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PLoS NEGLECTED TROPICAL DISEASES

Neutrophil Paralysis in *Plasmodium vivax* Malaria

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Abstract

Background: The activation of innate immune responses by *Plasmodium vivax* results in activation of effector cells and an excessive production of pro-inflammatory cytokines that may culminate in deleterious effects. Here, we examined the activation and function of neutrophils during acute episodes of malaria.

Materials and Methods: Blood samples were collected from *P. vivax*-infected patients at admission (day 0) and 30–45 days after treatment with chloroquine and primaquine. Expression of activation markers and cytokine levels produced by highly purified monocytes and neutrophils were measured by the Cytometric Bead Assay. Phagocytic activity, superoxide production, chemotaxis and the presence of G protein-coupled receptor (GRK2) were also evaluated in neutrophils from malaria patients.

Principal Findings: Both monocytes and neutrophils from *P. vivax*-infected patients were highly activated. While monocytes were found to be the main source of cytokines in response to TLR ligands, neutrophils showed enhanced phagocytic activity and superoxide production. Interestingly, neutrophils from the malaria patients expressed high levels of GRK2, low levels of CXCR2, and displayed impaired chemotaxis towards IL-8 (CXCL8).

Conclusion: Activated neutrophils from malaria patients are a poor source of pro-inflammatory cytokines and display reduced chemotactic activity, suggesting a possible mechanism for an enhanced susceptibility to secondary bacterial infection during malaria.

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Introduction

Malaria is a complex disease that affects approximately 300 million people every year. Among the different *Plasmodium* species that infect humans, *P. falciparum* is the main cause of deaths in sub-Saharan Africa. On the other hand, *P. vivax* is responsible for approximately 60–80% of the malaria cases in the world [1,2], and contributes to significant political, social and economic instability in the developing countries of Latin America and Asia [1].

The innate immune system recognizes *Plasmodium* sp. by different pattern-recognition receptors and initiates a broad spectrum of defense mechanisms that mediate host resistance to infection [3,4]. However, the innate immune response is the classic "two-edged sword", and clinical malaria is associated with high levels of circulating pro-inflammatory cytokines. The outcome of infection depends on a balance between pro- and anti-inflammatory responses allowing the formation of an effective immune response, while limiting its pathogenic potential [5,6,7].

Toll like receptors (TLR) play an important role in recognition of pathogens through distinct pathogen-associated molecular patterns (PAMPs). Activation of TLR on monocytes, dendritic cells and neutrophils can induce changes in the expression of surface proteins and release inflammatory mediators such as cytokines and chemokines. The production of cytokines amplifies innate immune responses and shapes the development of acquired immunity. In addition, activated myeloid cells release high levels of reactive oxygen species (ROS) and antimicrobial peptides that efficiently kill invading pathogens [8,9].

Author Summary

Plasmodium vivax is responsible for approximately 60–80% of the malaria cases in the world, and contributes to significant social and economic instability in the developing countries of Latin America and Asia. The pathogenesis of *P. vivax* malaria is a consequence of host derived inflammatory mediators. Hence, a better understanding of the mechanisms involved in induction of systemic inflammation during *P. vivax* malaria is critical for the clinical management and prevention of severe disease. The innate immune receptors recognize *Plasmodium sp*. and initiate a broad spectrum of host defense mechanisms that mediate resistance to infection. However, the innate immune response is the classic "two-edged sword", and clinical malaria is associated with high levels of circulating pro-inflammatory cytokines. Our findings show that both monocytes and neutrophils are highly activated during malaria. Monocytes produced high levels of IL-1β, IL-6 and TNF-α during acute malaria. On the other hand, neutrophils were a poor source of cytokines, but displayed an enhanced phagocytic activity and superoxide production. Unexpectedly, we noticed an impaired chemotaxis of neutrophils towards an IL-8 (CXCL8) gradient. We proposed that neutrophil paralysis is in part responsible for the enhanced susceptibility to bacterial infection observed in malaria patients.

