Comparative Medicine
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Vol 66, No 6
December 2016
Pages 474–479

Original Research

Using Hematology Data from Malaria Vaccine Research Trials in Humans and Rhesus Macaques (*Macaca mulatta*) To Guide Volume Limits for Blood Withdrawal

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Guidelines on safe volume limits for blood collection from research participants in both humans and laboratory animals vary widely between institutions. The main adverse event that may be encountered in large blood volume withdrawal is iron-deficiency anemia. Monitoring various parameters in a standard blood panel may help to prevent this outcome. To this end, we analyzed the Hgb and MCV values from 43 humans and 46 macaques in malaria vaccine research trials. Although the percentage of blood volume removed was greater for macaques than humans, macaques demonstrated an overall increase of MCV over time, indicating the ability to respond appropriately to frequent volume withdrawals. In contrast, humans showed a consistent declining trend in MCV. These declines in human MCV and Hgb were significant from the beginning to end of the study despite withdrawals that were smaller than recommended volume limits. Limiting the volume withdrawn to no more than 12.5% seemed to be sufficient for macaques, and at 14% or more individual animals tended to fail to respond appropriately to large-volume blood loss, as demonstrated by a decrease in MCV. The overall positive erythropoietic response seen in macaques was likely due to the controlled, iron-fortified diet they received. The lack of erythropoietic response in the human subjects may warrant iron supplementation or reconsideration of current blood volume withdrawal guidelines.

Abbreviations: IDA, iron-deficiency anemia; IRB, Institutional Review Board

Many of the vaccines and pharmacotherapeutics used for public health disease prevention programs are the result of extensive research involving human and laboratory animal participants. In both clinical trials and laboratory animal research, phlebotomy-induced anemia is a clinically relevant safety concern, particularly in studies involving large-volume collections. Researchers often experience a conflict between keeping volunteers and animal subjects safe, maximizing the blood volumes collected to achieve the most scientific benefit possible from a study, and reducing overall human and animal use.

Currently, few Institutional Review Board (IRB) or IACUC guidelines are available to inform researchers about appropriate phlebotomy volumes. In human-subjects research, a study may be eligible for expedited review by the IRB when it meets specific criteria designated as minimal risk. Regarding phlebotomy, the following criteria are considered "minimal risk" by the Department of Health and Human Services Office for Human Research Protections: for healthy adults weighing more than 110 pounds, "the amount drawn may not exceed 550 mL in an 8-week period, and collection may not occur more frequently than 2 times per week."[1] For adults weighing less than 110 pounds and children, the phlebotomy amounts my not exceed "the lesser of 50 mL or 3 mL/kg in an 8-week period" nor "occur more frequently than 2 times per week."[16] Phlebotomy amounts or frequencies that are outside of these criteria must go through a full IRB review, during which volumes will be considered according to the reviewing institution's guidelines regarding maximal blood draw limits, if available. The former American Association of Blood Banks (now known simply as AABB) instituted a statute with a similar limit: 525 mL in an 8-wk period.[1] This standard was adopted by the American Red Cross and is based on the estimate that a 110-pound adult will not lose more than 15% of the total blood volume at this sample size and frequency.[6] This standard bears similarity to the Office of Human Research Protections minimal risk standard, although the AABB standard is intended for a single-collection with an 8-wk recovery period. Some research protocols undergoing full IRB review use this single-collection standard, because it is even more conservative than the minimal risk standard, despite having multiple draws over the 8-wk period.

In animal research, each IACUC is responsible for reviewing research animal protocols prior to approval. Each facility's IACUC may follow different internal policies and regulate phlebotomy limits differently. In the *IACUC Handbook*, 228 (77.6%)

Received: 13 Apr 2016. Revision requested: 21 May 2016. Accepted: 12 Jun 2016.
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of the 293 institutions surveyed had a policy regarding maximal single blood-withdrawal limits, and a smaller number (n = 132; 45%) had a policy for repeated sampling limits.[14] In both cases, the blood withdrawal limits were either set as a percentage of body weight or as specific blood volumes. Of the institutions that reported having a policy, the 10% limit was reported most commonly, both as a single-sample and multiple-sample collection.[14] Because circulating blood volumes vary among species, a percentage basis is more appropriate than a volumetric measure from an animal research perspective.

