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P. O. Box 333-40105
Maseno, Kenya

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BACKGROUND

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Nasal carriage of methicillin-resistant *Staphylococcus sciuri* by residents of an urban informal settlement in Kenya

Charchil Onyango Ayodo[1,2], Robert Mugoh[2], Bernard Guya[1], Sylvia Omulo[2,3,4*]

[1]Department of Biomedical Sciences and Technology, Maseno University, Kenya.

[2]Washington State University Global Health-Kenya, Nairobi, Kenya.

[3]Paul G. Allen School for Global Health, Pullman, WA, US.

[4]University of Nairobi Institute of Tropical and Infectious Diseases, Nairobi, Kenya.

*Corresponding author

Sylvia.omulo@wsu.edu (SO)

Abstract

Background: *Staphylococcus sciuri* is an animal-associated group of bacteria but can constitute up to 4% of coagulase-negative staphylococci isolated from human clinical samples. These bacteria are reservoirs of resistance genes that are transferable to other staphylococci, including *Staphylococcus aureus*. Nevertheless, limited data exist regarding the distribution of *S. sciuri* in communities in sub-Saharan Africa despite the clinical importance of methicillin-resistant *Staphylococcus aureus* in the region.

Objectives: We characterized methicillin-resistant *S. sciuri* group bacteria isolated from nasal swabs of presumably healthy people living in an informal settlement in Nairobi to identify their resistance patterns, and carriage of *mec*A and *mecC* genes.

Method: Presumptive methicillin-resistant *S. sciuri* isolates were recovered from HardyCHROM™ methicillin-resistant *Staphylococcus aureus* (MRSA) media. Isolates were confirmed and tested for antibiotic susceptibility using the VITEK®2 Compact. DNA was extracted from positively-confirmed isolates using the ISOLATE II genomic kit and analysed for presence of *mecA* and *mecC* genes by polymerase chain reaction.

Results: Out of 37 presumptive isolates, 16 were confirmed to be *S. sciuri* (50%; 8/16), *S. lentus* (31%; 5/16) or *S. vitulinus* (19%; 3/16). All isolates were susceptible to ciprofloxacin, gentamycin, levofloxacin, moxifloxacin, nitrofurantoin and tigecycline. Resistance was observed to clindamycin and tetracycline (each 63%), erythromycin (56%), sulfamethoxazole/trimethoprim (25%), daptomycin (19%), rifampicin (13%), doxycycline, linezolid, and vancomycin (each 6%). Most isolates (88%; 14/16) were resistant to at least two antibiotic combinations, including methicillin. The *mecA* and *mecC* genes were identified in 75% and 50% of isolates, respectively.

Conclusion: Colonizing strains of *S. sciuri* group bacteria can carry resistance to methicillin and other therapeutic antibiotics. These traits can be transferred to clinically relevant staphylococci, highlighting the potential for these bacteria to facilitate antimicrobial resistance transmission in community and hospital settings. Surveillance to monitor emergent multidrug resistant strains should be considered in high transmission settings where human-animal interactions are prevalent.

Key words: *Staphylococcus sciuri*, informal settlement, antibiotic resistance, nasal colonization.

Introduction

The *Staphylococcus sciuri* group (*S. sciuri*, *S. lentus*, and *S. vitulinus*) consists of coagulase-negative staphylococci that are distinguishable from other staphylococci by a positive oxidase test [1]. These bacteria can be isolated from the environment, animals, and dairy products. People may be colonized in the nasopharynx and skin [3] following repeated contact with colonized livestock and pets [3] or through contact with food animal products [5]. Nevertheless, human infection with the *S. sciuri* group does occur; they can constitute up to 4% of coagulase-negative staphylococci isolated from clinical samples, and can cause endocarditis, peritonitis, septic shock, urinary tract infections, pelvic inflammatory disease, and wound infections [1].

Over the last decade, oxacillin/methicillin-resistant staphylococcal strains have emerged, increasing the medical relevance of the *S. sciuri* group [5]. These bacteria can transfer resistance genes to human pathogens such as *Staphylococcus aureus* [6] and can carry a *mecA* gene that is closely related to the methicillin-resistant *S. aureus* (MRSA) *mecA* gene [6]. The *mecA* gene encodes broad-spectrum beta-lactam resistance [6]. A novel *mecA* homolog – *mecC* – which also confers resistance to methicillin, has emerged in staphylococci isolated from animals, humans, and the environment [14].

We characterized *S. sciuri* group isolates from nasal swabs collected from presumably healthy people to determine their antibiotic resistance profiles and the proportion harbouring *mecA* and *mecC* genes.

Materials and Methods

During a 2019 population–based study on antimicrobial resistance in communities and hospitals in Kenya (KNH/UoN ERC# P164/03/2018), which targeted colonizing MRSA strains from presumably healthy people in Kibera—an informal settlement in Nairobi—we unexpectedly cultured *S. sciuri* group bacteria on HardyCHROM™ MRSA media (Hardy diagnostics, CA). A single small blue colony, presumptively identified as methicillin-resistant *S. sciuri* group, was collected from each positive agar plate pre-incubated at 37°C overnight (18-24 hrs). Isolates were sub-cultured on tryptic soy agar plates (KEMRI Production Department, Nairobi) and incubated overnight.

Species identification and antibiotic susceptibility testing (AST) was done using the VITEK®2 Compact (Biomerieux, Marcy-l'Étoile). For this process, bacterial suspensions were prepared by adding discreet colonies into 3 mL of 0.5% (w/v) normal saline and adjusting turbidity to 0.5 McFarlands. Isolate suspensions were tested against 15 antibiotics i.e. ciprofloxacin (≥4 mg/L), clindamycin (≥4 mg/L), daptomycin (8 mg/L), doxycycline (≥16 mg/L), erythromycin (≥8 mg/L), gentamycin (≥16 mg/L), levofloxacin (≥8 mg/L), linezolid (≥8 mg/L), moxifloxacin (≥2 mg/L), nitrofurantoin (≥128 mg/L), rifampicin (≥4 mg/L), sulfamethoxazole/ trimethoprim (≥4/76 mg/L), tetracycline (≥16 mg/L), tigecycline (2 mg/L) and vancomycin (≥32 mg/L). Minimum inhibitory concentration (MIC) values were interpreted following the 2020 Clinical Laboratory Standards Institute standards [2]. Isolates with intermediate resistance were considered susceptible. We defined multidrug resistance as resistance to three or more antibiotic classes.

DNA extraction from confirmed isolates was completed following manufacturer instructions using the ISOLATE II genomic kit (Bioline, FL), and stored at -20 ^{o}C until tested. The presence of *mecA* and *mecC* genes was determined using the VeritiPro Thermal Cycler (Thermo Fisher scientific, MA). Separate reaction mixes were prepared for *mecA* and *mecC*. Each 25 µL reaction mix consisted of 0.5 µL of each [0.2 µM] primer pair i.e., *mecA*F—5'GT AGA AAT GAC TGA ACG TCC GAT AA3', *mecA*R—5'CCA ATT CCA CAT TGT TTC GGT CTA A3' (310 bp), and *mecC*F—5'G CTC CTA ATG CTA ATG CA3', *mecC*R—5'TAA GCA ATA ATG ACT ACC3' (304 bp), respectively, 12.5 µL of 2X MyTaq™ Red Mix (Bioline, FL), 2 µL of DNA template and 9.5 µL of PCR-grade water. Thermocycling proceeded as follows: 95 ^{o}C, 1 min; 95 ^{o}C, 15 s; 51 ^{o}C, 15 s (30 cycles), 72 ^{o}C, 10 s. Amplified DNA (5 µL) was stained with 2 µL cyber-green dye and run in a 1% agarose gel alongside a 1 kB ladder. The gel was run in 5X Tri-acetate EDTA buffer (90 volts, 65mA and 6 watts) for 35 min. Two positive controls, ATCC 33591 (MRSA) and ATCC BAA 2312 (*S. aureus)* - were included to confirm *mecA* and *mecC* gene fragments. Bands corresponding to 310 bp and 304 bp under UV light confirmed the presence of *mecA* and *mecC* genes, respectively.

Results

In total, 37 presumptive methicillin–resistant *S. sciuri* group isolates were collected from HardyCHROM™ MRSA plates. Of these, 43.2% (16/37) were positively identified by the Vitek2 as methicillin-resistant *S. sciuri* (50%; 8/16), *S. lentus* (31%; 5/16) or *S. vitulinus* (19%; 3/16). Antibiotic susceptibility data confirmed that all isolates were susceptible to ciprofloxacin, gentamycin, levofloxacin, moxifloxacin, nitrofurantoin and tigecycline. More than half of all isolates were, however, resistant to clindamycin (63%), erythromycin (56%), and tetracycline (63%), with less than one-third resistant to the remaining antibiotics (Table 1).

Table 1. Antibiotic resistance among methicillin-resistant *S. sciuri* group isolates.

Antibiotic tested	*S. sciuri* (n = 8)	*S. lentus* (n = 6)	*S. vitulinus* (n = 4)	*S. sciuri* gp (n = 16)
Clindamycin	6 (75%)	4 (67%)	0 (0%)	10 (63%)
Daptomycin	1 (13%)	2 (33%)	0 (0%)	3 (19%)
Doxycycline	1 (13%)	0 (0%)	0 (0%)	1 (6%)
Erythromycin	7 (88%)	0 (0%)	2 (50%)	9 (56%)
Linezolid	1 (13%)	0 (0%)	0 (0%)	1 (6%)
Rifampicin	2 (25%)	0 (0%)	0 (0%)	2 (13%)
Sulfamethoxazole-trimethoprim	3 (38%)	1 (17%)	0 (0%)	4 (25%)
Tetracycline	6 (75%)	0 (0%)	4 (100%)	10 (63%)
Vancomycin	1 (13%)	0 (0%)	0 (0%)	1 (6%)

Most isolates (88%; 14/16) were resistant to several antibiotic combinations in addition to methicillin. Four (25%) were resistant to one antibiotic, 4 (25%) to two antibiotics, 4 (25%) to three antibiotics, 1 (6%) to five antibiotics and 1 (6%) to eight antibiotics. Clindamycin-daptomycin (CLI-DAP), clindamycin-erythromycin-tetracycline (CLI-ERY-TET) and erythromycin-tetracycline (ERY-TET) were common multidrug resistant phenotypes (Table 2).

The *mec*A gene was identified in 75% (12/16) of isolates, while *mecC* in 50% (8/16). Overall, 44% (7/16) of isolates carried the *mecA* gene only, 19% (3/16) carried *mecC* only, 31% (5/16) carried *mecA* and *mecC*, while 6% (1/16) had neither gene (Table 2).

Table 2. Carriage of *mecA* and *mecC* genes and phenotypic resistance among confirmed methicillin-resistant *S. sciuri* group isolates.

Isolate	*mecA*	*mecC*	CLI	DAP	DOX	ERY	LZD	RIF	SXT	TET	VAN	Resistance phenotype
1	Y	Y	+	-	-	+	-	-	+	-	-	CLI-ERY-SXT
2	N	Y	-	-	-	+	-	-	+	+	-	ERY-SXT-TET
3	Y	N	+	+	+	+	+	+	-	+	+	CLI-DAP-DOX-ERY-LNZ-RIF-TET-VAN
4	Y	Y	+	-	-	-	-	-	-	-	-	CLI
5	Y	Y	+	-	-	+	-	-	-	+	-	CLI-ERY-TET
6	Y	N	-	-	-	+	-	-	-	+	-	ERY-TET
7	Y	Y	+	-	-	+	-	-	-	+	-	CLI-ERY-TET
8	Y	N	-	-	-	-	-	-	-	-	-	-
9	Y	N	+	+	-	-	-	-	-	-	-	CLI-DAP
10	N	N	+	+	-	-	-	-	-	-	-	CLI-DAP
11	N	Y	+	-	-	-	-	-	-	-	-	CLI
12	Y	N	-	-	-	-	-	-	-	+	-	TET
13	Y	N	-	-	-	+	-	-	-	+	-	ERY-TET
14	N	Y	-	-	-	-	-	-	-	-	-	-
15	Y	N	-	-	-	-	-	-	-	+	-	TET
16	Y	Y	+	-	-	+	-	+	+	+	-	CLI-ERY-RIF-SXT-TET
# R	-	-	10	3	1	9	1	2	4	10	1	
% R	-	-	56	17	6	50	6	11	22	56	6	

CLI, clindamycin; DAP, daptomycin; DOX, doxycycline; ERY, erythromycin; LZD, linezolid; RIF, rifampicin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline; VAN, vancomycin. Resistant (+); Susceptible (-); Y, present; N, absent; R, Resistant.

Discussion

Focus on the *Staphylococcus sciuri* group has increased in recent years owing to its implication in opportunistic human and veterinary infections [7, 8], food contamination [9, 10], and its potential for zoonotic transmission [12]. *S. sciuri* group members are natural reservoirs of methicillin-resistant genes, which can be transferred to *Staphylococcus aureus*—an important human pathogen—and can carry virulence genes that promote pathogenicity in coagulase-negative staphylococci [8, 13].

Colonizing strains of methicillin-resistant *S. sciuri* group isolates in nasal cavities of presumptively healthy individuals in sub-Saharan Africa have not widely been reported. Consequently, little is known about the distribution of these bacteria within communities, despite their potential to transfer resistance genes to pathogenic staphylococci. Previous studies have reported low prevalence (~5%) of *S. sciuri* infections in hospitals—presumably transmitted through cross-transmission between patients and healthcare workers [11]—and in communities, presumably via close contact with animals [3]. It is likely that colonizing strains, as those found in our study, are transmitted via bioaerosols, with dust mediating the transfer of environmental bacteria when inhaled [16]. Informal settlements are commonly characterized by poor environmental hygiene, which can facilitate the thriving of *S. sciuri* within the environment. Unpublished data from the parent study indicate that 81% of sampled households keep domestic animals, the majority (76%) of which are dogs—a known reservoir of *S. sciuri*.

The susceptibility of *S. sciuri* group isolates to ciprofloxacin, gentamycin, levofloxacin, moxifloxacin, nitrofurantoin and tigecycline is consistent with a Serbian study that tested isolates from hospitalised patients and healthcare workers [11]. This suggests that these antibiotics can be used in the management of *S. sciuri* infections. Conversely, we identified an isolate that was resistant to eight antibiotics, including vancomycin—used to treat *S. sciuri* infections [16]—highlighting the potential public health threat that can arise if such strains become amplified in communities and hospitals. While the clinical significance of *S. sciuri* may be unappreciated, the capacity of these bacteria to carry multidrug resistance is well established [3] and has been reported in clinical studies in Serbia [5, 11] and Nigeria [4].

The observed distribution of *mecA* and *mecC* genes in our sample is consistent with findings from a study in Tunisia [12]. The absence of *mecA* and *mecC* genes in one methicillin-resistant isolate and presence of several resistance phenotypes suggests that *S. sciuri* group bacteria carry multiple resistance elements that mediate resistance to beta-lactams and other antibiotics [12, 15]. This can present a public health threat since *S. sciuri* can serve as reservoirs for resistant genes that can be transferred to *S. aureus* [6]; clindamycin, linezolid, and sulfamethoxazole-trimethoprim are used in the management of *S. aureus* infections [17]. Similar resistance transfers have been demonstrated *in vitro* for erythromycin [19], which is an important therapeutic agent for penicillin-allergic patients suffering from staphylococcal infections [18].

Conclusion

Given the limited scope of this study, we were unable to identify and report the presence and molecular determinants of the resistance phenotypes we identified. This study served as a first step towards characterizing colonizing strains of a potentially emerging antimicrobial resistance threat. Larger studies that address the prevalence and risk factors for colonization and infection with *S. sciuri* group are required in hospital and community settings to complement surveillance efforts.

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Conflict of interest

None declared.

