MiRNA-146a Polymorphism Was Not Associated with Malaria in Southern India

Welmoed van Loon,^{1*} Prabhanjan P. Gai,¹ Suyamindra S. Kulkarni,² Rashmi Rasalkar,² Konrad Siebert,¹ Jakob Wedam,¹ Archith Boloor,^{3,4} Shantaram Baliga,^{3,4} Arun Kumar,⁵ Animesh Jain,^{3,4} Chakrapani Mahabala,^{3,4} Damodara Shenoy,^{3,4} Rajeshwari Devi,⁶ Pramod Gai,² and Frank P. Mockenhaupt¹

¹Institute of Tropical Medicine and International Health Berlin, Charité–Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin, Germany; ²Karnataka Institute for DNA Research, Dharwad, India; ³Kasturba Medical College, Mangalore, India; ⁴Manipal Academy of Higher Education, Manipal, India; ⁵District Vector Borne Disease Control Programme Office, Mangaluru, India; ⁶Wenlock Hospital, Mangaluru, India

Abstract. Micro-RNAs (miRNAs) play a crucial role in immune regulation, and a common miRNA-146a polymorphism (rs2910164) increased the odds of falciparum malaria in pregnant African women. Here, we examined whether this association holds true in a different population, that is, 449 mainly male and adult malaria patients and 666 community controls in southwestern India. *Plasmodium vivax* malaria (67%) predominated over falciparum malaria (11%) and mixed species infections (22%). Overall, 59% of the study participants carried the miRNA-146a polymorphism. However, it was not associated with the odds of malaria, irrespective of parasite species. This underlines the importance of considering the complexities of clinical manifestations of malaria, genetic background, and parasite species when disentangling the role of human genetic variation, including those of miRNAs in malaria.

Understanding the immune components that predispose individuals to susceptibility to and severe manifestation of malaria is a key component of controlling and eliminating this complex disease. Micro-RNAs (miRNAs) are small, noncoding RNA molecules in a hairpin structure, and they are involved in gene regulation on a posttranslational level.¹ There is increasing interest in miRNAs because of their regulatory role at various steps in the innate and adaptive immune network, which could open up novel clinical applications. Micro-RNA-146a is an important regulator in pattern recognition receptor signaling, such as the Toll-like receptor machinery.^{1,2} Micro-RNA-146a suppresses NF- κ B activation by a negative regulation loop, thereby altering several inflammatory cytokines.^{3,4} It also participates in hematopoietic cell regulation,⁵ which might be relevant in malaria, considering its strong interference with blood cell homeostasis.

A common single-nucleotide polymorphism (SNP) in miRNA-146a, rs2910164 G>C, has been associated with modified risks for various types of cancer,⁶ autoimmune disorders,⁷ and infectious diseases.^{8–10} The presence of the polymorphism results in reduced amounts of mature miRNA-146a in vitro.¹¹ Also, the SNP gives rise to a miRNA-146a molecule that is thought to target another set of genes than wild-type miRNA-146a, and, therefore, SNP heterozygosity might have a very different functional effect compared with either of the homozygous genotypes.¹²

Previously, we found that this SNP greatly increased the odds of malaria in pregnancy, particularly in primigravidae.⁸ Malaria in pregnancy has a distinct pathophysiology and immunity, which differs from those of nonpregnant hosts.¹³ Also, miRNAs have been shown to play a specific role at the maternal–fetal interface,^{14,15} that is, the predilection site of malaria in pregnancy. Here, we examined whether miRNA-146a rs2910164 G>C also affects susceptibility to and/or manifestation of malaria in nonpregnant hosts including those with *Plasmodium vivax* infection.

The present study was conducted from June to December 2015 in Mangaluru, Karnataka, coastal southwestern India.

Mangaluru is a harbor city of some 500,000 inhabitants exhibiting the peculiarity of urban malaria in the otherwise less affected state of Karnataka. Wenlock Hospital is the largest governmental health facility in Mangaluru particularly serving the socioeconomically deprived portion of the population of the Mangaluru conglomeration. Patients presenting at the outpatient department (OPD) of the Wenlock Hospital with malaria-like symptoms were referred to the malaria diagnostic unit for diagnosis using Giemsa-stained thick blood films. Patients with confirmed malaria diagnosis were consecutively recruited during the operating hours (08:00–16:00) of the OPD. Patients presenting beyond that time were not regarded. Detailed recruitment procedures, clinical and laboratory examinations, and patient characteristics have been published elsewhere.¹⁶ Afebrile, unmatched community controls were randomly recruited across Mangaluru City Corporation in parallel, and they provided a finger-prick blood sample.¹⁷ Informed written consent was obtained from all adult participants and from parents or legal guardians of minors. The study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University, and permission to conduct the study was granted by the Directorate of Health and Family Welfare Services, Government of Karnataka. In brief, a medical history was obtained, and a clinical examination was performed for all patients. Parameters assessed or calculated included weight, height, body mass index, axillary temperature, fever (> 37.5°C), hemoglobin, white blood cell and thrombocyte counts, geometric mean parasite density, parasite species (polymerase chain reaction [PCR]-confirmed), and concentrations of creatinine and bilirubin.¹⁶ Severe malaria was defined based on the current WHO definition with some modifications,¹⁶ and severe thrombocytopenia as < 50,000/ μ L.¹⁸ DNA was extracted (Qiamp blood mini kit, Qiagen, Hilden, Germany), and miRNA-146a rs2910164 was genotyped by melting curve analysis using commercially available primers and probes (TIB Molbiol, Berlin, Germany). Analysis was limited to patients and controls originating from Karnataka avoiding potential geographic impact on the genetic association study. Data analysis was performed using R version 3.4.3. Genotype frequencies in patients and controls were

