Low Levels of RANTES Are Associated with Mortality in Children with Cerebral Malaria

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Background. In children with cerebral malaria (CM), serum chemokine levels and associated morbidity and mortality have not been characterized.

Methods. Serum levels of the cytokines interleukin (IL)–1β, IL-6, IL-10, interferon (IFN)–γ, and tumor necrosis factor–α and the chemokines macrophage inflammatory protein (MIP)–1α, MIP-1β, and regulated upon activation, normal T cell expressed and secreted (RANTES) were measured in Ugandan children with CM, in children with uncomplicated malaria (UM), and in healthy children from the community, as control subjects (CCs).

Results. Children with CM had lower levels of RANTES and higher levels of all other cytokines and chemokines than CCs (all $P < .0001$), and they had lower levels of RANTES ($P = .004$) and higher levels of IL-10 ($P = .003$), IFN-γ ($P = .007$), and IL-1β ($P = .05$) than children with UM. Children with CM who died had lower levels of RANTES ($P = .006$) and higher of levels of IL-6 ($P = .006$), IL-10 ($P = .01$), IFN-γ ($P = .03$), and MIP-1β ($P = .008$) than children who survived. After adjustment for other cytokine and chemokine levels, only low levels of RANTES were independently associated with mortality ($P = .016$). Levels of RANTES correlated with platelet count but were associated with mortality independently of platelet count.

Conclusions. The serum cytokine and chemokine profile of children who die of CM is similar to that of individuals who die of sepsis. Levels of RANTES are significantly lower in children with CM, and very low levels of RANTES are associated with mortality, independently of other cytokine and chemokine levels.

Cerebral malaria (CM) is one of the deadliest complications of *Plasmodium falciparum* infection, with mortality rates of 15%–40% [1]. Although the pathogenesis of CM is likely multifactorial, the balance between specific cytokines and chemokines produced in response to infection with *P. falciparum* is thought to play an important role in CM and other forms of severe malaria. Inflammatory Th1-type cytokines (e.g., tumor necrosis factor [TNF]–α, interferon [IFN]–γ, interleukin [IL]–1β, and IL-6) are thought to be critical to the control of exoerythrocytic and erythrocytic *P. falciparum* infection [2–7], but overvigorous responses may also contribute to organ damage, particularly in the brain. For example, in experimental mouse models of CM, TNF-α [8] and IFN-γ [9] have both been implicated in the pathogenesis of CM. Conversely, the regulation of Th1 cytokine levels by Th2-type cytokines such as IL-10 has been shown, in some animal models, to prevent CM [10], and regulation of TNF-α levels by IL-10 appears to contribute to the prevention of severe malarial anemia in humans [11, 12]. However, the role that IL-10 plays may depend on its levels: very high levels of IL-10 have been associated with severe malaria [13, 14], and some animal studies have suggested that high levels of IL-10 may also play a role in the pathogenesis of severe malaria [3]. Thus, in malaria, cytokines appear to mediate a delicate balance between the control of infection and contribution to disease.

Chemoattractant cytokines, or chemokines, are less well studied in severe malaria, but chemokines of the C-C or α-subfamily—including macrophage inflammatory protein (MIP)–α, MIP-1β, and, particularly, RANTES—are increasingly recognized as being important in the immune response to viral and bacterial in-

Received 24 March 2006; accepted 2 May 2006; electronically published 16 August 2006.

Potential conflicts of interest: none reported.

Financial support: National Institutes of Health (Fogarty Institute grant R21 TW-006794 to C.C.J.); Institute of International Education (Fulbright African Regional Research Award to M.J.B.).

This work was conducted while C.C.J. was at Case Western Reserve University and M.J.B. was at Indiana Wesleyan University.

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The Journal of Infectious Diseases 2006;194:837–45

0022-1899/2006/19406-0017$15.00

fection. Increased production of RANTES has been associated with resistance to HIV infection [15, 16], whereas low levels of RANTES correlate with disease severity and mortality in individuals with sepsis [17] and meningococcal disease [18, 19]. Levels of these C-C chemokines may also be important in severe malaria, but the data to date have been seemingly contradictory. Decreased plasma levels of RANTES and increased levels of MIP-1α and MIP-1β were documented in a study of children with severe malarial anemia or hyperparasitemia [20], but, in another study, increased mRNA expression of RANTES and CCR5 was found the brains of children who died of CM [21]. One potential reason for low levels of RANTES in severe malaria may be the thrombocytopenia frequently seen in this condition, given that platelets are a major reservoir of RANTES in the peripheral circulation [22]. To our knowledge, serum levels of MIP-1α, MIP-1β, and RANTES, which may mediate systemic disease and differ from protein expression in the central nervous system, have not yet been assessed in children with CM. In addition, the relationship between these chemokines and previously described cytokines and the association of each with mortality have not been characterized. The present study was designed to assess the serum levels of cytokines and chemokines in children with CM, in children with uncomplicated malaria (UM), and in healthy children from the community, as control subjects (CCs) and to determine the relationship between cytokine and chemokine levels and mortality in children with CM.