Author contributions

Conceptualization: Charchil Onyango Ayodo, Sylvia Omulo, Bernard Guya

Data curation: Charchil Onyango Ayodo, Sylvia Omulo, Robert Mugoh

Formal analysis: Charchil Onyango Ayodo, Sylvia Omulo, Bernard Guya,

Investigation: Charchil Onyango Ayodo, Sylvia Omulo, Robert Mugoh

Methodology: Charchil Onyango Ayodo, Sylvia Omulo, Robert Mugoh

Project administration: Charchil Onyango Ayodo, Sylvia Omulo

Resources: Charchil Onyango Ayodo, Sylvia Omulo, Robert Mugoh

Supervision: Sylvia Omulo, Bernard Guya

Validation: Charchil Onyango Ayodo, Sylvia Omulo, Bernard Guya

Visualization: Charchil Onyango Ayodo, Sylvia Omulo, Bernard Guya

Writing – original draft: Charchil Onyango Ayodo

Writing – review and editing: Charchil Onyango Ayodo, Sylvia Omulo, Bernard Guya

References

1. Stepanovic S, Dakic I, Morrison D, Hauschild T, Jezek P, Petrás P, et al. (2005) Identification and characterization of clinical isolates of members of the Staphylococcus sciuri group. J Clin Microbiol. 2005;43(2):956-8.
2. CLSI. (2020) *Performance Standards for Antimicrobial Susceptibility Testing*. 30th ed. CLSI supplement M100. Wayne, PA: Clinical and laboratory Standards Institute; 2020.
3. Couto I, Sanches IS, Sá-Leão R, de Lencastre H. (2000) Molecular characterization of Staphylococcus sciuri strains isolated from humans. J Clin Microbiol. 2000;38(3):1136-43.
4. Shittu A, Lin J, Morrison D, Kolawole D. (2004) Isolation and molecular characterization of multiresistant Staphylococcus sciuri and Staphylococcus haemolyticus associated with skin and soft-tissue infections. J Med Microbiol. 2004;53(Pt 1):51-5.

5. Dakić I, Morrison D, Vuković D, Savić B, Shittu A, Jezek P, et al. (2005) Isolation and molecular characterization of Staphylococcus sciuri in the hospital environment. J Clin Microbiol. 2005;43(6):2782-5.
6. Severin JA, Lestari ES, Kuntaman K, Pastink M, Snijders SV, Lemmens-den Toom N, et al. (2010) Nasal carriage of methicillin-resistant and methicillin-sensitive strains of Staphylococcus sciuri in the Indonesian population. Antimicrob Agents Chemother. 2010;54(12):5413-7.
7. Meservey A, Sullivan A, Wu C, Lantos PM. (2020) Staphylococcus sciuri peritonitis in a patient on peritoneal dialysis. Zoonoses Public Health. 2020;67(1):93-5.
8. Nemeghaire S, Argudín MA, Feßler AT, Hauschild T, Schwarz S, Butaye P. (2014) The ecological importance of the Staphylococcus sciuri species group as a reservoir for resistance and virulence genes. Vet Microbiol. 2014;171(3-4):342-56.
9. Salazar-Llorente E, Morales M, Sornoza I, Mariduena-Zavala M, Gu G, Nou X, et al. (2020)Microbiological quality of high-demand foods from three major cities in Ecuador. J Food Prot. 2020.
10. Neyaz L, Karki AB, Fakhr MK. (2020) Draft Genome Sequence of Megaplasmid Bearing Staphylococcus sciuri Strain B9-58B, Isolated from Retail Pork. Microbiol Resour Announc. 2020;9(1).
11. Cirkovic I, Trajkovic J, Hauschild T, Andersen PS, Shittu A, Larsen AR. (2017) Nasal and pharyngeal carriage of methicillin-resistant Staphylococcus sciuri among hospitalised patients and healthcare workers in a Serbian university hospital. PLoS One. 2017;12(9):e0185181.
12. Dhaouadi S, Soufi L, Campanile F, Dhaouadi F, Sociale M, Lazzaro L, et al. (2020) Prevalence of meticillin-resistant and -susceptible coagulase-negative staphylococci with the first detection of the mecC gene among cows, humans and manure in Tunisia. Int J Antimicrob Agents. 2020;55(1):105826.
13. Chon JW, Lee UJ, Bensen R, West S, Paredes A, Lim J, et al. (2020) Virulence Characteristics of *mecA*-Positive Multidrug-Resistant Clinical Coagulase-Negative Staphylococci. Microorganisms. 2020 May 1;8(5):659. doi: 10.3390/microorganisms8050659. PMID: 32369929; PMCID: PMC7284987.

14. Paterson GK, Harrison EM, Holmes MA. (2014) The emergence of mecC methicillin-resistant Staphylococcus aureus. Trends Microbiol. 2014;22(1):42-7.
15. Frey Y, Rodriguez JP, Thomann A, Schwendener S, Perreten V. (2013) Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. J Dairy Sci. 2013;96(4):2247-57.
16. Lu Y, Lu Q, Cheng Y, Wen G, Luo Q, Shao H, et al. (2020) High concentration of coagulase-negative staphylococci carriage among bioaerosols of henhouses in Central China. BMC Microbiol. 2020;20(1):21.
17. Davis JS, Van Hal S, Tong SY. (2015) Combination antibiotic treatment of serious methicillin-resistant Staphylococcus aureus infections. Semin Respir Crit Care Med. 2015;36(1):3-16.
18. Gatermann SG, Koschinski T, Friedrich S. (2007) Distribution and expression of macrolide resistance genes in coagulase-negative staphylococci. Clin Microbiol Infect. 2007;13(8):777-81.
19. Spigaglia P, Barbanti F, Mastrantonio P. (2005) Horizontal transfer of erythromycin resistance from Clostridium difficile to Butyrivibrio fibrisolvens. Antimicrob Agents Chemother. 2005;49(12):5142-5.

Human *NCR3* gene variants -109 C/G and 132 C/T condition malaria outcomes in a pediatric population in western Kenya

Clinton O. Onyango[1,2*], Elly O. Munde[2,3], Samuel B. Anyona[2,4], Evans Raballah[2,5], Qiuying Cheng[6], Kristan A. Schneider[7], Christophe G. Lambert[6], Patrick Onyango[8], Douglas J. Perkins[2,6] and Collins Ouma[1,2]

Affiliations:

1. Department of Biomedical Sciences and Technology, School of Public Health and Community Development, Maseno University, Maseno, Kenya;
2. UNM-Maseno Global Health Programs Laboratories, Kisumu and Siaya, Kenya;
3. Department of Clinical Medicine, School of Health Science, Kirinyaga University, Kerugoya, Kenya;
4. Department of Medical Biochemistry, School of Medicine, Maseno University, Maseno, Kenya;
5. Department of Medical Laboratory Sciences, School of Public Health Biomedical Sciences and Technology, Masinde Muliro University of Science and Technology, Kakamega, Kenya;
6. University of New Mexico, Centre for Global Health, Internal Medicine, New Mexico, USA;
7. Department Applied Computer- and Bio-Sciences, University of Applied Sciences Mittweida, Mittweida, Germany;
8. Department of Zoology, School of Physical and Biological Sciences, Maseno University, Maseno Kenya.

Running title: *NCR3* -109 C/G and 132 C/T and malaria outcomes

Key Words: *Plasmodium falciparum*, malaria, severe malaria anemia (SMA), Natural cytotoxicity-triggering receptor 3 gene (*NCR3*), genotypes and haplotypes

ABSTRACT

Background: *Plasmodium falciparum* malaria is a major cause of pediatric morbidity and mortality in holoendemic transmission areas. Pediatric clinical outcomes vary despite exposure to similar transmission intensity with severe malaria anemia (SMA, Hb<5.0g/dL) being the most common form of severe malaria in holoendemic areas such as Siaya County in western Kenya. Genetic variations in immune response genes can alter susceptibility to SMA. As such, we examined the impact of variations on the *NCR3* gene, encoding an innate immune receptor, on malaria outcomes at enrolment and over a 36-month follow-up period.

Methods: We investigated the association between *NCR3* -109 C/G (rs2736191) and *NCR3* 132 C/T (rs11575837) variants, and susceptibility to malaria, severe malarial anemia (SMA) and all-cause mortality controlling for anemia-promoting covariates. The study monitored *P. falciparum* infections in children enrolled at Siaya County Referral Hospital (n=1,515, aged 1.9-40 months at enrollment) for up to 36 months.

Results: A binary logistic regression model revealed no association with susceptibility to both malaria and SMA at enrollment for *NCR3* (-109 C/G and 132 C/T) genotypes and haplotypes. However, a Poisson regression modeling of the risk to develop either malaria or SMA episodes over the 36-month follow-up period, revealed increased risk of susceptibility to malaria episodes for the 132 TT genotype (RR=1.540, 95% CI=1.114–2.129, *P*=0.009). Contrastingly, the additive effect (presence of 1 or 2 copies) of -109 C/132 C (CC) haplotype was protective against SMA in both Poisson and Cox regression models, (RR=0.823, 95% CI=0.711-0.952, *P*=0.009) and (HR=0.841, 95% CI=0.724-0.977, *P*=0.023) respectively. The -109 C/132 T (CT) haplotype was associated with increased risk to SMA (HR=1.660, 95% CI=1.080-2.550, *P*=0.020). The Analysis of all-cause mortality outcomes using Cox regression revealed no association between genotypes or haplotypes and malaria or SMA episodes.

Conclusion: Our results reveal that *NCR3* gene mutations influence susceptibility to malaria and SMA during the acquisition of naturally-acquired malarial immunity.

INTRODUCTION

Plasmodium falciparum, the most prevalent malaria parasite in Sub-Saharan Africa, causes 99.7% of estimated malaria cases in the region (WHO, 2019). Children under the age of 5 years remain the most vulnerable group to malaria morbidity and mortality, with reports showing a 6% increase in malaria mortality globally (WHO, 2019). Life-threatening indicators of malaria include hyper-parasitemia, hypoglycemia, hyper-lactatemia, kidney failure, metabolic acidosis, cerebral malaria (CM), severe malaria anemia (SMA), and respiratory distress (Marsh *et al.*, 1995). In a region holoendemic for *P. falciparum* malaria such as western Kenya, high density parasitemia (HDP, ≥10,000 parasites/μL) and SMA (defined as Hb<5.0g/dL) are the most common clinical manifestations (Bloland *et al.*, 1999; Ong'echa *et al.*, 2006). SMA has remained one of the major causes of mortality in children aged 3-36 months in western Kenya (Obonyo *et al.*, 2007).

The selective pressure of malaria on the human genome is epitomized by the sickle hemoglobin (HbS) allele of hemoglobin beta gene (*HBB*), which is protective against severe malaria in areas of high malaria prevalence (Flint *et al.*, 1998; Tishkoff & Williams, 2002). More human genetic variants have been shown to influence host susceptibility to malaria infection (Verra *et al.*, 2009; Weatherall, 2008), and outcomes (Adel *et al.*, 2011; Kwiatkowski, 2005; Mackinnon *et al.*, 2005). One such factor is the polymorphic major histocompatibility complex (MHC) locus in the chromosome 6p21.3 region, which has been linked to mild malaria in an endemic malaria transmission area (Flori *et al.*, 2003). However, the influence of the natural cytotoxicity-triggering receptor 3 gene (*NCR3*), located in the same region of MHC (Moretta *et al.*, 2001), on malaria outcomes in a holoendemic area remains unknown.

The *NCR3* encodes for natural cytotoxicity-triggering receptor 3 (NCR3, also known as NKp30 or CD337) that is expressed constitutively on the surface of natural killer (NK) cells (Moretta *et al.*, 2001). The NCR3 directly recognizes parasitized red blood cells (pRBCs) and activates NK cells to initiate cell cytotoxicity and release cytokines (Artavanis-Tsakonas *et al.*, 2003; Korbel *et al.*, 2004). The direct recognition of pRBCs occurs when NCR3 binds to Duffy Binding Like (DBL1-α) domain of *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1), specifically in the absence of accessory molecules such as MHC class 1 molecules (Mavoungou *et al.*, 2007). NCR3/NKp30 has been shown to play an important role in NK cell activation, degranulation and cytotoxicity (Wang, 2012). NK cell activation in turn leads to the production of cytokines: interferon-γ (IFN-γ) tumor necrosis factor (TNFα), granulocyte colony stimulating factor (G-CSF), macrophage inflammatory protein (MIP-1α/β), and regulated on activation, normal T cell expressed and secreted (RANTES) (Baratin *et al.*, 2007; Fauriat *et al.*, 2010) that influence malaria disease outcomes. Upon activation, NK cells also release granzymes and perforins for cytotoxicity of pRBCs (Artavanis-Tsakonas *et al.*, 2003).

Previous studies have demonstrated the importance of NCR3 in conferring immunity against intracellular pathogens such as influenza virus, hepatitis C virus (De Maria *et al.*, 2007), West Nile virus (Hershkovitz *et al.*, 2009), and Ebola virus (Fuller *et al.*, 2007). Moreover, *NCR3* variant (rs2736191) showed association with mild malaria and parasitemia in malaria endemic populations of Senegal (Thiam *et al.*, 2018), Congo (Baaklini *et al.*, 2017) and Burkina Faso (Delahaye *et al.*, 2007). Currently, no literature links rs2736191 or other *NCR3* variants to malaria outcomes in immune-naïve children in a holoendemic malaria-transmission area, such as in western Kenya. Several studies have identified polymorphisms in multiple immune response genes as being associated with the risk of developing SMA and functional changes in respective gene products in a holoendemic *P. falciparum* transmission area of western Kenya (Achieng *et al.*, 2019; Anyona *et al.*, 2019; Awandare *et al.*, 2009; Munde *et al.*, 2017; Okeyo *et al.*, 2013; Ong'echa *et al.*, 2011; Ouma *et al.*, 2008). As an extension, we investigated whether rs2736191 (-109 C/G) and rs11575837 (132 C/T), two human *NCR3* SNPs, are associated with susceptibility to malaria, SMA and all-cause mortality in a pediatric population.

METHODS

Study site and study participants: The study was conducted at Siaya County Referral Hospital (SCRH) in western Kenya. SCRH is located in a region of holoendemic *P. falciparum* malaria transmission (Ong'echa *et al.*, 2006). In this region, the common malaria vectors are *Anopheles gambiae s.s, A. arabiensis*, and *A. funestus* (Minakawa *et al.*, 2012), and severe malaria majorly manifests as SMA and/or HDP (Bloland *et al.*, 1999; Obonyo *et al.*, 2007). Children (n=1,515, aged 1.9-40 months) were recruited at SCRH during their first reported hospital visit for treatment and/or vaccinations. Written informed consent in the language of choice (i.e., English, Swahili or Dholuo) was obtained from the parent or legal guardian of all children participating in the study. Questionnaires were used to collect demographic and clinical information. Based on *P. falciparum* parasite density in peripheral blood and WHO’s definition of SMA, study participants were grouped into three categories: aparasitemic (n=289), non-SMA (Hb>5.0 g/dL, n=962), and SMA (Hb≤5.0 g/dL, n=264) for cross-sectional data analysis at enrollment. Children were excluded from enrollment for any of the following reasons: children with cerebral malaria (CM, a rare occurrence in this holoendemic area); clinical evidence of acute respiratory infection; and prior hospitalization. Patients were treated according to the Ministry of Health (MOH)-Kenya guidelines. This included the administration of oral artemether/lumefantrine (Coartem®) for uncomplicated malaria and intravenous quinine (and when indicated, blood transfusion) for severe malaria.

Longitudinal follow-ups: Study participants were required to report to the hospital every three months during the 36 months longitudinal follow-up period after enrollment, and during any acute infections (unscheduled visits). All laboratory tests required for proper clinical management of the patients were performed at each acute visit and quarterly visit, and all-cause mortality data collected throughout the 36 months follow-up period. The precise location of residence of each child was registered in our GIS/GPS surveillance system. Failure to report during follow-up visits occasioned a visit to the child's residence by our field staff to determine the health status of the child, and to document cases of mortality.