When repeated sampling occurs in a study, safety laboratory tests measuring clinical pathology parameters are often written into protocols. For human vaccine trials, the US Food and Drug Administration has provided guidance defining the magnitude of the change in a hematologic parameter value that constitutes an adverse event and has a toxicity grading scale for such.[18] Conversely, in laboratory animal research, the policies regarding safety lab requirements and interpretation are often left to the veterinary staff's discretion.[13]

However, most institutional protocols do not take into consideration sex- and age-associated differences in hematology parameters or hematopoietic response times. Therefore, comparative study of the effect of repeated phlebotomy RBC parameters in both human subjects and NHP is of regulatory and practical value, because the information gained can be used to interpret the applicability of safe-volume limits among research participants in human and animal models.

The published literature involving analysis of RBC parameters in both humans and NHP is sparse, and the articles available tend to be dated. For example, a MeSH term search of the subjects "Blood Specimen Collection"[Mesh] AND ("Anemia, hypochromic microcytic"[Supplementary Concept] OR "Anemia, Iron-Deficiency"[Mesh]) yielded 21 results, most of which do not include the analysis of effects of frequent sampling. Much of the focus for literature involving clinical safety labs is done in an effort to recognize and prevent adverse events. In the case of repeated and large-volume blood withdrawals, this adverse event would be iron-deficiency anemia (IDA), which is also the most common form of anemia.[8] The diagnostic evaluation for IDA might include CBC analysis, a reticulocyte count, a peripheral blood smear, serum iron levels, serum ferritin levels, and transferrin levels.[2]

In animals including humans, IDA occurs through a specific physiologic process. The oxygen-carrying heme molecule (a component of hemoglobin) in RBC requires iron to function. As RBC are formed in the bone marrow through erythropoiesis, iron stores in the body are mobilized. When there is a lack of iron stores (iron deficiency), either through increased clearance (for example, blood loss secondary to menorrhea), decreased intake (for example, nutrient-poor diet), metabolic disorders, or a combination thereof, RBC parameters may fall outside of normal physiologic levels. When erythropoiesis is impaired and fewer RBC are formed, a disproportionate number of older RBC is present. This situation has an additional effect on cellular heoglobin, given that aged RBC lose 15% to 20% of their hemoglobin throughout their 115-d lifespan.[4] In the presence of sufficient iron stores, the marrow of a healthy animal will respond appropriately to RBC loss by synthesizing new RBC and releasing reticulocytes, immature RBC, into the periphery. Reticulocytes can be distinguished from mature RBC by the presence of RNA (detected with appropriate stain) and by their larger volume. The RBC MCV metric captured in a routine CBC analysis can be used to detect changes in the RBC fraction. This parameter enables observation of the appropriate erythropoietic response to blood losses by means of detecting an increase in the RBC MCV as reticulocytes are produced. If this response does not happen, such as in the case of iron deficiency, the larger reticulocytes are not produced at the same rate, mature RBC are smaller, and therefore the average RBC volume will fail to increase or might even decrease. In fact, nearly 70% of IDA patients have a decreased MCV (microcytosis).[2] Therefore, the absolute values and kinetic responses of MCV and Hgb are important correlates of appropriate erythropoietic responses.

A correlate analyte within the standard CBC that was predictive of subjects that might be at risk of phlebotomy-induced anemia secondary to low iron stores would be very useful to clinicians and researchers. However, there is no 'gold standard' test for IDA, making diagnosis and especially prevention challenging.[2] Although some have suggested bone marrow biopsy as a potential gold standard, others have questioned its utility, and the invasiveness of the test renders it impractical. The use of various hematologic parameters and a detailed patient history remain critical parts of the diagnostic toolkit. Here we review a set of hematologic data from 2 long-term protocols, one in humans and one in rhesus macaques, to evaluate the utility of a change in MCV as a useful biomarker of impending IDA.

