gender where appropriate and categorical variables were compared using chi-square tests. Variables with non-normal distributions were inverse-normally transformed before analysis.

Table 4. Factors associated with fALOD4 binding in 164 healthy subjects (Group 2)

Factor	Beta	SE	P-value	Partial R^2 (%)
Age	-0.044	0.034	0.20	1.40
Male Gender	-0.008	0.068	0.91	0.01
Ethnicity (AA vs. HA)	-0.442	0.077	6.9E-08	22.44
Triglycerides	-0.192	0.033	4.6E-08	22.96
LPE	-0.131	0.040	0.0014	8.55
SM	-0.005	0.045	0.91	0.01
PA	0.076	0.036	0.040	3.62
Total				56.25

Beta coefficients are in SD units. For quantitative variables (age, lipids and CBCs), betas are given per 1 SD change in the predictor. Partial R^2 indicates the proportion of variance in fALOD4 binding explained by a given predictor after adjusting for other factors. LPE, lysophosphatidylethanolamine ; SM, sphingomyelin; PA, phosphatidic acid

Table 5. Lipid levels in families with Tangier Disease and LCAT deficiency.

Tangier Disease						
Subject	Age	Genotype	Cholesterol (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)
control	26	+/+	192	98	110	62
1	42	+/+	174	107	84	69
2	46	-/-	42	140	34	<3
3	15	+/-	99	75	35	49
4	13	+/-	101	77	53	33
5	47	+/+	186	241	92	46
6	50	-/-	97	145	63	5
7	24	+/-	100	145	50	21
8	22	+/-	131	95	88	24
LCAT						
Subject						
control	26	+/+	192	98	110	62
1	24	+/+	173	62	116	45
2	31	-/-	220	325	150	<5
3	26	-/-	183	116	154	<5
4	68	+/-	133	127	78	30
5	60	+/-	178	111	121	35

Plasma lipid and lipoprotein levels were measured on plasma from fasting blood samples as described in the *Methods*.TG, triglycerides; LDL-C, low density-cholesterol; HDL-C, high density lipoprotein-cholesterol.

Table 6. Demographics and clinical characteristics of blood group donors

n	161
Age	42 ± 1
Male (%)	51
Female (%)	49
Blood Groups	
A (%)	42
B (%)	10
AB (%)	3
O (%)	45
Rh positive (%)	80
Rh negative (%)	20
Total cholesterol (mg/dL)	180 ± 3
HDL-C (mg/dL)	50 ± 1
LDL-C (mg/dL)	99 ± 3
TG (mg/dL)	155± 8
Hb (g/dL)	13.5 ± 0.2
Hct (%)	41.4 ± 0.5
MCV (fL)	88 ± 0.6
RBC count (10^6/uL)	4.7 ± 0.05

Blood samples were obtained from healthy subjects after fasting for at least 8 h. ABO blood group and presence of Rh antigen was determined by Carter BloodCare as described in Methods. Ethnicity, age, and gender were self-reported. Plasma lipids and lipoproteins and RBC indices were measured by Quest Diagnostics as described in the Methods. Numerical values are mean ± SD.

Table 7. Characteristics of 123 subjects in whom RBC membrane lipids were measured.

Characteristic	Black (n=41)	Hispanic (n=82)	P-value
Age, years	42.2 ± 11.5	34.8 ± 9.5	0.00087
Female, n (%)	34 (82.9)	55 (67.1)	0.10
Male, n (%)	7 (17.1)	27 (32.9)	0.10
fALOD4 binding (nRFU)	1.4 (1.1 – 1.7)	1.7 (1.3 – 2.0)	1.3 (1.0 – 1.5)
RBC membrane lipids			
PC (mol %)	40 ± 2.4	44 ± 3.6	2.7E-11
SM (mol %)	19 ± 4.1	19 ± 3.5	18 ± 4.4
PE (mol %)	15 ± 1.8	12 ± 1.8	6.6E-07
ePC (mol %)	5.7 ± 0.61	6.1 ± 0.92	0.0028
PS (mol %)	6.0 ± 0.94	6.2 ± 1.1	5.9 ± 0.81
LPE (mol %)	5.1 ± 1	4 ± 1.4	0.0027
Ceramides (mol %)	2.9 ± 0.45	2.8 ± 0.57	0.61
PI (mol %)	2.7 ± 0.63	2.7 ± 0.8	0.18
ePE (mol %)	1.7 ± 0.35	1.3 ± 0.26	8.3E-06
LPC (mol %)	1.4 ± 0.31	1.2 ± 0.29	2.3E-08
PA	0.44 ± 0.13	0.38 ± 0.099	0.0024

Data are mean ± SD or median (25th – 75th percentile), unless otherwise indicated. All RBC lipids are expressed as mol% of total phospholipids. The lipid mole percentages were determined by mass tandem mass spectrometry. Continuous variables were compared between groups using ANOVA adjusted for age and gender where appropriate, categorical variables - using chi-square tests. Variables with non-normal distributions were inverse-normally transformed before analysis. HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; ePC, ether-phosphatidylcholine; PS, phosphatidylserine; LPE, lysophosphatidylethanolamine; ePE, ether-phosphatidylethanolamine; LPC, lysophosphatidylcholine; PA, phosphatidic acid

Table 8. Factors associated with fALOD4 binding in subjects from Table 4.

Factor	Beta	SE	P-value	Partial R^2 (%)
Age	-0.044	0.034	0.20	1.40
Male Gender	-0.008	0.068	0.91	0.01
Ethnicity (AA vs. HA)	-0.442	0.077	6.9E-08	22.44
Triglycerides	-0.192	0.033	4.6E-08	22.96
LPE	-0.131	0.040	0.0014	8.55
SM	-0.005	0.045	0.91	0.01
PA	0.076	0.036	0.040	3.62
Total				56.25

Beta coefficients are in SD units. For quantitative variables (age, lipids and CBCs), betas are given per 1 SD change in the predictor. Partial R^2 indicates the proportion of variance in fALOD4 binding explained by a given predictor after adjusting for other factors. LPE, lysophosphatidylethanolamine; SM, sphingomyelin; PA, phosphatidic acid

Table 9. Demographics of patients with chronic kidney disease on hemodialysis

	All		White		Black		Hispanic	
	Control	Dialysis	Control	Dialysis	Control	Dialysis	Control	Dialysis
N	48	50	26	8	10	32	9	10
Age	44 ± 2	54 ± 2	48 ± 2	52 ± 6	51 ± 3	57 ± 2	27 ± 4	48 ± 4
Male (%)	46	58	54	71	30	50	56	80
Total cholesterol (mg/dL)	182 ± 6	142 ± 5	191 ± 8	180 ± 3	153 ± 12	156 ± 6	184 ± 13	116 ± 5\5
HDL-C (mg/dL)	48 ± 2	45 ± 2	45 ± 3	40 ± 4	54 ± 4	49 ± 2	48 ± 3	38 ± 4
LDL-C (mg/dL)	103 ± 6	72 ± 4	111 ± 7	84 ± 15	83 ± 10	75 ± 5	100 ± 7	54 ± 4
TG (mg/dL)	153 ± 15	122 ± 11	172 ± 22	144 ± 20	78 ± 9	109 ± 15	180 ± 30	147 ± 22
Hb (g/dL)	13.8 ± 0.2	11.8 ± 0.8	14 ± 0.3	16 ± 3	13 ± 0.5	10.4 ± 0.4	13.5 ± 0.4	12.6 ± 2
Hct (%)	42 ± 1	32 ± 1	43 ± 1	31 ± 1	41 ± 2	32 ± 1	41 ± 1.2	31 ± 1
MCV (fL)	87 ± 1	90 ± 1	86 ± 2	87 ± 6	90 ± 2	92 ± 1	86 ± 2	88 ± 4
RBC count (10^6/μL)	4.8 ± 0.1	3.7 ± 0.2	4.8 ± 0.2	3.4 ± 0.1	4.7 ± 0.3	3.8 ± 0.3	4.8 ± 0.2	3.5 ± 0.1

Data are mean ± SD, unless otherwise indicated. Blood samples were obtained either from hemodialysis patients immediately prior to dialysis or from healthy subjects after a minimum of 8 h of fasting. Ethnicity, age, and gender were self-reported. Plasma lipids and lipoproteins and RBC indices were measured by Quest Diagnostics as described in the *Methods.*

FIGURES

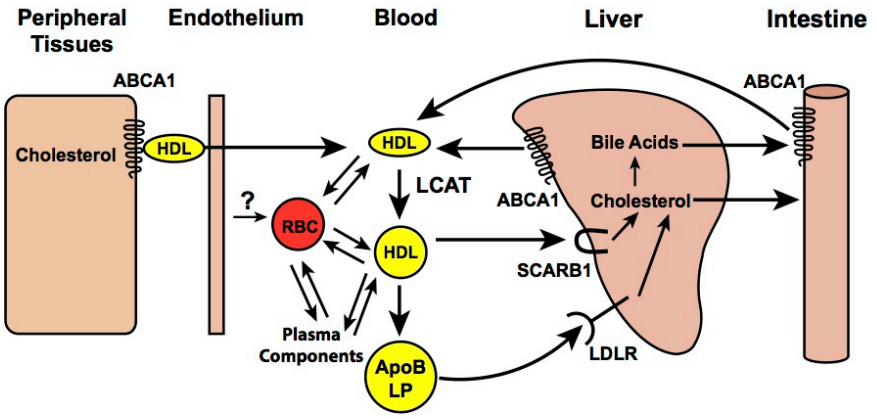

Figure 1: Schematic of reverse cholesterol transport (RCT) in the blood.