Laboratory investigations: Heel or finger-prick blood samples (<100 μL) were obtained and used to determine key variables such as parasitemia and Hb concentrations according to previous published methods (Ong'echa *et al.*, 2006). Complete blood counts (CBC) were assessed using the Beckman Coulter ACT diff2™ (Beckman-Coulter Corporation, Miami, FL, USA). To account for the common causes of severe anemia in the region, anemia-promoting conditions including HIV-1, bacteremia, sickle-cell trait (HbAS) status, alpha-thalassemia, and glucose-6-phosphate dehydrogenase deficiency (G6PD) were determined. Pre- and post-test HIV counseling was provided to all participants. HIV-1 exposure was determined serologically (i.e., Unigold™ and Determine™) and HIV-1 infection was determined by pro-viral DNA PCR testing according to our previous methods (Otieno *et al.*, 2006). Bacteremia was determined according to our published methods (Were *et al.*, 2011). G6PD deficiency was determined by a fluorescent spot test using the manufacturer's methods (Trinity Biotech Plc., Bray, Ireland), while presence of the sickle cell trait (HbAS) was determined by cellulose acetate electrophoresis as per manufacturer's instructions (Helena Bio-Sciences, Oxford, United Kingdom).

Selection of *NCR3* SNPs: The *NCR3* SNPs (rs2736191 and rs11575837) were selected based on the following criteria: a) proximity to *NCR3* transcription start site (TSS); b) minor allele frequencies (MAF) >5% from the 1000 genomes project; c) likelihood of corresponding SNP to potentially create or ablate transcription factor binding sites (TFBS), determined using Alggen-Promo Version 3.0.2 software (Messeguer *et al.*, 2002), and d) strong linkage disequilibrium (LD) exists for the SNPs using Multiallelic Interallelic Disequilibrium Analysis (MIDAS) software version 1.0 (Gaunt *et al.*, 2006).

Genotyping of rs2736191 and rs11575837 variants: Genomic DNA was extracted from buccal swabs using the MasterAmp™ Buccal swab DNA Extraction kit (Epicentre Biotechnologies, Madison, WI, USA) and subjected to amplification using GenomiPhi® system (GE Healthcare, 174 NJ, USA) to obtain sufficient quantities for genotyping. The *NCR3* -109 C/G and 132 C/T polymorphisms were genotyped using the TaqMan® 5' allelic discrimination Assay-By-Design high-throughput method according to the manufacturer's instructions [Assay ID: C_16286876_10 for rs2736191 and C_27834902_10 for rs11575837; Thermofisher Scientific, Carlsbad, CA, USA). PCR was performed in a total reaction volume of 10 μL with the following amplification cycles: initial denaturation (60 °C for 30s and 95 °C for 10 min) followed by 40 cycles of (95 °C for 15 s and 60 °C for 1 min) and a final extension (60 °C for 30 s) using allele-specific fluorescence on the StepOnePlus™ Real-Time PCR Systems. StepOne™ Software (Version 2.3) was used for allelic discrimination (Thermofisher Scientific, Carlsbad, CA, USA).

Data analysis: SPSS® statistical software package version 23.0 (IBM SPSS Inc., Chicago, IL, USA) was used to analyze cross-sectional data. Demographic and clinical data were compared using Pearson's Chi square (χ^2) test and Kruskal Wallis test. Differences in parasitological variables between SMA and non-SMA were computed using Mann–Whitney U test. *NCR3* (-109 C/G and 132 C/T) haplotypes were constructed using HPlus software program, Version 2.5 (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). The proportions of alleles, genotypes and haplotypes of the *NCR3* variants were compared across the study groups using Chi-Square (χ^2) and Hardy–Weinberg Equilibrium (HWE) was computed using a χ^2 goodness of fit test. For the 132 C/T SNP with the rare TT genotype (n=8), the HWE was confirmed using HWE exact test in R (version 3.6.1). The association between genotypes and haplotypes and episodes of malaria and SMA at enrollment was determined by logistic regression analysis with age, sex, co-infections (HIV-1 and bacteremia), G6PD deficiency, sickle-cell trait and alpha-thalassemia as covariates in the regression model (Aidoo *et al.*, 2002; Otieno *et al.*, 2006; Preuss *et al.*, 2012; Were *et al.*, 2011).

The association between *NCR3* genotypes/haplotypes and longitudinal clinical outcomes were analyzed in R (version 3.6.1) using a bidirectional elimination stepwise Poisson regression model selected using the Akaike Information Criterion (AIC), controlling for the covariates as mentioned above. In the log-linear models (Poisson regression), the varying length of observation was accounted for by treating the logarithm of the length of the observational window as an offset to the logarithm of the expected number of events. Log-linear regression with an additive model for the *NCR3* genotypes/haplotypes was also carried out i.e., susceptibility to longitudinal clinical outcomes was investigated in individuals stratified into 0, 1, and 2 copies of the genotypes/haplotypes to determine whether there was an additive impact of genotypic/haplotypic carriage. Survival analyses were performed by a Cox regression analysis modeling the risk of all-cause mortality that is associated with *NCR3* genotypes/haplotypes over a 36-month follow-up period. For each model, Holm-Bonferroni correction to control for familywise error rates in multiple comparison. The statistical significance for all analyses was set at $P \leq 0.05$.

RESULTS

Clinical, demographic, and laboratory characteristics of the study participants at enrollment

Clinical, demographic, and laboratory characteristics of the study participants, grouped into three categories: aparasitemic (n=289), non-SMA (n=962) and SMA (n=264) are presented in Table 1. Sex distribution was comparable among the three groups (P=0.752). Children with SMA were significantly younger than those in the aparasitemic individuals and non-SMA individuals (P=0.003). Among the three groups, children with SMA exhibited the lowest hemoglobin (g/dL) concentrations, hematocrit levels and RBC counts (P<0.001, P<0.001 and P<0.001, respectively) but the highest WBC counts (P<0.001). Non-SMA children presented a higher parasite density than SMA children (P=0.043). The distributions of confounding factors bacteremia, alpha-thalassemia and sickle-cell trait were significantly different (P=0.036, P=0.014, and P<0.001, respectively) while presence of HIV-1 and glucose-6-phosphate (G6PD) deficiency was comparable among the study groups (P=0.116 and P=0.387, respectively).

Selection of *NCR3* SNPs

NCR3 is located on chromosome 6p21.33 (GRCh38.p12; Figure 1A). The location and analysis of the MAF, TFBS and LD of the *NCR3* variants selected for this study (see Methods) are shown in Figure 1. The MAF reported in our studies were comparable with those observed for the Luhya (LWK) on the 1000 genome project (Figure 1B). The TFBS analysis identified the glucocorticoid receptor-α for the *NCR3* -109 major and minor alleles. The glial cells missing variants were documented for the *NCR3* 132 major allele, while the farnesoid X receptor and C/EBP-β, CCAAT-enhancer binding protein were substituted for in the minor allele (Figure 1B). Linkage disequilibrium analysis between the selected *NCR3* SNPs showed departure (D': 0.882, LOD: 6.61, r2 squared: 0.011; Figure 1C).

Distribution of *NCR3* -109 C/G and 132 C/T genotypes and haplotypes within the study groups

The distribution of haplotypes is shown in Table 2, alongside the frequency distribution of the *NCR3* -109 C/G and *NCR3* 132 C/T genotypes. The frequency of the *NCR3* -109 C/G genotypes in the overall population were 0.503 for CC (n=762), 0.386 for CG (n=585) and 0.111 for GG (n=168), displaying significant departure from Hardy-Weinberg Equilibrium (HWE, $\chi 2$=11.58, P<0.001). Similarly, the observed frequency of the *NCR3* 132 C/T genotypes in the overall population were 0.941 for CC (n=1426), 0.053 for CT (n=81) and 0.005 for TT (n=8), in significant departure from Hardy-Weinberg Equilibrium in the population (HWE, $\chi 2$=28.57, P<0.001). Further analysis by the HWE exact test also showed a significant departure from the HWE (P<0.001) (Table 2).

Associations between *NCR3* -109 C/G or 132 C/T genotypes and haplotypes and malaria or SMA at enrollment

A logistic regression was conducted to determine the associations between the *NCR3* variants and disease outcomes at enrollment. We used *NCR3* -109 CC or 132 CC wild-type variants as reference groups, while controlling for the confounding variables described earlier. As shown in Table 3, no significant association was found between *NCR3* -109 C/G or 132 C/T genotypes and malaria or SMA. Similarly, using logistic regression analysis, controlling for the potential confounders, we found no association between the *NCR3* haplotypes (-109 C/G or 132 C/T) and malaria or SMA episodes (Table 3).

Longitudinal effects of *NCR*3 -109 C/G and 132 C/T variants on malaria and SMA episodes

First, the relationship between NCR3 -109 C/G and 132 C/T variants and longitudinal occurrence of malaria episodes was determined. In particular, a log-linear regression model (Poisson regression) was used to determine the effect of *NCR*3 -109 C/G and 132 C/T genotype and haplotype carriage on malaria episodes during the 36-month follow-up, controlling for covariates. There were 7,228 occurrences of malaria episodes in carriers of both the *NCR*3 -109 C/G and *NCR*3 132 C/T genotypes, during the longitudinal follow-up period. Though carriage of *NCR3* 132 TT genotype increased the risk of having multiple malaria episodes (RR=1.540, 95% CI=1.114–2.129, *P*=0.009) (significant after Holm-Bonferroni correction), no other *NCR*3 -109 C/G or 132 C/T genotype demonstrated any significant risk factors for the number of malaria episodes (Table 4). On the other hand, no haplotype showed significantly different susceptibility to multiple malaria episodes (Table 4).

Since children are most vulnerable to SMA in *P. falciparum* malaria holoendemic transmission regions (Obonyo *et al.*, 2007), we also established the relationship between *NCR*3 -109 C/G and 132 C/T variants and longitudinal episodes of SMA (Table 4). Carriage of the *NCR*3 -109 CG genotype (RR=1.269, 95% CI=1.009-1.597, *P*=0.041) and GG (RR=1.378, 95% CI=0.991-1.915, *P*=0.057) genotypes increased the risk of presenting with SMA during the 36-month follow-up. However, the increased risks were not significant after Holm-Bonferroni correction for multiple tests. The additive model of the *NCR3* -109 C/G showed an increased risk (not significant after Holm-Bonferroni adjustment) to SMA during the follow-up period (RR=1.198, 95% CI=1.030-1.393, *P*=0.019). In contrast, *NCR3* 132 C/T genotypes were not risk factors for SMA over 36-months follow-up (Table 4). The GC haplotype significantly increased susceptibility to SMA (RR=1.276, 95% CI=1.030-1.581, *P*=0.026), with the additive model showing enhanced risk

(RR=1.182, 95% CI=1.018-1.372, *P*=0.029) (both not significant after Holm-Bonferroni adjustment). Although carriage of the CC haplotype reduced the risk of SMA (RR=0.850, 95% CI=0.637-0.929, *P*=0.002) (significant after Holm-Bonferroni adjustment), no other haplotype showed any effect on SMA (Table 4).

In an effort to identify host risk factors that contribute to malaria severity, and to understand host risk factors that make a malaria episode an SMA case, we modelled both age and number of previous malaria episodes as predictors of SMA, since these factors are related to the maturation of the immune system. Log-linear regression analysis revealed an exacerbated risk of SMA at a hospital visit for the -109 CG (RR=1.242, 95% CI=0.969-1.591, *P*=0.087) and GG (RR=1.493, 95% CI=1.043-2.138, *P*=0.029) genotypes, with the additive model augmenting the variant effects (RR=1.225, 95% CI=1.038-1.446, *P*=0.016) but upon Holm-Bonferroni adjustment, both *p*-values were not significant. As observed earlier, co-inheritance of the GC haplotype significantly increased the risk to cases of SMA at hospital visits (RR=1.284, 95% CI=1.019-1.617, *P*=0.034), further compounded by the additive effect of the G allele (RR=1.215, 95% CI=1.031-1.432, *P*=0.019). However, only the additive effect remained significant after Holm-Bonferroni adjustment. In contrast, the additive model of the CC haplotype protected children against SMA (RR=0.810, 95% CI=0.690-0.952, *P*=0.011) (not significant after Holm-Bonferroni adjustment). None of the other genotypes and haplotypes altered risk to SMA at hospital visits over 36-month longitudinal follow-ups (Table 4).

Longitudinal effects of *NCR*3 -109 C/G and 132 C/T variants on survival against SMA and all-cause mortality

In trying to understand the probability of an individual acquiring SMA outcomes as a function of time, while considering the force of infection from the environment, the effect from the maturation of the immune system and the impact of individual risk factors, we employed the Cox proportional hazard model to predict acquisition of SMA hazards and mortality as outcomes. The relationship between *NCR*3 -109 C/G and 132 C/T variants and acquisition of SMA over time was investigated (Table 5). Results presented here show that the association between -109 C/G and 132 C/T genotypes and SMA could not be determined. However, 109 C/G and 132 C/T additive models showed increased hazard for SMA with only 132 C/T additive model remaining significant after Holm-Bonferroni adjustment (HR=1.470, 95% CI=1.040-2.080, *P*=0.030). Similarly, genotype (CT) at *NCR3* 132 increased for SMA (HR=1.760, 95% CI1.110-2.800, *P*=0.016), as did the additive model (HR=1.470, 95% CI=1.040-2.080, *P*=0.030) (significant after Holm-Bonferroni adjustment). Analysis of the haplotypes revealed an increase in hazard risk for the CT (HR=1.660, 95% CI=1.080-2.550, *P*=0.020, Holm-Bonferroni significant) and GC (HR=1.240, 95% CI=0.998-1.550, *P*=0.051, non-significant upon Holm Bonferroni adjustment) haplotypes but a reduced hazard risk in the additive model for the CC haplotype (HR=0.841, 95% CI=0.724-0.997, *P*=0.023, Holm-Bonferroni significant). None of the other genotypes and haplotypes had a significant effect of hazard risk (Table 5).

The effect of the two *NCR*3 variants on all-cause mortality was determined by a Cox regression model. Results revealed that the *NCR*3 -109 C/G and 132 C/T genotypes and haplotypes were not significant predictors of survival against all-cause mortality in our study participants (Table 5).

DISCUSSION

Pediatrics in holoendemic areas are exposed to the same infection rates and transmission intensity of *Plasmodium falciparum* malaria yet develop different malaria outcomes. This population is especially susceptible to malaria when maternal immunity begins to abate but the adaptive immunity, strengthened by multiple infections, is not fully developed (WHO, 2019). Since immunity to malaria develops progressively in the children upon recurrent *P. falciparum* infections (Langhorne *et al.*, 2008), the dynamics of this process is best studied longitudinally in a patient (Kwiatkowski, 2005). To the best of our knowledge, we report for the first-time a study on the impact of two *NCR3* variants, rs2736191 in proximal promoter and rs11575837 in 5'-UTR (5'-untranslated region), on the longitudinal risk of clinical malaria and SMA in pediatric populations resident in western Kenya, a holoendemic *P. falciparum* malaria transmission area.

SNPs with a minor allele frequency of 0.05 (5%) or greater in the HapMap project were targeted for investigation (HapMap, 2003). The minor allele frequencies of the variants under investigation were higher in the study participants than that of the Luhya ethnic group (Webuye) from the 1000 Genome Project (Auton *et al.*, 2015), although both populations are resident in western Kenya (Figure 1). Additionally, the overall distribution of *NCR3* -109 C/G and 132 C/T genotypes in our study population displayed significant departure from Hardy-Weinberg Equilibrium, indicative of the influence of evolutionary forces on the human genome of this population. One example of such evolutionary force is malaria (Kwiatkowski, 2005; Tishkoff & Williams, 2002). There is mounting evidence that natural selection defines the frequencies of disease-associated genetic variants in a population (Gorlov *et al.*, 2015). Specifically, malaria exerts an immense selective pressure on the host genome causing risk-associated alleles to shift to high frequencies (Kwiatkowski, 2005). The HWE proportions for 132 C/T genotypes was further analyzed using HW exact test since the rare genotype (TT) could render the χ^2-test inaccurate (Engels, 2009).

