

Inhaled Nitric Oxide and Cerebral Malaria: Basis of a Strategy for Buying Time for Pharmacotherapy

Brian Bergmark,* MD, Regan Bergmark, MD, PhD,† Pierre De Beaudrap, MD, PhD,‡ Yap Boum, PhD,§ Juliet Mwanga-Amumpaire, MD,¶ Ryan Carroll, MD, MPH,|| and Warren Zapol, MD||

Abstract: There are approximately 225–600 million new malaria infections worldwide annually, with severe and cerebral malaria representing major causes of death internationally. The role of nitric oxide (NO) in the host response in cerebral malaria continues to be elucidated, with numerous known functions relating to the cytokine, endovascular and cellular responses to infection with *Plasmodium falciparum*. Evidence from diverse modes of inquiry suggests NO to be critical in modulating the immune response and promoting survival in patients with cerebral malaria. This line of investigation has culminated in the approval of 2 phase II randomized prospective clinical trials in Uganda studying the use of inhaled NO as adjuvant therapy in children with severe malaria. The strategy underlying both trials is to use the systemic antiinflammatory properties of inhaled NO to “buy time” for chemical antiparasite therapy to lower the parasite load. This article reviews the nexus of malaria and NO biology with a primary focus on cerebral malaria in humans.

Key Words: inhaled nitric oxide, cerebral malaria, nitric oxide synthase, *Plasmodium falciparum*

(*Pediatr Infect Dis J* 2012;31: e250–e254)

Plasmodium falciparum has coevolved with *Homo sapiens* and its predecessors for at least 70,000 years. Nitric oxide (NO), originally considered as solely an environmental toxin, was discovered to be a physiologically critical signaling molecule in 1988. Subsequently, NO was studied, among an enormous amount of worldwide research, as an inhaled gas with eventual approval in the United States for the treatment of term neonates with persistent pulmonary hypertension. Over the past 2 decades, a growing number of studies have examined the role of endogenous NO production in the pathophysiology of malarial disease in humans, particularly severe and cerebral malaria (CM).¹

These efforts have generated controversy regarding the role of NO in malaria. Initial findings were interpreted to suggest that high NO levels contributed to the development of CM and severe anemia, but tended to be retrospective chemical pathology correlations. More recent work has indicated that NO is, in fact, likely to be critical in

allowing survival in patients with severe and cerebral malaria. The debate has culminated in the approval of 2 phase II randomized prospective clinical trials in Uganda studying the use of inhaled NO (iNO) as adjuvant therapy in children with severe malaria. The strategy underlying both trials is to use the antiinflammatory properties of iNO in the lung and systemic circulation to “buy time” for chemical antiparasite therapy to lower the parasite load. This article reviews the nexus of malaria and NO biology with a primary focus on CM in humans.

MALARIA

There are approximately 225–600 million new malaria infections worldwide each year.² Severe malaria is defined by the World Health Organization as vital organ dysfunction and/or high parasite burden in the setting of demonstrated parasitemia.³ *P. falciparum* is the organism primarily responsible for severe malaria.⁴ Common perturbations in severe malaria include anemia, metabolic acidosis, hypoglycemia, respiratory failure and CM.⁴ CM is typically defined as coma in the setting of parasitemia in the absence of other causes of reduced consciousness, such as other central nervous system infections, hypoglycemia, a postictal state or ongoing seizure.⁴ As is true with severe malaria, definitions of CM must balance sensitivity and specificity in clinical and research contexts. In locations such as sub-Saharan Africa in which incidental parasitemia is common and the resources necessary to definitively rule out other causes of altered mental status are often limited, reliable diagnosis of CM is difficult.⁵ Severe and cerebral malaria tend to occur in young children who have not yet developed effective immunity or in adult travelers from nonendemic regions. The overall fatality rate for patients with CM who seek medical attention is roughly 10–40%⁴ and remains roughly 9% among children who receive the most effective and rapidly acting therapy, intravenous artesunate.⁶ Children who survive CM have an approximately 1 in 10 chance of suffering long-term neurologic sequelae⁴ and a 1 in 4 chance of persistent cognitive impairment.⁷

