ARIC Manuscript Proposal # 1167

PC Reviewed: __06/_20_/06	**Status: _A_**	**Priority: _2_**
SC Reviewed: __06/23/06_	**Status: _A_**	**Priority: _2_**

1.a. Full Title: Reliability of Flow Cytometric Parameters

b. Abbreviated Title (Length 26 characters): Reliability of Flow Cytometry

2. Writing Group: Diane Catellier, Nena Aleksic

I, the first author, confirm that all the coauthors have given their approval for this manuscript proposal. __DJC_____ **[please confirm with your initials electronically or in writing]**

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3. Timeline: Begin analyses when approved by Pubs committee

4. Rationale: The purpose of this paper is to report on the reliability of flow cytometry measurement of antigen expression in stabilized blood samples in a multicenter study. An extensive series of monoclonal antibodies (platelet markers: CD41, CD61, CD62P, CD154; monocytes markers: CD14, TLR2, TLR4, CD162, MPO, COX-2; pan-leukocyte marker: CD45) were tested using 2-color or 3-color protocols. A description of the basic aspects of multi-color flow cytometry protocols is given by Baumgarth et al. (1); Michelson et al. (2) and Li et al. (3) provide specific details of evaluation of platelet markers; Sabroe and colleagues (4) describe the analysis of TLR2 and TLR4; and Hazen discusses the link between MPO and atherosclerotic plaque (5). The variables derived from flow cytometry are proportion of cells expressing the antigen of interest, and the relative level of antigen expression (mean or median fluorescence intensity).

We can think of an individual's measurement as being made up of two components, the true value and random error. Reliability is the proportion of "truth" in the measurement, or the ratio of the true score variance to the observed score variance. Reliability can be estimated by taking repeated

measurements of the same group of people to determine how much their measurements fluctuate. Fluctuations from one person's measurements are attributed to error. If reliability is low, the ability to differentiate between the subjects with different risk factors or disease states decreases. The ARIC Carotid MRI quality control program was designed to monitor the reliability of flow cytometry measurements over time, and to identify factors that might affect reliability.

Potential sources of error in measurement include variability in the drawing or processing of blood (e.g., fasting status, time between drawing and processing), variation in quality of the blood sample after shipping to the central laboratory, variation in the reagent (from one lot to the next), variation between technicians performing the measurements, and variation over time within an individual. Three sub-studies were designed to evaluate additional sources of variation. The first study has the fewest sources of variation. A convenience sample of 20 tubes of blood was selected at the laboratory for replicate testing. Each tube of blood was split into two aliquots and measures obtained from independent flow cytometry analysis were compared. Measurement error variation estimated from this data cannot be attributed to variation in blood drawing, processing, or shipment procedures, or within-subject variation over time. In the second study, a random sample of 50 subjects was selected to have a second tube of blood drawn by the same technician during venipuncture and sent to the flow cytometry laboratory. These additional QC specimens are labeled with a *phantom* participant ID that is indistinguishable from other ID numbers, so that the laboratory is blinded to the QC process. Measurement error variation estimated from this second study will include the analysis sources of error present in Study 1 and variation in blood drawing, processing, or shipment procedures. In the third study, 60 participants repeated the entire clinic visit within one month of their original visit. Again, the replicate sample was labeled with a *phantom* participant ID so that the laboratory was blinded to the QC process. Measurement error variation from this study will include measurement variability that is due to errors in the analysis process, blood drawing, processing and shipping, as well as variability within-subject variation over time.

5. Main Hypothesis/Study Questions: What is the reliability of flow cytometry for determination of antigen expression in stabilized blood samples in a large multicenter study? Are there factors that are associated with reliability (e.g., quality of the sample)? Using results from all three sub-studies, estimate the component of variation that is attributable to laboratory error, sample drawing and processing error, and short-term within-subject variation in antigen expression.

6. Data (variables, time window, source, inclusions/exclusions): Internal QC pool: 20 unblinded replicates selected (non-randomly) at the laboratory. External QC pool: 50 replicates selected at random, unblinded to the venipuncture technician but blinded to laboratory staff. Repeatability study data: repeated venipuncture one month part on 60 participants selected (non-randomly) at the field centers.