Materials and Methods

Hematology data sets were obtained for analysis from malaria vaccine trials in humans and in rhesus macaques. The macaque dataset included hematologic profiles of blood collected from 46 colony-bred male and female rhesus macaques (*Macaca mulatta*) enrolled in a malaria immunogenicity study.[15] This study was conducted under an approved IACUC protocol and was part of an AAALAC-accredited program. Animals were housed with conspecifics in rooms with a 12:12-h light cycle and environmental parameters within the standards outlined in the *Guide for the Care and Use of Laboratory Animals*.[9] Inclusion criteria were determined by those adult, nonpregnant, macaques deemed healthy by physical examination and having baseline clinical pathologic data parameters within normal reference ranges. Blood samples for the study were drawn under injectable sedation (ketamine and either acepromazine or diazepam as a cosedative) at specific intervals throughout the study. Concomitant hematologic parameters were obtained as part of pre- and postvaccination clinical pathology monitoring. These parameters included RBC indices (quantities and morphologies), WBC count, platelet count, BUN, serum creatinine, AST, ALT, GGT, and creatine kinase.

The human dataset involved hematologic data from a malaria vaccine trial evaluating safety and efficacy in human volunteers.[12] This clinical trial was approved by the conducting facility's Institutional Review Board, and all participants provided written informed consent. Human subjects were deidentified by an uninterested party by reviewing the dataset and removing subjects' initials; each subject was identifiable by number only. The protection of personal health data, in accordance with the Health Insurance Portability and Accountability Act of 1996 (HIPAA) Privacy Rule, was achieved through deidentification. The data were provided free of the 18 types of identifiers to meet the Safe Harbor criteria in statute §164.514(b)(2). No residual information remained that could identify subjects, although they were coded in

such a way that would allow reidentification if necessary. However, that code was not disclosed in the course of this current investigation. Inclusion criteria were healthy, nonpregnant adults 18 to 50 y of age, able to participate within the scheduled timelines of the study. Exclusion criteria included immune-compromised persons and those with preexisting immune responses to malaria. Fixed-volume blood samples were collected at specified intervals throughout the study, although collection intervals varied. Similar clinical pathologic safety data were collected: RBC indices (quantities and morphologies), WBC count, platelet count, BUN, serum creatinine, AST, ALT, and creatine kinase. Data from this set were collected on a fixed schedule with rolling enrollment.

Both datasets were similar in that they had large prestudy and periodic within-study blood draws (represented by the bar graph axes in Figures 1 and 2). Data analysis was performed under the hypothesis that blood volumes removed during clinical and lab animal trials cause detectable changes in hematologic parameters. A secondary hypothesis was that some of these parameters may be useful predictors of potential incipient IDA.

For the purposes of simplicity and clinical utility, RBC values of Hgb and MCV were included in analysis. Along with these CBC parameters, demographic variables of sex, weight, and age were transferred from their original spreadsheet format into SPSS 22 (IBM, Armonk, NY). Separate corresponding human and macaque SPSS datasets were created with a similar variable order and format. Because of the magnitude of data points generated at each time point and because some weeks had multiple blood draws and others none at all, aggregate datasets were computed on a per-study-week basis. Data was truncated by converting each 7-d period into a corresponding 1-wk variable and running an aggregate data command to calculate each associated variable as a mean value for the week. However, blood withdrawal amounts were aggregated as weekly totals (per subject) rather than mean values. In an attempt to compare changes in parameters across time, the percentage change from initial baseline amount was calculated for each case CBC parameter at each time point (Figures 1 and 2); in addition, an individual example was chosen for discussion purposes (Figure 3).

After variables were compiled and calculated, data were analyzed to plot responses of the hematologic parameters as they may pertain to blood volume limits. The human dataset was examined according to the percentage change from baseline during the three 8-wk periods we created: weeks –6 through 1, 2 through 9, and 10 through 12 (no data were available after week 12). The corresponding cases with parameters were selected and transferred to a new dataset. Mean MCV and Hgb values from the first and last week of each 8-wk period were compared with baseline. The total volume of blood collected over each 8-wk period was summed separately. The beginning and ending week data from each interval were analyzed for differences by paired *t* tests. In addition, paired *t* tests were performed from the beginning week to end week. The differences were charted and were compared relative to FDA regulations and AABB guidelines.