NITRIC OXIDE

NO is a free radical with protean effects in the human body. Initially described in the vasculature as the endothelium-derived relaxing factor, it is an important signaling molecule in diverse processes including neurotransmission, immune system and cytokine modulation, platelet inhibition, vascular homeostasis and regulation of hematopoiesis. Endogenous production occurs via 3 NO synthase (NOS) isoform enzymes: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3).⁸ Exogenous delivery by inhalation has been used therapeutically since the 1990s for a growing number of indications.⁹ NO exists as a gas at physiologic temperature and pressure and has a half-life estimated to be under 2 ms in whole blood.¹⁰

L-arginine is converted into NO and L-citrulline by NOS1, NOS2 and NOS3. NOS1 and NOS3 are constitutively expressed and are calcium/calmodulin-dependent,⁸ whereas NOS2 is only expressed when its transcription is activated.⁸

NO has many sites of action. Made in the endothelium, it diffuses into subjacent smooth muscle cells where it activates soluble guanylate cyclase, increases intracellular levels of cyclic

Accepted for publication May 01, 2012.

From the *Department of Medicine, †Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; ‡Institut de Recherche pour le Développement, Marseille, France; §Epicentre Research Base; ¶Mbarara University, Mbarara, Uganda; and ||Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

The authors are members of the team leading one of the clinical trials that will evaluate the use of inhaled nitric oxide as adjunctive therapy in cerebral malaria in Uganda. The authors have no other funding or conflicts of interest to disclose.

BB and RB contributed equally to this manuscript.

Address for Correspondence: Brian Bergmark, MD, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115.
E-mail: bbergmark@partners.org.

Copyright © 2012 by Lippincott Williams & Wilkins
ISSN: 0891-3668/12/3112-e250

DOI: 10.1097/INF.0b013e318266c113

guanosine monophosphate and, thereby, lowers the intracellular calcium concentration causing vasorelaxation. In the vascular lumen, NO inhibits platelet aggregation, also through a cyclic guanosine monophosphate-dependent mechanism, and has numerous effects on endothelial adhesion molecule expression and endothelial homeostasis. Two other mechanisms by which NO exerts intracellular effects are through activation of adenylate cyclase and inhibition of phospholipase C, both effected via adenosine diphosphate ribosylation.¹¹ Soluble guanylate cyclase activation by NO in bone marrow-derived cells, principally monocytes and other leukocytes, is also a critical pathway.¹² Of less importance, a minority of the NO intracellular signaling effect is due to posttranslational S-nitrosylation of cysteine thiols on target proteins.¹¹

Initial use of iNO was directed at pulmonary effects rather than systemic. More recent work has demonstrated systemic effects of iNO, initially in animals, such as reducing cardiac ischemia-reperfusion injury in mice.¹² In humans, evidence of systemic effects of iNO has been seen in hepatic ischemia-reperfusion injury.¹³ As in CM, low NO bioavailability in hepatic ischemia mediates hepatic ischemia-reperfusion injury via cytokine dysregulation, increased leukocyte adhesion and decreased endothelial function with increased microcirculatory tone.¹³ Among patients undergoing orthotopic liver transplantation, the use of iNO at 80 ppm was associated with more rapid restoration of liver function posttransplant, decreased hospital length of stay and a 75% reduction in hepatocellular apoptosis.¹³

Catabolism of NO takes place through multiple pathways. NO reacts with oxyhemoglobin to produce methemoglobin and nitrates, and also reacts with the superoxide anion to create peroxynitrite.¹¹ Additionally, NO is degraded enzymatically by peroxidases.¹¹ Nitrates and nitrites, typically denoted NO_x, are frequently measured as the breakdown products of NO.

Numerous challenges exist in the reliable and physiologically relevant study of NO. Because of its short half-life and propensity for diffusion, NO tends to exert effects near the site of production. It is difficult, however, to measure NO levels directly at the site of action *in vivo*. Additionally, nitrate and nitrite levels in plasma or other body fluids, such as cerebrospinal fluid are influenced not only by NO production, but also by diet, renal function and the array of catabolic pathways available to NO. Debate continues regarding the relevance of transport *in vivo* of NO and its potential for distant effects, including transport bound to hemoglobin or thiol residues.¹⁴