SUBJECTS, MATERIALS, AND METHODS

Study population and recruitment. The study was conducted at Mulago Hospital, Kampala, Uganda. Children 4–12 years old were recruited as part of 2 studies assessing the complications of CM. Children were recruited in 3 categories: CM, UM, and CCs without evidence of acute illness. A total of 88 children with CM, 76 children with UM, and 100 CCs were recruited.

Children with CM were enrolled if they were admitted to Mulago Hospital and met the World Health Organization criteria for CM: coma (Blantyre coma scale $\leq$2 or Glasgow coma scale $\leq$8), *P. falciparum* on blood smear, and no other cause for coma. Lumbar punctures were performed to rule out meningitis and encephalitis. Ugandan Ministry of Health national guidelines for drug treatment of CM (quinine for 7 days) were followed. Children with UM were enrolled from the acute-care clinic or an outpatient malaria clinic at the hospital that is sponsored by the University of California, San Francisco (UCSF). Children were considered to have UM if they had signs and symptoms of malaria (fever, chills, vomiting, and headache), *P. falciparum* infection on blood smear, and no evidence of malaria complications (e.g., seizures, respiratory distress, severe anemia, or coma) or other acute illness. Children with UM were treated in accordance with the Ugandan Ministry of Health guidelines in the acute-care clinic (chloroquine plus sulfadoxine-pyrimethamine) or with combination therapy at the UCSF outpatient clinic (either amodiaquine plus sulfadoxine-pyrimethamine or amodiaquine plus artesunate). CCs were recruited from the extended household areas of children with CM or UM. CCs and children with UM were recruited to be in the same age range (4–12 years) as children with CM; participants were not matched by sex. A medical history was obtained and a physical exam performed to ascertain that the CCs were healthy at the time of enrollment. Exclusion criteria for enrollment in all 3 groups included (1) a history of meningitis, encephalitis, or any brain disorder, including CM; (2) a history of developmental delay; (3) prior admission to the hospital for malnutrition; or (4) a history of chronic illness. In addition, children recruited for the CC cohort were excluded if they had evidence of acute illness at the physical exam, had been treated for an acute illness during the preceding month, or had been admitted for malaria during the preceding 6 months. All CCs were tested for asymptomatic *P. falciparum* infection by microscopic examination of peripheral-blood smears; 6 children were infected and were treated with chloroquine and sulfadoxine-pyrimethamine. These children were not excluded from the study.

Blood samples of 2–5 mL were obtained by venipuncture at the time of admission for children with CM and at the initial clinic visit for children with UM and for CCs. Follow-up blood samples were obtained from children with CM at 72 h after admission. Blood samples for serum testing were collected in a Vacutainer serum separator tube (BD Diagnostics), gently inverted 4–5 times, allowed to clot in a horizontal position for 30 min, and then centrifuged for 10 min at 1000 *g*. The separated serum was pipetted into aliquots and frozen at -70°C until testing was performed. Serum samples from 80 children with CM and 74 children with UM were available for testing. Serum samples from 63 of 100 CCs were randomly chosen for testing. Written, informed consent was obtained from the parents or guardians of study participants. Ethics approval for the study was granted by the institutional review boards for human studies at Makerere University Faculty of Medicine, University Hospitals of Cleveland, Case Western Reserve University, and Indiana Wesleyan University.

Cytokine testing. Levels of 5 cytokines (IFN-γ, IL-10, IL-1β, IL-6, and TNF-α) and 3 chemokines (MIP-1α, MIP-1β, and RANTES) that have been shown to be important in human and/or animal studies of CM (see above) were assessed in each sample. Cytokine and chemokine levels other than IL-10 were determined by colorimetric bead assay using the Luminex system and human-specific bead sets (R&D Systems). Results were interpolated from 5-parameter-fit standard curves generated using the relevant recombinant human proteins (R&D Systems). Levels of IL-10 were measured by standard cytokine ELISA, using human-specific primary and secondary antibodies

(BD Pharmingen). Results were interpolated from 5-parameter-fit standard curves generated with recombinant human IL-10 (BD Pharmingen). Samples were tested at a 1:8 dilution.