The linkage disequilibrium (LD) analysis revealed the two SNPs are highly co-inherited (88% of the time). In consideration that D' values of LD are known to fluctuate upwards when rare alleles are examined (Mueller, 2004), we further determined the confidence intervals of D' values. SNP rs11575837 (132 C/T) was also included in our current study since the C to T conversion creates a potential binding site for transcription factor CCAAT-enhancer binding protein alpha (C/EBP-α), though 132T turns out to be a rare allele (3%) in the population. C/EBP-α is a basic leucine zipper-class transcription factor that drives myeloid cell differentiation from hematopoietic stem cells by interacting with other proteins, such as the ETS family transcription factor PU.1, the ATP dependent chromatin remodeling complex SWI/SNF and the DNA modifying enzyme TET2 (Kallin *et al.*, 2012; Koschmieder *et al.*, 2005; Leutz *et al.*, 2011; McNagny *et al.*, 1998). Furthermore, C/EBP-α was shown to be essential for differentiation of granulocytes and monocytes, independent of granulocyte colony-stimulating factor (G-CSF) (Porse *et al.*, 2005; Radomska *et al.*, 1998). Specifically, up-regulation of interleukin 6 receptor (IL-6R) and granulocyte colony-stimulating factor receptor (G-CSFR) by C/EBP-α is critical for granulopoiesis (Zhang *et al.*, 1998). Earlier studies from our group and others have demonstrated that IL-6, the ligand of IL-6R and an important player in IL-6 signaling pathway that is primarily produced by peripheral blood mononuclear cells (PBMC) during acute malaria in children (Aubouy *et al.*, 2002), provides protection against malaria infection (Harpaz *et al.*, 1992) but contributes to the development of SMA by suppressing erythropoiesis (Perkins *et al.*, 2011). Further investigation is thus warranted to determine if rs11575837 is involved in the modulation of *NCR3* expression by C/EBP-α and if *NCR3* mediates the effect of C/EBP-α on granulopoiesis.

In contrast to an earlier report that rs2736191 was associated with mild malaria (Delahaye *et al.*, 2007), we found no association between rs2736191 and malaria outcomes at enrollment in our study population. The inconsistency may be attributed to differences in study design and population compositions: as noted, the previous study focused on family-based approaches, whilst our current study only focused on pediatric populations aged 1.9-40 months at enrollment with a 36-months longitudinal follow-up. Moreover, the populations in the previous study presented mixed clinical phenotypes as opposed to the current study in which the most severe form of disease is SMA. In the same study (Delahaye *et al.*, 2007), rs11575837 was left out of further analysis due to its low allele frequency (<1%) in the study population. In the current study, the allele frequency was higher (3%) and was included in the data analysis. Interestingly, this SNP was associated with Sjögren's syndrome in parts of Europe (allele frequency of 2%) (Auton *et al.*, 2015; Rusakiewicz *et al.*, 2013). The observation that there is no significant association between either rs2736191 or rs11575837 and malaria outcomes cross-sectionally at enrollment, even with haplotypes composed of alleles of these SNPs underscores the fact that host genetics may not regulate susceptibility to clinical malaria in this population. However, genes may set in at play following malaria infection and dictate whether or not a child in this population becomes more susceptible to malaria disease outcomes.

Longitudinally, carriers of *NCR* 132 TT genotype had increased risk of having multiple malaria episodes. The minor allele T has been associated with lowered *NCR3* expression (Rusakiewicz *et al.*, 2013) but NCR3 activate NK cells to clear malaria infection by directly recognizing pRBCs (Baratin *et al.*, 2007; Mavoungou *et al.*, 2007; Wang, 2012). Thus, we infer that the increased malaria episodes could be as a result of this effect of the T allele on *NCR3* expression. Carriage of *NCR*3 132 C/T SNP also led to increased episodes of SMA and mortality cases, though not significantly. Even though C to T conversion up regulates IL-6R expression through transcription factor C/EBP-α, hence more binding sites for IL-6, thereby suppressing erythropoiesis, this SNP was not significantly associated with SMA longitudinally.

Pediatrics are at an increased risk of rapid disease progression to SMA once immunity acquired from the mother starts to wane (WHO, 2019). Therefore, host genetic variations contributing to malaria severity during this critical period when adaptive immunity is maturing are critical.

Carriage of -109 GG genotypes was significantly linked to increased risk of SMA while CG marginally increased the risk of SMA and at hospital visits during the follow-up period. The GC haplotype also displayed a similar effect. However, these observations are attributed to familywise error rate as the increased risk failed to remain significant after Holm-Bonferroni correction. Log-linear regression modelling for the predictors of SMA episodes longitudinally extended the observations that genotypes that increased the risk of cases of SMA at hospital visits were also associated with increased risk to the development of frequent SMA episodes during the follow up period however not significantly.

In contrast, the CC haplotype was not significantly protective against SMA at hospital visits and against developing multiple episodes of SMA longitudinally but carriage of more copies of the CC haplotype revealed significant protection against SMA in the additive model, and the protection remained significant after Holm-Bonferroni correction. The *NCR3* C allele of the rs2736191 (-109 C/G) SNP has been linked with susceptibility to mild malaria with the CC haplotype significantly associated with risk to mild malaria in previous studies (Delahaye *et al.*, 2007). The differences between the current and the previous studies may be attributed to the different study design and population composition as explained before. Functional polymorphic allele interactions either amplifies or moderates their individual effects (Ouma *et al.*, 2008). Both the T and C alleles of the rs11575837 and rs2736191, respectively, have been linked to decreased *NCR3* expression yet CT allele combination in the CT haplotype was not significantly associated with risk to malaria or SMA, thus suggesting that they may combine to moderate their individual effects. The GT haplotype was not significantly associated with any malaria outcome. We hypothesize that this observation may be due to the low numbers of individuals with the GT haplotype.

Secondary analyses using an additive model of inheritance of the variants revealed that the additive effect of the inheritance of -109 C/G and 132 C/T genotypes non-significantly increased risk to malaria and SMA episodes though -109 C/G significantly increased risk to SMA episodes longitudinally but this increased risk was not significant upon Holm-Bonferroni adjustment. The additive models also revealed that both the individual effect and the sum of the individual effects of genotypes predispose carriers to increased SMA episodes. Carriage of 1 or 2 copies of the GC haplotype also displayed a similar trend by showing an increased susceptibility to SMA episodes longitudinally. The conversion of the -109 C to G was associated with increased risk to SMA longitudinally despite the fact that -109 C to G conversion does not create any transcription factor binding site. Thus, functional analyses are warranted to link this particular SNP to any form of impaired erythroid response.

The hazard rate for SMA events of individuals with *NCR3* -109 C/G and 132 C/T genotypes was not determined since they were too few to generate any association. We infer that the low number of individuals displaying SMA events is attributed to the clinical management of the study participants underscoring the effectiveness of clinical management in curtailing the escalation of a malaria case to an SMA event. However, the additive effect of the *NCR3* -109 C/G and 132 C/T genotypes increased hazard rates with 132 C/T model being Holm-Bonferroni significant. Consistent with the log-linear regression model, the Cox regression model revealed that *NCR3* -109 C/G genotypes and GC haplotype linked to increased risk to SMA, showed a higher hazard rate for SMA episodes. Therefore, individuals with increased risk to SMA episodes had a higher rate of occurrence of SMA episodes during the follow-up period. Both models are consistent in the case of CC haplotype revealing that carriage of 1 or 2 copies of the CC haplotype increased risk to SMA episodes and increased hazard for SMA episodes. The carriage of the CT haplotype predisposed individuals to increased hazard for SMA episodes though its carriage doesn't significantly increase the risk for SMA in the log-linear regression model. The C and T allele combination (CT haplotype) showing an increased hazard for SMA may be through decreased *NCR3* expression (Delahaye *et al.*, 2007; Rusakiewicz *et al.*, 2013).

Although SMA is a major cause of mortality in children with malaria in a holoendemic area (Obonyo *et al.*, 2007), survival analysis for mortality (Cox regression), revealed -109 C/T and 132 C/T genotypes and haplotypes were not significantly associated with hazard for mortality. We postulate that the clinical interventions employed by the study i.e., provision of medicare during hospital visits for acute malaria and monitoring of patients 14 days after an acute visit lowered the risk of pediatric mortality due to malaria outcomes. This fact may be true despite the fact that variants under investigation have been significantly associated with risk to SMA, a major cause of mortality in this study area.

In conclusion, we have demonstrated for the first time that human *NCR3* gene variants rs2736191 and rs11575837 condition malaria outcomes in this pediatric population resident in western Kenya. This novel finding thus warrants further studies to define molecular mechanisms and pathways involved in SMA pathogenesis to facilitate development of better strategies for the control and clinical management of SMA in malaria patients.

COMPETING INTEREST

All authors declared no competing interests in the submitted work.

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References

Achieng, A. O., Hengartner, N. W., Raballah, E., Cheng, Q., Anyona, S. B., Lauve, N., . . . Perkins, D. J. (2019). Integrated OMICS platforms identify LAIR1 genetic variants as novel predictors of cross-sectional and longitudinal susceptibility to severe malaria and all-cause mortality in Kenyan children. *EBioMedicine, 45*, 290-302. doi:10.1016/j.ebiom.2019.06.043

Adel, D., Jacqueline, M., Nana, O., Shareen, A., Thomas, V., & Jonathan, K. (2011). Genetic Polymorphisms Linked To Susceptibility To Malaria. *Malar J, 10*(271). doi:10.1186/1475-2875-10-271

Aidoo, M., Terlouw, D., Kolczak, M., McElroy, P., Kuile, F., Kariuki, S., . . . Udhayakumar, V. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet, 359*(9314), 1311-1312.

Anyona, S. B., Hengartner, N. W., Raballah, E., Ong'echa, J. M., ,, Lauve, N., Cheng, Q., . . . Perkins, D. J. (2019). Cyclooxygenase-2 haplotypes influence the longitudinal risk of malaria and severe malarial anemia in Kenyan children from a holoendemic transmission region. . *J Hum Genet,, 65*(2), 99-113. doi:10.1038/s10038-019-0692-3

Artavanis-Tsakonas, K., Eleme, K., McQueen, K. L., Cheng, N. W., Parham, P., Davis, D. M., & Riley, E. M. (2003). Activation of a subset of human NK cells upon contact with Plasmodium falciparum-infected erythrocytes. *J Immunol, 171*(10), 5396-5405. doi:10.4049/jimmunol.171.10.5396

Aubouy, A., Deloron, P., & Migot-Nabias, F. (2002). Plasma and in vitro levels of cytokines during and after a Plasmodium falciparum malaria attack in Gabon. *Acta Trop., 83*(3), 195-203.

Auton, A., Abecasis, G. R., Altshuler, D. M., Durbin, R. M., Abecasis, G. R., Bentley, D. R., . . . National Eye Institute, N. I. H. (2015). A global reference for human genetic variation. *Nature*, *526*(7571), 68-74. doi:10.1038/nature15393

Awandare, G. A., Martinson, J. J., Were, T., Ouma, C., Davenport, G. C., Ong'echa, J. M., . . . Perkins, D. J. (2009). MIF (macrophage migration inhibitory factor) promoter polymorphisms and susceptibility to severe malarial anemia. *J Infect Dis, 200*(4), 629-637. doi:10.1086/600894

Baaklini, S., Afridi, S., Nguyen, T. N., Koukouikila-Koussounda, F., Ndounga, M., Imbert, J., . . . Rihet, P. (2017). Beyond genome-wide scan: Association of a cis-regulatory NCR3 variant with mild malaria in a population living in the Republic of Congo. *PLoS One, 12*(11), e0187818-e0187818. doi:10.1371/journal.pone.0187818

Baratin, M., Roetynck, S., Pouvelle, B., Lemmers, C., Viebig, N. K., Johansson, S., . . . Ugolini, S. (2007). Dissection of the role of PfEMP1 and ICAM-1 in the sensing of Plasmodium-falciparum-infected erythrocytes by natural killer cells. *PLoS One, 2*(2), e228. doi:10.1371/journal.pone.0000228

Bloland, P. B., Boriga, D. A., Ruebush, T. K., McCormick, J. B., Roberts, J. M., Oloo, A. J., . . . Campbell, C. C. (1999). Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II. Descriptive epidemiology of malaria infection and disease among children. *Am J Trop Med Hyg, 60*(4), 641-648.

De Maria, A., Fogli, M., Mazza, S., Basso, M., Picciotto, A., Costa, P., . . . Moretta, L. (2007). Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol, 37*(2), 445-455. doi:10.1002/eji.200635989

Delahaye, N. F., Barbier, M., Fumoux, F., & Rihet, P. (2007). Association analyses of NCR3 polymorphisms with P. falciparum mild malaria. *Microbes Infect, 9*(2), 160-166. doi:S1286-4579(06)00385-6 [pii] 10.1016/j.micinf.2006.11.002

Engels, W. R. (2009). Exact Tests for Hardy–Weinberg Proportions. *Genetics, 183*(4), 1431-1441. doi:10.1534/genetics.109.108977

Fauriat, C., Long, E. O., Ljunggren, H. G., & Bryceson, Y. T. (2010). Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood, 115*(11), 2167-2176. doi:10.1182/blood-2009-08-238469

Flint, J., Harding, R. M., Boyce, A. J., & Clegg, J. B. (1998). The population genetics of the haemoglobinopathies. *Baillieres Clin Haematol, 11*(1), 1-51.

Flori, L., Sawadogo, S., Esnault, C., Delahaye, N., Fumoux, F., & Rihet, P. (2003). Linkage of mild malaria to the major histocompatibility complex in families living in Burkina Faso. *Hum Mol Genet., 12*, 375-378.

Fuller, C. L., Ruthel, G., Warfield, K. L., Swenson, D. L., Bosio, C. M., Aman, M. J., & Bavari, S. (2007). NKp30-dependent cytolysis of filovirus-infected human dendritic cells. *Cell Microbiol, 9*(4), 962-976. doi:CMI844 [pii] 10.1111/j.1462-5822.2006.00844.x

Gaunt, T. R., Rodriguez, S., Zapata, C., & Day, I. N. M. (2006). MIDAS: software for analysis and visualisation of interallelic disequilibrium between multiallelic markers. *BMC Bioinformatics, 7*(1), 227. doi:10.1186/1471-2105-7-227

Gorlov, I. P., Gorlova, O. Y., & Amos, C. I. (2015). Allelic Spectra of Risk SNPs Are Different for Environment/Lifestyle Dependent versus Independent Diseases. *PLoS Genet, 11(7)*(e1005371).

HapMap. (2003). The International HapMap Project. *Nature, 426*, 789–796.