Multiple pathways lead to low NO bioavailability in malaria infection, including hemolysis with release of NO-scavenging cell-free oxyhemoglobin and arginase,¹⁵ as well as inflammatory production of superoxide, which reacts with NO to produce peroxynitrite. Anstey et al¹⁶ demonstrated an inverse relationship between disease severity and monocyte NOS2 activity in the presence of high levels of interleukin (IL)-10. Concurrently, however, tumor necrosis factor (TNF) and interferon-gamma induce NOS2 mRNA expression in bone marrow-derived cells.¹⁷

PATHOPHYSIOLOGY OF CEREBRAL MALARIA

The mechanisms by which *P. falciparum* infection leads to altered mental status and coma are not completely understood, although both disruption of microcirculatory flow and the host immune response appear to be central processes. Although initial attention was drawn to parasite sequestration within the cerebral vasculature and the resulting microcirculatory flow reduction, this explanation is no longer considered sufficient. Now under investigation are the consequences of the exuberant cytokine response.

SEQUESTRATION

The term sequestration describes the tendency of parasitized erythrocytes to adhere to the endothelium as well as to other

parasitized erythrocytes (agglutination), uninfected erythrocytes (rosetting) or platelets (platelet-mediated clumping), a finding that is particular to *falciparum* malaria among the human malaria species.¹ The coma of CM can resolve, with permanent neurologic sequelae seen in only 10% of pediatric patients who survive and persistent measurable cognitive deficits seen in one quarter,^{4,7,18} which are lower rates than would be expected after ischemic injury to the brain. Additionally, the extent and location of sequestration found on autopsy does not correspond with high fidelity to the severity of clinical malaria.¹⁹

CYTOKINE RESPONSE

Recent study of the pathophysiology of CM has focused on the cytokine and inflammatory response to the parasite and the interaction between the immune system and the endothelium. The balance between proinflammatory signals (TNF, interferon-gamma, IL-1 and IL-12) and immunomodulatory signals (IL-10, NO) is of particular importance.²⁰ Disease severity correlates with TNF and IL-1 concentrations independent of degree of parasitemia.²¹ Furthermore, patients with a TNF promoter mutation associated with elevated TNF activity are at increased risk for mortality or serious neurologic sequelae.²² NO is known to inhibit cytochrome C oxidation via competition with oxygen and could theoretically inhibit the neuronal respiratory cycle in the setting of low partial pressure of oxygen.²³ Angiotensin-1 (Ang-1) depletion and Angiotensin-2 (Ang-2) elevation have been investigated as diagnostic and prognostic markers in severe and cerebral malaria. Ang-1 and Ang-2 regulate endothelial activation. Ang-1 is produced by endothelial cells constitutively and appears to maintain endothelial homeostasis via activation of the Tie-2 receptor.²⁴ Ang-1 levels fall during inflammation and endothelial activation, as is seen in sepsis or severe *falciparum* malaria.²⁴ Ang-2 is stored in endothelial Weibel-Palade bodies from where it is released, displaces Ang-1 from the Tie-2 receptor and propagates endothelial activation, including through adhesion molecule expression and sensitization of the endothelium to TNF.²⁴ Weibel-Palade bodies exocytosis is inhibited by NO, limiting the release of Ang-2.²⁵ Additionally, erythrocyte cytoadherence is reduced *in vitro* by NO via inhibition of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 expression.²⁶

INTRACRANIAL HYPERTENSION

Some authors have additionally posited a central role of increased intracranial pressure in the pathophysiology of mental status changes and death in CM. Possible mechanisms of intracranial hypertension development include increased intracranial blood volume, decreased adenosine triphosphate availability leading to adenosine triphosphate-dependent ion pump failure and extracellular fluid shift and blood-brain barrier breakdown.¹

Intracranial hypertension is undoubtedly responsible for some patients' neurologic deficits and deaths, and in fact papilledema and other findings of intracranial hypertension are significantly associated with poor outcome.²⁷ Evidence has not supported a critical effect of elevated intracranial pressure in all patients, however. Cerebral edema is commonly seen on autopsy, although studies have failed to show evidence of herniation regularly in patients with CM who have died.²⁸ Similarly, opening pressure on lumbar puncture and low cerebral perfusion pressure have not been shown to be associated with mortality.²⁹

NITRIC OXIDE IN CEREBRAL MALARIA

Accumulating evidence suggests a protective role for NO in severe and cerebral malaria attributed to NO's modulating the inflammatory response.

