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Babesia microti and Malaria Infection in Africa: A Pilot Serosurvey in Kilosa District, Tanzania

Evan M. Bloch,[1]* Mabula Kasubi,[2] Andrew Levin,[3] Zakayo Mrango,[4] Jerusha Weaver,[5] Beatriz Munoz,[5] and Sheila K. West[5]

[1]*Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland;* [2]*Department of Microbiology, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania;* [3]*Immunetics, Inc., Boston, Massachusetts;* [4]*National Institute for Medical Research, Kilosa, Tanzania;* [5]*Dana Center for Preventive Ophthalmology, Johns Hopkins School of Medicine, Baltimore, Maryland*

Abstract. *Babesia* is a tick-borne intraerythrocytic parasite that is clinically and diagnostically similar to malaria parasite, conferring risk of misdiagnosis in areas where both parasites are endemic. Data on *Babesia* in humans in Africa are lacking, despite evidence that it is present in regional animal populations. Samples that were collected in November 2014 to July 2015 in Kilosa district, Tanzania, were evaluated for evidence of malaria and *Babesia* infection. Clinical data and laboratory samples (i.e., hemoglobin, rapid diagnostic testing [RDT] for malaria, peripheral blood smear, and dried blood spots) from a routine survey were available for analysis. Dried blood spots were tested using an investigational enzyme linked immunosorbent assay (ELISA) against *Babesia microti*. A total of 1,030 children aged 1 month to < 5 years were evaluated; 186 (18.1%) were malaria RDT positive, 180 (96.8%) of whom had peripheral smears reviewed; 70/180 (38.9%) were smear positive for parasites. The median (inter quartile range) and range of *B. microti* ELISA signal to cutoff (S/C) ratio was 0.10 (0.06–0.15) and 0.01–1.65, respectively; the S/C ratios were significantly higher in subjects ≥ 1 year as compared with those < 1 year old ($P < 0.001$). There was also a statistically significant association between a positive RDT for malaria and the *Babesia* S/C (median 0.09 versus 0.13 in RDT negative versus RDT positive, respectively; $P < 0.001$). The highest S/C ratios were disproportionately clustered in a few hamlets. The findings suggest that *Babesia* may be present in Kilosa district, Tanzania. However, serological cross-reactivity and false positivity, notably between *Babesia* and *Plasmodium* spp., cannot be definitively excluded and have implications for testing in other settings.

INTRODUCTION

Babesia is a genus of tick-borne intraerythrocytic apicomplexan parasites that is responsible for a range of zoonotic diseases in animals worldwide, and includes several species that are known to infect humans. *Babesia* infection in immunocompetent human hosts is often subclinical or mild; by contrast, individuals with asplenia, at extremes of age, and/or who are immunocompromised are at risk of severe disease, which includes hemolytic anemia, cardiorespiratory-failure, renal failure, and even death.[1] Although natural acquisition of *Babesia* is via tick bite (Ixodes or hard bodied ticks), *Babesia* is also transfusion transmissible, thus posing a risk to the blood supply given the ability to establish chronic asymptomatic infection that goes unnoticed at the time of donation.[2,3]

Babesia is endemic in the United States. *Babesia microti*, which has been responsible for most of the reported cases of human babesiosis, is widely distributed in the Northeastern and Upper Midwestern United States.[1] By contrast, *Babesia duncani*, which has only rarely been implicated in cases of human infection, is thought to be endemic in California and the Pacific Northwest.[4] Although most cases of human babesiosis have been reported in the United States, *Babesia* is the most ubiquitous genus of vertebrate parasites worldwide.[5,6] It is plausible that lack of awareness, in part because of historically limited diagnostic tools, has impeded greater recognition of its role in human disease. Although still a nascent emerging infectious disease, new reports of human babesiosis have been described in South America,[7] Europe,[8–10] Asia,[11–13] and Australia.[14] Furthermore, the risk to the US blood supply has spurred development of highly sensitive and specific, automated screening assays that offer an opportunity for broadening epidemiological surveillance of *Babesia*.