Harpaz, R., Edelman, R., Wasserman, S. S., Levine, M. M., Davis, J. R., & Sztein, M. B. (1992). Serum cytokine profiles in experimental human malaria. Relationship to protection and disease course after challenge. *J Clin Invest., 90*(2), 515-523. doi:10.1172/JCI115889

Hershkovitz, O., Rosental, B., Rosenberg, L. A., Navarro-Sanchez, M. E., Jivov, S., Zilka, A., . . . Porgador, A. (2009). NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. *J Immunol, 183*(4), 2610-2621. doi:jimmunol.0802806 [pii]10.4049/jimmunol.0802806

Kallin, E. M., Rodríguez-Ubreva, J., Christensen, J., Cimmino, L., Aifantis, I., Helin, K., . . . Graf, T. (2012). Tet2 facilitates the derepression of myeloid target genes during CEBPα-induced transdifferentiation of pre-B cells. *Mol Cell, 48*(2), 266-276. doi:10.1016/j.molcel.2012.08.007

Korbel, D. S., Finney, O. C., & Riley, E. M. (2004). Natural killer cells and innate immunity to protozoan pathogens. *Int J Parasitol, 34*(13-14), 1517-1528. doi:S0020-7519(04)00211-5 [pii]10.1016/j.ijpara.2004.10.006

Koschmieder, S., Rosenbauer, F., Steidl, U., Owens, B. M., & Tenen, D. G. (2005). Role of Transcription Factors C/EBPα and PU.1 in Normal Hematopoiesis and Leukemia. *Int. J. Hematol, 81*(5), 368-377. doi:10.1532/IJH97.05051

Kwiatkowski, D. P. (2005). How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Hum Genet, 77*(2), 171-192. doi:S0002-9297(07)62909-7 [pii]10.1086/432519

Langhorne, J., Ndungu, F. M., Sponaas, A. M., & Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nat Immunol, 9*(7), 725-732. doi:10.1038/ni.f.205

Leutz, A., Pless, O., Lappe, M., Dittmar, G., & Kowenz-Leutz, E. (2011). Crosstalk between phosphorylation and multi-site arginine/lysine methylation in C/EBPs. *Transcription, 2*(1), 3-8.

Mackinnon, M. J., Mwangi, T. W., Snow, R. W., Marsh, K., & Williams, T. N. (2005). Heritability of Malaria in Africa. *PLoS Med, 2*(12), e340.

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., . . . et al. (1995). Indicators of life-threatening malaria in African children. *N Engl J Med, 332*(21), 1399-1404. doi:10.1056/NEJM199505253322102

Mavoungou, E., Held, J., Mewono, L., & Kremsner, P. G. (2007). A Duffy binding-like domain is involved in the NKp30-mediated recognition of Plasmodium falciparum-parasitized erythrocytes by natural killer cells. *J Infect Dis, 195*(10), 1521-1531. doi:JID37754 [pii]10.1086/515579

McNagny, K. M., Sieweke, M. H., Doderlein, G., Graf, T., & Nerlov, C. (1998). Regulation of eosinophil-specific gene expression by a C/EBP-Ets complex and GATA-1. *EMBO J, 17*(13), 3669-3680.

Minakawa, N., Dida, G. O., Sonye, G. O., Futami, K., & Njenga, S. M. (2012). Malaria vectors in Lake Victoria and adjacent habitats in western Kenya. *PLoS One, 7*(3), e32725. doi:10.1371/journal.pone.0032725 PONE-D-11-18095 [pii]

Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., . . . Moretta, L. (2001). Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol, 19*, 197-223. doi:19/1/197 [pii] 10.1146/annurev.immunol.19.1.197

Mueller, J. C. (2004). Linkage disequilibrium for different scales and applications. *Brief Bioinform, 5*(4), 355-364.

Munde, E. O., Okeyo, W. A., Raballah, E., Anyona, S. B., Were, T., Ong'echa, J. M., . . . Ouma, C. (2017). Association between Fcγ receptor IIA, IIIA and IIIB genetic polymorphisms and susceptibility to severe malaria anemia in children in western Kenya. *BMC infectious diseases, 17*(1), 289-289. doi:10.1186/s12879-017-2390-0

Obonyo, C. O., Vulule, J., Akhwale, W. S., & Grobbee, D. E. (2007). In-hospital morbidity and mortality due to severe malarial anemia in western Kenya. *Am J Trop Med Hyg, 77*(6 Suppl), 23-28. doi:77/6_Suppl/23 [pii]

Okeyo, W. A., Munde, E. O., Okumu, W., Raballah, E., Anyona, S. B., Vulule, J. M., . . . Ouma, C. (2013). Interleukin (IL)-13 promoter polymorphisms (−7402 T/G and -4729G/A) condition susceptibility to pediatric severe malarial anemia but not circulating IL-13 levels. *BMC Immunol., 14*(15).

Ong'echa, J. M., Keller, C. C., Were, T., Ouma, C., Otieno, R. O., Landis-Lewis, Z., . . . Perkins, D. J. (2006). Parasitemia, anemia, and malarial anemia in infants and young children in a rural holoendemic *Plasmodium falciparum* transmission area. *Am J Trop Med Hyg, 74*(3), 376-385. doi:74/3/376 [pii]

Ong'echa, J. M., Raballah, E. O., Kempaiah, P. M., Anyona, S. B., Were, T., Davenport, G. C., . . . Perkins, D. J. (2011). Polymorphic variability in the 3' untranslated region (UTR) of IL12B is associated with susceptibility to severe anaemia in Kenyan children with acute Plasmodium falciparum malaria. *BMC Genet, 12,* 69. doi:10.1186/1471-2156-12-69

Otieno, R. O., Ouma, C., Ong'echa, J. M., Keller, C. C., Were, T., Waindi, E. N., . . . Perkins, D. J. (2006). Increased severe anemia in HIV-1-exposed and HIV-1-positive infants and children during acute malaria. *AIDS, 20*(2), 275-280. doi:10.1097/01.aids.0000200533.56490.b700002030-200601090-00017 [pii]

Ouma, C., Davenport, G. C., Awandare, G. A., Keller, C. C., Were, T., Otieno, M. F., . . . Perkins, D. J. (2008). Polymorphic variability in the interleukin (IL)-1beta promoter conditions susceptibility to severe malarial anemia and functional changes in IL-1beta production. *J Infect Dis, 198*(8), 1219-1226. doi:10.1086/592055

Perkins, D. J., Were, T., Davenport, G. C., Kempaiah, P., Hittner, J. B., & Ong'echa, J. M. (2011). Severe malarial anemia: innate immunity and pathogenesis. *Int J Biol Sci., 7*(9), 1427-1442. doi:10.7150/ijbs.7.1427

Porse, B. T., Bryder, D., Theilgaard-Mönch, K., Hasemann, M. S., Anderson, K., Damgaard, I., & Jacobsen, S. E. (2005). Loss of C/EBP alpha cell cycle control increases myeloid progenitor proliferation and transforms the neutrophil granulocyte lineage. *J Exp Med, 202*(1).

Preuss, J., Jortzik, E., & Becker, K. (2012). Glucose-6-phosphate metabolism in Plasmodium falciparum. . *IUBMB Life, 64*(7), 603–611.

Radomska, H. S., Huettner, C. S., Zhang, P., Cheng, T., Scadden, D. T., & D.G., T. (1998). CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. . *Mol Cell Biol., 18*(7), 4301-4314. .

Rusakiewicz, S., Nocturne, G., Lazure, T., Semeraro, M., Flament, C., Caillat-Zucman, S., . . . Mariette, X. (2013). NCR3/NKp30 Contributes to Pathogenesis in Primary Sjogren's Syndrome. *Science translational medicine, 5*, 195ra196. doi:10.1126/scitranslmed.3005727

Thiam, A., Baaklini, S., Mbengue, B., Nisar, S., Diarra, M., Marquet, S., . . . Rihet, P. (2018). NCR3 polymorphism, haematological parameters, and severe malaria in Senegalese patients. *PeerJ, 6*, e6048-e6048. doi:10.7717/peerj.6048

Tishkoff, S. A., & Williams, S. M. (2002). Genetic analysis of African populations: human evolution and complex disease. *Nat Rev Genet, 3*(8), 611-621. doi:10.1038/nrg865nrg865 [pii]

Verra, F., Mangano, V. D., & Modiano, D. (2009). Genetics of susceptibility to Plasmodium falciparum: from classical malaria resistance genes towards genome-wide association studies. *Parasite Immunol, 31*(5), 234-253. doi:PIM1106 [pii]10.1111/j.1365-3024.2009.01106.x

Wang, H., Zheng, X, Wei, H, Tian, Z, & Sun, R. (2012). . (2012). Important role for NKp30 in synapse formation and activation of NK cells. . *Immunol Invest, 41*(4), 367-381.

Weatherall, D. J. (2008). Genetic variation and susceptibility to infection: the red cell and malaria. *Br J Haematol, 141*(3), 276-286. doi:BJH7085 [pii]10.1111/j.1365-2141.2008.07085.x

Were, T., Davenport, G. C., Hittner, J. B., Ouma, C., Vulule, J. M., Ong'echa, J. M., & Perkins, D. J. (2011). Bacteremia in Kenyan children presenting with malaria. *J Clin Microbiol, 49*(2), 671–676.

WHO. (2019). World malaria report. *WHO Report*.

Zhang, P., Iwama, A., Datta, M., Darlington, G., Link, D., & Tenen, D. (1998). Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis. *J Exp Med, 188*(6), 1173-1184.

TABLES

Table 1: Clinical, demographic and laboratory characteristics of study participants

Characteristics	Total	Aparasitemic (MPS Negative)	non-SMA (Hb>5.0 g/dL)	SMA (Hb≤5.0 g/dL)	*P*
No. of participants	**1,515**	**289**	**962**	**264**	
Sex [n, (%)]					
Male	760	145 (50.2)	488 (50.7)	137 (51.9)	0.752[a]
Female	755	144 (49.8)	474 (49.3)	127 (48.1)	
Age, months	1,515	11.0 (13.4)	12.7 (10.4)	10.1 (10.7)	**0.003[b]**
Axillary temperature, °C	1,503	37.0 (1.5)	38.0 (1.5)	38.0 (1.1)	**<0.001[b]**
Hematological parameters					
Hemoglobin, g/dL	1,508	10.3 (2.8)	7.7 (2.8)	4.3 (1.1)	**<0.001[b]**
Hematocrit, %	1,507	32.9 (8.2)	25.2 (9.1)	14.1 (3.8)	**<0.001[b]**
Red Blood Cells, × 10^6/μL	1,504	4.7 (1.1)	3.8 (1.4)	1.9 (0.6)	**<0.001[b]**
White Blood Cells, × 10^3/μL	1,503	11.0 (7.3)	11.7 (6.5)	14.9 (9.8)	**<0.001[b]**
Parasitological Indices					
Parasite density, MPS/μL	1,226	0.0 (0.0)	28,595 (79,256)	24,831 (66,014)	**0.043[c]**
Co-infections					
HIV-1, Negative [n, (%)]	1,457	275 (95.8)	933 (97.2)	249 (94.7)	0.116 [a]
Positive [n, (%)]	53	12 (4.2)	27 (2.8)	14 (5.3)	
Bacteremia, Negative [n, (%)]	1,402	257 (89.9)	902 (94.2)	243 (92.0)	**0.036 [a]**
Positive [n, (%)]	106	29 (10.1)	56 (5.8)	21 (8.0)	
Genetics Variants					
α^+-Thalassemia, αα/αα [n, (%)]	554	105 (42.9)	355 (42.4)	94 (39.7)	
-α/αα [n, (%)]	505	75 (30.6)	329 (39.3)	101 (42.6)	**0.014[a]**
-α/-α [n, (%)]	261	65 (26.5)	154 (18.4)	42 (17.7)	
G6PD, Normal, [n, (%)]	1,078	194 (75.8)	678 (75.8)	206 (80.5)	
Intermediate, [n, (%)]	266	51 (19.9)	178 (19.9)	37 (14.5)	0.387[a]
Deficiency, [n, (%)]	63	11 (4.3)	39 (4.4)	13 (5.1)	
Sickle cell trait, Hb AA [n, (%)]	1,241	223 (79.1)	785 (82.5)	233 (90.7)	
Hb AS [n, (%)]	239	55 (19.5)	164 (17.2)	20 (7.8)	**<0.001[a]**
Hb SS [n, (%)]	10	4 (1.4)	2 (0.2)	4 (1.6)	

Data are presented as median (interquartile range, IQR) and [n (%)] unless stated otherwise. Children (n=1,515) were categorized into aparasitemic controls (n=289; no parasitemia), and either non-SMA (n=962; Hb>5.0 g/dL) or SMA (n=264; Hb≤5.0 g/dL). Non-SMA and SMA patients are collectively categorized as parasitemic (n=1,226). Abbreviations: MPS- malaria parasites; HIV, human immunodeficiency virus; G6PD, glucose-6-phosphate dehydrogenase deficiency;

[a] Statistical significance was determined by Chi-square analysis.

[b] Statistical significance determined by Kruskal-Wallis test.

[c] Statistical significance determined by Mann-Whitney U test.

***P*-values ≤0.05 were considered significant, and are indicated in bold.**

Table 2: Distribution of *NCR3* -109 C/G and *NCR3* 132 C/T genotypes and haplotypes

Variants	Total	Aparasitemic (MPS Negative)	Non-SMA (Hb>5.0g/dL)	SMA (Hb≤5.0g/dL)	*P*
No. of participants	**1,515**	**289**	**962**	**264**	
***NCR3* -109 C/G**					
CC, n (%)	762 (50.3)	153 (52.9)	490 (50.9)	119 (45.1)	
CG, n (%)	585 (38.6)	101 (34.9)	372 (38.7)	112 (42.4)	0.280
GG, n (%)	168 (11.1)	35 (12.1)	100 (10.4)	33 (12.5)	
HWE (X^2, *P*)	11.58, **<0.001**	7.51, **<0.001**	5.34, **0.020**	0.681, 0.409	
***NCR3* 132 C/T**					
CC, n (%)	1,426 (94.1)	272 (94.1)	908 (94.4)	246 (93.2)	
CT, n (%)	81 (5.3)	17 (5.9)	46 (4.8)	18 (6.8)	0.169
TT, n (%)	8 (0.5)	0 (0.0)	8 (0.8)	0 (0.0)	
HWE ($\chi 2$, *P*)	28.57, **<0.001**	0.27, 0.606	52.39, **<0.001**	0.33, 0.566	
Haplotypes					
Non-CC, n (%)	196 (12.9)	38 (13.1)	121 (12.6)	37 (14.0)	0.821
CC, n (%)	1,319 (87.1)	251 (86.9)	841 (87.4)	227 (86.0)	
Non-CT, n (%)	1,426 (94.1)	272 (94.1)	908 (94.4)	246 (93.2)	0.762
CT, n (%)	89 (5.9)	17 (5.9)	54 (5.6)	18 (6.8)	
Non-GC, n (%)	764 (50.4)	153 (52.9)	492 (51.1)	119 (45.1)	0.139
GC, n (%)	751 (49.6)	136 (47.1)	470 (48.9)	145 (54.9)	
Non-GT, n (%)	1,513 (99.9)	289 (100.0)	960 (99.8)	264 (100.0)	0.562
GT, n (%)	2 (0.1)	0 (0.0)	2 (0.2)	0 (0.0)	

Data are presented as proportions [n, (%)] unless otherwise stated for *NCR3* variants −109 C/G and 132 C/T. Children were categorized into aparasitemic (n=289), non-SMA (Hb>5.0g/dL, n=962) and SMA (Hb≤5.0g/dL, n=264). Non-SMA and SMA patients are collectively categorized as parasitemic (n=1,226). Statistical significance was determined by Chi-square analysis across groups. *P*-values ≤0.05 were considered significant, and are indicated in bold. HWE, Hardy-Weinberg Equilibrium. $\chi 2$ - Hardy-Weinberg Equilibrium Chi-square.