Statistical analysis. Demographic variables were compared across the 3 study groups using analysis of variance (for continuous variables) and χ^2 testing (for categorical variables). Cytokine levels in unmatched comparison groups (e.g., levels in children who survived vs. those who died) were compared using the Mann-Whitney 2-sample *U* test, adjusted for multiple comparisons according to the method of Bonferroni. Cytokine levels in matched comparison groups (e.g., levels in the same children at 0 and 72 h) were compared using the Wilcoxon matched-pairs signed-rank test. Correlations between cytokine levels were assessed using Spearman's rank correlation. The relationship between mortality and high-level (highest quartile) or low-level (lowest quartile, for RANTES only) cytokine production was assessed using the χ^2 test, with the Mantel-Haenszel adjustment for the other cytokines. (Logistic-regression analysis could not be performed because high levels of IL-6 and low levels of RANTES perfectly predicted mortality.)

RESULTS

Demographic and hematological characteristics of children with CM, children with UM, and CCs. Children with CM were slightly younger than children with UM and CCs, and there was a higher frequency of boys in the CM group than in the UM and CC groups (table 1). As expected, children with CM had lower hemoglobin levels and platelet counts than children with UM, who in turn had lower levels than CCs (table 1). The median parasite density was higher in children with CM and UM than in CCs (most CCs were uninfected) but did not differ significantly between children with CM and UM (37,970 and 54,840, respectively; P = NS, Mann-Whitney *U* test).

Cytokine and chemokine levels in children with CM, children with UM, and CCs. All serum cytokine and chemokine levels differed significantly in children with CM and in CCs. Increased levels of IL-1β, IL-6, IL-10, IFN-γ, TNF-α, MIP-1α, and MIP-1β and decreased levels of RANTES were observed in children with CM, compared with those in CCs (all $P <$ .0001) (figure 1). In addition, significantly higher levels (median values) of IL-10 (706 vs. 250 pg/mL; $P = .003$), IFN-γ (87 vs. 34 pg/mL; $P = .007$), and IL-1β (3 vs. 0 pg/mL; $P = .05$) and significantly lower levels of RANTES (23,503 vs. 31,162 pg/mL; $P = .004$) were observed in children with CM, compared with those in children with UM (figure 1). Cytokine and chemokine levels did not differ significantly by age, sex, hemoglobin level, or parasite density in any study group, and CCs with asymptomatic parasitemia had cytokine and chemokine levels similar to those of nonparasitemic CCs (data not shown).

Cytokine and chemokine levels in children with CM over time. For all cytokines and chemokines except RANTES, levels in children with CM at 72 h after admission were similar to levels in CCs (figure 2). Levels of RANTES 72 h after admission were significantly higher than those at the time of admission but were comparable to those in children with UM and were still not as high as those in CCs.

Correlation between cytokine and chemokine levels in children with CM. Levels of RANTES correlated weakly and inversely with levels of IL-10, IFN-γ, and TNF-α only (Spearman's $\rho = -0.35$ to -0.31; $P = .01$–$.03$). Similarly, levels of IL-1β correlated weakly with levels of MIP-1α and MIP-1β only (Spearman's $\rho = 0.31$–0.33; all $P = .01$). By contrast, levels of all other cytokines and chemokines correlated strongly with each other (Spearman's $\rho = 0.45$–0.82; all $P <$ .0001).

Correlation between cytokine and chemokine levels and platelet counts in children with CM. A strong positive correlation was seen between platelet count and levels of RANTES (Spearman's $\rho = 0.49$; $P <$ .0001). A moderately strong inverse correlation was seen between platelet count and levels of IL-1β, IL-10, MIP-1α, and TNF-α (Spearman's $\rho = -0.36$ to -0.30; $P = .0005$–$.007$), and a weak inverse correlation was seen between platelet counts and levels of IL-6 (Spearman's $\rho = -0.24$; $P = .03$). A nonsignificant trend toward increased mortality was seen with thrombocytopenia ($P = .11$), but this

Table 1. Demographic and hematological characteristics of study participants.

Characteristic	CM (n = 80)	UM (n = 74)	CC (n = 63)	P[a]
Sex, % male	57.9	40.5	47.6	.05
Age, years	6.1 ± 2.5	7.1 ± 2.4	7.7 ± 2.1	.003
Weight, kg	19.4 ± 7.2	20.8 ± 7.1	21.2 ± 6.8	NS
Hemoglobin level, mg/dL	8.5 ± 2.0	11.2 ± 1.8	12.4 ± 1.3	<.0001
Platelet count, cells/mL	102 ± 75	165 ± 83	345 ± 103	<.0001
Parasite density, median (IQR)	39,790 (143–560)	54,840 (118–220)	0 (0)	<.0001

NOTE. Data are mean ± SD, unless otherwise indicated. CC, control children from the community; CM, cerebral malaria; IQR, interquartile range; NS, not significant; UM, uncomplicated malaria.