For NHP, which typically have a percentage-based limit regarding sample volume, our focus shifted to the large blood draws that approached these limits. In addition, because of the physiologic lag effect in MCV after blood withdrawal, the effect of the change had to be calculated by using the percentage change in the value from the sampling day compared with data obtained 4 wk after blood drawing. This practice allowed the measurement of

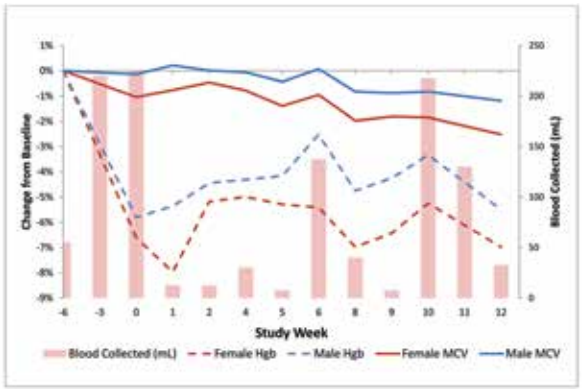

Figure 1. Dual-axis graph of human hematology data. The two-tailed Spearman ρ is –0.266 for MCV percentage change ($P < 0.001$) and –0.142 for Hgb percentage change ($P < 0.01$).

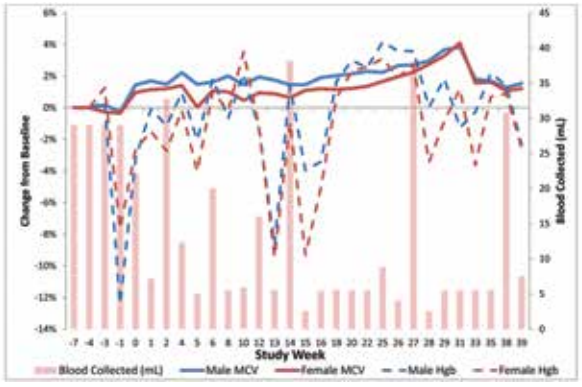

Figure 2. Dual-axis graph of macaque hematology data. The two-tailed Spearman ρ is 0.279 for MCV percentage change ($P < 0.001$) and 0.080 for Hgb percentage change ($P < 0.01$).

the mean change in MCV in macaques that were below or above a particular blood-volume-removed cutoff value. We chose the cutoff values of 10%, 12.5%, 14%, and 15% because they reflect common IACUC-acceptable withdrawal maximums.[15] First, the percentage of blood removed was calculated for each macaque case by taking the blood withdrawal amount multiplied by a species-specific constant (in mL/kg/%); for macaques, we used 56 mL/kg, as recommended previously.[5] For example: a 10-kg macaque from which 56 mL blood was removed that week experienced the loss of 10% of her blood volume, that is:

$$\text{Blood volume removed (\%)} = \frac{56 \text{ mL blood removed}}{10 \text{ kg} \times 56 \text{ mL/kg}}$$

$$= 10\%$$

We then chose the weeks of 0, 2, 6, 14, and 27 were chosen for the postcollection MCV analysis. These time points were selected because all were associated with large collection volumes, and all laboratory values at 4 wk after sample collection were available. A total of 146 data points were available at these time points. Independent *t* tests were performed for each cutoff value, and levels of significance were noted (Figure 4).

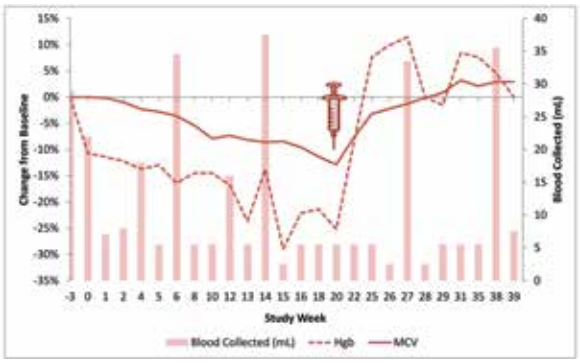

Figure 3. Dual-axis graph of an individual macaque's hematology data. Iron supplementation was requested during week 17 of the study and provided during week 20 (intramuscular injection of iron dextran; indicated by syringe).