Table 3: Association of *NCR3* -109 C/G and 132 C/T genotypes and haplotypes with malaria and SMA at enrollment

Variants	Malaria				SMA			
	n	**OR**	**95% CI**	***P***	**n**	**OR**	**95% CI**	***P***
***NCR3* -109 C/G**								
CC	603	Ref			488	Ref		
CG	478	1.242	0.898-1.717	0.190	404	1.211	0.875-1.675	0.248
GG	141	0.943	0.592-1.504	0.806	113	1.401	0.865-2.270	0.170
***NCR3* 132 C/T**								
CC	1,154	Ref			949	Ref		
CT	62	0.857	0.443-1.659	0.648	50	1.775	0.943-3.341	0.075
TT	6	x	x	x	6	x	x	x
Haplotypes								
Non-CC	162	Ref			131	Ref		
CC	1,060	1.120	0.730-1.716	0.604	874	0.833	0.540-1.287	0.411
Non-CT	1,154	Ref			949	Ref		
CT	68	0.973	0.507-1.868	0.934	56	1.508	0.813-2.797	0.192
Non-GC	605	Ref			490	Ref		
GC	617	1.150	0.854-1.548	0.356	515	1.263	0.932-1.711	0.132
Non-GT	1,220	Ref			1003	Ref		
GT	2	x	x	x	2	x	x	x

Data are presented as odds ratio (OR) with 95% confidence intervals (CI). Logistic regression analysis was used to determine the odds ratio (OR) and 95% Confidence Interval (CI), controlling for the confounding effects of age, sex, G6PD deficiency, sickle-cell trait (HbAS), HIV-1, alpha-thalassemia and bacteremia in the model to determine susceptibility to malaria and SMA. X shows the sample size was too small to generate any meaningful association. The homozygous wild-type genotypes and the absence of the haplotype were used as the reference group for the logistic regression analyses. Statistical significance was set at *P*≤0.05 and was indicated in bold.

Table 4: Log-linear regression analysis of malaria episodes, SMA episodes and the presence of SMA at a visit based on genotypic and haplotypic models for *NCR3* -109 C/G and *NCR3* 132 C/T polymorphisms

Genotypes		**Malaria episodes**				**SMA episodes**					**Presence of SMA at a visit**		
		n	**RR**	**95% CI**	***P***	**n**	**RR**	**95% CI**	***P***		**RR**	**95% CI**	***P***
NCR3	CC	3,575	Ref			184	Ref			184	Ref		
-109	CG	2,832	1.011	0.957-1.067	0.700	170	1.269	1.009-1.597	**0.041**	170	1.242	0.969-1.591	0.087
C/G	GG	821	1.014	0.936-1.100	0.731	52	1.378	0.991-1.915	**0.057**	52	1.493	1.043-2.138	**0.029**
	Additive	7,228	1.010	0.974-1.048	0.590	406	1.198	1.030-1.393	**0.019**	406	1.225	1.038-1.446	**0.016**
NCR3	CC	6,809	Ref			379	Ref			379	Ref		
132	CT	370	0.957	0.853-1.074	0.454	24	1.294	0.828-2.020	0.258	24	1.289	0.793-2.097	0.306
C/T	TT	49	1.540	1.114-2.129	**0.009***	3	1.911	0.609-6.000	0.267	3	1.226	0.330-4.555	0.761
	Additive	7,228	1.031	0.853-1.074	0.454	406	1.330	0.929-1.904	0.119	406	1.219	0.824-1.804	0.321
Haplotypes													
	Non-CC	978	Ref			61	Ref			61	Ref		
CC	CC	2,971	0.976	0.908-1.048	0.503	181	0.850	0.637-1.135	0.271	181	0.799	0.583-1.095	0.163
	Additive	6,250	0.990	0.955-1.026	0.571	345	0.823	0.711-0.952	**0.009***	345	0.810	0.690-0.952	**0.011***
	Non-CT	6,809	Ref			379	Ref			379	Ref		
CT	CT	385	0.995	0.893-1.108	0.920	25	1.271	0.839-1.925	0.257	25	1.190	0.755-1.875	0.454
	Additive	419	1.015	0.918-1.123	0.769	27	1.295	0.886-1.894	0.182	27	1.190	0.790-1.792	0.407
	Non-GC	3,590	Ref			185	Ref			185	Ref		
GC	GC	2,817	1.009	0.960-1.061	0.726	169	1.276	1.030-1.581	**0.026**	169	1.284	1.019-1.617	**0.034**
	Additive	3,638	1.007	0.971-1.044	0.709	221	1.182	1.018-1.372	**0.029**	221	1.215	1.031-1.432	**0.019***
	Non-GT	7,213	Ref			405	Ref			405	Ref		
GT	GT	15	1.574	0.947-2.619	0.080	1	1.196	0.166-8.627	0.859	1	0.667	0.068-6.543	0.728
	Additive	15	1.574	0.947-2.619	0.080	1	1.196	0.166-8.627	0.859	1	0.667	0.068-6.543	0.728

Data are presented as relative risk (RR) and odds ratio (RR) with 95% confidence intervals (CI) using log-linear regression and logistic regression respectively with the following covariates in the models: age at enrollment, sex, HIV-1, bacteremia, sickle cell trait, alpha thalassemia, and G6PD deficiency to determine susceptibility to malaria and SMA. Homozygous wild-type genotypes and non-carriers of haplotypes were used as references in the analyses. *P* value of ≤ 0.050 is indicated in bold. *Significant after Bonferroni correction for multiple comparisons.

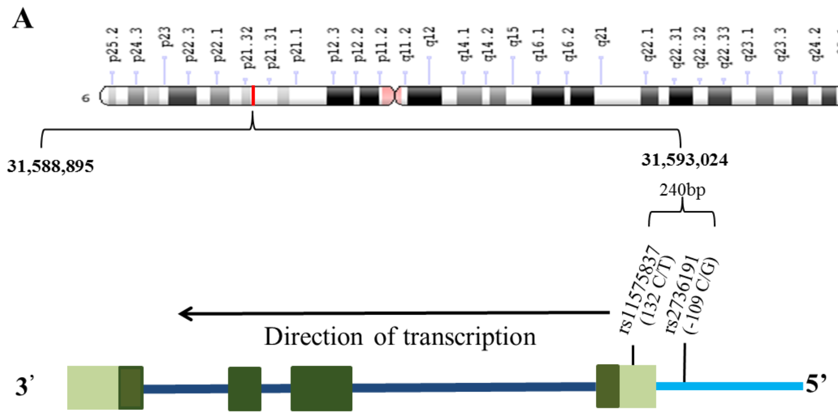
Table 5: Survival analysis for SMA episodes and all-cause mortality

Genotypes		SMA episodes				All-cause mortality			
		n	**HR**	**95% CI**	***P***	**n**	**HR**	**95% CI**	***P***
***NCR3* -109 C/G**	CC	184	Ref			46	Ref		
	CG	170	ND	ND	ND	35	1.480	0.865-2.520	0.153
	GG	52	ND	ND	ND	7	0.934	0.354-2.460	0.890
	Additive	406	1.170	1.000- 1.360	**0.049**	88	1.140	0.783-1.650	0.502
***NCR3* 132 CT**	CC	379	Ref			83	Ref		
	CT	24	ND	ND	ND	3	1.130	0.348-3.650	0.842
	TT	3	ND	ND	ND	2	2.860	0.371-22.10	0.313
	Additive	406	1.470	1.040- 2.080	**0.030***	88	1.430	0.630-3.260	0.390
Haplotypes									
CC	Non-CC	61	Ref			9	Ref		
	CC	181	0.821	0.615-1.100	0.182	37	1.410	0.604-3.280	0.428
	Additive	345	0.841	0.724-0.977	**0.023***	79	0.871	0.608-1.250	0.452
CT	Non-CT	379	Ref			83	Ref		
	CT	25	1.660	1.080-2.550	**0.020***	4	1.310	0.472-3.630	0.605
	Additive	27	1.370	0.961-1.960	0.081	5	1.240	0.467-3.310	0.664
GC	Non-GC	185	Ref			47	Ref		
	GC	169	1.240	0.998-1.550	**0.051**	34	1.300	0.778-2.170	0.318
	Additive	221	1.130	0.973-1.320	0.110	41	1.090	0.753-1.590	0.641
GT	Non-GT	405	Ref			88	Ref		
	GT	1	2.620	0.365-18.80	0.338	1	5.430	0.688-42.80	0.108
	Additive	1	2.620	0.365-18.80	0.338	1	5.430	0.688-42.80	0.108

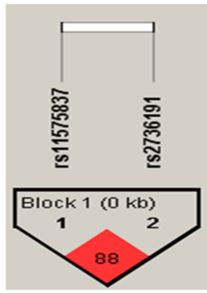
Data are presented as hazard ratios (HR) with 95% confidence intervals (CI). Analysis was done using Cox regression model with covariates: age at enrollment, sex, HIV-1, bacteremia, sickle cell trait, alpha thalassemia, and G6PD deficiency in the model. Homozygous wild-type genotypes and non-carriers of haplotypes were used as references in the analyses. Statistical significance was set at *P*≤0.050 and was indicated in bold. ND-not determined. *Significant after Bonferroni correction for multiple comparisons.

FIGURES

A



C



D': 0.882
D' CI:0.650-0.960
LOD:6.61
R^2 squared: 0.011

- UTRs
Promoter
Introns

B

Gene variants	Minor Allele Frequency		HWE *P*-value			TFBS	
	dbSNP LWK	Study Population	Aparasitemic	Non-SMA	SMA	Major allele	Minor allele
-109 C/G	0.23	0.30	<0.001	0.020	0.409	GR-α	GR-α
132 C/T	0.01	0.03	0.606	<0.001	0.566	GCM GCMα GCMβ	C/EBPα FXR

Fig. 1 Chromosome location of *NCR3* and linkage disequilibrium of SNPs under investigation. (A) Human natural cytotoxicity-triggering receptor 3 gene (*NCR3*) is located on chromosome 6p21.33 (GRCh38.p12). The SNPs under investigation were rs2736191 (-109 C/G) and rs11575837 (132 C/T), respectively. rs2736191 is located in the proximal promoter region, while rs11575837 is a 5' UTR variant and located in exon 1. (B) *NCR3* minor allele frequencies (MAF) for the Luhya (LWK) and Luo populations. Transcription factor binding analyses of the *NCR3* variants. GR-α, glucocorticoid receptor-α, C/EBP-β, CCAAT-enhancer binding protein, GCM, glial cells missing and FXR, farnesoid X receptor. (C) Linkage disequilibrium between the selected *NCR3* SNPs (D': 0.882, LOD: 6.61, r2 squared: 0.011).

Evaluating the Potential of Differentiation-related gene-1 as a Biomarker for Metastasis of Estrogen Receptor-positive Breast Cancer

Bor H.[1*], Nyambega B.[2], Patel K.T[3], Olwal C.O[1], Nalyanya W.[4]

1. Department of Zoology, Maseno University, P.O. Box 333-40105, Maseno, Kenya
2. Department of Medical Biochemistry, Maseno University, P.O. Box 333-40105, Maseno, Kenya
3. Department of Immunology, Moi University, P.O. Box 4606-30100, Eldoret, Kenya
4. Department of Pathology, Moi University, P.O. Box 4606-30100, Eldoret, Kenya

Abstract

Background: Breast cancer is a major public health burden worldwide. The majority of breast cancers express estrogen receptors (ER) suggesting a high dependence on estrogen hormone. While age is a major predictor of breast cancer development, the age distribution of ER-positive breast cancer in western Kenya has not been studied. Differentiation-related gene-1 (DRG1) is a metastasis suppressor gene hence, is a potential biomarker for predicting the level of metastasis. However, its potential application in assessing the extent of metastasis in ER-positive breast cancer has not been explored. Hence, this study investigated the age distribution, and the potential of DRG1 expression in assessing metastasis in ER-positive breast cancer.

Methods: Breast cancer tumour blocks archived in safe cabins in the histology laboratory section, Moi Teaching and Referral Hospital, Eldoret, Kenya were used. Clinico-pathological parameters associated with metastatic cancer, such as histology grade and tumor size were obtained from clinico-pathological reports and/or histological analysis of the tumour blocks. Whereas, expression of DRG1 and Ki-67 proteins were determined using immunohistochemistry.

Results: ER-positive breast cancer was predominant among women aged 40 years. No association was observed between immunohistochemical expression of DRG1 and parameters such as histology grade, tumor size or expression of Ki-67 protein expressed DRG1 ($p > 0.05$).

Conclusions: The findings suggest that the expression of DRG1 protein is not associated with parameters that indicate breast cancer metastasis. Thus, DRG1 expression is not a potential biomarker candidate for ER-positive breast cancer metastasis. However, since the study sample

size used was small, further research using a larger prospective study is necessary to confirm the present findings.

Keywords: Breast cancer, Differentiation related gene-1, Estrogen receptor, Immunohistochemical expression, Biomarker, Metastasis, Age

Introduction

Globally, breast cancer is the commonest malignancy and the leading cause of cancer-related deaths for women (1). Developing countries are majorly affected and continue to report increased breast cancer incidences worldwide (2). Annually, breast cancer accounts for 2553 deaths in Kenya (3) with the majority of the deaths being attributed to late detection (3).

Two-thirds of breast cancers express estrogen receptor (ER) α and/or progesterone receptor, which are known to stimulate breast cancer growth (4). Over 52.5% of hormone receptor breast cancers express ER, which typically indicates a high degree of estrogen dependence for growth and survival (4). About 50% of patients with metastatic ER-positive breast cancer are non-response to first-line endocrine treatment due to primary, *de novo* resistance (5) or secondary acquired resistance (6) making ER-positive breast cancer a major concern. Nevertheless, early cancer detection remains a major challenge in many developing countries, including Kenya (2). Thus, more studies are required to better predict the breast cancer metastasis level, which is an essential determinant of prognosis.

One of the critical factors that determine the development of breast cancer is age (7,8). It has been shown that breast cancer incidences and death-related cases increase with age. Among African women, breast cancer incidence peaks approximately 10–15 years earlier than peak incidence for western countries outside of the western Africa region (8). In addition to age, racial origin also seems to play a role in the incidence of breast cancer. The disease is more aggressive among Chinese women aged 40 to 50 years (7). In addition, African-American women are more predisposed to breast cancer than Caucasians as from 18 years old (9). Kenya has the highest risk of breast cancer among African countries (10). The majority of breast cancers in western Kenya are ER-positive (11). Despite age being an important factor in breast cancer development and management, age distribution of ER breast cancer in western Kenya is largely undocumented. Understanding the age distribution of ER breast cancer could inform the type of therapeutic approaches to be used for breast management. This could lead to targeted procurement of equipment and therapeutic materials for the most affected age group leading to reduced mortality.

Breast cancer-related mortality is majorly linked to metastasis (12). *Differentiation-related gene-1* (*DRG1*) is a metastasis suppressor gene, which controls the metastasis spread without affecting the growth of the primary tumor (13). It is a metastasis suppressor in breast cancer that affects the step of invasion through the extracellular matrix. Studies have reported DRG1 as a potentially good biomarker for determining the level of metastasis in *in vitro* cell lines (13,14). In most countries, only clinico-pathological characteristics are used to assess the level of breast cancer metastasis. However, these features are not detailed in evaluating and predicting metastasis of breast cancer. DRG1 protein is a potentially more accurate biomarker for predicting the extent of metastasis. This is so because the protein will be expressed right away more in the cytoplasm when metastasis begins. However, the potential role of immunohistochemical expression of DRG1 protein in determining metastasis level of ER breast cancer is largely unexplored. Parameters, such as histology grade, lymph node metastasis, tumor size, expression of proliferation markers, Ki-67, or survival rates, are predictors of metastases that may be used to predict metastatic cancer, including breast cancer (13,15).

Therefore, the present study sought to better understand the age distribution of ER breast cancer, and the association between immunohistochemical expression of DRG1 and the predictors of metastasis among ER-positive breast cancer women. This information will reduce the cost of and improve the accuracy of predicting ER breast cancer metastasis levels in resource strain facilities.

Materials and methods

Biological samples and medical data

This retrospective study targeted archived breast cancer tumour blocks from female patients at Moi Teaching and Referral Hospital (MTRH), a primary academic hospital in western Kenya region (11). Breast tissue samples had been collected previously during surgery or biopsy and were fixed and stored at the hospital as tumour blocks using standard procedures. Tissue blocks of normal cerebellum and normal breast tissue were used as controls for DRG 1 ER and Ki67 respectively. Inclusion criteria for breast tumour blocks were as follows: tissues obtained from women who are 18-55 years, ER-positive, HIV negative, had no history of any other types of tumours, and had not undergone chemotherapy, radiotherapy, or any other cancer-related treatments. HIV status and clinico-pathological data, namely tumour size, survival rates, and age, were obtained from the clinical records and pathology reports. The record officers and pathology department helped in providing the medical records and identifying tumour blocks, respectively.