Free cholesterol in peripheral tissues or the intestine is effluxed by ABCA1 transporters to high-density lipoprotein (HDL), and transported into the vascular space. Lecithin cholesterol acyl transferase (LCAT), present on HDL, esterifies free cholesterol. The cholesterol esters formed by the LCAT reaction are either taken up by SCARB1 receptors on hepatocytes or transferred to apolipoprotein B containing lipoproteins (ApoB LP) and taken up into hepatocytes by the low density lipoprotein receptor (LDLR). Red blood cells (RBCs) may also participate in reverse cholesterol transport by accepting cholesterol from vascular endothelial cells and participating in cholesterol exchange with lipoproteins and plasma components such as albumin.

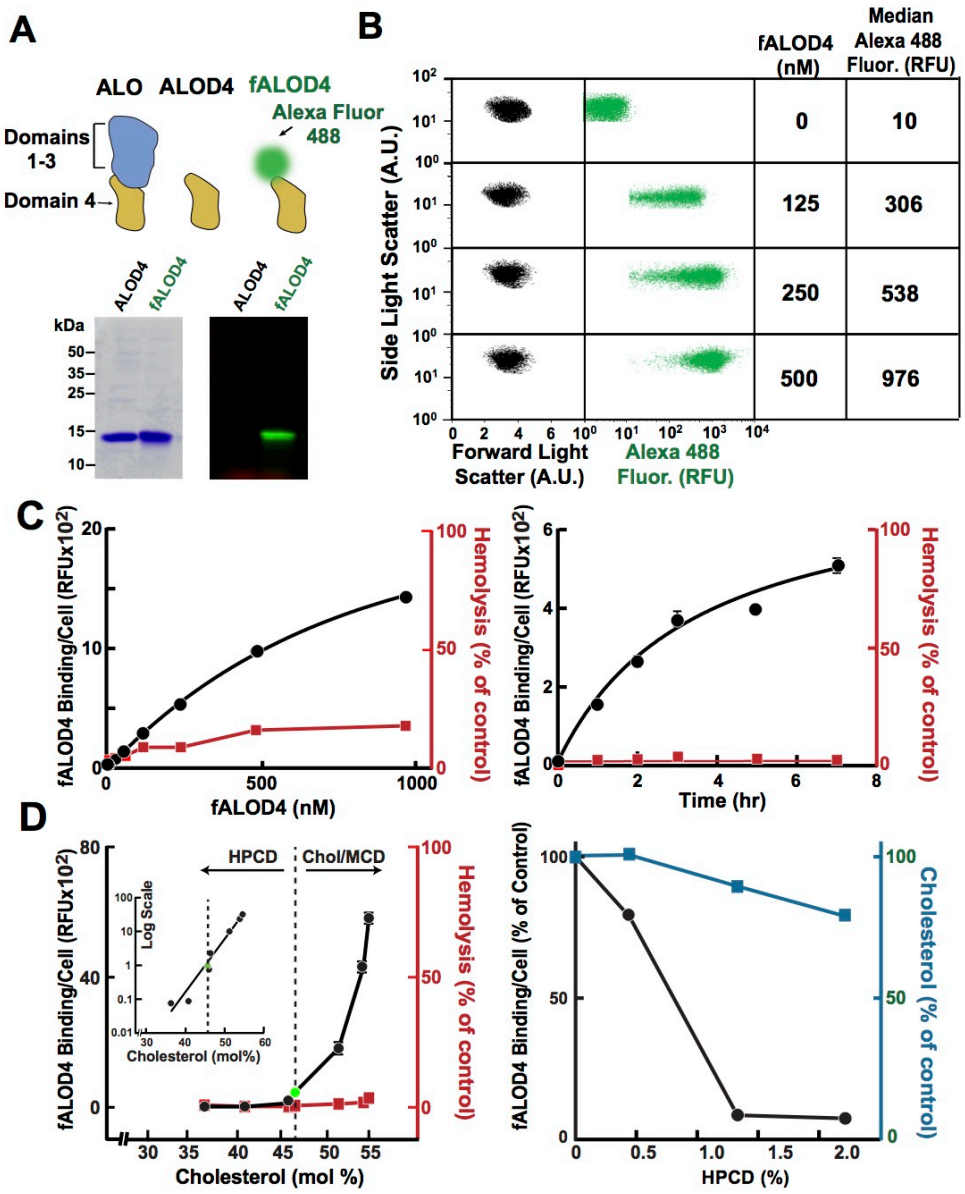

Figure 2: Assay for red blood cell (RBC) cholesterol accessibility. (**A**) Schematic of anthrolysin O (ALO) domains. Domain 4 (ALOD4) binds cholesterol but does not oligomerize or form membrane-lysing pores. In fALOD4, Alexa Fluor 488 dye is covalently attached to an engineered cysteine near the NH_2-terminus of domain 4. fALOD4 retains cholesterol binding activity and is nonlytic (20). Purified proteins (5 µg) were subjected to SDS-PAGE (15%) and visualized by Coomassie staining *(left)* or fluorescence scanning (LI-COR) at 600 nm *(right)*. (**B**)

Flow cytometry analysis of fALOD4 binding to RBCs. RBCs ($2.5x10^5$ RBCs in 500 μl buffer D) were incubated for 3 h at 4°C with fALOD4 at the indicated concentrations. Fluorescence was measured by a FACSCalibur flow cytometer as described in the *Methods*. Forward light scatter (FSC), side light scatter (SSC), and Alexa 488 fluorescence measurements for 10,000 RBCs were acquired on the flow cytometer. Median Alexa 488 fluorescence per cell was calculated using FlowJo software. (**C**) Dose response (*left*) and time course *(right)* of fALOD4 binding to RBCs. Each reaction was set up as described above using either the indicated concentrations of fALOD4 *(right) or* 250 nM fALOD4 *(left)*. After incubation at 4°C for 3 h (l*eft*) or for the indicated times (*right*), fALOD4 RBC binding was measured by flow cytometry. Hemolysis during fALOD4 binding reactions was determined by measuring release of hemoglobin as described in the *Methods*. 100% hemolysis is defined as the amount of hemoglobin released after treatment of RBCs with 1% (w/v) Triton X-100. Data points represent means of three independent measurements. Error bars represent the SEM. AU, arbitrary units; RFU*,* relative fluorescence units. (**D**) Effect of RBC cholesterol modulation on RBC fALOD4 binding. (*Left*) RBCs were not treated (green circle) or treated with either hydroxypropyl-β-cyclodextrin (HPCD) or cholesterol/methyl-β-cyclodextrin (MCD) to reduce or increase the cholesterol content of RBCs. The fALOD4 binding assay using 250 nM fALOD4 and fluorescence quantification was performed as described in the Methods. Lipids were extracted from ghost membranes isolated from the RBCs, and the amounts of cholesterol and choline-containing phospholipids were measured enzymatically and expressed as moles percentage. The dashed line indicates cholesterol content of untreated RBCs. Hemolysis was measured as described in the Methods. *Inset:* Data for the experiment plotted on a logarithmic-linear scale. (*Right*) RBCs were treated with HPCD to remove cholesterol and both fALOD4 binding and cholesterol content were measured as described in the *Methods*. The fALOD4 binding per cell and cholesterol content (mole %) in untreated RBCs was set to 100%. Data points represent the mean of three

independent measurements of fALOD4 binding and a single measurement of RBC cholesterol content. Error bars represent the SEM. RFU, relative fluorescence units. The experiments were repeated three times and the results were similar.

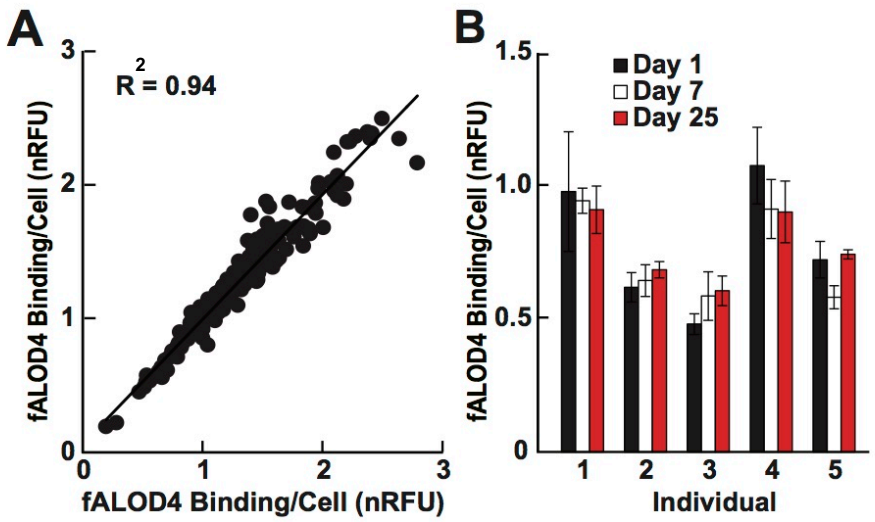

Figure 3. Characterization of RBC cholesterol accessibility assay. (**A**) Intra-assay correlation was determined by measuring fALOD4 binding to two aliquots of RBCs from 164 unrelated healthy individuals as described in the *Methods*. . Data points represent the mean of three independent measurements from each of the RBC aliquots. (**B**) Intra-individual variability of fALOD4 binding to RBCs from 5 individuals. Blood samples from 5 unrelated individuals were collected on 3 separate days over a month and fALOD4 binding was measured. fALOD4 binding values in **A** and **B** were normalized to the binding values obtained from the reference blood sample. The reference blood sample was collected from the same healthy volunteer prior to each experiment in this study and the sample was processed and assayed concurrently. Error bars represent the mean ± SEM.