It is noteworthy that glycosylphosphatidylinositol anchors and DNA from *Plasmodium* parasites are important PAMPs that activate TLR during malaria [10,11,12,13]. Some *in vitro* studies show that phagocytosis of opsonized hemozoin (HZ) decreases expression of HLA-DR in monocytes [14,15]. On the other hand, study has demonstrated that DNA bound to HZ induces monocytes to produce high levels of cytokines and contribute to dendritic cell maturation [16]. While other report evaluated activation of polymorphonuclear cells and observed elevated levels of myeloperoxidase, lysozyme and lipocalin in patients with severe malaria [17], the involvement of neutrophils in malaria pathogenesis has been poorly investigated.

Our findings show that both monocytes and neutrophils are highly activated during malaria. Monocytes produced high levels of IL-1β, IL-6 and TNF-α in response to TLR agonists during acute malaria and seem to be the main source of pro-inflammatory cytokines in the blood. On the other hand, neutrophils were a poor source of cytokines, but displayed an enhanced phagocytic activity and superoxide production. Interestingly, we noticed an enhanced expression of G-protein receptor protein kinase (GRK2) associated with decreased levels of CXCR2 and impaired chemotaxis of neutrophils towards an IL-8 (CXCL8) gradient. Our findings indicate a mechanism by which malaria patients may become more susceptible to bacterial infection.

Methods

Ethics statement

All protocols and consent forms were approved by the Institutional Research Board from University of Massachusetts Medical School (IRB-UMMS 10268), the Ethical Committees on Human Experimentation from Centro de Pesquisa em Medicina Tropical (CEP-CEPEM 095/2009) and Centro de Pesquisas René Rachou – Fundação Oswaldo Cruz (CEP-CPqRR 2004), as well as by the National Ethical Committee (CONEP 15652) from Ministry of Health, Brazil. A signed informed consent was obtained from each subject prior to enrollment in the study.

Patients

Patients were recruited and examined at CEPEM in Porto Velho, a malaria endemic area in the Amazon region of Brazil. Up to 100 ml of peripheral blood was collected immediately after confirmation of *P. vivax* infection by thick blood smear film and 30–45 days after chemotherapy (n = 26, ranging from 18 to 66 years old [35+9.5]). Patients were treated for 10 days with chloroquine and primaquine. *P. vivax* infection was confirmed by polymerase chain reaction (PCR) analysis [18]. The clinical manifestations of malaria were fever, myalgia, chills, arthralgia, nausea, vomiting or diarrhea, but no patient had complicated malaria. Peripheral blood was also collected from 15 healthy donors (HD) ranging from 21 to 56 years old [32+8] living Porto Velho and negative for *P. vivax* infection by thick blood film and PCR. All the experiments were done using fresh cells.

Cellular immunophenotyping

Whole blood was stained with antibodies from ebioscience: anti-CD14-FITC (clone 61D3), anti-TLR2-PE (clone T2.5), anti-TLR4-PE (clone HTA125), anti-HLA-DR-PE (clone L243), anti-CD14-APC (clone 61D3), anti-CD62L-APC (clone DREG56); BD Bioscience-Pharmingen: anti-CD66b-FITC (clone G10F5), anti-CD88-PE (clone C85-4124), CXCR2-PE (clone 6C6), anti-CD16-PercpCy5.5 (clone3G8), anti-CD15-FITC (clone HI98); R&D: CCR2-PE (clone 48607) Subsequently, red blood cells (RBC) were lysed with FACS lysing solution (BD Biosciences) following manufacturer's instructions, washed with phosphate buffered saline (PBS) and maintained in paraformaldehyde 2% (PFA) until the acquisition on an FACScan upgraded with a second laser (5 colors). The software used for acquisition was CellQuest Pro from BD and Rainbow from Cytek. Data analyzed using FlowJo version 9.3.2 (TreeStar)