HYPOARGININEMIA

L-arginine concentrations fall in the settings of inflammation, hemolysis and malaria infection, and L-arginine levels vary inversely with malaria disease severity. It is hypothesized that the low bioavailability of arginine for NO synthesis contributes to pathogenesis. L-arginine levels are low in patients with CM, moderately low in patients with uncomplicated malaria and normal in healthy controls.³⁰ Hypoargininemia caused by arginase release from hemolyzed erythrocytes is significantly associated with fatal outcome in patients with CM.³⁰ Exogenous L-arginine rescues reactive hyperemia-peripheral artery tonometry, a noninvasive measurement of vascular NO production, in patients with severe malaria.³¹ Asymmetric dimethylarginine levels are higher in patients with severe malaria than in those with moderately severe malaria and each micromolar increase of this endogenous inhibitor of NOS conversion of L-arginine to NO is associated with an 18-fold increase in mortality.³²

MURINE CEREBRAL MALARIA

Mice provide a reliable, although imperfect, model of CM.⁴ Mice deficient in NOS2 or NOS3 have equivalent courses of CM as do controls when infected with *Plasmodium berghei* ANKA, and delivery of an exogenous NO donor to NOS-deficient mice prevents CM and reduces the inflammatory response.³³ Similarly, CM has been reported in a NOS2-deficient murine model with pharmaceutical NOS antagonism, suggesting that NO is not necessary for CM.³⁴ Exogenous NO as compared with saline placebo in *Plasmodium berghei* ANKA-infected mice leads to improved pial blood flow, fewer hemorrhagic foci and less leukocyte adherence.³⁵ Most recently, iNO was shown to reduce cerebral erythrocyte sequestration, endothelial activation and systemic inflammation as well as improve survival in *Plasmodium berghei* ANKA-infected mice.³⁶ Taken together, these findings demonstrate that NO is not necessary for the development of CM and that restoring NO bioavailability reduces the inflammatory response and improves clinical status.

POPULATION AND GENETIC STUDIES

A number of early studies found a positive correlation between NOx in the blood, cerebrospinal fluid or urine and disease severity, yet many of these studies did not control for dietary intake or renal function or compare results with healthy controls within the same population.^{37–39} Later studies have demonstrated lower levels of NOS2 activity, NO and NOx in children with severe malaria compared with those with mild malaria or asymptomatic parasitemia, suggesting that higher NOS2 activity and NO production protect children against severe malaria.^{16,40,41} Although it does appear that people in malaria-endemic regions have higher NOS2 expression at baseline and that low NOS2 levels may be associated with severe malaria, the differences in and weaknesses of methodology preclude definitive conclusions as to whether NOS2 expression is correlated with disease severity.

Genetic studies have investigated whether haplotypes resulting in higher or lower levels of NO production lead to variable susceptibility to severe forms of malaria. The gene for NOS2 is NOS2A. Attention has been focused on single nucleotide polymorphisms (SNPs) in NOS2A, G954C and C1173T, as well as a microsatellite pentanucleotide repeat, CCTTT(n).⁴² Additional SNPs and promoter site mutations continue to be identified.

The NOS2 promoter SNP at site G954C in the promoter region was first discovered in 1998 and found to be associated with less severe malaria. This polymorphism was originally described as the Lambarene mutation at site 969, but was subsequently referred to as the 954 mutation by the same group.⁴³ This SNP is

more prevalent among malaria-exposed populations than among controls.⁴⁴ Ex vivo studies demonstrated a 7-fold higher NOS2 activity level in patients with this mutation,⁴³ and in vivo studies demonstrated higher NO production in response to 15N-arginine infusion.⁴⁵ G954C has also been shown to be protective against infection with malaria⁴⁶ and associated with high NOx levels and lower symptomatology in malaria infection.⁴⁷ Not all studies have corroborated these findings, however.^{47–49} An additional NOS2 SNP, C1173T, is associated with less symptomatic malaria and increased fasting urine and plasma NOx.⁵⁰

Large diversity in the CCTTT(n) repeat number is observed, and although shorter alleles were initially associated with worse malaria symptoms and CM death, more recent findings demonstrate increased disease severity with greater allele length^{51,52} or no correlation between length and disease severity,^{42,50} suggesting a complex relationship between the microsatellite repeat and disease outcome.