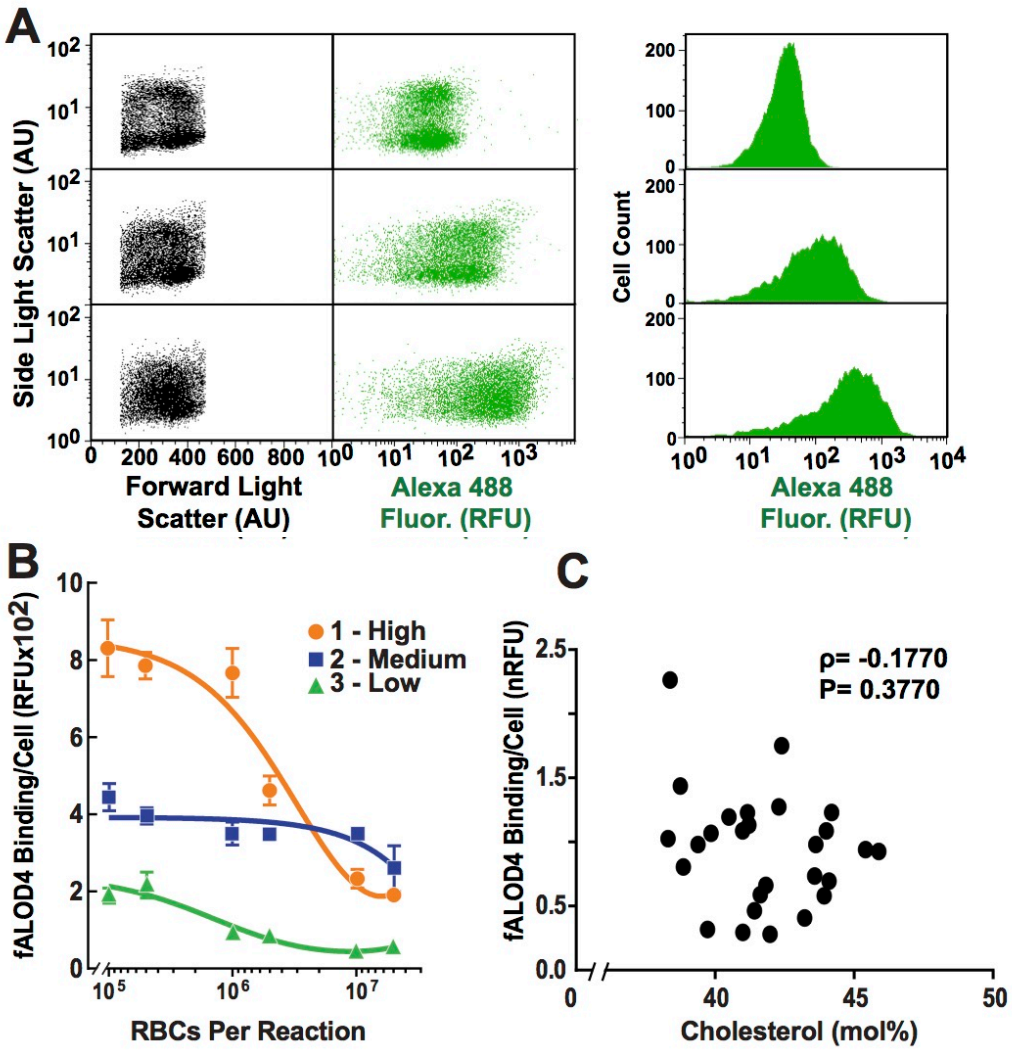

Figure 4. Distribution of fALOD4 binding to RBCs in individuals with low, medium and high binding and relationship with RBC cholesterol. Flow cytometry analysis of fALOD4 binding to RBCs from three individuals with low (top), medium (middle) and high (bottom) fALOD4 binding. Forward light scatter, side light scatter, and Alexa 488 fluorescence measurements of 10,000 RBCs were acquired using a FACSCalibur flow cytometer as described in the *Methods.* Fluorescence data is presented as both a dot plot (middle) and histogram (right). AU, arbitrary units; RFU, relative fluorescence units. (**B**) Relationship between RBC number and fALOD4 binding in three individuals with low (green), medium (blue) and high (orange) binding. Samples were collected on the same day and the fALOD4 binding assay was

performed as described in the legend to ***Figure 1***. Error bars represent the mean ± SEM of three measurements from each blood sample. The experiment was repeated once and the results were similar. (**C**) RBC cholesterol content and fALOD4 binding to RBCs. RBC ghost membranes were prepared from RBCs of 73 healthy, unrelated individuals. Total lipids were extracted from the membranes and the molar percentage of cholesterol was measured as described in the *Methods*. fALOD4 binding values were normalized to the binding values obtained from the reference blood sample. Shown is the Spearman correlation between fALOD4 binding, normalized to the reference blood sample, and RBC membrane total cholesterol expressed as mole % of total lipids. Data points represent the means of three independent measurements of fALOD4 binding and a single measurement of RBC cholesterol content. The experiment was repeats once and the results were similar. nRFU, normalized relative fluorescence units.

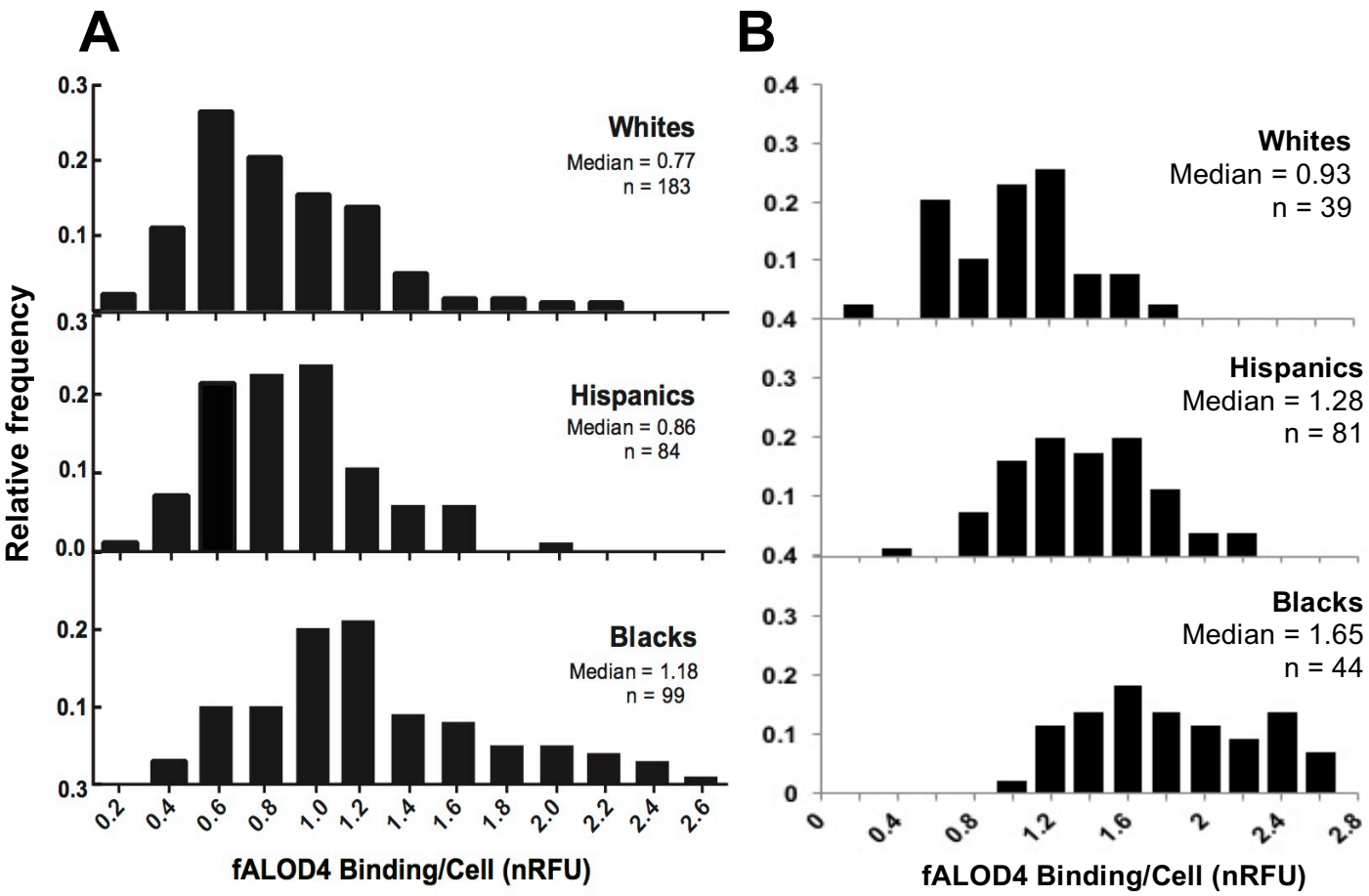

Figure 5. Distribution of fALOD4 binding to RBCs from healthy, unrelated individuals. (**A**) Blood samples were collected from 366 individuals (Group 1) and RBCs were isolated as described in the *Methods*. Each measurement was performed in triplicate. fALOD4 binding values were normalized to those of the reference blood sample. Data are plotted as frequency histograms of median fALOD4 binding. (**B**) fALOD4 binding to RBCs of 164 additional individuals (Group 2). Each measurement was performed in duplicate and normalized as described above.