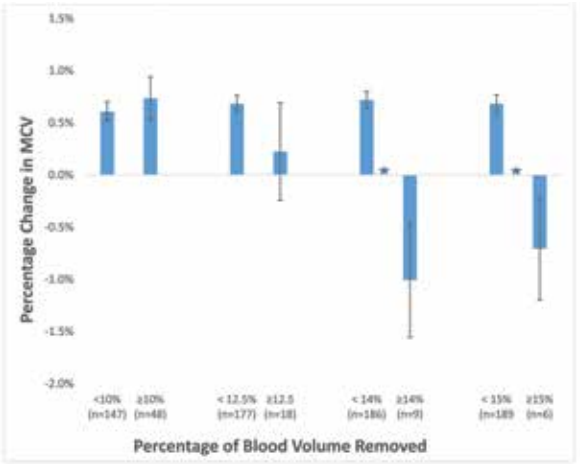

Figure 4. Mean change (error bar, 1 SE) in macaque MCV at 4 wk after blood collection according to percentage of blood volume removed. Stars represent statistical significance. *P* values by independent samples *t* test for each percentage grouping, with equal variances assumed: 10%, $P = 0.526$ (±1 SE, 0.09 to 0.21); 12.5%, $P = 0.124$ (±1 SE 0.08 to 0.47); 14%, $P < 0.001$ (±1 SE, 0.08 to 0.55); 15%, $P < 0.01$ (±1 SE, 0.09 to 0.49).

Results

The 21 female and 25 male macaques in the study ranged in age from 37 to 168 mo and in weight from 3.8 to 11.9 kg. Of 102 human volunteers, 47 were enrolled in the original study, and hematologic data were obtained from 25 men and 18 women, who ranged in age from 21 to 50 y old and in weight from 117 to 273 pounds. The remaining 4 volunteers could not be included in this hematologic analysis because their hematologic data were incomplete: 2 volunteers had served as infectivity controls only, with different blood collection schedules, and the other 2 were lost to follow up.

Group hematologic change over time. To best conceptualize the effect of changes in MCV and Hgb over time from their baseline values, we used the weekly mean parameter values by sex for each group to create a dual-axis graph that includes the actual amount of blood collected (in mL; Figures 1 and 2). The effect of large blood draws on the CBC parameters is evident by large decreases in Hgb after sampling. The overall MCV response differs between macaques and humans. Despite having an overall larger amount of blood removed per week on a percentage-removed basis, the macaque MCV tended to increase as the study progressed, whereas humans did not experience such an increase. This difference was tested by using the Spearman correlation coefficient (2-tailed). For macaques, the Spearman ρ was 0.279 for week and MCV percentage change ($P < 0.001$) and 0.080 for week and Hgb percentage change ($P < 0.01$). For humans, the Spearman ρ was –0.266 for week and MCV percentage change ($P < 0.001$) and –0.142 for week and Hgb percentage change ($P < 0.01$).

The actual values corresponding to these percentage decreases are shown in (Table 1). for clinical relationship. According to the *FDA Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials*,[18] any decrease to a maximum of 1.5 g/dL below the baseline value constitutes a mild (grade 1) adverse event, meaning that there is no interference with activity at this level.[18] Both females and males fell into this category, given that neither recovered or surpassed their baseline hemoglobin levels.

Individual example analysis. The individual macaque example in Figure 3 shows an overall decline in both MCV and HGB throughout the first half of the study. This macaque was a 10-y-old, 4.3-kg menstruating adult female that ranks 5th in the 46 macaques according to increasing weight. Because the same blood volume amounts were collected regardless of signalment, lightweight females might have been affected disproportionately, in light of their size and metabolic differences (for example, menstruation losses). Because of this subject's continual decline in parameters, a single iron injection was administered during week 20. The hemapoietic response was quite dramatic: once the hemoglobin molecules were able to function due to appropriate iron, Hgb improved rapidly. Because MCV is based on the proportion of new to old RBC, the response in this parameter was slower but remained positive.