Ethical consideration

Ethical clearance to conduct this study was obtained from the Institutional Research and Ethics Committee (IREC) of Moi University and MTRH, Eldoret, Kenya (Approval number: IREC 1203). Further, permission to conduct the study was also granted by the MTRH management. The study was exempted from the need for consent since it used archived samples, and was not in direct contact with the patients. All the tumor blocks were coded to conceal the identity of the source patients. Only the investigators accessed patients' medical files.

Sample processing

Upon identification of the tissue blocks meeting the inclusion criteria, the breast tumor blocks were retrieved from the safe cabins in the histology laboratory and placed on ice to cool. Then, tumor blocks were cut into 5 µm sections using a rotary microtome (Lerts Leica, W. Nuhsbaum, Inc., McHenry, Illinois). The sections were put to float on distilled water at 25°C for easy selection of suitable sections. Each section was then transferred to a glass slide and allowed to dry overnight at 25°C for histological grading and immunohistochemistry.

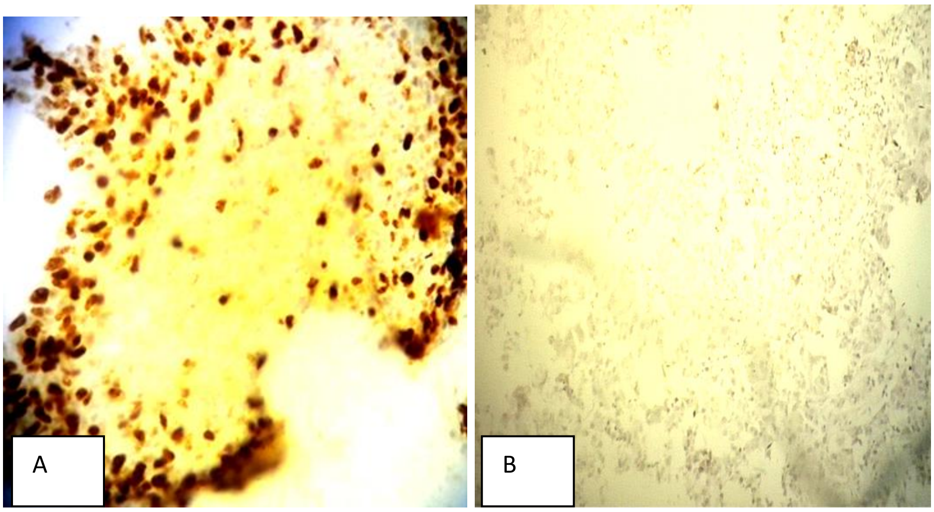
Histological grading

Slides processed in the preceding section were deparaffinized in xylene (Agilent Technologies Inc., Glostrup, Denmark) for x? minutes and transferred into three baths of ascending grades of ethanol ((80, 95, and 100%); Agilent Technologies Inc) for 3 minutes each. The slides were rinsed in tap water followed by hematoxylin (Merck KGart, Darmstadt, Germany) application for 5 minutes. The slides were thereafter washed in tap water. Eosin (Loba Chemical DVT. Ltd, Mumbai, India) was then applied for 1 minute followed by another rinse. Slides were then sequentially rinsed with 100% ethanol and xylene (Agilent Technologies Inc). Coverslips were placed, permount applied, and dried overnight then viewed using Olympus BH-2 microscope (Olympus Inc., Tokyo, Japan) at ×400 to assess the grade of the tumor. The slides were independently viewed and evaluated by two pathologists with specialty-level training. Histological grading was based on the degree of tubule or gland formation, nuclear pleomorphism, and mitotic count as previously described (16). The images of tissues stained for histological analysis are shown (Figure 1).

Immunohistochemistry and immunoscoring

Immunohistochemistry was performed as previously described (15) with some modifications to the scoring rate. Briefly, the slide with a 5 µm section was deparaffinized and hydrated. The sections were treated for 5 min with 100% methanol containing 3% hydrogen peroxide and incubated at 25°C for 10 minutes to block endogenous peroxidase activity. Non-specific binding was blocked by incubation in 1% normal swine serum (Dako) in phosphate-buffered saline. Following manufacturer's instructions, tissue sections were incubated with anti-ER (Cat no: GA084, Dako), anti-DRG1 (Cat no: HPA006881, Q92597, Sigma-Aldrich Inc., St. Louis, Missouri, USA,) or anti-Ki-67 (MIB-1, Dako) antibody (1:100 dilution) at 25°C for 1 hour, followed by incubation for 30 min at 25°C with horseradish peroxidase (HRP)-conjugated secondary antibody (Agilent technologies Inc) following manufacturers' instructions. Immunostaining was performed using a DAB substrate (Dako), and counterstaining was performed with haematoxylin. Positive control and negative control specimens were included for each antibody set as a quality control measure. Color of antibody ER, DRG1, and Ki-67 were viewed and evaluated independently by two pathologists with specialty level training using Olympus BH-2 microscope (Olympus Inc) at ×400.

Immunoscoring was based on stain intensity and was used to categorize the expression of DRG1, ER and Ki-67 in relation to their controls. The scores for DRG1 were 0 (negative), 1+, 2+ and 3+ as described previously (17). For the ER receptor and Ki-67, scoring was defined as negative (< 10%) or positive (≥ 10%) based on the percentage of stained cells based on criteria described by Koboyashi et al. (15). Images of tissues stained for DRG1 and ER (Figure 1).



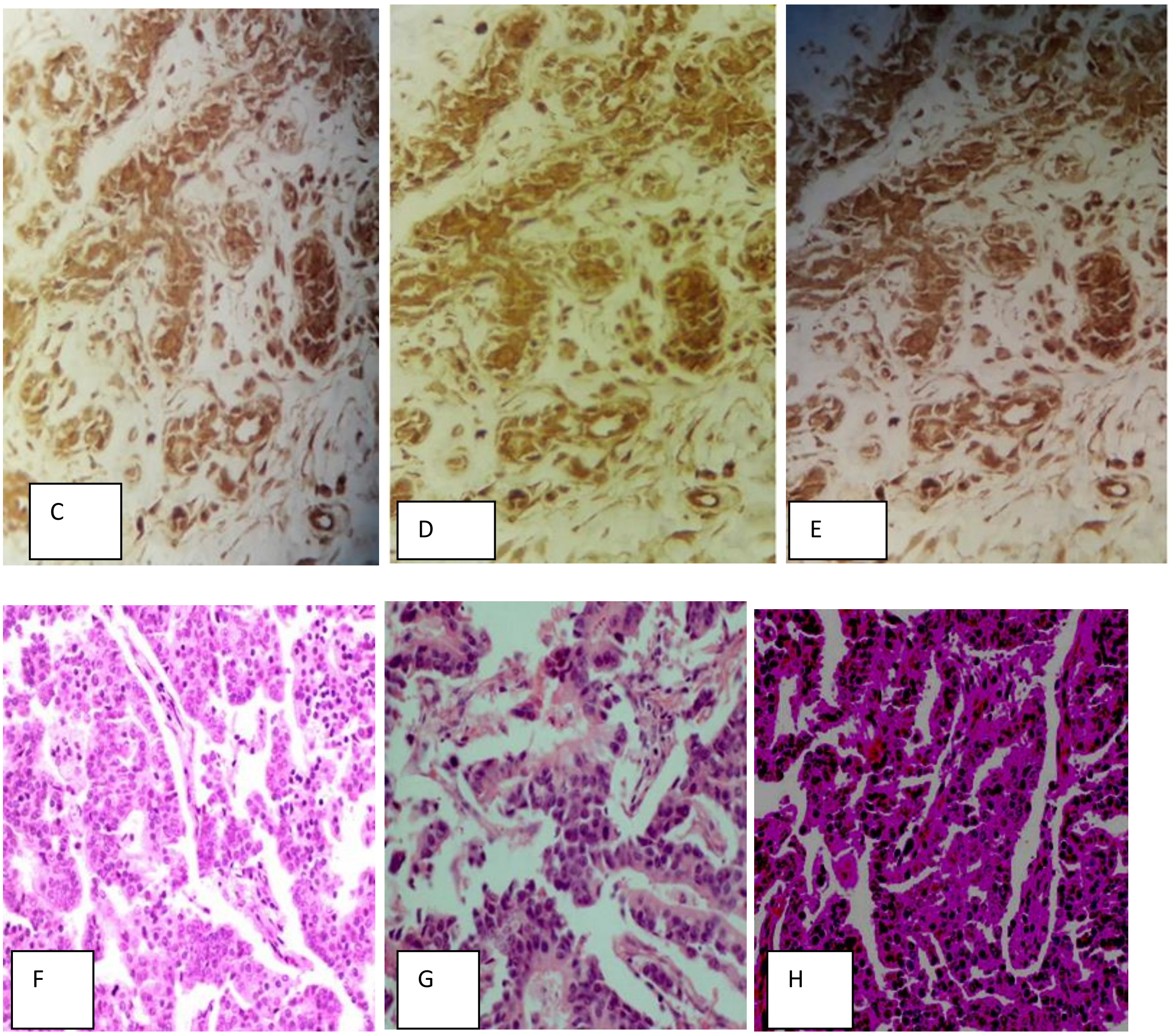


Figure 1. Representative images for immunochemistry and histology. ER-positive (**A**), and ER-negative (**B**). Anti-Rabbit monoclonal antibodies were used in the ratio 1:100 and staining intensity of ≥ 10% was considered positive as described previously (17). DRG1 expression intensity 1+ (**C**), intensity 2+ (**D**) and intensity 3+ (**E**). Anti-DRG1 Rabbit polyclonal was used in the ratio 1:100. Histology grade I (**F**), II (**G**), and III (**H**). Histology grading was based on the average tubule formation, mitotic count, and nuclear pleomorphism following the Nottingham grading system. The magnification of all figures is ×400.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad Software Inc., California, USA). Association between variables was analyzed using the chi-square test.

Where applicable, data are presented as mean ± standard deviation (SD). A p-value ≤ 0.05 was considered significant.

Results

Age distribution of ER positive breast cancer

Breast tumour blocks archived between 2012 and 2015 at MTRH and the corresponding medical records of 37 patients of African origin were used in this study. Among them, 16 either had inadequate samples or were ER negative, hence were excluded from this study (Table 1). The participants were aged between 26 and 55 years, with a mean age of 41.76 ± 7.71 years. Majority of the ER-positive breast cancer women were aged 40 and 50 years (Figure 2).

Table 1. Breast tumour blocks sample characteristics

Characteristic	**Number (n)**	**Percentage (%)**
ER receptor status		
Negative	2	5.4
Positive	*21*	*56.8*
Folded	1	2.7
No tissue	2	5.4
No tumor	8	22.2
Inadequate sample	3	8.1
Total (n)	37	

Note: Values in italics depict the samples used in this study.

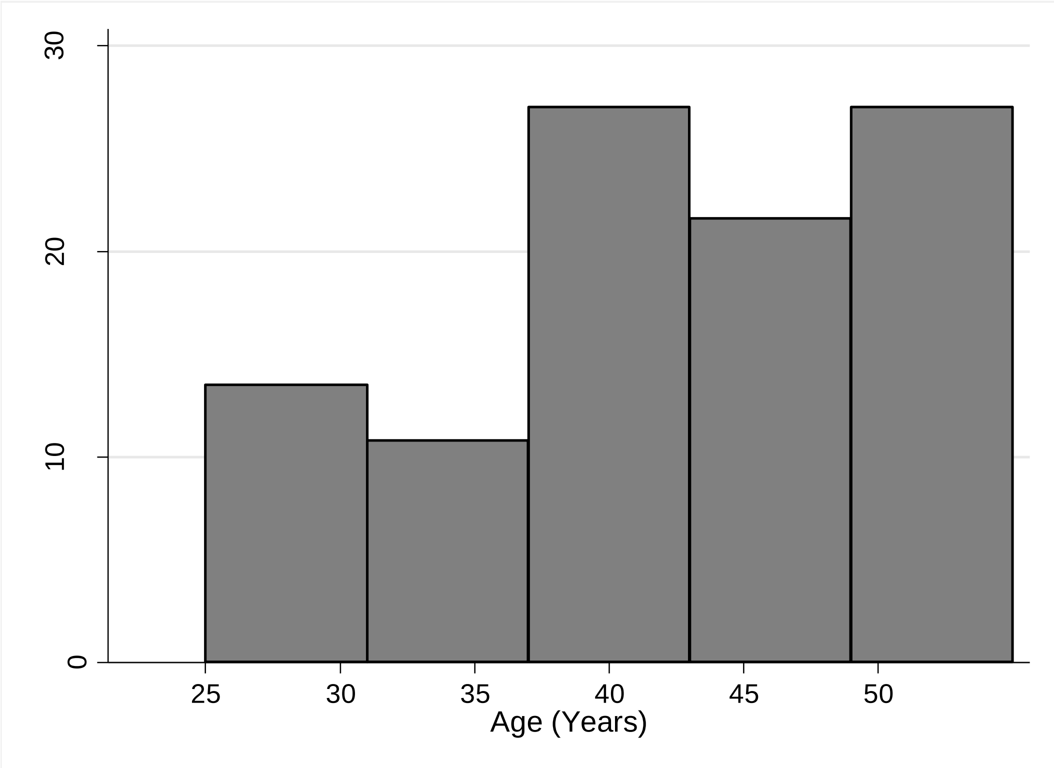


Figure 2. Age distribution of ER positive breast cancer**.**

Association between DRG1 expression and the indicators of cancer metastasis

The majority of the ER-positive breast tumors in histology grades I and II expressed DRG1. However, there was no significant association between DRG1 expression and histology grades (p = 0.316; Table 2). No significant association was observed between DRG1 expression and ER-positive breast tumor size (p = 1.000; Table 2). In addition, whereas most of the ER-positive breast tumor blocks were both positive for DRG1 and Ki-67 protein, there was no statistically significant association between DRG1 expression and Ki-67 protein expression among the patients (p = 0.387; Table 2).

Table 2. Association between DRG1 expression and various clinico-pathological parameters among ER positive breast cancer.

Parameter	Total (n = 21)	DRG1 I and II, n (%)	DRG1 III, n (%)	OR	95% CI	*p*-value
Histology grade						
I and II	17	8 (47)	9 (53)	2.667	0.229 to 31.09	0.603
III	4	1 (25)	3 (75)			
Tumour size						
< 5 cm	10	4 (40)	6 (60)	1.167	0.199 to 6.808	1.000
> 5 cm	11	4 (36)	7 (64)			
Ki-67 expression						
Positive	12	7 (58)	5 (42)	2.80	0.463 to 16.94	0.387
Negative	9	3 (33)	6 (67)			

Discussion

This study evaluated the age distribution of ER-positive breast cancer and the expression of DRG-1 protein as a potential biomarker for metastasis of ER-positive breast cancer.

ER-positive breast cancer patients had a mean age of 41.76 ± 7.71 years with the majority of them aged between 40 and 50 years. The present finding is consistent with previous reports in which breast cancer incidence was shown to peak between the ages of 35 and 45 years among African females (8) and Chinese women between the age of 40 and 50 years (7). However, the previous studies did not focus on ER-mediated breast cancer, hence, provided only a general age distribution of breast cancer.

ER is a nuclear receptor functioning as a transcriptional regulator that mediates the biological responses to the sex hormone, estrogen essential for reproduction (18). It is, however, not clear why women aged 50 years had a higher prevalence of ER-positive breast cancer. Further investigations may be necessary to establish the reason underlying the predominance of ER-positive breast cancer among women aged 50 years since this is menopausal age and women are expected to produce little estrogen.