[a] Frequencies were compared using the χ^2 homogeneity of odds test, means were compared using analysis of variance, and medians were compared using the Kruskal-Wallis test.

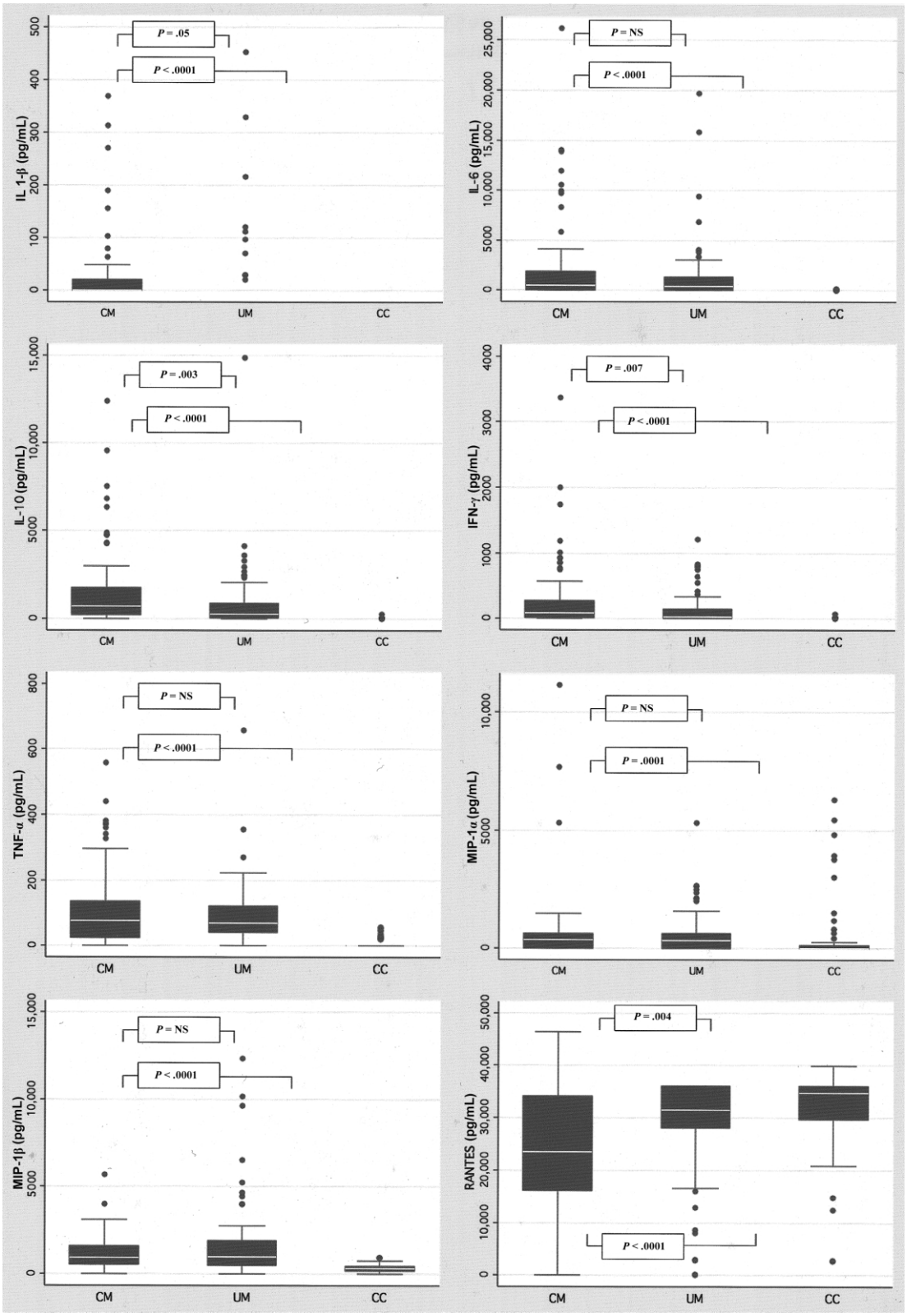

Figure 1. Serum cytokine and chemokine levels in children with cerebral malaria (CM; $n = 80$), in those with uncomplicated malaria (UM; $n = 74$), and in healthy children from the community, as control subjects (CC; $n = 63$). *P* values were calculated using the Mann-Whitney 2-sample *U* test. To allow display of median differences, the following outlier values are not depicted: interleukin (IL)–1β, 5795 pg/mL (UM) and 1065 pg/mL (CM); IL-6, 19,727 pg/mL (UM) and 26,228 pg/mL (CM); IL-10, 24,687 pg/mL (CM); interferon (IFN)–γ, 6114 pg/mL (UM) and 17,600 pg/mL (CM); macrophage inflammatory protein (MIP)–1α, 23,317 pg/mL (CC); MIP-1β, 22,256 pg/mL (CM). NS, not significant; TNF, tumor necrosis factor.