Analysis compared with current blood volume limit guidelines. To correlate with AABB and IRB guidelines, the human data were examined according to the three 8-wk periods (Figure 5). The results essentially mirror Figure 1, but even though the total blood volume collected followed the recommended guidelines, parameter values failed to remain constant or increase during each period. This effect was tested by using paired *t* tests for each interval and from the beginning to end of the study. All differences were significant, except for MCV during the first 8-wk interval and Hgb during the second interval.

Finally, the effect of large-volume withdrawals of blood on the MCV of macaques was analyzed by comparing the mean percentage change in MCV across groups according to the percentage of blood volume removed. For the 195 events in total, there were decreasing trends in MCV at the 14% and 15% levels. These levels had significant differences according to independent sample *t* tests, although the 12.5% and 10% levels did not (Figure 4).

Discussion

At first glance, the changes in MCV seem less severe than those of Hgb, but it is important to recall that MCV is a measurement of the mean volume of the total population of circulating RBC. As such, the MCV value changes very subtly because only a small fraction of new, normally macrocytic, RBC affect the overall mean volume of the current circulating population of new and old cells. Importantly, each subject's MCV has a tightly regulated physiologic set point, such that any drop from baseline value is indicative of a microcytic anemia of some degree.[7] Therefore

Table 1. Descriptive statistics of human Hgb and MCV values (mean ± 1 SD)

	Week	Hgb (g/dL)	MCV (fL)
Women			
	–6	12.92 ± 0.99	88.03 ± 7.87
	1	11.83 ± 1.05	87.33 ± 7.62
	2	12.15 ± 0.85	87.07 ± 7.57
	9	12.04 ± 1.14	86.41 ± 7.86
	10	12.19 ± 1.23	86.37 ± 7.86
	12	11.93 ± 1.05	85.73 ± 7.25
Men			
	–6	15.01 ± 0.71	88.87 ± 4.69
	1	14.20 ± 0.70	89.04 ± 4.38
	2	14.29 ± 0.74	88.76 ± 4.70
	9	14.31 ± 0.88	88.43 ± 4.91
	10	14.51 ± 0.82	88.18 ± 5.00
	12	14.18 ± 0.80	87.87 ± 5.25

Normal reference ranges: women, Hgb = 12–16 g/dL; MCV, 80–94 fL; men, Hgb = 14-18 g/dL; MCV = 81–99 fL.

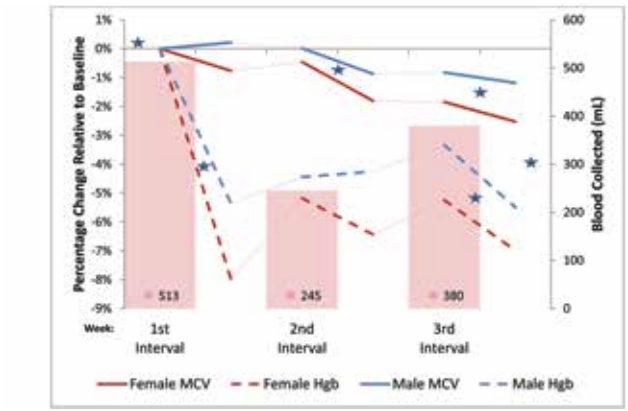

Figure 5. Dual-axis graph of hematology data according to 8-wk period. The bold lines indicate the mean percentage decreases in each parameter relative to baseline from the beginning to end of each 8-wk period. The actual total volume (in mL) of blood collected per 3-wk period is labeled at the bottom of each bar. Stars indicate significant mean differences by paired *t* testing (1st interval: Hgb, $P < 0.001$ [95% confidence interval {CI}, 0.738 to 1.128]; MCV, $P = 0.371$ [95% CI, –0.243 to 0.639]; 2nd interval: Hgb, $P = 0.540$ [95% CI, –0.131 to 0.247]; MCV, $P < 0.001$ [95% CI, 0.427 to 1.103]; 3rd interval: Hgb, $P < 0.01$ [95% CI, 0.106 to 0.488]; MCV, $P = 0.01$[95% CI, 0.115 to 0.784]; beginning week to end week: Hgb, $P < 0.001$ [95% CI, 0.704 to 1.095]; MCV, $P < 0.001$ [95% CI, 0.966 to 2.190]).

small, individual decreases of less than 1% should prompt concern, even when the measured value is still within the acceptable MCV reference range.