NO SYNTHASE 1 AND NO SYNTHASE 3

NOS1 and NOS3 have received less attention than NOS2 because they are constitutively expressed, although some evidence supports cytokine recruitment of NOS1 in viral encephalitis.⁵³ Yet, although significant enrollment of these enzymes in *P. falciparum* infection has not yet been demonstrated, variation in activity may be relevant. Two NOS1 SNPs are associated with altered NOS1 expression, G84A and C276T. The former is located in the promoter region of NOS1 and is associated with decreased basal activity⁵⁴ and higher risk for CM.⁵⁵ NOS3 mutations are correlated with protection against CM as well as plasma NOx levels.^{56,57}

Collectively, these findings provide strong biochemical evidence for a protective role of NO in CM via immune response modulation as well as robust, although not invariably positive, epidemiologic and genetic support for this effect. Coupled with this elucidation of the relationship between NO and *Plasmodium* infection is an increasing availability of iNO therapy worldwide. Although engineering challenges remain in the reliable production and transportation of NO to resource-limited settings, iNO can be produced cheaply via electrical and chemical mechanisms, and methods of production and delivery suited to the developing world are under active investigation.^{58,59}

CONCLUSION

Millennia of evolutionary pressure exerted by malaria have helped to shape the human organism. The role of NO in the host's response to infection has become a focus of intense investigation over the past 20 years as the interactions between NO, malaria, the immune system and the endothelium become increasingly understood. The weight of evidence suggests a protective role of NO produced by inducible NOS in severe and cerebral malaria. Demonstration of NO's immunomodulatory role as well as measurement of NOx levels, NOS promoter mutations and disease severity have provided compelling evidence that NO is essential in preventing or moderating the severe manifestations of *P. falciparum* infection. After more than 70,000 years of hominid-*Plasmodium* coevolution, the time has come to test directly whether inhaled NO can give infected children the time they need to survive cerebral malaria.

ACKNOWLEDGMENTS

The authors acknowledge the contributions of Elisabeth Kemigisha and David Bangsberg, as well as the support of the MSF Epicentre Mbarara Research Base. Authors' contributions: BB and RB contributed to the writing and editing of the manuscript. PDB, YB, JM, RC and WZ contributed to the planning of the project and editing of the manuscript.