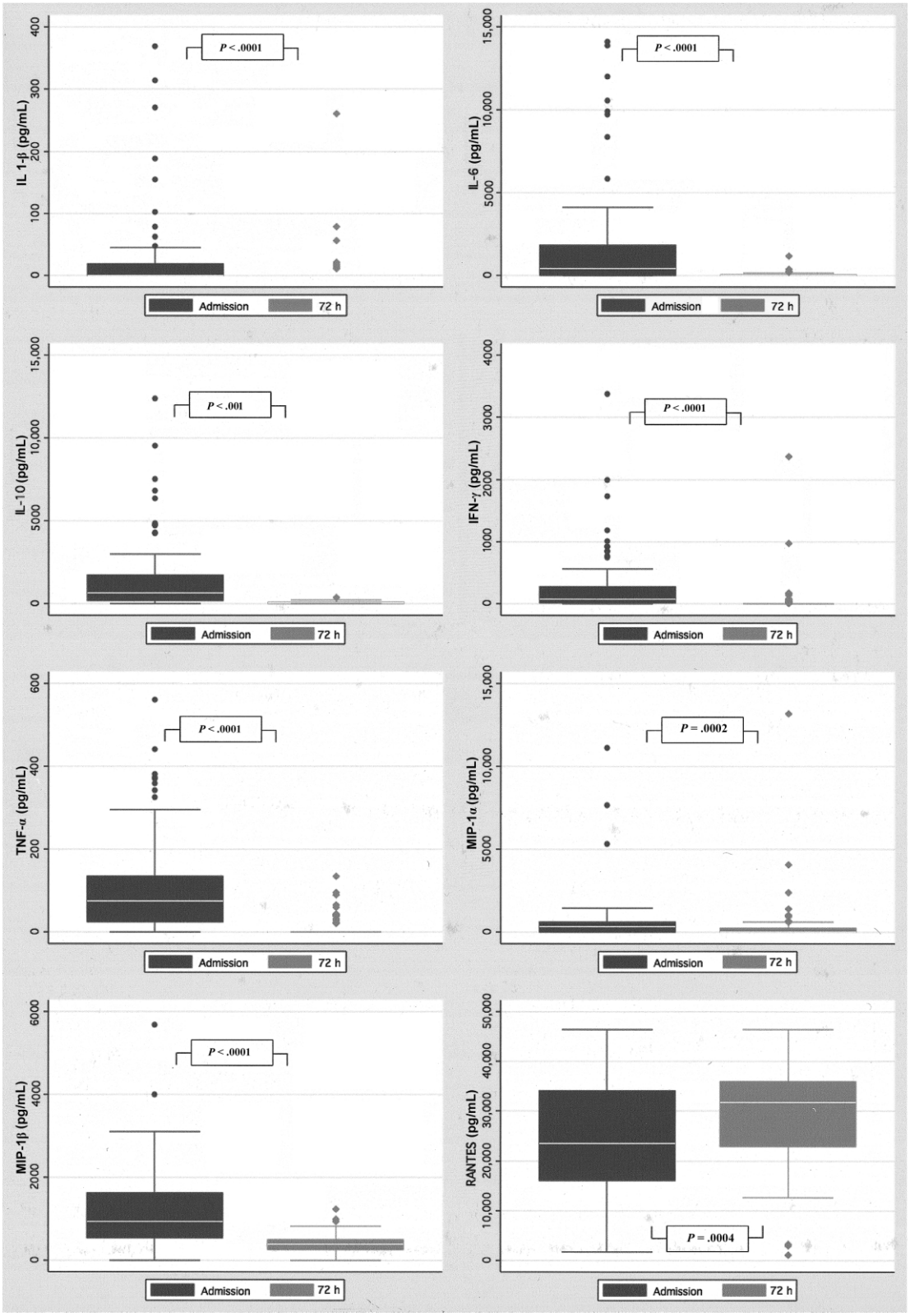

Figure 2. Serum cytokine and chemokine levels in children with cerebral malaria ($n = 72$) at admission and 72 h after admission. *P* values were calculated using the Wilcoxon matched-pairs signed-rank test. To allow display of median differences, the outlier values, all at admission, are not depicted: interleukin (IL)–1β, 1065 pg/mL; IL-6, 26,228 pg/mL; IL-10, 24,687 pg/mL; interferon (IFN)–γ, 17,600 pg/mL; macrophage inflammatory protein (MIP)–1β, 22,256 pg/mL. TNF, tumor necrosis factor.

disappeared completely when adjusted for levels of RANTES ($P = .53$). Repeat blood count and platelet testing was performed 72 h after admission at the discretion of the clinical officer. In the 10 study participants with CM in whom platelet counts were performed at 72 h, platelet counts and levels of RANTES had both increased significantly from levels at the time of admission (mean platelet count, 189×10^9 vs. 89×10^9 cells/mL; $P = .02$; median level of RANTES, 34,555 vs. 24,508 pg/mL; $P = .05$). However, both platelet counts and levels of RANTES remained lower than those in CCs.