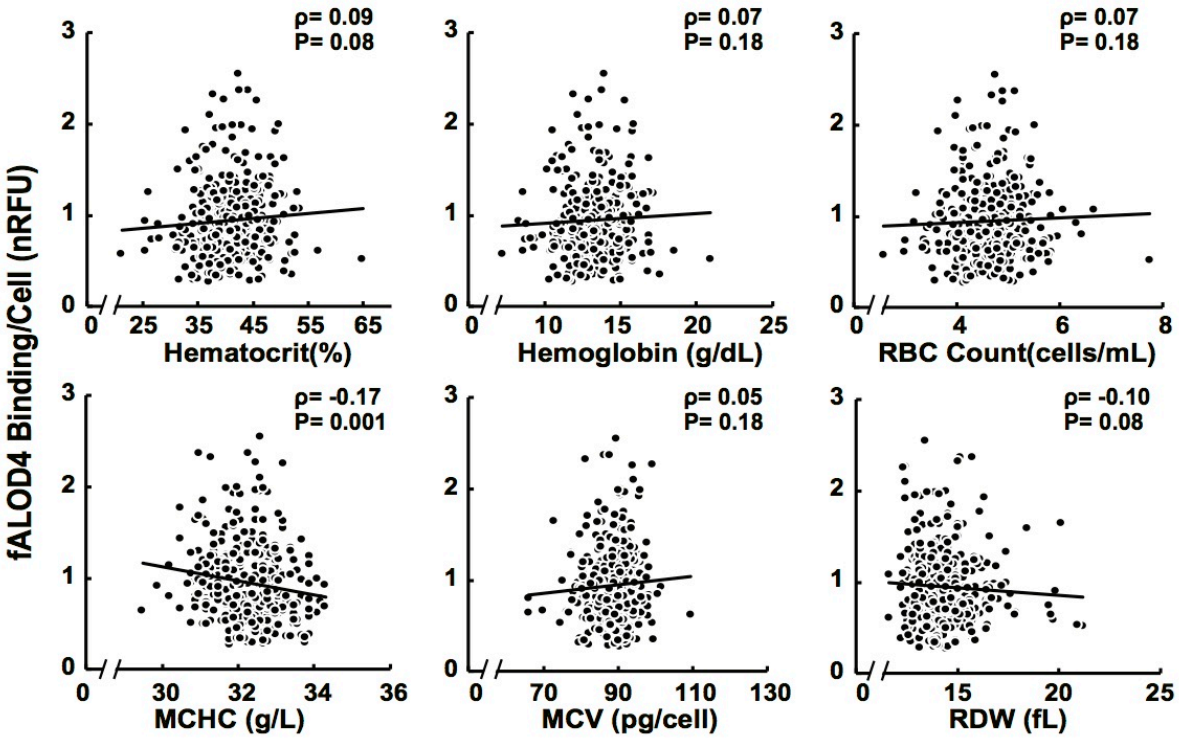

Figure 6. Association between fALOD4 binding and RBC indices. RBC indices including hematocrit, hemoglobin, RBC count, mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) and RBC distribution width (RDW), were determined for approximately 350 individuals (see Table 1 for exact numbers) by Quest diagnostics. The values were plotted against fALOD4 binding to RBCs, measured as described in the *Methods*. Spearman correlation coefficients and p-values were calculated using the software package Prism 6. nRFU, normalized relative fluorescence units.

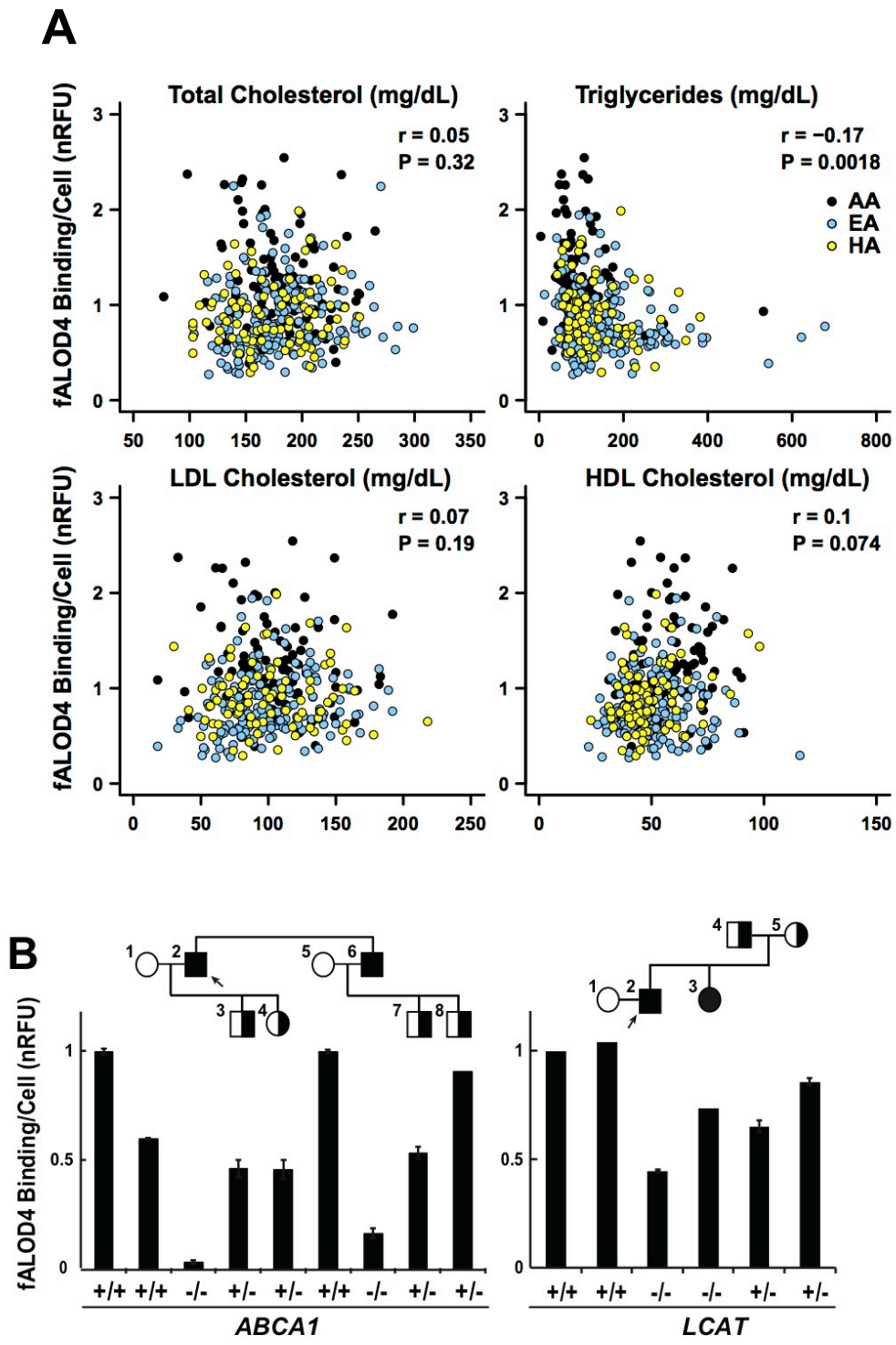

Fig 7. Relationship between fALOD4 binding to RBCs and plasma lipids. (**A**) Correlations between fALOD4 binding and serum lipids and lipoproteins. Total cholesterol, triglycerides, LDL-C and HDL-C were measured in approximately 350 individuals (see Table 1 for exact

numbers) by Quest Diagnostics. fALOD4 binding to RBCs was measured as described in the *Methods* and normalized to the reference blood sample. Spearman's correlation coefficients and two tailed P-values were calculated using the software package Prism 6. (**B**) RBCs were collected from 2 siblings with Tangier disease *(left)* and 2 siblings with LCAT deficiency *(right*) and from their family members. fALOD4 binding to RBCs was measured as described in *Methods* and normalized to the reference sample from an unrelated individual. Data points represent the mean of three independent measurements. Error bars represent SEM. nRFU, normalized relative fluorescence units.