There is a paucity of human surveillance data on *Babesia* in Africa, although its presence in ticks and its role as a significant veterinary pathogen is well established.[15–20] Of particular interest, *B. microti*[18] and *B. microti*–like parasites[21] have been recovered from nonhuman primates in Africa. *Babesia* overlaps clinically and diagnostically with *Plasmodium* spp. likely contributing to underreporting and/or misdiagnosis, particularly in areas where both infections are endemic.[12] *Babesia* is almost indistinguishable from *Plasmodium* on peripheral blood smear; the latter remains the most common means of diagnosis for both parasites, particularly in low resource settings where laboratory capacity is suboptimal. The limited experience with new assays in use for blood screening in the United States[2,22] raises questions surrounding the assay's performance in malaria-endemic settings.

Given a lack of human data on *Babesia* in Africa, particularly in areas that are malaria-endemic, we sought to conduct a pilot seroprevalence study in Kilosa district, Tanzania. Selection of Kilosa district was informed by the rural setting in which there is close contact between the residents and livestock, placing them at potential risk of tick-borne illness. Importantly, Kilosa district has a high prevalence of malaria and has regional proximity to areas where *B. microti* and *B. microti*–like organisms have been reported.[18,21]

MATERIALS AND METHODS

Setting and population. Mortality reduction after oral azithromycin (MORDOR) is a multinational, cluster-randomized clinical trial to evaluate the impact of mass distribution of azithromycin on mortality in children aged less than 5 years. Our study represents those participants in the Tanzanian arm of the MORDOR study, representing 32 randomly selected communities in Kilosa District, Tanzania (sampled from a total of 646 communities). A complete census was conducted of each community, after which 40 children (aged between 1 and 59 months) who had a guardian capable of providing

* Address correspondence to Evan M. Bloch, Department of Pathology, Johns Hopkins School of Medicine, 600 N. Wolfe St./Carnegie, Baltimore, MD 21287-2182. E-mail: ebloch2@jhmi.edu

consent were randomly selected to participate in the survey. In communities with < 40 eligible children, all of the children were invited to participate. Samples for this study were collected between November 2014 and July 2015 as part of the initial assessment for the MORDOR clinical trial. Given the random sampling of the communities, those children who participated were not necessarily febrile or ill at the time of assessment.

Data collection. A trained field team collected data including whether the subjects had current symptoms of malaria and any recent or ongoing treatment at the time of evaluation.

Laboratory procedures. A finger-stick test was performed on each of the participants; testing included a point of care hemoglobin evaluation using a Hemocue instrument, rapid diagnostic testing (RDT) for malaria, preparation of thick and thin peripheral blood smears, and dried blood spots (DBS). The RDTs were performed as per the manufacturer's instructions; a trained technologist reviewed Giemsa-stained peripheral blood smears on all RDT positive samples at the MORDOR study laboratory in Tanzania; a random sample of RDT-negative smears was also examined. The DBS were stored refrigerated with a desiccant, pending testing.

Laboratory testing. Dried blood spots were shipped to Immunetics, Inc. (Boston, MA) for testing. Each blood spot was eluted with 300 μL of kit sample buffer overnight at 4°C. The samples were subsequently tested using a commercial *B. microti* ELISA.[22] For the purposes of this study, the cutoff defined in the ELISA kit, which is intended for use on serum samples, was modified to equal the mean A_{450} of replicate-negative dried blood spot controls plus 3 standard deviations (SDs).

Outcomes. The outcome of interest is the distribution of seroreactivity as measured by the ELISA signal to cutoff (S/C) ratio, calculated as the ELISA absorbance of the sample at 450 nm divided by the cutoff.

Statistical analyses. Mean (SD), Median (inter quartile range [IQR]), and the range of the S/C signal are presented for the overall sample and stratified by factors of interest such as age and RDT positivity. To test for differences in the S/C signal between groups the Wilcoxon rank sum test was used. Three levels of seroreactivity were created using 95 and 99 percentiles of the S/C distribution as cutoffs. Contingency table analysis was used to examine factors related to high levels of seroreactivity; Fisher's exact test was used to test for differences. The intraclass correlation coefficient (ICC) with 95% confidence intervals (CIs) was used to describe the level of clustering of high seroreactivity at the community level. Data were analyzed with SAS software (SAS, Raleigh, NC).

Ethical review. Ethical approval for MORDOR was obtained from the Tanzanian National Institute for Medical Research and the Institutional Review Board of the Johns Hopkins School of Medicine. Written informed consent was obtained from guardians for all children who participated in the MORDOR study.