Cytokine and chemokine levels and mortality in children with CM. Levels of multiple cytokines and chemokines were markedly higher in children who died ($n = 4$), compared with those who survived ($n = 76$), including (median values) levels of IL-6 (6183 vs. 446 pg/mL; $P = .006$), IL-10 (3834 vs. 641 pg/mL; $P = .01$), IFN-γ (1007 vs. 80 pg/mL; $P = .03$), and MIP-1β (3001 vs. 899 pg/mL; $P = .008$) (figure 3). However, levels of RANTES were significantly lower in children who died than in children who survived (11,220 vs. 25,303 pg/mL; $P = .006$). High levels of IL-6 (>1884 pg/mL; $P = .006$), IL-10 (>2080 pg/mL; $P = .02$), and MIP-1β (>1638 pg/mL; $P = .02$) and low levels of RANTES (<16,858 pg/mL; $P = .0003$) were associated with death. Low levels of RANTES remained highly associated with death after controlling for thrombocytopenia (platelet count, $<100 \times 10^9$ cells/mL; $P = .005$). Because levels of most cytokines and chemokines were strongly correlated, we assessed the association of each of these 4 cytokines or chemokines with death, adjusting for levels of the other cytokines. Low levels of RANTES remained associated with death, independently of other cytokine responses ($P = .016$), whereas high levels of IL-6, IL-10, and MIP-1β were not associated with death after adjustment for the other cytokine levels.

DISCUSSION

Increasing evidence demonstrates the importance of the C-C or α-chemokines MIP-1α, MIP-1β, and RANTES in the mediation of the host immune response to viral [15], bacterial [17–19], and protozoal infections, including *P. falciparum* infection during pregnancy [23, 24] and *P. falciparum* infection complicated by severe anemia or hyperparasitemia [20]. However, serum levels of the C-C chemokines MIP-1α, MIP-1β, and RANTES and their relationship to other pro- and anti-inflammatory cytokines have not, to our knowledge, been previously described in children with CM. In the present study, we provide evidence that levels of multiple cytokines and chemokines are higher in children with CM but that decreased levels of RANTES may be particularly important, because these are the only ones that have not normalized in children with CM after 72 h and they are associated with mortality independently of other cytokine and chemokine responses.

RANTES production appears to be important in a variety of infectious diseases, most notably HIV infection [15, 16], but levels of RANTES and its expression in severe malaria have only recently been assessed. A study of Ghanaian children with CM by Sarfo et al. [21] demonstrated increased mRNA expression of CCR3, CCR5, and RANTES and of CCR5 and RANTES protein in post mortem samples of the cerebellum and cortex of children with CM but not children with other nonmalarial illness. The same group also recently demonstrated that mRNA expression of RANTES, CCR1, CCR3, and CCR5 is up-regulated and that plasma levels of RANTES are higher in the brains of mice infected with *P. yoellii* [25]. By contrast, a study in Kenyan children with severe malaria (anemia or hyperparasitemia) by Ochiel et al. [20] demonstrated increased levels of MIP-1α and MIP-1β but decreased levels of RANTES in children with severe malaria, compared with children with mild malaria. In addition, children with prior severe malaria had lower levels of RANTES than children with prior mild malaria, which suggests that children with severe malaria may be incapable of maintaining levels of RANTES in the face of *P. falciparum* infection. Our results in children with CM are consistent with the findings of the Kenyan study: levels of RANTES were low in children with CM, did not normalize after 72 h, and were associated with death independently of other cytokine and chemokine levels. Taken together, the findings suggest that impaired RANTES production may occur in both of the major forms of severe malaria and that the inability to produce RANTES may be a factor in mortality in children with CM. However, our findings do not exclude the possibility of RANTES up-regulation in localized areas of the brain itself, as was demonstrated by Sarfo et al. [21]. The cells that produce RANTES may be different in the brain (microglia, astrocytes, and monocytes) [26] and peripheral circulation (platelets, monocytes, and lymphocytes) [22], and the effects of RANTES may also be different in the 2 areas. In areas of parasite sequestration, such as the capillary endothelium of the brain in children with CM, RANTES-induced chemoattraction of large numbers of monocytes and activated T lymphocytes to a small area may manifest primarily as vascular and perivascular inflammation, even as it mediates the control of infection. In addition, RANTES binding to CCR5 on endothelial cells, microglia, and astrocytes—the 3 primary components of the blood-brain barrier—may lead to the activation of these cells and disruption of the blood-brain barrier [25]. By contrast, in the peripheral circulation and areas of nonsequestered parasitic infection, RANTES-induced chemoattraction of monocytes and activated T lymphocytes may be less likely to induce inflammation, because cell concentrations and local inflammatory mediator production may be less pronounced than in areas of sequestration. In these areas, the monocytes and activated T lymphocytes may serve primarily to combat infection, reduce parasite load, and control disease.