Purification of monocytes and neutrophils

Peripheral blood mononuclear cells (PBMC) and neutrophils were enriched on-site by gradient centrifugation over Ficoll-Paque™ plus (GE-Healthcare). Isolated PBMC were washed twice in RPMI, ressuspended at 5×10^7/ml in PBS supplemented with 2% heat- inactivated fetal bovine serum (FBS) and 1 mM EDTA. Monocytes were purified from PBMC with the Human Monocyte Enrichment Kit (STEMCELL-Technologies) following manufacturer's instructions. The cell layer containing the granulocytes were collected just above the red blood cells phase and the cell suspension was lysed with NH_4Cl 0.15 M with $KHCO_3$ 0.1 M and Na_2EDTA 0.1 mM. Cells were ressuspended in PBS (2% FBS, 1 mM EDTA) at final concentration 5×10^7/ml. Purification of neutrophils were performed with the Human Neutrophil Enrichment Kit (STEMCELL-Technologies) following manufacturer's instructions. Purity of monocytes and neutrophils was measured by flow cytometry after staining with mAbs specific for CD14, CD66b and CD16. Purified monocytes were $CD14^+CD16^-CD66b^-$ and neutrophils were $CD14^-CD16^+CD66b^+$. Cell preparations reached over 98% of purity and viability of 90–100%.

Tissue culture assays

Purified monocytes and neutrophils were plated in 96-well cell culture plates at a final concentration 2×10^5/well in RPMI1640 (Sigma Aldrich R6504) (10% FBS and 100 µg/ml streptomycin/100 U/ml penicillin) in the presence or absence of *Toll like receptors* agonists: 100 ng/ml lipopolysaccharide (LPS - TLR4 agonist, Sigma-Aldrich) or 100 ng/ml Pam2CSK4 (Pam - TLR2/TLR1 agonist, InvivoGen). Supernatants were harvested after 48 hours of culture and kept at −20ºC until cytokine measurement.

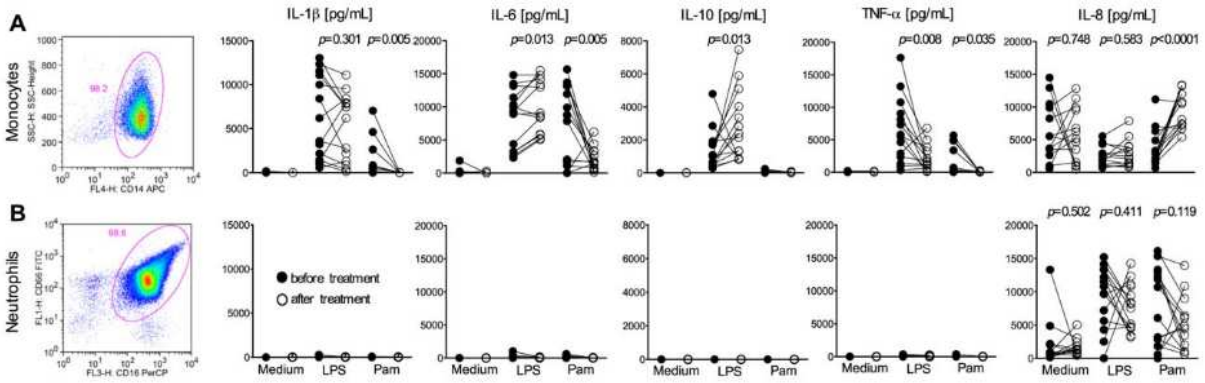

Figure 3. TLR agonists induce production of high IL-1β, IL-6 and TNF-α levels by monocytes from *P. vivax*-infected subjects. Purified monocytes (A) or neutrophils (B) from *P. vivax*-infected subjects before (closed circles; n = 13) and 30–45 days after treatment (open circles; n = 13) were cultured for 48 hours in the absence or presence of LPS or Pam. Levels of IL-1β, IL-6, IL-10, TNF-α, and IL-8 (CXCL8) were measured in supernatant of monocyte (A) and neutrophil (B) cultures. Levels of cytokines were measured employing the Cytometric Bead Array (CBA). Significant differences are indicated *p*-values using paired t test or Wilcoxon signed rank test when a normality test failed.
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pro-inflammatory cytokines during *P. vivax*-malaria? We found that in the blood, monocytes are an important source of cytokine compared with neutrophils. Despite the fact that neutrophils are an important source of cytokines [25], we found that except for IL-8 (CXCL8), the neutrophils from malaria patients produced none or very small amounts of pro-inflammatory cytokines (*i.e.*, IL-1β, IL-6, and TNF-αin response to TLR agonists. Importantly, PBMCs from individuals experimentally or naturally infected with *P. falciparum* are hyperresponsive and produce high amounts of pro-inflammatory cytokines once activated with TLR agonists [6,7]. Here, we observed that highly purified monocytes (but not neutrophils) derived from *P. vivax* malaria patients were primed and produce high levels of IL-1β, IL-6 and TNF-α upon TLR stimulation. In contrast, the monocytes from malaria patients produced low levels of IL-10, even when activated with TLR agonists. Thus, we favor the hypothesis that during malaria monocytes differentiate into an inflammatory stage producing high levels of pro-inflammatory cytokines and low levels of IL-10.