RESULTS

A total of 1,049 subjects participated in the study of whom 527 (51%) were male; 856 (83.1%) were aged ≥ 1 to 5 years.

Babesia ELISA. A total of 1,030 (98.2%) subjects had DBS available for *Babesia* evaluation (Table 1). The overall distribution of the ELISA S/C ratio is presented in Figure 1. The S/C ratios increased by age: the median (IQR) and range were 0.06 (0.04, 0.10), 0.01–1.65, in children less than a year, and 0.11 (0.07, 0.17), 0.02–0.89 in children 3 years or older with a statistically significant difference between those < 1 year and those ≥ 1 year old ($P < 0.001$) (Table 2); consistent with this finding, high S/C values were more likely to be present in older children (Table 3). There was also a statistically significant association between a positive RDT result for malaria and *Babesia* S/C (median [IQR] 0.13 [0.08, 0.18]) versus RDT negative (median [IQR] 0.09 [0.06, 0.14]; $P < 0.001$). Although those subjects who displayed very high S/C values (≥ 30) were rare (Figure 1), they were disproportionately clustered in a few hamlets ICC (95% CI) 0.26 (0.07, 0.44) (Figure 2).

***Plasmodium* assessment.** A total of 186 (18.1%) subjects were malaria RDT positive at the time of evaluation; 194 peripheral blood smears were reviewed, representing 180 (97%) RDT-positive cases and a random sample of RDT-negative results ($N = 14$). A total of 70/180 (38.9%) RDT-positive cases were smear positive. Of the 14 RDT-negative cases, four

TABLE 1
Population overview

Number of children	1,030
Age group (year)	
< 1	174 (16.9)
1–3	426 (41.4)
3–5	430 (41.7)
Gender	
Females	503 (49.0)
Proportion (*n*/%) of children with malaria	
RDT+	186 (18.1)
Smear+/given RDT+	70/180 (38.9)*
Fever	66 (6.4)
Hemoglobin levels (g/dL)	
< 10 (anemia)	316 (30.7)
10–11	321 (31.2)
> 11	391 (38.0)

RDT = rapid diagnostic testing.
* Missing results for six RDT positives.

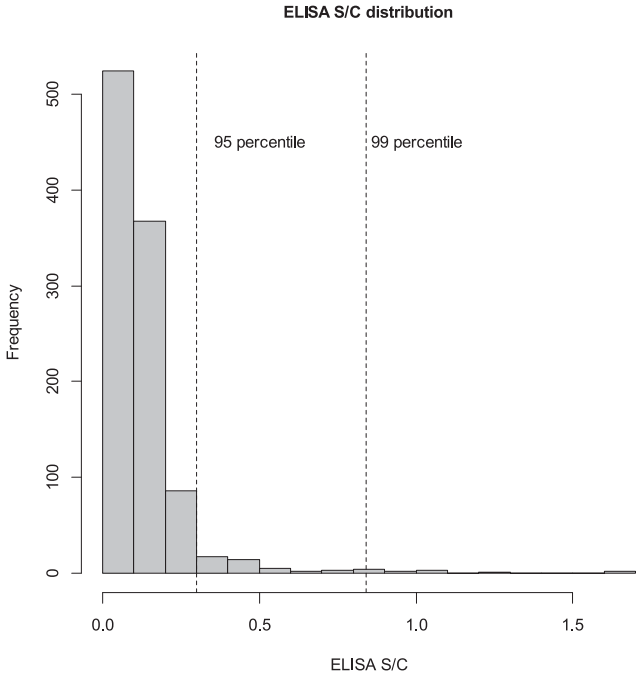

FIGURE 1. Distribution of signal to cutoff (S/C).