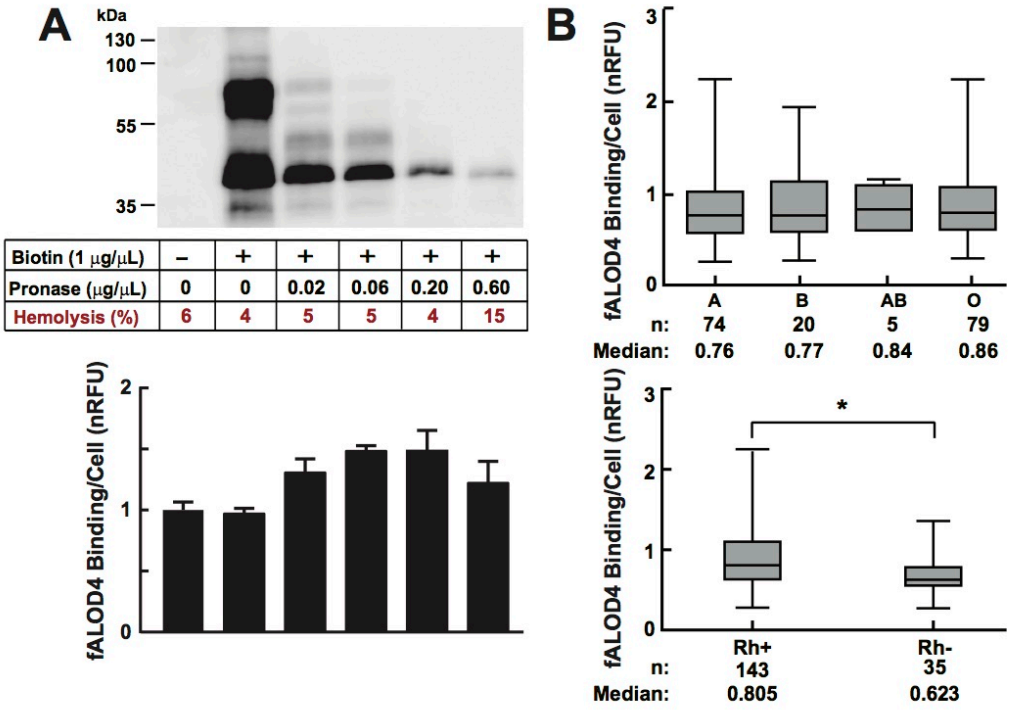

Figure 8. Effects of cell surface proteins on fALOD4 binding to RBCs. (**A**) Effect of proteolysis of RBC surface membrane proteins on fALOD4 binding to RBCs. RBCs isolated from a healthy individual were labeled with biotin and then treated with increasing amounts of pronase as indicated. An aliquot of the treated RBCs was used for fALOD4 binding assays and the remainder was subjected to 10% SDS/PAGE and probed with streptavidin-HRP (0.22 μg/mL) as described in the *Methods*. fALOD4 binding values were normalized to the untreated sample. Data points represent the mean of three independent measurements. Error bars represent the SEM. The experiment was repeated three times and the results were similar. (**B**) Relationship between fALOD4 binding and ABO blood group antigens (upper panel) and Rhesus blood group (Rh antigen) (lower panel). RBC fALOD4 binding was determined in triplicate using RBCs from 178 White blood donors as described in the *Methods*. Each fALOD4 binding measurement was performed in triplicate and values were normalized to the reference blood sample. Boxes represent the 25th and 75th percentiles and whiskers represent the

minimum and maximum measurements. *, $p < 0.005$, two-tailed t-test. nRFU, normalized relative fluorescence units.

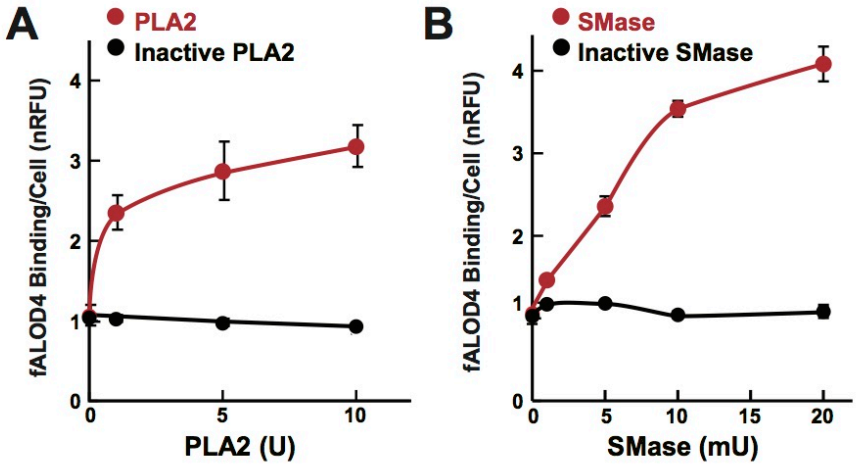

Figure 9. Effect of phospholipase and sphingomyelinase treatment of RBCs on fALOD4 binding. A total of 1.6×10^8 RBCs were incubated with indicated amounts of active or inactivated phospholipase A2 (PLA2) (***A***) or active or inactivated sphingomyelinase (SMase) (***B***) for 1 h at room temperature or 37°C, respectively. Cells were washed three times in PBS and an aliquot containing 2×10^5 RBCs was used to measure fALOD4 binding. Data points represent the mean of three independent measurements (± SEM). The experiment was repeated three times and the results were similar.

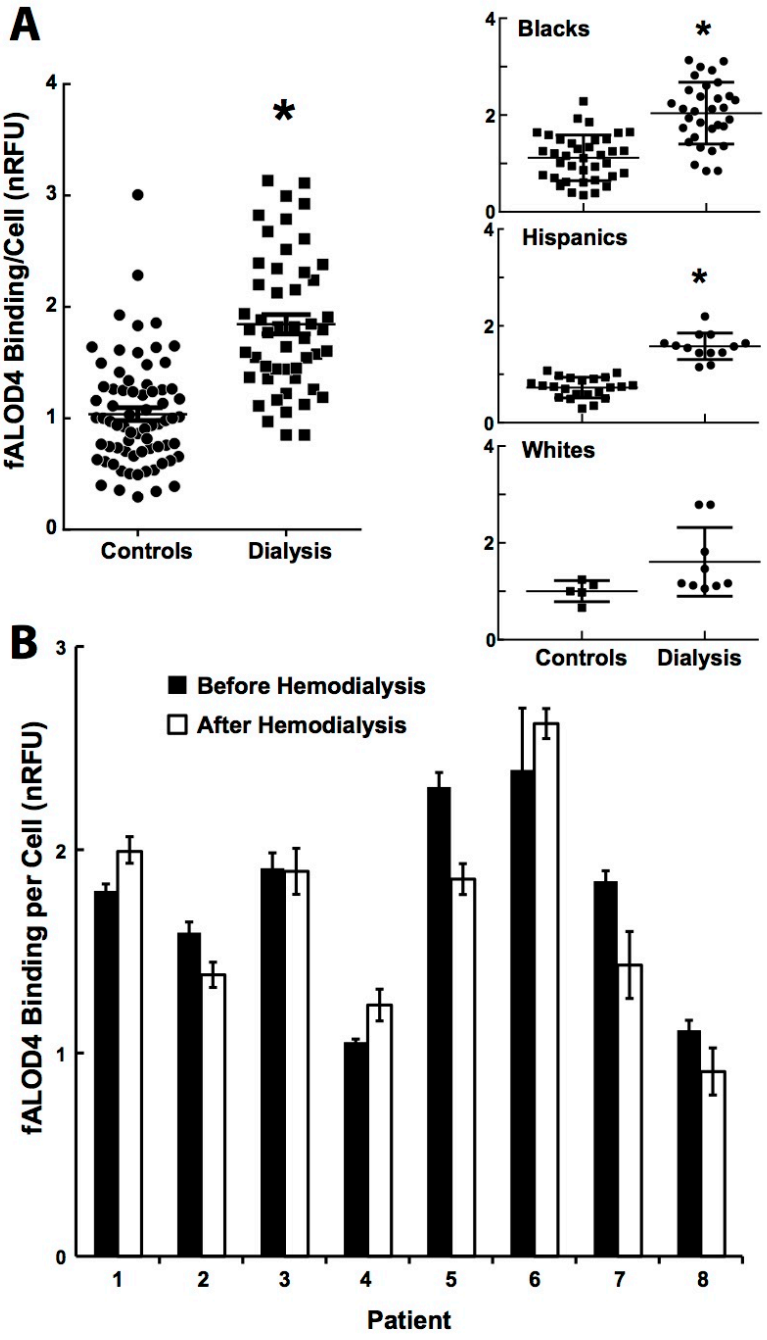

Figure 10. fALOD4 binding to RBCs of patients on hemodialysis. (**A**) Blood samples were collected from 50 hemodialysis patients immediately before dialysis was initiated and from 71 ethnicity-matched individuals who did not have chronic renal failure. RBCs were isolated and fALOD4 binding assays were performed as described in the *Methods*. Each measurement was

performed in triplicate. fALOD4 binding values were normalized to the binding values obtained for the reference blood sample that was collected from the same healthy subject for each experiment. Data is plotted as the mean of each normalized value. *, $p < 0.0001$, two-tailed t-test. (**B**) fALOD4 binding measurements were made exactly as described above using RBCs isolated from blood samples collected before and after dialysis in eight individuals undergoing hemodialysis. Blood samples were collected once from each patient before and after hemodialysis in eight patients. Data is plotted as the mean ± SEM.