* Address correspondence to Welmoed van Loon, Institute of Tropical Medicine and International Health, Charité – Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, Berlin 13353, Germany. E-mail: welmoed.vanloon@gmail.com

compared by a two-tailed Fisher's exact test, and odds ratios, 95% CIs and *P*-values were produced. Clinical parameters of patients were compared between genotypes using a two-tailed Fisher's exact test, Welch's *t*-test, Wilcoxon signed-rank test, or Kruskal–Wallis test as applicable. Adjusted odds ratios (aORs) for malaria, clinical parameters, and self-reported symptoms were derived from a logistic regression model including age and gender. In addition, all analyses were stratified by *Plasmodium* spp., that is, *P. vivax* mono-infection, *Plasmodium falciparum* mono-infection, and mixed spp. infection. A *P*-value < 0.05 was considered statistically significant.

The mean age (SD) of the 449 malaria patients was 33.7 (13.0) years, and it was 35.0 (15.2) years in 666 community controls. Among the patients, only 11.8% (53/449) were female, but 47.9% (319/666) among the controls (*P* < 0.001). *Plasmodium vivax* malaria was present in 67.5% (303/449) of patients, *P. falciparum* malaria in 10.5% (47/449), and 22.0% (99/449) had a mixed *P. vivax*–*P. falciparum* infection. Overall, 58.9% (657/1115) of the study participants were carriers of the miRNA-146a SNP (46.8% heterozygous and 12.1% homozygous). Of note, the SNP distribution did not differ between patients and controls, irrespective of stratification by parasite species (Tables 1 and 2). Geometric mean parasite density (95% CI) in individuals with a wild-type genotype, and heterozygous and homozygous polymorphism was 3,422 (2,734–4,283), 4,057 (3,310–4,971), and 3,256 (2,092–5,068), respectively (*P* = 0.4). Likewise, absolute thrombocyte counts were similar in malaria patients with three genotypes (median [range]: 110,000 [6,000–273,000], 103,000 [6,000–658,000], and 113,000 [10,000, 299,000]; *P* = 0.6), but severe thrombocytopenia was least common in wild-type patients (9.6%, 17/177) and more common in heterozygous (19.1%, 36/188) and homozygous patients (21.2%, 11/52, *P* = 0.01). In multivariate analysis, severe thrombocytopenia was increased in heterozygous (aOR [95% CI], 2.3 [1.2–4.3]; *P* = 0.01) and homozygous patients (aOR [95% CI], 2.4 [1.0–5.5]; *P* = 0.04). After stratification by parasite species, this risk increase became nonsignificant. Severe malaria was present in 3.4% (15/443) of all patients. It tended to occur more frequently in heterozygous (3.5%, 7/202) and homozygous patients (7.3%, 4/55) than those with wild-type alleles (2.2%, 4/186; *P* = 0.2). No further differences with respect to clinical parameters and self-reported symptoms were observed (data not shown).

We have previously shown that carriage of the miRNA-146a rs2910164 SNP is present in 67% of a study population of pregnant women in Ghana. It is associated with 1.6-fold increased odds of malaria and with almost 6-fold increased odds in homozygous primigravidae and primiparae.⁸ In the present Indian study population, the prevalence was 59%, but the SNP was not associated with malaria. Several reasons