TABLE 2
Factors associated with ELISA S/C

	N	ELISA S/C (mean (SD), median (IQR), range	Test
Age group (year)			Comparing < 1 year vs. 1 year or older
< 1	174	0.10 (0.17), 0.06 (0.04, 0.10), 0.01–1.65	**Wilcoxon rank-sum test *P* value < 0.001**
1–3	426	0.14 (0.16), 0.10 (0.07, 0.15), 0.01–1.60	
3–5	430	0.14 (0.10), 0.11 (0.07, 0.17), 0.02–0.89	
Fever			Wilcoxon rank-sum test *P* value 0.47
Yes	66	0.11 (0.08), 0.09 (0.05, 0.14), 0.02–0.44	
No	960	0.13 (0.15), 0.10 (0.06, 0.15), 0.01–1.65	
Hemoglobin (g/dL)			Comparing anemia vs. no anemia
< 10 (anemia)	316	0.14 (0.16), 0.10 (0.05, 0.16), 0.01–1.60	Wilcoxon rank-sum test *P* value 0.85
10–11	321	0.12 (0.14), 0.09 (0.06, 0.14), 0.01–1.65	
> 11	391	0.13 (0.13), 0.10 (0.06, 0.15), 0.02–1.28	
RDT status			**Wilcoxon rank-sum test *P* value < 0.001**
Negative	840	0.12 (0.14), 0.09 (0.06, 0.14), 0.01–1.65	
Positive	186	0.16 (0.15), 0.13 (0.08, 0.18), 0.02–1.01	
Malaria smear*/RDT+			Wilcoxon rank-sum test *P* value 0.62
Positive	70	0.14 (0.11), 0.13 (0.09, 0.17), 0.02–0.82	
Negative	110	0.17 (0.17), 0.13 (0.07, 0.18), 0.02–1.01	
Overall	1,030	0.13 (0.14), 0.10 (0.06, 0.15), 0.01–1.65	–

IQR = inter quartile range; RDT = rapid diagnostic testing; S/C = signal to cutoff; SD = standard deviation. Bold text highlights statistical significance.
* Malaria smear: 194 slides were read, 180 RDT positives and 14 RDT negatives: 70/180 (38.9%) of the RDT positives were positive for malaria, and 4/14 (28.6) of the RDT negative were positive for malaria. Results presented in Table 2 are for RDT-positive children.

(28.6%) were smear positive for malaria. A total of 316 (30.7%) subjects were anemic (hemoglobin < 10 g/dL), and 66 (6.4%) were febrile at time of evaluation.

DISCUSSION

There is a paucity of *Babesia* surveillance data in humans in Africa, despite evidence that *Babesia* is present in the region.[16–18] A newly developed *B. microti* ELISA was used to conduct pilot surveillance in Kilosa district where the presence of *Babesia* is plausible, and high prevalence of malaria poses a risk of misdiagnosis and underreporting. The study findings are mixed. The finding of high S/C ratios in a small percentage of subjects suggests that *B. microti* may well be present. Furthermore, the disproportionate clustering of subjects with high S/C ratios in a relatively small number of hamlets is in keeping with a localized exposure, whereas an increase in seroreactivity with age is reflective of a progressive increase in exposure. Follow-up and repeat sampling are needed to confirm the findings.

The results highlight the challenges of Interpretation when conducting pilot surveillance. There are several possible explanations for the findings. Ecological clustering, as reflected by the observation of the highest S/C values in a relatively small group of hamlets, is in keeping with tick-borne illness and could suggest local tick exposure. On the other hand, although malaria is generalized across the hamlets, including those hamlets where high S/C ratios are not observed, an association between *B. microti* seroreactivity and a positive malaria RDT result was found. Cross-reactivity with antibodies to *Plasmodium* in the *B. microti* ELISA may be one possible explanation. The *B. microti* ELISA that was used in

TABLE 3
Factors associated with high signal-to-cutoff levels

Characteristics	SC < 0.30 *n* (%)	SC 0.30–0.83 *n* (%)	SC ≥ 0.84 *n* (%)	*P* value Fisher's exact
Age group (year)				0.027
< 1	168 (96.6)	3 (1.7)	3 (1.7)	
1–3	404 (94.8)	15 (3.5)	7 (1.6)	
3–5	405 (94.2)	24 (5.6)	1 (0.2)	
Fever				0.88
Yes	63 (95.5)	3 (4.6)	0 (0.0)	
No	910 (94.8)	39 (4.1)	11 (1.1)	
Hemoglobin (g/dL)				0.38
< 10 (anemia)	294 (93.0)	18 (5.7)	4 (1.3)	
10–11	308 (96.0)	9 (2.8)	4 (1.2)	
> 11	373 (95.4)	15 (3.8)	3 (0.8)	
RDT status				0.004
Negative	805 (95.8)	26 (3.1)	9 (1.1)	
Positive	168 (90.3)	16 (8.6)	2 (1.1)	
Malaria smear/RDT+				0.38
Positive	66 (94.3)	4 (5.7)	0 (0.0)	
Negative	97 (88.2)	11 (10.0)	2 (1.8)	
Malaria smear/RDT–				–
Positive	4 (100.0)	0 (0.00)	0 (0.00)	
Negative	10 (100.0)	0 (0.00)	0 (0.00)	
Overall	977 (94.9)	42 (4.1)	11 (1.0)	–