REFERENCES

- Clark IA, Alleva LM, Mills AC, et al. Pathogenesis of malaria and clinically similar conditions. *Clin Microbiol Rev*. 2004;17:509–39, table of contents.
- World Health Organization. *World Malaria Report 2010*. Geneva, Switzerland: World Health Organization, 2010.
- World Health Organization. *WHO Guidelines for the Treatment of Malaria*. Geneva, Switzerland: World Health Organization, 2010.
- Idro R, Jenkins NE, Newton CR. Pathogenesis, clinical features, and neurological outcome of cerebral malaria. *Lancet Neurol*. 2005;4:827–840.
- Taylor TE, Fu WJ, Carr RA, et al. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat Med*. 2004;10:143–145.
- Dondorp AM, Fanello CI, Hendriksen IC, et al. AQUAMAT group. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet*. 2010;376:1647–1657.
- John CC, Bangirana P, Byarugaba J, et al. Cerebral malaria in children is associated with long-term cognitive impairment. *Pediatrics*. 2008;122:e92–e99.
- Lin S, Fagan KA, Li KX, et al. Sustained endothelial nitric-oxide synthase activation requires capacitative Ca²⁺ entry. *J Biol Chem*. 2000;275:17979–17985.
- Bloch KD, Ichinose F, Roberts JD Jr, et al. Inhaled NO as a therapeutic agent. *Cardiovasc Res*. 2007;75:339–348.
- Rassaf T, Preik M, Kleinbongard P, et al. Evidence for *in vivo* transport of bioactive nitric oxide in human plasma. *J Clin Invest*. 2002;109:1241–1248.
- Tuteja N, Chandra M, Tuteja R, et al. Nitric Oxide as a Unique Bioactive Signaling Messenger in Physiology and Pathophysiology. *J Biomed Biotechnol*. 2004;2004:227–237.
- Nagasaka Y, Buys ES, Spagnoli E, et al. Soluble guanylate cyclase-1 is required for the cardioprotective effects of inhaled nitric oxide. *Am J Physiol Heart Circ Physiol*. 2011;300:H1477–H1483.
- Siriussawakul A, Zaky A, Lang JD. Role of nitric oxide in hepatic ischemia-reperfusion injury. *World J Gastroenterol*. 2010;16:6079–6086.
- Rassaf T, Kleinbongard P, Preik M, et al. Plasma nitrosothiols contribute to the systemic vasodilator effects of intravenously applied NO: experimental and clinical Study on the fate of NO in human blood. *Circ Res*. 2002;91:470–477.
- Yeo TW, Lampah DA, Tjitra E, et al. Relationship of cell-free hemoglobin to impaired endothelial nitric oxide bioavailability and perfusion in severe falciparum malaria. *J Infect Dis*. 2009;200:1522–1529.
- Anstey NM, Weinberg JB, Hassanali MY, et al. Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J Exp Med*. 1996;184:557–567.
- Maciejewski JP, Selleri C, Sato T, et al. Nitric oxide suppression of human hematopoiesis *in vitro*. Contribution to inhibitory action of interferon-gamma and tumor necrosis factor-alpha. *J Clin Invest*. 1995;96:1085–1092.
- Newton CR, Krishna S. Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. *Pharmacol Ther*. 1998;79:1–53.
- Clark IA, Awburn MM, Whitten RO, et al. Tissue distribution of migration inhibitory factor and inducible nitric oxide synthase in falciparum malaria and sepsis in African children. *Malar J*. 2003;2:6.
- Stevenson MM, Riley EM. Innate immunity to malaria. *Nat Rev Immunol*. 2004;4:169–180.
- Kwiatkowski D, Hill AV, Sambou I, et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet*. 1990;336:1201–1204.
- McGuire W, Hill AV, Allsopp CE, et al. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature*. 1994;371:508–510.
- Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta*. 2001;1504:46–57.
- Fiedler U, Reiss Y, Scharpfenecker M, et al. Angiotensin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med*. 2006;12:235–239.
- Matsushita K, Morrell CN, Cambien B, et al. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. *Cell*. 2003;115:139–150.
- Serirom S, Raharjo WH, Chotivanich K, et al. Anti-adhesive effect of nitric oxide on *Plasmodium falciparum* cytoadherence under flow. *Am J Pathol*. 2003;162:1651–1660.
- Beare NA, Southern C, Chalira C, et al. Prognostic significance and course of retinopathy in children with severe malaria. *Arch Ophthalmol*. 2004;122:1141–1147.
- White VA, Lewallen S, Beare NA, et al. Retinal pathology of pediatric cerebral malaria in Malawi. *PLoS ONE*. 2009;4:e4317.
- Waller D, Crawley J, Nosten F, et al. Intracranial pressure in childhood cerebral malaria. *Trans R Soc Trop Med Hyg*. 1991;85:362–364.
- Lopansri BK, Anstey NM, Weinberg JB, et al. Low plasma arginine concentrations in children with cerebral malaria and decreased nitric oxide production. *Lancet*. 2003;361:676–678.
- Yeo TW, Lampah DA, Gitawati R, et al. Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciparum malaria. *J Exp Med*. 2007;204:2693–2704.
- Yeo TW, Lampah DA, Tjitra E, et al. Increased asymmetric dimethylarginine in severe falciparum malaria: association with impaired nitric oxide bioavailability and fatal outcome. *PLoS Pathog*. 2010;6:e1000868.
- Gramaglia I, Sobolewski P, Meays D, et al. Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. *Nat Med*. 2006;12:1417–1422.
- Favre N, Ryffel B, Rudin W. The development of murine cerebral malaria does not require nitric oxide production. *Parasitology*. 1999;118(Pt 2):135–138.
- Cabrales P, Zanini GM, Meays D, et al. Nitric oxide protection against murine cerebral malaria is associated with improved cerebral microcirculatory physiology. *J Infect Dis*. 2011;203:1454–1463.
- Serghides L, Kim H, Lu Z, et al. Inhaled nitric oxide reduces endothelial activation and parasite accumulation in the brain, and enhances survival in experimental cerebral malaria. *PLoS ONE*. 2011;6:e27714.
- Al Yaman FM, Mokela D, Genton B, et al. Association between serum levels of reactive nitrogen intermediates and coma in children with cerebral malaria in Papua New Guinea. *Trans R Soc Trop Med Hyg*. 1996;90:270–273.
- Weiss G, Thuma PE, Biemba G, et al. Cerebrospinal fluid levels of bioperin, nitric oxide metabolites, and immune activation markers and the clinical course of human cerebral malaria. *J Infect Dis*. 1998;177:1064–1068.
- Agina AA, Abd-Allah SH. Plasma levels of nitric oxide in association with severe *Plasmodium falciparum* in Yemen. *J Egypt Soc Parasitol*. 1999;29:215–222.
- Cot S, Ringwald P, Mulder B, et al. Nitric oxide in cerebral malaria. *J Infect Dis*. 1994;169:1417–1418.
- Perkins DJ, Kremsner PG, Schmid D, et al. Blood mononuclear cell nitric oxide production and plasma cytokine levels in healthy gabonese children with prior mild or severe malaria. *Infect Immun*. 1999;67:4977–4981.
- Boutlis CS, Hobbs MR, Marsh RL, et al. Inducible nitric oxide synthase (NOS2) promoter CCTTT repeat polymorphism: relationship to *in vivo* nitric oxide production/NOS activity in an asymptomatic malaria-endemic population. *Am J Trop Med Hyg*. 2003;69:569–573.
- Kun JF, Mordmüller B, Perkins DJ, et al. Nitric oxide synthase 2(Lam-baréné) (G-954C), increased nitric oxide production, and protection against malaria. *J Infect Dis*. 2001;184:330–336.
- Levesque MC, Hobbs MR, Anstey NM, et al. Nitric oxide synthase type 2 promoter polymorphisms, nitric oxide production, and disease severity in Tanzanian children with malaria. *J Infect Dis*. 1999;180:1994–2002.
- Planche T, Macallan DC, Sobande T, et al. Nitric oxide generation in children with malaria and the NOS2G-954C promoter polymorphism. *Am J Physiol Regul Integr Comp Physiol*. 2010;299:R1248–R1253.
- Parikh S, Dorsey G, Rosenthal PJ. Host polymorphisms and the incidence of malaria in Ugandan children. *Am J Trop Med Hyg*. 2004;71:750–753.
- Cramer JP, Nüssler AK, Ehrhardt S, et al. Age-dependent effect of plasma nitric oxide on parasite density in Ghanaian children with severe malaria. *Trop Med Int Health*. 2005;10:672–680.
- Burgner D, Usen S, Rockett K, et al. Nucleotide and haplotypic diversity of the NOS2A promoter region and its relationship to cerebral malaria. *Hum Genet*. 2003;112:379–386.
- Mombo LE, Ntoumi F, Bisseye C, et al. Human genetic polymorphisms and asymptomatic *Plasmodium falciparum* malaria in Gabonese schoolchildren. *Am J Trop Med Hyg*. 2003;68:186–190.