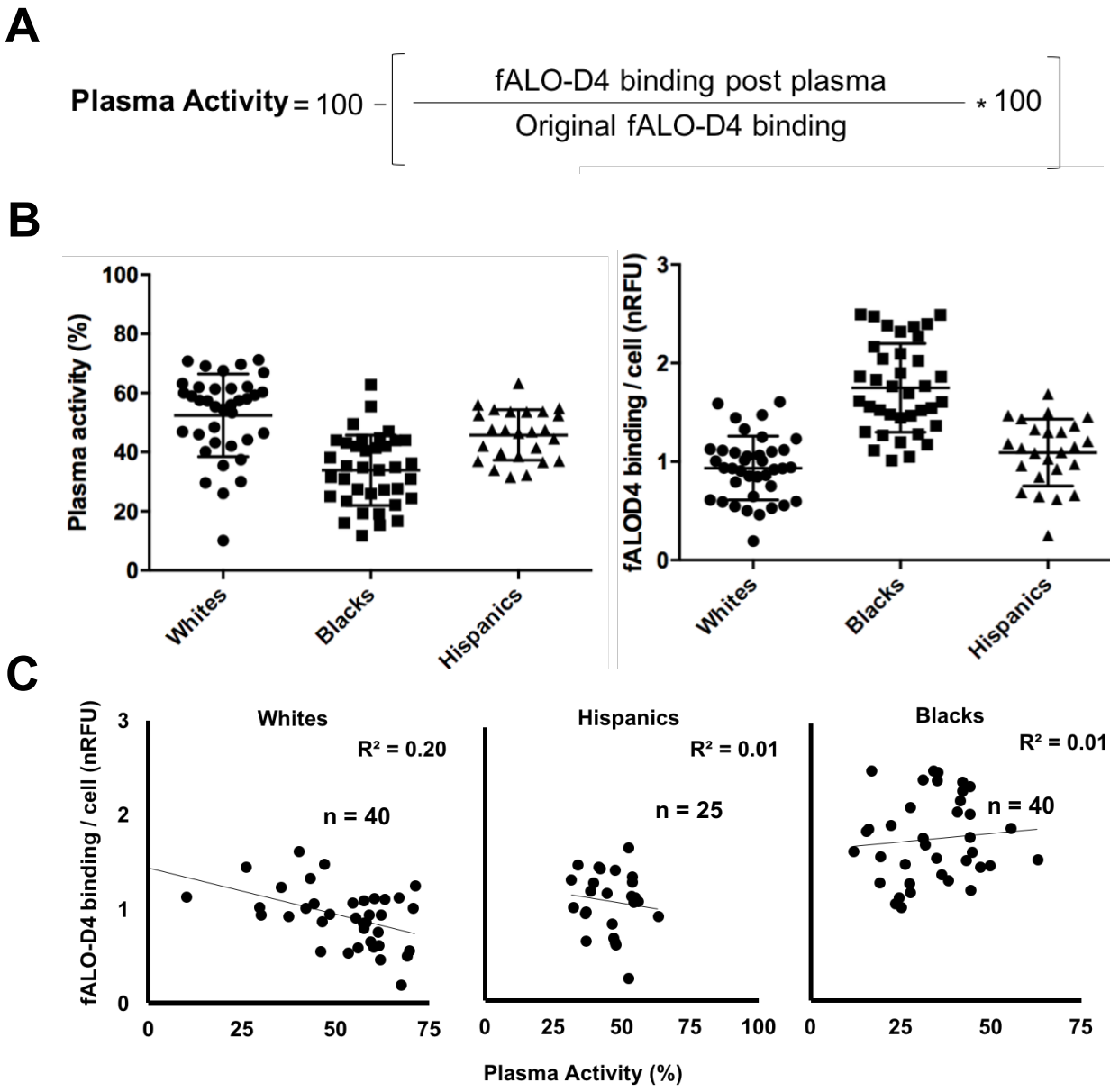

Figure 11. Plasma activity of healthy, unrelated individuals. (**A**) Plasma activity is calculated using fALOD4 binding to one individual's RBCs and fALOD4 binding to the same RBCs after incubating the RBCs with other individuals' plasma. from. (**B**) Plasma activity was calculated for 105 individuals (left panel) and is displayed next to fALOD4 binding to RBCs from the same individuals. (**C**) fALOD4 binding to RBCs of 105 individuals was plotted against plasma activity and separated by ethnicity.

DISCUSSION

The major finding of this study is that the amount of accessible cholesterol in RBC membranes is stable within an individual but varies >10-fold among individuals. The high degree of inter-individual variation was unrelated to the total cholesterol content of the RBC membrane, but was associated with differences in RBC phospholipid composition of the RBC membrane (Table 4). Cholesterol accessibility in RBCs was also correlated with the levels of HDL-C (positively) and TG (inversely) in plasma (***Figure 7A***). RBCs from two subjects with ABCA1 deficiency (Tangier disease) had the lowest level of accessible cholesterol among the 528 individuals in whom it was measured, and yet patients with LCAT deficiency, who have similarly low levels of HDL-C, have a more modest reduction in fALOD4 binding to RBCs (***Figure 7B***). Thus, the accessibility is determined in part by genetic factors. Additionally, plasma from different individuals is able to modulate RBC cholesterol accessibility differentially, suggesting that circulating factors extrinsic to the RBC partly determine cholesterol accessibility. Taken together, our data indicate that RBC accessible cholesterol, as determined by fALOD4 binding, is a stable phenotype that is independent of total cholesterol levels in RBCs but varies with the phospholipid content of the RBC membrane and with plasma lipid and lipoprotein levels. The significance of this variability on the trafficking of cholesterol among plasma components and tissues awaits further elucidation.

Among individuals, relative fALOD4 binding to RBCs did not correlate with the total cholesterol content of the RBC membranes (***Figure 4C***). Thus, the cholesterol in the RBC membrane that is accessible to fALOD4 represents a distinct pool (or state) of cholesterol, which is not reflected by direct assays of RBC cholesterol mass. The striking reproducibility of relative fALOD4 binding to RBCs within a given individual (***Figure 3***) indicates that the factors that govern RBC cholesterol accessibility must be relatively stable. Here we show for the first time that the level of accessible cholesterol in RBCs varies significantly among individuals in a

healthy population and identify several intrinsic and extrinsic factors associated with variation in RBC cholesterol accessibility.

The relationship between RBC cholesterol content and fALOD4 binding was nonlinear (***Figure 2D***), as has been reported previously for RBC membranes (21) and for other cellular membranes like the ER and PM (25, 26). The lack of a relationship between cholesterol content and cholesterol activity in RBCs is like what has been observed in membranes from different mammalian cell organelles. For example, despite the wide differences in cholesterol content of plasma membranes (PM) and endoplasmic reticulum (ER) (40-50 mole% vs. 5 mole% respectively) both membranes similar cholesterol chemical activity (32). This equivalence is achieved because non-cholesterol lipids in PM and ER differ widely and the affinity of cholesterol for phospholipids varies depending on the phospholipid structure (32-34). Fluctuations in membrane lipid composition leads to changes in the chemical activity of cholesterol and result in cholesterol transport between membranes, either internally or externally. In the case of the RBC, changes in cholesterol activity in the plasma membrane must equilibrate with cholesterol in lipoproteins, other plasma proteins, or with other cells, such as circulating leukocytes or endothelial cells (35).

Why should RBC cholesterol accessibility be maintained at levels that closely bracket the threshold point? At cholesterol concentrations below the inflection point, the capacity of the membrane to incorporate cholesterol into complexes with phospholipids is high and the propensity for cholesterol to leave the membrane is low, therefore cholesterol will tend to accumulate in the RBC. At cholesterol concentrations above the inflection point, the capacity of the membrane to accommodate additional cholesterol in complexes with phospholipids is low, and cholesterol is poised to leave the RBC membrane when suitable acceptors become available. Thus, the cholesterol content of the RBC membranes is maintained close to equilibrium with respect to cholesterol exchange.

Since the fALOD4 assays were performed on isolated RBCs, the variation in fALOD4 binding likely reflects differences in one or more components of the RBC membrane, which is comprised of proteins and lipids. Depletion of membrane proteins by pronase digestion had only a modest effect on fALOD4 binding (***Figure 8A***), although we cannot exclude the possibility that in our experiments pronase treatment failed to digest a protein that contributes to the inter-individual differences in cholesterol accessibility. Nor was association found between ABO blood groups and fALOD4 binding (***Figure 8B***), but fALOD4 binding was reproducibly lower in those who were negative for the Rh antigen, a transmembrane protein of unknown function (36). The effect of the Rh antigen on fALOD4 binding may be direct or indirect. Rh antigen may compete with or directly inhibit fALOD4 binding. Alternatively, the antigen may alter cholesterol accessibility in the RBC membrane. More extensive studies of the relationship between Rh antigenicity and cholesterol accessibility will be needed to elucidate the basis for this relationship.