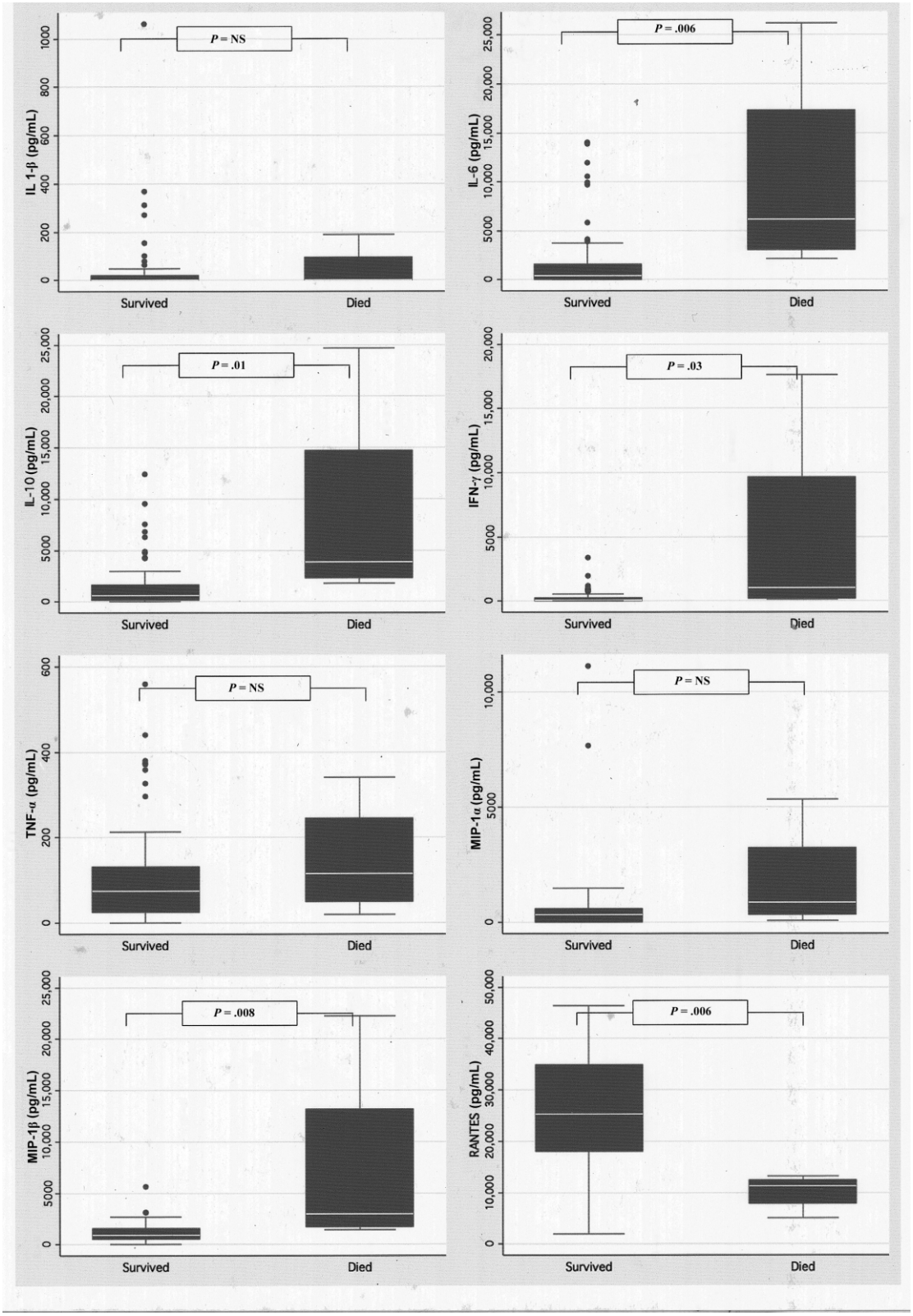

Figure 3. Cytokine and chemokine levels in children with cerebral malaria who survived ($n = 76$) and those who died ($n = 4$). *P* values were calculated using the Mann-Whitney 2-sample *U* test. IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; NS, not significant; TNF, tumor necrosis factor.