On the other hand, circulatory neutrophils from malaria-patients displayed enhanced phagocytic activity and constitutively

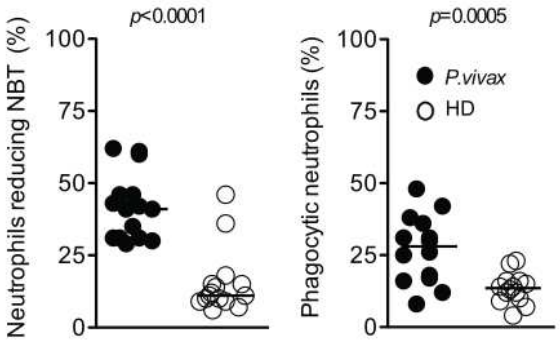

Figure 4. Neutrophils from *P. vivax*-infected patients produce high levels of superoxide and display enhanced phagocytic function. Neutrophils were isolated from *P. vivax*-infected patients (closed circles; n = 15) or healthy donors (open circles; n = 15), and the frequencies of neutrophils reducing NBT (left panel) as well as cell containing zymosan (right panel) were quantified. Significant differences are indicated with *p*-values using unpaired t test or Mann-Whitney test when a normality test failed.
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released high levels of superoxide. The process of neutrophil activation could involve phagocytosis of opsonized parasites, which would in turn trigger the antibody dependent respiratory burst [26]. Alternatively, phagocytosis of the malaria pigment (hemozoin) may also activate neutrophils [27]. The enhanced effector function of neutrophils may account for a more efficient up-take and destruction of free parasites and infected erythrocytes [26]. On the other hand, activated neutrophils have been shown to cause damage of endothelial cells, in a process that is mediated by sera of malaria patients [28]. Therefore, enhanced effector functions in neutrophils could be involved in both host resistance and pathogenesis of *P. vivax* malaria.

Unexpectedly, we found an altered migration towards IL-8 gradient, which was associated with a decreased expression of CXCR2 on neutrophils from *P. vivax*-infected patients. Functional studies showed that upon phagocytosis of bacteria by neutrophils there is reduction in expression of CXCR1 and CXCR2 [29]. Down-regulation of CXCR2 in severe sepsis also results in failure of neutrophil migration that is associated with enhanced susceptibility to bacterial infection [30]. Furthermore, expression of CD88 (C5a receptor) and CD62L (L-selectin) is decreased on the surface of neutrophils from *P. vivax*-malaria patients. CD88 is a G protein-coupled receptor involved in recruitment [19,31], whereas CD62L is a key molecule that mediates cytoadherence of leukocytes [32]. Thus, altogether, our data strongly suggest that systemic activation neutrophils, leads to failure of extravasation and chemotaxis from blood to the tissues. Since IL-8 mediates chemotaxis and stimulate neutrophils to release specific granules and proteases to fight microbial infections [33,34], the impairment of neutrophil migration to the site of infection would prevent front line cells to promote an inflammation to effectively kill infectious pathogens allowing secondary infections.

Production of IL-8 has been assessed in several cell type upon stimulation, several medical conditions and even constitutively [35,36,37]. The observed high levels of circulatory IL-8 may mediate desensitization and/or down-regulation of CXCR2 in acutely infected malaria patients. In addition, TNF-α [38], nitric oxide [30], heme-oxygenase products [39] and TLR ligands [20], cause the heterologous desensitization of CXCR2 via GRK2 induction. As previous described in *vivax* malaria [40,41], we did not find a high level of TNFα in serum, the monocyte stimulated with TLR agonists produced high amounts of TNF-α