Although the cholesterol content of RBCs did not affect fALOD4 binding, the non-cholesterol lipids contributed significantly to differences in cholesterol accessibility. Previous studies have shown that membrane phospholipid composition systematically influences cholesterol accessibility (30, 37, 38). In the present study, differences in lipid composition were identified that were associated with fALOD4 binding (Table 4) but identifying exactly which lipid species contributed to inter-individual variation in cholesterol accessibility was not straightforward. The complex population of phospholipids in the RBC membrane and the high degree of correlation between different phospholipid classes confounded the analysis. In the multivariate model adjusted for age, sex, lipid and lipoprotein levels, only LPE, SM and PA were associated with fALOD4 binding. Taken together, differences in these membrane lipids accounted for 17.6% of the variability in fALOD4 binding. Studies in which the RBC membrane content of individual phospholipids can be selectively altered will be required to elucidate the effect of membrane phospholipids on cholesterol accessibility. We found that treatment of RBCs

with either PLA2 or SMase increased cholesterol accessibility (***Figure 9***), a finding that is consistent with prior studies (29, 39).

Factors extrinsic to the RBC were also associated with differences in RBC cholesterol accessibility. Lipoprotein lipids accounted for 5.5% of the variation in fALOD4 binding in a multivariate model that included gender and race. RBC cholesterol accessibility was positively correlated with plasma levels of HDL-C, but not with levels of LDL-C. This finding is consistent with prior studies that have documented a propensity of cholesterol to move from HDL to RBC membranes (40). In contrast, the plasma levels of TG and RBC cholesterol accessibility were inversely correlated. This correlation may be a direct result of movement of accessible cholesterol from RBCs to very low density lipoproteins (VLDL) (41). It is also possible that circulating lipoproteins influence RBC cholesterol accessibility by transferring phospholipids to (or from) the RBC membrane (42). We cannot exclude the possibility that the correlation of fALOD4 binding to RBC and VLDL-C levels is secondary to confounding with HDL levels, which are strongly negatively correlated with VLDL levels.

The lowest level of fALOD4 binding to RBCs in our study was observed in individuals with Tangier disease, a recessive disease caused by absence of ABCA1 (43). ABCA1 exports free cholesterol from cells and is required for the formation of HDL. Individuals with Tangier Disease have extremely low levels of HDL, but the negligible binding of fALOD4 to RBCs in these individuals cannot be fully explained by their low HDL levels. An equivalent reduction in HDL levels were seen in patients with LCAT deficiency, yet these individuals had more moderate reductions in fALOD4 binding to RBCs. A potential contributing factor to the low fALOD4 binding observed in these patients is differences in RBC membrane phospholipid composition: Tangier patients' RBCs have notably high phosphatidylcholine and low sphingomyelin relative to control individuals (56, 57).

It remains possible that ABCA1 transfers cholesterol directly from cells to RBCs, and thus contributes to the flux of cholesterol from extrahepatic tissues to the liver. RBCs do not

have access to peripheral tissues unless there is loss of vascular integrity, but they can interact indirectly with peripheral tissues through cells, such as macrophages, and lipoproteins that enter tissues and return to the circulation. RBCs have been shown to be excellent acceptors of free cholesterol from macrophages (44). Brown and Goldstein found that RBCs were 6-fold more potent than whole serum as cholesterol acceptors from macrophages (44). Lipoprotein-depleted plasma is as efficient an acceptor as whole serum or plasma from cholesterol-loaded macrophages (44). Although here we focused on the relationship between RBC accessible cholesterol and lipoprotein levels, it is also possible that blood components other than lipoproteins contribute to differences in cholesterol accessibility of the RBC membrane (44). RBCs may also exchange cholesterol with endothelial cells, thus indirectly transporting cholesterol from tissues. It is unlikely that RBCs deliver cholesterol directly to hepatocytes since they are too large to enter the space of Disse.

Significant and systematic differences in RBC cholesterol activity were found among individuals of White, Black, and Hispanic descent. Median fALOD4 binding was highest in African-Americans (1.18 nRFU), intermediate in Hispanics (0.86 nRFU) and lowest in European-Americans (0.77 nRFU). These differences were seen in two independent samples collected at different times (***Figure 5***). The increased RBC accessibility in Blacks was not fully explained by the measured differences in RBC lipid composition or by the plasma lipoprotein levels. Significant differences in plasma activity among individuals of European, African, and Hispanic descent, with plasma activity highest in Whites, lowest in Blacks and intermediate in Hispanics (***Figure 11B***). This suggests that factors in plasma separate from lipoprotein content contribute to the ethnic differences observed in RBC cholesterol accessibility. It would be informative to measure fALOD4 binding to RBC in a larger multiethnic sample and use genetic association to identify potential factors accounting for the observed differences between both individuals and ethnic groups.

It remains possible that the interethnic differences in cholesterol accessibility may be the consequences of historic differences in selective pressure among the three ethnic groups. RBCs with higher fALOD4 binding would be anticipated to be more susceptible to microorganisms that produce cholesterol-binding toxins. ALO, produced by *Bacillus Anthracis*, is a member of the cholesterol-dependent cytolysin family, which is a family of pore-forming toxins produced by a myriad of different gram positive bacteria, including species of *Streptococcus*, *Listeria* and *Clostridium (45)*. Perhaps individuals with lower fALOD4 binding are less susceptible to the hemolytic effects of these toxins, thus providing them with a survival advantage.

Genetic and pharmacological studies have provided compelling evidence that increased circulating levels of HDL, widely viewed as the vehicle for reverse cholesterol transport, do not confer protection against coronary heart disease (46). RBCs may be a conduit for transporting cholesterol from peripheral tissues to the liver. Mice made anemic had reduced fecal excretion of cholesterol from macrophages (14), and anemia has been shown to be associated with increased coronary artery disease (Hazard Ratio of 1.41 over 6 years) (47). To determine if RBC cholesterol accessibility provides clinically relevant biomarker, we measured cholesterol accessibility in patients with chronic renal failure on dialysis, who have a dramatically increased risk of atherosclerosis that is not explained by known risk factors (23). We found a systematic increase in cholesterol accessibility in RBCs in patients on dialysis when compared to healthy age- and race-matched controls. The cause of these differences and whether they are related to the increase in atherosclerosis seen in this population is not known at this time and will require further study.

ACKNOWLEDGEMENTS

I want to express my deepest appreciation for the mentorship and guidance of Dr. Helen Hobbs, Dr. Jonathan Cohen, and Dr. Arun Radhakrishan. They provided for me an environment that granted both independence and structured guidance, which resulted in a productive and rigorous research experience. They constantly challenged me to think critically and creatively, which are skills that will be invaluable as I move forward in my academic research career as a neurologist. I am also grateful to my committee members Dr. Jay Horton and Dr. Sandra Hofmann for their support during this process.

Additionally, I want to thank Sally Ingham, a fellow Howard Hughes Medical Institute (HHMI) year-long fellow, who validated and moved this project forward after I completed my two-year HHMI fellowship.

Funding for this work was provided by the HHMI year-long fellowship and I am grateful that the organization renewed my research funding for a second year. The Howard Hughes Medical Institute has been pivotal in my growth as a physician-scientist and I especially want to thank Melanie Daub for being a dedicated advocate for the medical fellow's program.

Finally, I want to thank my husband, Gaurab Chakrabarti, for his unwavering support in all my professional endeavors, brilliant insights during our discussions of my project, and shared enthusiasm for making the world a better place. I am also deeply appreciative of my parents, whose unremitting love and guidance are my constant source of energy and whose sacrifices and life choices are the reasons why I have the opportunity to write this paper and pursue my dream of becoming a physician-scientist.

REFERENCES

1. Goldstein JL, Helgeson JA, Brown MS. Inhibition of cholesterol synthesis with compactin renders growth of cultured cells dependent on the low density lipoprotein receptor. J Biol Chem. 1979;254(12):5403-9.
2. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science. 2000;290:1771-75.
3. Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res. 2009;50 Suppl:S120-5.
4. Glomset JA. The plasma lecithin: cholesterol acyltransferease reaction. J Lipid Res. 1968;9:155-67.
5. Bailey JM. Lipid Metabolism in Cultured Cells. 3. Cholesterol Excretion Process. Am J Physiol. 1964;207:1221-5.
6. Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, et al. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J Clin Invest. 1999;104:R25-31.
7. Stoffel W, Zierenberg O, Tunggal B, Schreiber E. 13C nuclear magnetic resonance spectroscopic evidence for hydrophobic lipid-protein interactions in human high density lipoproteins. Proc Natl Acad Sci U S A. 1974;71(9):3696-700.
8. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science. 1996;271(5248):518-20.
9. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ Res. 2005;96(12):1221-32.
10. Xie C, Turley SD, Dietschy JM. ABCA1 plays no role in the centripetal movement of cholesterol from peripheral tissues to the liver and intestine in the mouse. J Lipid Res. 2009;50(7):1316-29.
11. Jolley CD, Woollett LA, Turley SD, Dietschy JM. Centripetal cholesterol flux to the liver is dictated by events in the peripheral organs and not by the plasma high density lipoprotein or apolipoprotein A-I concentration. J Lipid Res. 1998;39(11):2143-9.
12. Turner S, Voogt J, Davidson M, Glass A, Killion S, Decaris J, et al. Measurement of reverse cholesterol transport pathways in humans: in vivo rates of free cholesterol efflux, esterification, and excretion. J Am Heart Assoc. 2012;1(4):e001826.
13. Hagerman JS, Gould RG. The in vitro interchange of cholesterol between plasma and red cells. Proc Soc Exp Biol Med. 1951;78(1):329-32.
14. Hung KT, Berisha SZ, Ritchey BM, Santore J, Smith JD. Red blood cells play a role in reverse cholesterol transport. Arterioscler Thromb Vasc Biol. 2012;32(6):1460-5.
15. McConnell HM, Radhakrishnan A. Condensed complexes of cholesterol and phospholipids. Biochim Biophys Acta. 2003;1610(2):159-73.
16. Radhakrishnan A, McConnell HM. Chemical activity of cholesterol in membranes. Biochemistry. 2000;39(28):8119-24.
17. Ahn KW, Sampson NS. Cholesterol oxidase senses subtle changes in lipid bilayer structure. Biochemistry. 2004;43(3):827-36.
18. Lange Y, Tabei SM, Ye J, Steck TL. Stability and stoichiometry of bilayer phospholipid-cholesterol complexes: relationship to cellular sterol distribution and homeostasis. Biochemistry. 2013;52(40):6950-9.
19. Heuck AP, Hotze EM, Tweten RK, Johnson AE. Mechanism of membrane insertion of a multimeric beta-barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. Mol Cell. 2000;6(5):1233-42.