may explain these discrepant findings. First, all pregnant African women in the previous study had *P. falciparum* infection, whereas *P. vivax* predominated in the Indian patients who largely were adult males. The observed absence of an effect of the SNP may consequently stem from the scarcity of *P. falciparum* among the Indian patients. Considering the sample size, the present study was powered to detect increased odds of falciparum malaria, at a level of almost double the value previously observed among pregnant African women.⁸ Moreover, acquired immune mechanisms in the Indian adult patients may have blurred an effect of an innate immune mechanism provided by miRNA-146a variation. Malaria in pregnancy is caused by specific placenta-adhering strains of *P. falciparum*, which induce strain-specific immune mechanisms. These are absent or low in first pregnancies.¹³ Potential concealment of a SNP effect is consequently less likely, particularly in first-time pregnant women who can be considered relatively immune-naïve. In addition, the malaria-related effect of miRNA-146a might be pronounced in malaria in pregnancy: placental tissue exhibits a specific miRNA expression pattern, and placental miRNAs take part in gene regulation and inflammation control at the fetal–maternal interface.^{14,15} Micro-RNA-146a has been hypothesized to be involved in the reaction on cell stress in the placenta.¹⁹ In pregnancy, *P. falciparum* adheres to the placental syncytiotrophoblast, thereby evading splenic elimination.¹³ This epithelium lining the intervillous space is known to produce extracellular vesicles containing miRNAs.¹⁵ This peculiarity may partially explain the discrepant finding of the miRNA-146a SNP affecting malaria risk in pregnant women but not in predominantly adult Indian males. Furthermore, considering both, genetic diversity between Africans and Indians and the complex interaction of miRNA-146a in immune regulation,^{1,3,4} it is possible that the SNP yields different effects per se in these two populations. Last, human miRNA transcripts have been shown to impair *Plasmodium* function via gene regulation²⁰ even though the role of the miRNA-146a SNP in this context is unknown. The scarcity of data on the actual function of this polymorphism also impedes the interpretation of the weak associations of the polymorphism with thrombocytopenia (nonsignificant following Bonferroni correction for 16 comparisons) and even more so with respect to severe malaria. Conflicting findings on thrombocyte counts in miRNA-146a knockdown and knockout mice have been reported.⁵ Assuming that miRNA-146a negatively regulates inflammatory processes, a SNP-dependent alteration might result in more intense pro-inflammatory response to infection. This in turn may increase the probability of thrombocytopenia and severe malaria. However, this interpretation should be considered with care because we did not assess inflammatory mediators in the present study. Definitely, this as well as the

TABLE 1
Distribution of miRNA-146a rs2910164 in malaria patients and controls from Karnataka, India

MiRNA-146a single nucleotide polymorphism rs2910164	Total (N = 1115)	Controls (N = 666)	Patients (N = 449)	Odds ratio (95% CI)	<i>P</i> -value*	Adjusted odds ratio (95% CI)†	<i>P</i> -value‡
Wild type	458 (41.1%)	271 (40.7%)	187 (41.6%)	1	–	1	–
Heterozygote	522 (46.8%)	316 (47.4%)	206 (45.9%)	0.9 (0.7–1.2)	0.7	0.9 (0.7–1.2)	0.5
Homozygote	135 (12.1%)	79 (11.9%)	56 (12.5%)	1.0 (0.7–1.5)	0.9	1.0 (0.7–1.5)	0.9

Micro-RNA = miRNA.

* By two-tailed Fisher's exact test.

† By logistic regression model including gender and age in years.

TABLE 2

Distribution of miRNA-146a rs2910164 in malaria patients and controls from Karnataka, India, stratified by parasite species

miRNA-146a single nucleotide polymorphism rs2910164	<i>Plasmodium vivax</i> patients (N = 303)	<i>Plasmodium falciparum</i> patients (N = 47)	Mixed spp patients (N = 99)
Wild type	129 (42.6%)	21 (44.7%)	37 (37.4%)
Heterozygote	140 (46.2%)	19 (40.4%)	47 (47.5%)
Homozygote	34 (11.2%)	7 (14.9%)	15 (15.2%)

Micro-RNA = miRNA.

absence of an effect on malaria risk as seen here needs to be confirmed in larger study samples and different populations, and functional studies are needed to decipher the underlying mechanisms. Still, the present work emphasizes the role of various factors, which might influence the results of genetic association studies with respect to malaria.

Received November 10, 2019. Accepted for publication January 11, 2020.

Published online March 2, 2020.

Acknowledgments: We thank the patients, staff, and doctors and administration at the Wenlock Hospital and Kasturba Medical College, Mangalore. We also thank the control individuals from Mangaluru, staff, and field-workers of the Mangaluru City Corporation and the District Vector Borne Disease Control Programme Office.