Despite the greater proportional amount of blood drawn, the macaque mean MCV change for both sexes trended higher than baseline over the length of the study. In contrast, MCV did not respond appropriately over the course of the study in humans, especially women. The decreases observed in human volunteers (Figure 5) demonstrate an erythropoietic failure to return to baseline even under the recommended volume limit of 525 mL per 8-wk period. Because the data were obtained during an outpatient clinical trial where participants were in control of their own diets, insufficient dietary intake of iron likely potentiated this effect. In contrast, the macaque diet was a controlled, standard lab diet (Monkey Diet 5038, Purina Mills International, St Louis, MO). This diet provides 220 ppm (220 mg/1 kg) of iron on a dry-matter basis,[10] which exceeds the 100-mg/kg recommendation for growing macaques noted in the *Nutrient Requirements of Nonhuman Primates*.[11]

The relative ability of MCV to respond to serial blood losses is important to note, because even with decreased Hgb levels, a patient is much less likely to become anemic if the body is able to produce new RBC on a continual basis. However, issues regarding the analysis of group data arise regarding individual responses for hematology safety testing. In this context, individually assessing the changes in a patient's parameters from baseline is useful for attending clinicians and veterinarians. When analyzing the hemogram to monitor for potential adverse events such as IDA throughout a study, each subject's values should be compared with their respective baseline or prestudy values. This practice may help guide the decision to iron-supplement an individual. The individual macaque example (Figure 3) shows the importance of observing a negative trend from baseline and the subsequent physiologic advantage of supplementation.

Regarding large-volume blood withdrawals in macaques, the removal of volumes as large as 12.5% generally appears to be safe, whereas withdrawal levels of 14% and 15% likely warrant close monitoring and supplementation as necessary. The macaque dataset was collected under a recommended limit of a 1 ml/kg daily, which translates to approximately a 12.5% blood volume removed weekly. As seen in Figure 4, this rate seems appropriate, given that at higher levels, MCV decreased significantly at 4 wk after the collection.

Because this study involved a secondary analysis of existing data, the main limitation was lack of control over study designs, in particular that both studies were evaluating vaccines for a parasite that affects RBC. Although we attempted to standardize methodologies in each dataset, study design and length and blood volumes removed varied markedly. One effort involved truncating data to a weekly format, and this approach likely led to loss of fidelity.

Previous research showed that even when large volumes of blood were withdrawn from a population of girls, concomitant iron supplementation prevented decreases in MCV and Hgb.[3] Although additional research would be useful, a randomized clinical trial involving iron supplementation and placebo groups might border on unethical. Because of the known benefits of iron-supplementation, the variable iron content of the human diet, and the lack of a 'gold standard' test for iron deficiency, iron supplementation should be considered even in study populations that fall under minimal risk withdrawal guidelines. Iron supplements are inexpensive, effective, well-tolerated, and readily available in various formulations without prescription.[13] Checking a subject's MCV and comparing it with baseline may be an efficient way to target those most at risk for an inappropriate hematopoietic response. In addition, limiting iron supplementation therapy to those patients who fail to maintain or increase their MCV may avoid any potential risks of oversupplementation.

The clinical management of research participants is complex, regardless of species. The underlying goal, however, is to minimize harmful changes to subjects, so that scientific advancements are

not made at the expense of the participant. To this end, keeping the subject safe must be balanced against obtaining sufficient samples for research.

Acknowledgments

We thank Cara Olsen, DrPH, who provided invaluable biometric input in the data-analysis process, and program director Tomoko Hooper, MD, who helped shape the direction of the project.

The content of this publication is the sole responsibility of the author(s) and does not necessarily reflect the views or policies of the Uniformed Services University of the Health Sciences (USUHS), the Department of Defense (DoD), or the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organizations does not imply endorsement by the US Government.

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