20. Gay A, Rye D, Radhakrishnan A. Switch-like responses of two cholesterol sensors do not require protein oligomerization in membranes. Biophys J. 2015;108(6):1459-69.
21. Lange Y, Cutler HB, Steck TL. The effect of cholesterol and other intercalated amphipaths on the contour and stability of the isolated red cell membrane. J Biol Chem. 1980;255(19):9331-7.
22. Bourdeau RW, Malito E, Chenal A, Bishop BL, Musch MW, Villereal ML, et al. Cellular functions and X-ray structure of anthrolysin O, a cholesterol-dependent cytolysin secreted by Bacillus anthracis. J Biol Chem. 2009;284(21):14645-56.
23. Levey AS, Beto JA, Coronado BE, Eknoyan G, Foley RN, Kasiske BL, et al. Controlling the epidemic of cardiovascular disease in chronic renal disease: what do we know? What do we need to learn? Where do we go from here? National Kidney Foundation Task Force on Cardiovascular Disease. Am J Kidney Dis. 1998;32(5):853-906.
24. Nelson LD, Johnson AE, London E. How interaction of perfringolysin O with membranes is controlled by sterol structure, lipid structure, and physiological low pH: insights into the origin of perfringolysin O-lipid raft interaction. J Biol Chem. 2008;283(8):4632-42.
25. Das A, Goldstein JL, Anderson DD, Brown MS, Radhakrishnan A. Use of mutant 125I-perfringolysin O to probe transport and organization of cholesterol in membranes of animal cells. Proc Natl Acad Sci U S A. 2013;110(26):10580-5.
26. Sokolov A, Radhakrishnan A. Accessibility of cholesterol in endoplasmic reticulum membranes and activation of SREBP-2 switch abruptly at a common cholesterol threshold. J Biol Chem. 2010;285(38):29480-90.
27. Besenicar MP, Bavdek A, Kladnik A, Macek P, Anderluh G. Kinetics of cholesterol extraction from lipid membranes by methyl-beta-cyclodextrin--a surface plasmon resonance approach. Biochim Biophys Acta. 2008;1778(1):175-84.
28. Slotte JP, Bierman EL. Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts. Biochem J. 1988;250(3):653-8.
29. Subbaiah PV, Billington SJ, Jost BH, Songer JG, Lange Y. Sphingomyelinase D, a novel probe for cellular sphingomyelin: effects on cholesterol homeostasis in human skin fibroblasts. J Lipid Res. 2003;44(8):1574-80.
30. Ohvo H, Olsio C, Slotte JP. Effects of sphingomyelin and phosphatidylcholine degradation on cyclodextrin-mediated cholesterol efflux in cultured fibroblasts. Biochim Biophys Acta. 1997;1349(2):131-41.
31. Leidl K, Liebisch G, Richter D, Schmitz G. Mass spectrometric analysis of lipid species of human circulating blood cells. Biochim Biophys Acta. 2008;1781(10):655-64.
32. Radhakrishnan A, McConnell HM. Electric field effect on cholesterol-phospholipid complexes. Proc Natl Acad Sci U S A. 2000;97(3):1073-8.
33. Finean JB. Phospholipid-cholesterol complex in the structure of myelin. Experientia. 1953;9(1):17-9.
34. Wattenberg BW, Silbert DF. Sterol partitioning among intracellular membranes. Testing a model for cellular sterol distribution. J Biol Chem. 1983;258(4):2284-9.
35. Lange Y, Steck TL. Active membrane cholesterol as a physiological effector. Chem Phys Lipids. 2016;199:74-93.
36. Huang CH, Liu PZ, Cheng JG. Molecular biology and genetics of the Rh blood group system. Semin Hematol. 2000;37(2):150-65.
37. Ohvo H, Slotte JP. Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. Biochemistry. 1996;35(24):8018-24.
38. Lund-Katz S, Laboda HM, McLean LR, Phillips MC. Influence of molecular packing and phospholipid type on rates of cholesterol exchange. Biochemistry. 1988;27(9):3416-23.

39. Slotte JP, Hedstrom G, Rannstrom S, Ekman S. Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior. Biochim Biophys Acta. 1989;985(1):90-6.
40. Quarfordt SH, Hilderman HL. Quantitation of the in vitro free cholesterol exchange of human red cells and lipoproteins. J Lipid Res. 1970;11(6):528-35.
41. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, et al. Single verticla spin density gradient ultracentrifugation. Methods in Enzymol. 1986;128:181.
42. Reed CF. Phospholipid exchange between plasma and erythrocytes in man and the dog. J Clin Invest. 1968;47(4):749-60.
43. Hobbs HH, Rader DJ. ABC1: connecting yellow tonsils, neuropathy, and very low HDL. J Clin Invest. 1999;104(8):1015-17.
44. Ho YK, Brown MS, Goldstein JL. Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. J Lipid Res. 1980;21(4):391-8.
45. Palmer M. The family of thiol-activated, cholesterol-binding cytolysins. Toxicon. 2001;39(11):1681-9.
46. Siddiqi HK, Kiss D, Rader D. HDL-cholesterol and cardiovascular disease: rethinking our approach. Curr Opin Cardiol. 2015;30(5):536-42.
47. Sarnak MJ, Tighiouart H, Manjunath G, MacLeod B, Griffith J, Salem D, et al. Anemia as a risk factor for cardiovascular disease in The Atherosclerosis Risk in Communities (ARIC) study. J Am Coll Cardiol. 2002;40(1):27-33.
48. Goldstein JL, Basu SK, Brown MS. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol. 1983;98:241-60.
49. Brown AJ, Sun L, Feramisco JD, Brown MS, Goldstein JL. Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol Cell. 2002;10(2):237-45.
50. Dodge JD. Chromosome structure in the Dinophyceae. I. The spiral chromonema. Arch Mikrobiol. 1963;45:46-57.
51. Ferru E, Pantaleo A, Turrini F. A new method for the capture of surface proteins in Plasmodium falciparum parasitized erythrocyte. J Infect Dev Ctries. 2012;6(6):536-41.
52. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226(1):497-509.
53. Wilund KR, Yu L, Xu F, Vega GL, Grundy SM, Cohen JC, et al. No association between plasma levels of plant sterols and atherosclerosis in mice and men. Arterioscler Thromb Vasc Biol. 2004;24(12):2326-32.
54. Dodge JT, Phillips GB. Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. J Lipid Res. 1967;8(6):667-75.
55. Dinkla S, Wessels K, Verdurmen WP, Tomelleri C, Cluitmans JC, Fransen J, et al. Functional consequences of sphingomyelinase-induced changes in erythrocyte membrane structure. Cell Death Dis. 2012;3:e410.
56. Frohlich J, Hoag G, McLeod R, Hayden M, Godin DV, Wadsworth LD, Critchley JD, Pritchard PH. Hypoalphalipoproteinemia resembling fish eye disease. Acta Med Scand. 1987;221(3):291-8.
57. Reinhart WH, Gössi U, Bütikofer P, Ott P, Sigrist H, Schatzmann HJ, Lutz HU, Straub PW. Haemolytic anaemia in analpha-lipoproteinaemia (Tangier disease):morphological, biochemical, and biophysical properties of the red blood cell. Br J Haematol. 1989 Jun;72(2):272-7.

VITAE

Rima Shah Chakrabarti (November 2nd, 1988-present) was born in Scranton, Pennsylvania. She attended Brown University where she concentrated in neuroscience and dance. She met her husband at Brown and after graduation, she joined him at UT Southwestern Medical Center for medical school, where she is currently a fourth-year medical student. Upon graduation, Rima will begin residency in Neurology at the University of Pennsylvania. In her free time, Rima practices classical Indian dance, ballet, and hip hop and choreographs dances that fuse these dance forms. She also enjoys singing a capella, inventing meal recipes with whatever is lying around in the kitchen, reading non-fiction, and 'talking science' with her husband, Gaurab.

Permanent Address: 115 Whitetail Run

Waverly Township, PA 18414