Financial support: W. v. L. is supported by grant GRK2046 from the German Research Foundation (DFG), and P. P. G. by DFG grant GRK1673 and a stipend of the Sonnenfeld-Foundation, Berlin. The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' addresses: Welmoed van Loon, Prabhanjan P. Gai, Konrad Siebert, Jakob Wedam, and Frank P. Mockenhaupt, Institute of Tropical Medicine and International Health Berlin, Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Germany, E-mails: welmoed.vanloon@gmail.com, prabhanjangai@hotmail.com, konrad.siebert@charite.de, jakob.wedam@charite.de, and frank.mockenhaupt@charite.de. Suyamindra S. Kulkarni, Rashmi Rasalkar, and Pramod Gai, Karnataka Institute for DNA Research, Dharwad, India, E-mails: suyamindrask@gmail.com, rashmmi.ng.rasalkar@gmail.com, and pramodbgai@gmail.com. Archith Bolor, Shantaram Baliga, Animesh Jain, Chakrapani Mahabala, and Damodara Shenoy, Kasturba Medical College, Mangalore, India, and Manipal Academy of Higher Education, Manipal, India, E-mails: archith_bolor@yahoo.co.in, baliga.bs@manipal.edu, animesh_jain@manipal.edu, chakrapani.m@manipal.edu, and drshenoy2001@hotmail.com. Arun Kumar, District Vector Borne Disease Control Programme Office, Mangaluru, India, E-mail: arun.kumar@manipal.edu. Rajeshwari Devi, Wenlock Hospital, Mangaluru, India, E-mail: rajeshwaridevimangalore@gmail.com.

REFERENCES

- Mehta A, Baltimore D, 2016. MicroRNAs as regulatory elements in immune system logic. *Nat Rev Immunol* 16: 279–294.
- O'Neill LA, Sheedy FJ, McCoy CE, 2011. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat Rev Immunol* 11: 163–175.

- Taganov KD, Boldin MP, Chang KJ, Baltimore D, 2006. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 103: 12481–12486.
- Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X, 2009. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J Immunol* 183: 2150–2158.
- Labbaye C, Testa U, 2012. The emerging role of MIR-146A in the control of hematopoiesis, immune function and cancer. *J Hematol Oncol* 5: 13.
- Hao X, Xia L, Qu R, Yang X, Jiang M, Zhou B, 2018. Association between miR-146a rs2910164 polymorphism and specific cancer susceptibility: an updated meta-analysis. *Fam Cancer* 17: 459–468.
- Park R, Lee WJ, Ji JD, 2016. Association between the three functional miR-146a single-nucleotide polymorphisms, rs2910164, rs57095329, and rs2431697, and autoimmune disease susceptibility: a meta-analysis. *Autoimmunity* 49: 451–458.
- van Loon W, Gai PP, Hamann L, Bedu-Addo G, Mockenhaupt FP, 2019. MiRNA-146a polymorphism increases the odds of malaria in pregnancy. *Malar J* 18: 7.
- Li D, Wang T, Song X, Qucuo M, Yang B, Zhang J, Wang J, Ying B, Tao C, Wang L, 2011. Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population. *Hum Immunol* 72: 598–602.
- Cezar-de-Mello PF et al., 2014. Pre-miR-146a (rs2910164 G>C) single nucleotide polymorphism is genetically and functionally associated with leprosy. *PLoS Negl Trop Dis* 8: e3099.
- Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A, 2008. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. *Proc Natl Acad Sci USA* 105: 7269–7274.
- Jazdzewski K, Liyanarachchi S, Swierniak M, Pachucki J, Ringel MD, Jarzab B, de la Chapelle A, 2009. Polymorphic mature microRNAs from passenger strand of pre-miR-146a contribute to thyroid cancer. *Proc Natl Acad Sci USA* 106: 1502–1505.
- Fried M, Duffy PE, 2017. Malaria during pregnancy. *Cold Spring Harb Perspect Med* 7: a025551.
- Karnity R, Sharma S, Hanna N, 2019. MicroRNA-mediated control of inflammation and tolerance in pregnancy. *Front Immunol* 10: 718.
- Morales Prieto DM, Markert UR, 2011. MicroRNAs in pregnancy. *J Reprod Immunol* 88: 106–111.
- Gai PP et al., 2018. Manifestation of malaria in Mangaluru, southern India. *Malar J* 17: 313.
- Gai PP et al., 2019. Duffy antigen receptor for chemokines gene polymorphisms and malaria in Mangaluru, India. *Malar J* 18: 328.
- WHO, 2010. *WHO Guidelines Approved by the Guidelines Review Committee. Guidelines for the Treatment of Malaria*. Geneva, Switzerland: World Health Organization.
- Maccani MA, Avissar-Whiting M, Banister CE, McGonnigal B, Padbury JF, Marsit CJ, 2010. Maternal cigarette smoking during pregnancy is associated with downregulation of miR-16, miR-21, and miR-146a in the placenta. *Epigenetics* 5: 583–589.
- LaMonte G et al., 2012. Translocation of sickle cell erythrocyte microRNAs into *Plasmodium falciparum* inhibits parasite translation and contributes to malaria resistance. *Cell Host Microbe* 12: 187–199.