50. Hobbs MR, Udhayakumar V, Levesque MC, et al. A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. *Lancet*. 2002;360:1468–1475.
51. Ohashi J, Naka I, Patarapotikul J, et al. Significant association of longer forms of CCTTT Microsatellite repeat in the inducible nitric oxide synthase promoter with severe malaria in Thailand. *J Infect Dis*. 2002;186:578–581.
52. Cramer JP, Mockenhaupt FP, Ehrhardt S, et al. iNOS promoter variants and severe malaria in Ghanaian children. *Trop Med Int Health*. 2004;9:1074–1080.
53. Komatsu T, Bi Z, Reiss CS. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. *J Neuroimmunol*. 1996;68:101–108.
54. Saur D, Vanderwinden JM, Seidler B, et al. Single-nucleotide promoter polymorphism alters transcription of neuronal nitric oxide synthase exon 1c in infantile hypertrophic pyloric stenosis. *Proc Natl Acad Sci USA*. 2004;101:1662–1667.
55. Dhangadamajhi G, Mohapatra BN, Kar SK, et al. Genetic variation in neuronal nitric oxide synthase (nNOS) gene and susceptibility to cerebral malaria in Indian adults. *Infect Genet Evol*. 2009;9:908–911.
56. Dhangadamajhi G, Mohapatra BN, Kar SK, et al. Endothelial nitric oxide synthase gene polymorphisms and *Plasmodium falciparum* infection in Indian adults. *Infect Immun*. 2009;77:2943–2947.
57. Dhangadamajhi G, Mohapatra BN, Kar SK, et al. A new allele (eNOS4e) in the intron 4 (VNTR) of eNOS gene in malaria infected individuals of the population of Orissa (an eastern Indian state). *Nitric Oxide*. 2010;22:58–59.
58. Hu H, Liang H, Li J, et al. Study on production of inhaled nitric oxide for medical applications by pulsed discharge. *IEEE Trans Plas Sci*. 2007;35:619.
59. Carpenter AW, Schoenfisch MH. Nitric oxide release: part II. Therapeutic applications. *Chem Soc Rev*. 2012;41:3742–3752.