Exclusions/Inclusions: none

Variables:

1. Antigens: CD61, CD62P, CD41, CD154, COX2, TLR2, TLR4, CD14, CD45, CD162, MPO

- the proportion of cells expressing the antigen of interest
- the relative level of antigen expression (mean or median fluorescence intensity)

2. Blood processing and shipping variables:
- technician
- problems during venipuncture (non-fasting state, multiple venipuncture attempts, tourniquet reapplied, needle movement, excessive bleeding)
- blood processing variables (volume collected, time sample left at room temperature before shipping)
- quality of sample upon shipping (clotted, hemolyzed, lipemic, other contamination)
- quality when received at the laboratory

3. Repeatability study variables:
- time between visits
- technician (same or different on two visits)

For each of the 3 sub-studies, we will compute standard indices of reliability including: (1) mean, standard deviation of paired measurements; (2) the mean difference, and associated confidence interval, between paired measurements on the same subject; (3) variances (within- and between-subject); (4) proportion of total variance attributable to measurement error (e.g., reliability).

We will estimate reliability (R) from a one way analysis of variance with subject as the only factor. That is, $R = (MS_b - MS_w)/ (MS_b + MS_w)$, where using the MS_b and MS_w are the between and within-subject mean square values, respectively. We can also estimate reliability by treating subject as a random effect in a mixed model. Using this model, the total variance is partitioned between the variance of the random effect parameter (within-subject) and residual components of variation. Using this modeling framework, we will also examine the effect of various factors on reliability. In particular, we will examine whether inclusion of fixed effects such as field center, technician, sample quality, reagent lot number, time trends significantly reduces or "explains" the within-subject component of variance. Note that the proportion of cells expressing the antigen of interest is not normally distributed (i.e., it is constrained to lie between 0 and 1). Therefore, generalized logistic mixed models (rather than linear mixed models) will be used to estimate the variance components and to explore whether the measurement error component differs as a function of field center, technician, sample quality, etc (6).

7.a. Will the data be used for non-CVD analysis in this manuscript? ____ Yes _X_ No

b. If Yes, is the author aware that the file ICTDER02 must be used to exclude persons with a value RES_OTH = "CVD Research" for non-DNA analysis, and for DNA analysis RES_DNA = "CVD Research" would be used? ____ Yes ____ No
(This file ICTDER02 has been distributed to ARIC PIs, and contains the responses to consent updates related to stored sample use for research.)

8.a. Will the DNA data be used in this manuscript? ____ Yes _X_ No

8.b. If yes, is the author aware that either DNA data distributed by the Coordinating Center must be used, or the file ICTDER02 must be used to exclude those with value RES_DNA = "No use/storage DNA"? **_X_ Yes ____ No**

9.The lead author of this manuscript proposal has reviewed the list of existing ARIC Study manuscript proposals and has found no overlap between this proposal and previously approved manuscript proposals either published or still in active status. ARIC Investigators have access to the publications lists under the Study Members Area of the web site at: http://www.cscc.unc.edu/ARIC/search.php

_X_ Yes _____ No

10. What are the most related manuscript proposals in ARIC (authors are encouraged to contact lead authors of these proposals for comments on the new proposal or collaboration)?
none

11. a. Is this manuscript proposal associated with any ARIC ancillary studies or use any ancillary study data? **____ Yes _X_ No**

11.b. If yes, is the proposal

___ A. primarily the result of an ancillary study (list number* _____)

___ B. primarily based on ARIC data with ancillary data playing a minor role (usually control variables; list number(s)* _____ _____ _____)

*ancillary studies are listed by number at http://www.cscc.unc.edu/aric/forms/

12. Manuscript preparation is expected to be completed in one to three years. If a manuscript is not submitted for ARIC review at the end of the 3-years from the date of the approval, the manuscript proposal will expire.

References:

1. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Meth*, 2000;243:77-97.
2. Michelson AD, Barnard MR, Krueger LA, Frelinger AL, Furman MI. Evaluation of platelet function by flow cytometry. *Methods*, 2000;21:259-279.
3. Li N et al. A sensitive flow cytometric assay for circulating platelet-leukocyte aggregates *British J Haematology*, 1997;99:808-816.
4. Sabroe I, Jones EC, Usher LR, Whyte MKB, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *The Journal of Immunology*, 2002;168:4701-4710.
5. Hazen LH. Myeloperoxidase and plaque vulnerability. *Arterioscler Thromb Vasc Biol*, 2004;24:1142-1146.
6. Liao JG, Lipsitz SR. A type of restricted maximum likelihood estimator of variance components in generalized linear mixed models. Biometrika 2002;89:401-409.