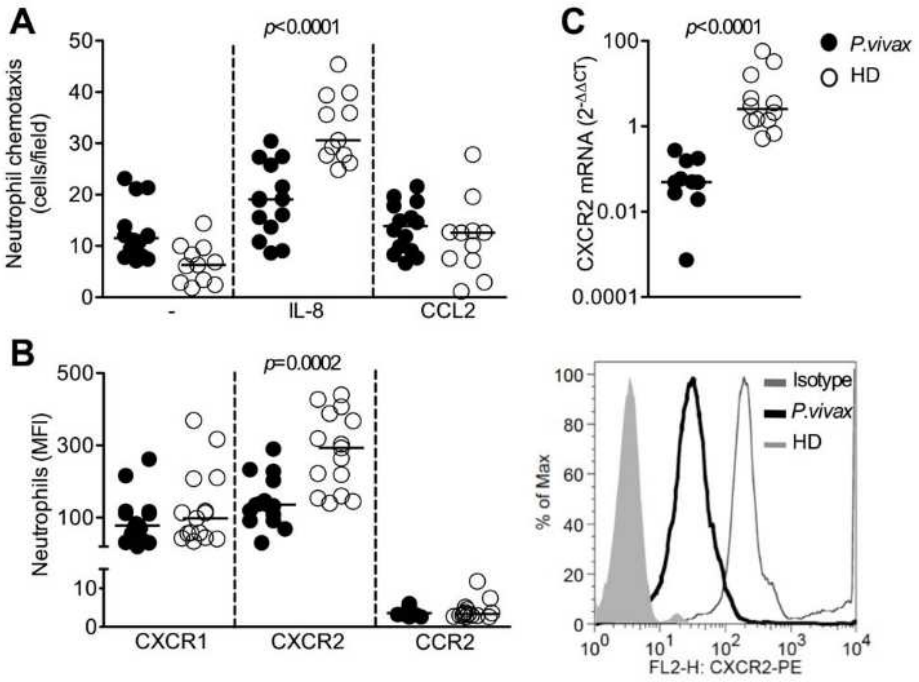

Figure 5. Malaria impairs neutrophils response to CXCR1 and CXCR2 ligand. Neutrophils were isolated from *P. vivax*-infected patients (closed circles; n = 15) or healthy donors (open circles; n = 15), and chemotaxis towards IL-8 (CXCL8) and CCL2 was assessed (A). MFI of CXCR1, CXCR2 and CCR2 on neutrophils were evaluated by flow cytometry and representative histograms of CXCR2 expression are shown (B). CXCR2 message was measured by qPCR (C). Significant differences are indicated with *p*-values using unpaired t test or Mann-Whitney test when a normality test failed.
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that may contribute to CXCR2 desensitization. In addition, *Plasmodium* can be recognized by TLR2, TLR4 and TLR9 [10,42] and induce down-regulation of CXCR2 via GRK2. Importantly, CD88 is also desensitized via GRK2 [43]. Thus, decreased expression of CXCR2 and CD88 on neutrophils from malaria patients may be a consequence of an enhanced expression of GRK2.

GRKs constitute a group of serine/threonine protein kinases that are key modulators of protein-coupled receptor signaling (GPCR) [44]. A major mechanism for desensitization of activated GPCR is their phosphorylation by GRKs [45]. Of note both CD88 and CXCR2 are GPCRs. Deficient expression of GRK and regulation of chemokine receptors in GRK2$^{-/-}$ mice results in enhanced migration of lymphocytes and chemotaxis toward