RDT = rapid diagnostic testing.

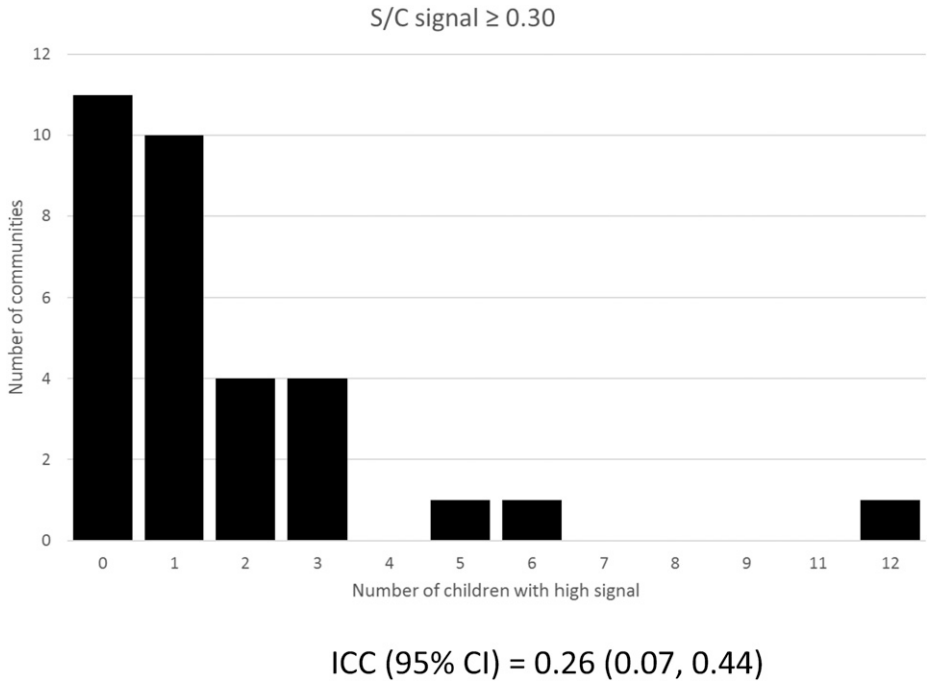

FIGURE 2. Distribution of subjects with a high S/C ratio by community.

the study contains four immunodominant peptide antigens that were shown to be highly specific to *B. microti* and did not exhibit reactivity with sera from *Plasmodium falciparum* or *Plasmodium vivax* infections in validation studies,[22] although the numbers tested were relatively small. However, an earlier study of *B. microti* peptides derived from the same gene family as those used in the ELISA found varying levels of cross-reactivity depending on the peptide sequence.[23] In addition, in the setting of malaria (*P. falciparum*) infection, (i.e., as reflected by RDT positivity) there is stimulation of a low avidity, polyclonal antibody response against the individual's past microbial exposures.[24,25] If there were to be past exposure to *B. microti*, it is plausible that there would be an increase in antibody titers, accounting for seroreactivity even in the absence of active infection with *B. microti*. Another possibility is that of cross-reactivity with other pathogens that are encountered locally. This could include *Babesia* species other than *B. microti* such as *Babesia bigemina* and *Babesia bovis* that are regionally endemic,[19,20] as well as *Entopolypoides macaci*, a parasite of baboons which is genetically highly homologous to *B. microti*.[26] *Babesia* are also phylogenetically related to *Theileria*, which have been reported in the region.[27,28] *Babesia bigemina*, *B. bovis*, and *Theileria* infect cattle and wild buffalo primarily and have little evidence of zoonotic potential; nonetheless, their effect on serologic testing for *B. microti* is unknown.