Thrombocytopenia has been associated in some studies of severe malaria with increased mortality [27, 28], and the majority of our patients were thrombocytopenic (63% had a platelet count of $<100 \times 10^9$ cells/mL). Intriguingly, platelets are a major reservoir of RANTES in the peripheral circulation [29], and levels of RANTES in our study correlated strongly with platelet count. We found a nonsignificant trend toward increased mortality with thrombocytopenia that disappeared when we adjusted for levels of RANTES, whereas low levels of RANTES were associated with mortality, and the association remained strong after adjustment for thrombocytopenia ($P = .005$). The findings suggest that one mechanism for the increased mortality associated with thrombocytopenia in some studies of CM may be the impaired production of RANTES when the major peripheral-blood reservoir of RANTES production—platelets—is significantly decreased. Further support for this idea comes from a recent study from the United Arab Emirates of children with hematological malignancies. In that study, levels of RANTES paralleled platelet counts, and the 4 children who died of bacterial or fungal sepsis had extremely low levels of RANTES, compared with 4 children with sepsis who survived [29]. The authors noted that most febrile episodes occurred after thrombocytopenia, which suggests that thrombocytopenia, through its effect on levels of RANTES, might be the cause of an immunodeficient state in these children rather than a reflection of response to infection. In the present study, platelet counts and levels of RANTES increased at 72 h in the children with CM in whom they were tested, but the counts and levels remained below those of CCs. These findings also support to the idea that the normalization of levels of RANTES may require the normalization of platelet counts.

Studies of cytokine levels and disease severity in individuals with meningococcal disease have demonstrated a pattern like that seen in the present study of children with CM: a correlation of very high levels of IL-10 [30, 31] and IL-6 [31] with disease severity [31] and death [30]. Similarly, recent studies of chemokine levels have demonstrated associations between decreased levels of RANTES and disease severity [18, 19] and death [19]. Similarly, a recent study of individuals with bacterial sepsis (not limited to meningococcal disease) was also associated with very high levels of IL-10 and IL-6 and very low levels of RANTES with mortality [17]. The similar findings in our study suggest that, despite the often very different clinical presentations of CM and bacterial sepsis, the cytokine and chemokine profile in individuals who die of the 2 syndromes is similar, and IL-6, IL-10, and RANTES are related to disease severity and death in both syndromes. In light of earlier studies that documented that increased levels of IL-6 and IL-10 [13, 14] and decreased levels of RANTES [20] are seen in other forms of severe malaria, these associations may hold true for all forms of severe malaria and not only CM.

The mortality rate for children with CM in our study (5%) was lower than the rates reported in most of the literature on CM (a mean mortality rate of 18.6% was calculated in one review of African studies of CM [1]). However, the magnitude of differences in cytokine and chemokine levels between children who died and those who survived was so large that highly significant differences were seen, despite the small number of deaths. The study was limited by the lack of a comparator group with another manifestation of severe malaria, such as severe malarial anemia. As was noted above, without this comparator group, we cannot say whether the present study findings are limited to children with CM or are common to all children with severe malaria.

In conclusion, in children with CM, only levels of RANTES fail to normalize by 72 h after admission, and only low levels of RANTES are associated with death after adjustment for other cytokine levels. In addition, children who die of CM have a cytokine and chemokine profile similar to that of individuals who die of bacterial sepsis (increased levels of IL-6 and IL-10 and decreased levels of RANTES). Decreased production of RANTES may be due, in part, to the thrombocytopenia seen commonly in CM, given that platelets are a major reservoir of RANTES. Low levels of RANTES in individuals with thrombocytopenia may explain the association of thrombocytopenia with increased mortality in severe malaria. From the nature of our study design, we cannot exclude the possibility that RANTES is a marker for disease severity, but, taken together, the study findings suggest that RANTES may be involved in the pathogenesis of severe malaria.

Acknowledgments

We thank the study participants and their guardians, for their participation in the study; Chris Statt (in the laboratory of Thomas Kelly) and Marilyn McHugh, for assistance in cytokine testing; Paul Bangirana, for study coordination and data entry; Medical Officers Christine Mugasha, Judy Orikiiriza, and Moorine Sekkade, for patient enrollment and care; study nurses Jolly Rubarumbara and Carol Akumu, for patient enrollment and care; and the University of California, San Francisco–Makerere Outpatient Clinic, for assisting with the enrollment of patients and microscopic testing.

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