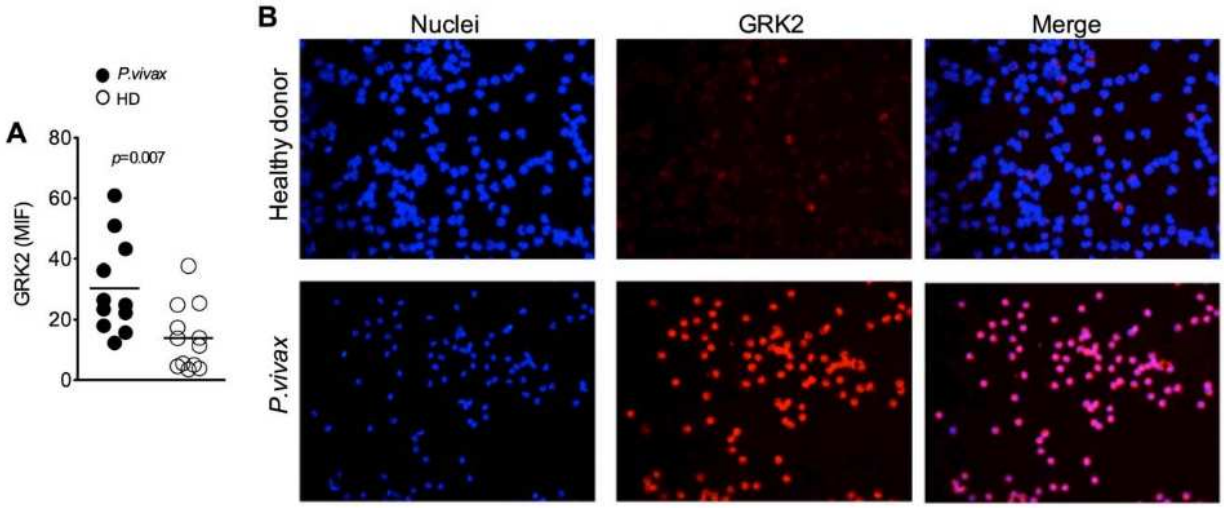

Figure 6. GRK2 expression is enhanced in neutrophils during acute malaria. Neutrophils isolated from *P. vivax*-infected patients (closed circles; n = 11) or healthy donors (closed circles; n = 12) were stained for GRK2 and mean fluorescence intensity (MFI) of GRK2 was quantified (A). Representative fluorescence microscopy illustrating GRK2 expression in neutrophils from a healthy donor and a *P. vivax*-infected patient (B). Significant difference is indicated with *p*-values using unpaired t test.
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CCL4, the CCR5 ligand [46]. It was also described that transcription of GRK2 and GRK5 is upregulated upon LPS-mediated activation, leading to reduced expression of chemokine receptor and neutrophils chemotaxis [47]. GRK2 and GRK5 expression are enhanced in sepsis patients [48] and in rodent models of severe sepsis [20], which are associated with impaired migration of neutrophils and enhanced susceptibility to secondary microbial infection.

For many years, *P. vivax* malaria was considered a benign and self-limited disease, especially when compared to *P. falciparum* infection [49]. However, recent studies highlighted the association of *P. vivax* malaria with life-threatening manifestations, such as respiratory distress, severe thrombocytopenia and anemia, as well as neurological manifestations [1,22,50,51,52,53,54]. A main hypothesis of our research group is that secondary infections, in malaria primed individuals, is a main cause of severe disease. In this regard, pro-inflammatory priming during malaria would result in dramatic decrease in the threshold to initiate a septic shock [7], due to an over-reaction to secondary infections, particularly in the case of bacteria that have extremely potent TLR agonists.

Importantly, areas of the world with the highest incidence and prevalence of malaria also have a high incidence of bacterial infections, including *Salmonella*, *Pneumococcus* and *Meningococcus* [23,55]. Furthermore, a recent study highlights that severe malaria as indicated by respiratory distress, anemia and mortality, is 8.5 times more elevated in children with both malaria and bacteremia as compared to infection with *P. falciparum* alone [24]. Thus, co-infection with bacteria is not only common, but as we might predict, it is an important factor influencing outcome of disease and development of severe disease [24,56]. Here, we demonstrate for the first time that circulatory neutrophils from malaria patients display a decreased expression of chemokine receptors and adhesion molecules, which culminates in impaired chemotaxis. Hence, our results suggest that a failure of these PMNs to migrate to peripheral tissues is an important mechanism leading to enhance susceptibility to bacterial infection during malaria.

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Author Contributions

Conceived and designed the experiments: FMSL LRVA RTG. Performed the experiments: FMSL LRVA SCT BCR PACC HDG. Analyzed the data: FMSL LRVA SCT. Contributed reagents/materials/analysis tools: FMSL LRVA RTG DTG FQC MST DBP. Wrote the paper: FMSL LRVA RTG. Assisted with patient care and case identification: MST DBP.

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