This study has several limitations. First, it was conceived as a pilot serosurvey to take advantage of an existing parent study. As such, the available samples (i.e., DBS) were quantitatively insufficient to conduct confirmatory and ancillary testing (e.g., indirect fluorescent antibody testing, molecular evaluation). As such, our findings are preliminary and lack comprehensive evaluation for *Babesia*. Similarly, treatment data for malaria were lacking, thus detracting from the interpretation of cases where a malaria RDT was positive yet blood smear was negative. Second, the *B. microti* ELISA had not been previously evaluated in this setting. It is uncertain to what extent performance is affected by a different population and microbiome, such that the contribution of false positivity and/or cross-reactivity with other local pathogens cannot be excluded. In a pilot study such as this, in the absence of true prevalence data obtained by other methods, the positive predictive value of the assay is unknown. The validation experiments for the ELISA did not include other *Babesia* species, such that potential cross-reactivities are likewise not known. Third, the assay in use was designed for serum or plasma samples, rather than DBS. The assay methods and procedures were adapted as a research endeavor specifically for this study and may not be optimal for the intended purpose. The ELISA has been authorized under investigational new drug for specific use on US blood donors; the latter typically selects for a low-risk, healthy subset of the population. It is uncertain as to how this impacts evaluation in a population of young children in rural Tanzania. Given that this was an exploratory study, a provisional ELISA cutoff was applied with little objective clinical basis, highlighting the need for further study to categorize samples as true positive or true negative. Unfortunately, the absence of results from an accepted gold standard method such as PCR for *Babesia* renders interpretation of the test results a challenge that is not unique to this study. Well-characterized *Babesia* samples (i.e., from known parasitemic donors in the setting of symptomatic infection) are rarely available even in known endemic areas (e.g., Northeastern USA) let alone in an entirely novel study population as in our study. Without an objective way of ascertaining positivity, the ELISA cutoff can only be provisional. Finally, evidence of animal infection alone should not be construed as implying human risk, although discovery of enzootic *B. microti* in the region would be highly significant, as would evidence of anthropophilic tick vectors for the parasite. Such underscores the need for an entomological survey whereby the

finding of competent tick vectors infected with *B. microti* would fill in a key missing element that would complement the study's findings.

Thus, the data offer a compelling reason to perform a comprehensive evaluation of *Babesia*, and specifically, to revisit those hamlets that displayed the highest rates of seroreactivity. It will be particularly important to characterize the performance of the described tests and conduct an environmental assessment. Rather than a deficiency, cross-reactivity could be exploited diagnostically. For example, tests that are capable of targeting both parasites could prove useful. In the context of blood donor screening, both genera of parasites (*Babesia* and *Plasmodium*) are transfusion transmissible, such that a combined platform would be advantageous.[3,29] Nonetheless, discrimination between *Babesia* and *Plasmodium* is important, given differences in therapy, risking treatment failure in the case of misdiagnosis.

In conclusion, our study offers serological evidence for *B. microti* infection and preliminary population-based estimates of seroprevalence in Kilosa, Tanzania. Broadly, the study underscores the need to further characterize the global epidemiology of *Babesia*, and likewise to better understand the potential for assay cross-reactivity in applications both within and outside the intended use, including blood donor screening in the United States.

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Authors' addresses: Evan M. Bloch, Department of Pathology, Johns Hopkins Medicine, Baltimore, MD, E-mail: ebloch2@jhmi.edu. Kasubi Mabula, Department of Microbiology, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania, E-mail: mkasubi68@gmail.com. Andrew Levin, Kephera Diagnostics, LLC, Boston, MA, E-mail: alevin@kepheradx.com. Zakayo Mrango, National Institute for Medical Research, Kilosa, Tanzania, E-mail: mrango@yahoo.com. Jerusha Weaver, Department of Ophthalmology, Johns Hopkins Wilmer Eye Institute, Baltimore, MD, E-mail: jerusha.u.weaver@gmail.com. Beatriz Munoz and Sheila K. West, Dana Center for Preventive Ophthalmology, Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD, E-mail: bmunoz@jhmi.edu and shwest@jhmi.edu.

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