ERRATUM

Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 Analysis: ERRATUM

In the article that appeared on page e213 of volume 31, issue 11, the figure legends were incorrect for Figures 1 and 2. The figures and legends are reprinted below correctly.

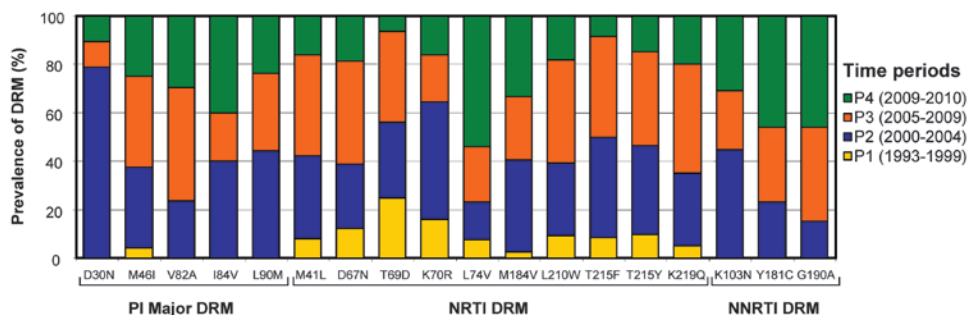


FIGURE 1. Prevalence of DRM positions for each drug class represented over 5% in the selected population.

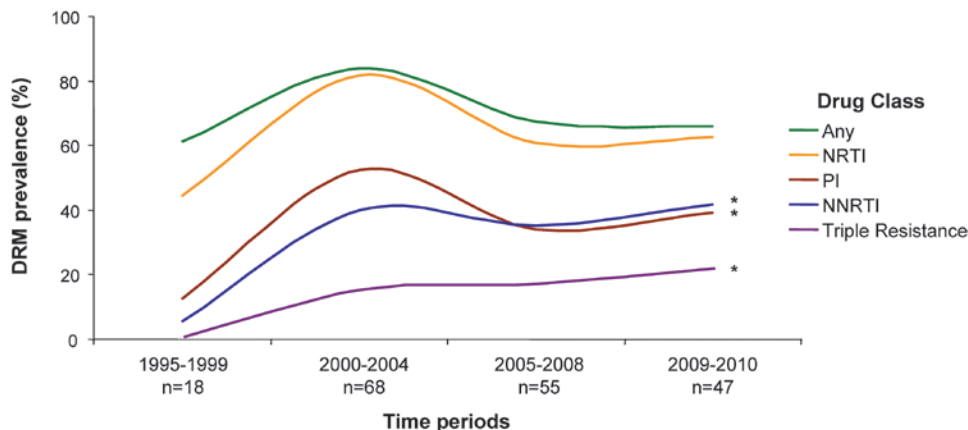


FIGURE 2. Temporal trends of DRM prevalence according to drug class. PI indicates protease inhibitors; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors. Asterisk indicates statistical differences between P1 (1993–1999) and P4 (2008–2010).

REFERENCE

de Mulder M, Yebra G, Navas A, et al. Trends in Drug Resistance Prevalence in HIV-1–infected Children in Madrid: 1993 to 2010 Analysis. *Pediatr Infect Dis* 2012;31:e213–Je221