Furthermore, this study did not reveal an association between DRG1 protein expression and the parameters indicative of cancer metastasis, such as histology grades, tumour size, and Ki-

67 protein expression among ER-positive breast cancer women. The present findings concur with a previous study (13) and partly agree with a previous study in which DRG1 was shown to have a correlation with lymph node metastasis, but not with tumour size or histology grades (14). Considering that individual breast tumours exhibit great variations in clinical presentation in different ethnic populations (19), it is probable that the discrepancy between the present and previous studies with regards to lymph node metastasis could be linked to the different genetic backgrounds of the study populations, i.e, Africans versus Caucasians. In addition, it is not clear whether the previous study used both males and female-derived specimens. This might also contribute to the discrepancy.

From the results obtained, DRG1 appears not to be suitable marker for breast cancer metastasis. However, our study could have been limited by the relatively small sample size. This might have contributed to the lack of significant association between the study variables. It is therefore difficult to entirely rule out the potential utility of DRG1 in breast cancer metastasis, and further, more robust studies will be needed to confirm or rule out this hypothesis.

Conclusions

In summary, the present study showed that women of productive age that is 40 years are the most affected by ER-positive breast cancer. Furthermore, no associations were observed between DRG1 and the parameters indicative of metastasis (histology grade, tumour size and Ki-67) that could be associated with metastasis of breast cancer.

Recommendations

Breast cancer is still a major challenge in most countries; therefore, effective techniques are needed to detect breast cancer at early stages. Although our study was not adequately powered to conclusively determine the utility of DRG1 as a marker of breast cancer metastasis, it contributes to the ongoing search for feasible biomarkers for metastasis of breast cancer ER-positive. Further studies involving larger number of participants and targeting DRG1 and other potential molecular markers are warranted to in the fight against breast cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HB, BN, KTP conceived and designed the experiments; HB, WN performed the experiments; BN, KTP supervised the experiments; COO analyzed the data, prepared figures and tables; HB, COO wrote the paper, BN, KTP, WN reviewed drafts of the paper. HB, BN, KTP, COO, WN reviewed and approved the final version of the manuscript.

Acknowledgements

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References

1. Chen S-H, Cheung CHA. (2018) Challenges in treating estrogen receptor-positive breast cancer. Estrogen [Internet]. 2018 Nov 5 [cited 2019 Jul 9]; Available from: https://www.intechopen.com/books/estrogen/challenges-in-treating-estrogen-receptor-positive-breast-cancer
2. Naanyu V, Asirwa CF, Wachira J, Busakhala N, Kisuya J, Otieno G, et al. (2015) Lay perceptions of breast cancer in Western Kenya. World J Clin Oncol. 2015 Oct 10;6(5):147–55.
3. Ministry of Health, Kenya. (2018) Kenya national cancer screening guidelines [Internet]. Ministry of Health, GoK; 2018 [cited 2019 Apr 20]. Available from: http://www.health.go.ke/wp-content/uploads/2019/02/National-Cancer-Screening-Guidelines-2018.pdf

4. Miller TW, Hennessy BT, González-Angulo AM, Fox EM, Mills GB, Chen H, et al. (2010) Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor–positive human breast cancer. J Clin Invest. 2010 Jul 1;120(7):2406–13.
5. Clarke R, Tyson JJ, Dixon JM. (2015) Endocrine resistance in breast cancer – An overview and update. Molecular and Cellular Endocrinology. 2015 Dec 15;418:220–34.
6. Johnston SRD. (2016) Targeted combinations for hormone receptor–positive advanced breast cancer: who benefits? JCO. 2016;34(5):393–5.
7. Su Y, Zheng Y, Zheng W, Gu K, Chen Z, Li G, et al. (2011) Distinct distribution and prognostic significance of molecular subtypes of breast cancer in Chinese women: a population-based cohort study. BMC Cancer. 2011 Jul 12;11:292.
8. Anyanwu SNC. (2000)Breast cancer in Eastern Nigeria: a ten-year review. West African Journal of Medicine. 2000;19(2):120–5.
9. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. (2008) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010 Dec 15;127(12):2893–917.
10. Parkin DM, Bray F, Ferlay J, Jemal A. (2014) Cancer in Africa 2012. Cancer Epidemiol Biomarkers Prev. 2014;23(6):953–66.
11. Sawe RT, Kerper M, Badve S, Li J, Sandoval-Cooper M, Xie J, et al. (2016)Aggressive breast cancer in western Kenya has early onset, high proliferation, andimmune cell infiltration. BMC Cancer. 2016 Mar 10;16:1–15.
12. Zhang XH-F, Giuliano M, Trivedi MV, Schiff R, Kent Osborne C. (2013) Metastasis dormancy in estrogen receptor-positive breast cancer. Clin Cancer Res. 2013 Dec 1;19(23):1–16.

13. Baig RM, Sanders AJ, Kayani MA, Jiang WG. (2012) Association of differentiation-related gene-1 (DRG1) with breast cancer survival and in vitro impact of DRG1 suppression. Cancers (Basel). 2012 Jul 10;4(3):658–72.

14. Bandyopadhyay S, Pai SK, Hirota S, Hosobe S, Takano Y, Saito K, et al. (2004) Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression. Oncogene. 2004 Jul;23(33):5675–5681.

15. Kobayashi T, Iwaya K, Moriya T, Yamasaki T, Tsuda H, Yamamoto J, et al. (2013) A simple immunohistochemical panel comprising 2 conventional markers, Ki67 and p53, is a powerful tool for predicting patient outcome in luminal-type breast cancer. BMC Clinical Pathology. 2013;13:1–11.

16. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, et al. (2010) Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res. 2010;12(4):1–12.

17. Fotovati A, Fujii T, Yamaguchi M, Kage M, Shirouzu K, Oie S, et al. (2006) 17β-estradiol induces down-regulation of Cap43/NDRG1/Drg-1, a putative differentiation-related and metastasis suppressor gene, in human breast cancer cells. Clin Cancer Res. 2006 May 15;12(10):3010–8.

18. Li X, Huang J, Yi P, Bambara RA, Hilf R, Muyan M. (2004) Single-chain estrogen receptors (ERs) reveal that the ERα/β heterodimer emulates functions of the ERα dimer in genomic estrogen signaling pathways. Mol Cell Biol. 2004 Sep;24(17):7681–94.

19. Wiencke JK. (2004) Impact of race/ethnicity on molecular pathways in human cancer. Nature Reviews Cancer. 2004 Jan;4(1):79.

Quality of diagnosis and treatment of malaria patients in rural hospitals in Kisumu County, Kenya

Wilfred Ouma Otambo[1, 2*], Julius Olumeh[2], Collins Ouma[3], Patrick Onyango[1], Harrysone Atieli[2], Chloe Wang[4], Ming-Chieh Lee[4], Andrew Githeko[5],Gofa Zhou[4], John Githure[2],James Kazura[6], Guiyan Yan[4],

[1]Department of Zoology, Maseno University, Kisumu, Kenya

[2]International Centre of Excellence for Malaria Research, Tom Mboya University College of Maseno University, Homa Bay, Kenya

[3]Department of Biomedical Sciences and Technology, Maseno University, Kisumu, Kenya

[4]Depatment of Population Health and Disease Prevention, University of California, Irvine, CA USA

[5]Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya

[6]Centre for Global Health and Diseases, Case Western University Reserve, Cleveland, Ohio

Keywords: Microscopic diagnosis, slide positivity, hospitalization, symptoms antimalarial treatment, sensitivity and specificity, discrepancy in slide reading, misdiagnosis, overtreatment, and presumptive clinical diagnosis treatment.

Abstract

Background: Accurate, reliable, and affordable malaria diagnosis followed by effective treatment at the health facilities is important for effective malaria management and control to help reduce the burden of infections and minimize the human reservoir of infectious gametocytes. However, cases of malaria misdiagnosis at the health facilities remains a serious concern which may in lead to erroneous treatment of malaria. As Kenya moves towards malaria elimination, there is need to examine routine practices in diagnosis and treatment of malaria cases in health facilities. The objective of the current study was to examine discrepancies in diagnosis and treatment of malaria in rural communities in Kisumu County, Kenya.

Method: Passive case detection was conducted in three hospitals in Kisumu County Kenya: two public hospitals and one private hospital. Two groups of study participants were enrolled for evaluate clinical diagnosis and treatment of malaria in out-patients attending these hospitals between November 2019 and March 2020. Febrile patients who exhibited clinical malaria symptoms had their finger-prick blood smears taken for examination; the accuracy of microscopy reading was compared between the hospital laboratory technicians, independent microscopists and polymerase chain reaction (PCR). The antimalarial prescribed to the patients was recorded. Blood smears from patients diagnosed with rapid diagnostic test (RDT) and in those patients presumptively treated with antimalarial and antibiotics were re-examined for the presence of malaria parasites.

Results: A total of 1,117 febrile cases consented to participate in the study; 936 assessed for slide positivity rates and malarial treatment. 126 assessed by RDT diagnosis, 55 examined for presumptive treatment prescribed by clinicians. Of the 936 febrile cases examined for clinical treatment, malaria slide positivity rate was 27.5% (257/936) with the two public hospitals slide positivity rate higher at 29.8%(n=624) than private hospital at 22.8%(n=312). There was a significant difference in symptoms between slide positive and negative groups (X^2 = 2165.42, df. = 13, $P < 0.0001$) with common symptoms of the slide positive patients of <5 years Old's being fever (97.6%), joint pains (53.7%) and upper respiratory tract infection symptoms (24.4%); 5-15 years Old's being headache (88.8%) and vomiting (30.2%); and the ≥15 years old being malaise (14.0%), joint pains (53.0%), diarrhea (21.0%) and abdominal pains (17.0%). Antimalarial treatment among the slide negative patients was higher in public hospitals at 14.6% compared with 3.7% in the private hospital. About 8.8% of slide positive patients missed out on antimalarial treatment. Among the slide positives; treatment with coartem was highest among the 5-15 years old at 75.9 % (88/116). Using qPCR as standard for parasite detection, the hospital microscopy sensitivity was at 38.3% and specificity at 91.2%. Using independent microscopy as standard, the hospital microscopy sensitivity was at 47.6% and specificity at 86.7%. The inter-reliability reading between qPCR and hospital microscopy showed a low level of agreement (Kappa = 0.26) while hospital microscopy and independent microscopy showed a fair level of agreement (Kappa = 0.41; P < 0.0001). Of the 1,117 febrile cases 387 cases were examined for slide reading accuracy and misdiagnosis was at 19.9% (77/387). Of the 77 misdiagnosed, PCR confirmed about 17.4 %(4/23) as false positive and 68.5% (37/54) as false negative. There was moderate level of agreement in malaria diagnosis between health facilities RDT (48.4% (61/126) and independent microscopy (41.3% (52/126) (Kappa = 0.633; *P*<0.0001). Of the 55 presumptively treated, 25% (14/55) were slide positive and 74.5 %(41/55) were slide negative.

Conclusion: The result of this study showed that the overall slide positivity rate in the three hospitals was 27.5% with about 25% discrepancy in slide reading and about 10% of the slide negative patients being treated with coartem. Misdiagnosis was higher in the private hospital than in the public hospitals. Febrile cases were presumptively treated mainly because of inadequate personnel in the laboratory, lack of laboratory reagents and affordability of diagnostic tests and treatment by the patients. The highlighted discrepancies between clinical malaria diagnosis and treatment carried out at these health facilities in reference to the guidelines provided by the Ministry of Health calls for an urgent need for the government to strengthen capacity for clinicians and laboratory technicians to enhance malaria diagnosis and treatment at all health facilities.

Introduction

Malaria remains a major public health problem and a major cause of morbidity and mortality worldwide. Approximately 229 million malaria cases were reported globally in 2019, with about 409,000 deaths annually; the African region reporting about 94% (215 million) cases and 384,000 deaths(1). In Kenya, approximately 70% of the country's 47 million people are at risk of the disease with the Lake Victoria region having the highest burden of infection of about 27% malaria prevalence (2). Kenya is currently scaling up malaria control interventions with the goal to reduce the disease burden and eventual elimination. However, demographics and socioeconomic factors are some of the challenges faced in malaria control. For instance, low-income groups are more likely to be infected by malaria due to limited chances of accessing malaria preventive methods than the high -income group (3). The most vulnerable groups at risk of malaria include <5 years old children and pregnant women (4,5). The school-going children aged 5-15 years and adults have high malaria prevalence and hence act as reservoirs of the parasites(6).

The main strategy for optimization of clinical malaria treatment is early and accurate diagnosis followed by effective treatment. According to the WHO guidelines for treatment of malaria, all patients suspected of malaria should be confirmed with microscopy or by rapid diagnostic tests (RDT) before treatment with an antimalarial, and clinical diagnosis should only be carried out when a parasitological diagnosis is not available (7). Artemisinin-based combination therapies (ACTs) is the recommended treatments for uncomplicated *P. falciparum* malaria and artesunate and quinine for treatment of severe malaria(7). However, cases of individuals being treated with antimalarial drugs despite negative parasitological results have been reported(8). The commonly employed malaria diagnostic techniques at the health facilities include; clinical evidence, microscopy, and RDT (9). Clinical diagnosis is usually not reliable since there is an overlap of malaria symptoms with other infectious diseases which may also vary with the level of malaria endemicity, malaria transmission season, and age group (10). Malaria microscopy reliability depends on the expertise of technicians with an advantage that it can differentiate blood stages and malaria parasite species.. Microscopy diagnosis also provides pathophysiological and prognostic information which serve as indicators for the severity of malaria infection, e.g. Parasite counts (11). On the other hand, malaria RDT kits which are simple to use at peripheral health facilities or the community level cannot determine parasite density or differentiate *Plasmodium* species. Its sensitivity is reduced in low parasitemia and false-positive results are common after parasites have been cleared from the host since the antigens remain in circulation (10). Despite the availability of these malaria diagnostic tools, health facilities are reported to use clinical diagnosis (12). For effective malaria management and control, it is important to have accurate, reliable, and affordable diagnosis followed by effective and timely treatment at the health facilities. However, there is a concern of patients presumptively treated with antimalarial without confirmed laboratory diagnosis despite the Kenya National Malaria Treatment Guidelines policy (13).

Despite microscopy being the gold-standard, there are challenges attributed to effective diagnosis at the health facilities such as limited equipment, supplies, working environment, training, and adequate supervision (14). Cases of patient overtreatment (patient given antimalarial but has no malaria) or under treatment (patients not given antimalarial but has malaria) is a growing concern. There is therefore a need to examine the discrepancies in diagnostic capabilities and treatment profiles of malaria at health facilities. Such information may help optimize the diagnosis and management of malaria cases hence overall reduction in malaria incidence. The current study aimed at evaluating clinical and parasitological practices carried out in the diagnosis and treatment of malaria in out-patients attending health facilities with reference to results obtained by PCR in Kisumu County, Kenya.

Materials and Methods

Study site

The study site is in Kisumu County located on the shores of Lake Victoria in Western Kenya an area of approximately 327 sq. km. According to the 2019 Kenya National Population Census(15), (Figure 1).The study was done in three main hospitals in rural area of Kisumu

County with a high catchment: Public hospital 1 with 16,123, Public hospital 2 with 8,250, and private hospital with 16,115 population. Public hospital 1 is in the Lake Victoria basin an area characterized by a flat plain and lower elevation at 1100-1200 m above sea level with fishing and maize farming as the main economic and is frequented by flooding during the rainy season. Public hospital 2 is located on the slopes that are hilly and rocky with an elevation between 1300-1450 m with ballast making as the main economic activity with larval habitats in this zone unstable and permanent large aquatic habitats are uncommon. The private hospital is in the plateau at 1450 –1600 m elevations with brick making and farming as the main economic activities with human density being higher than the two zones, and stable larval habitats are more common. The three hospitals were selected based on malaria prevalence and topographical features with the high catchment of the local residents’ population.

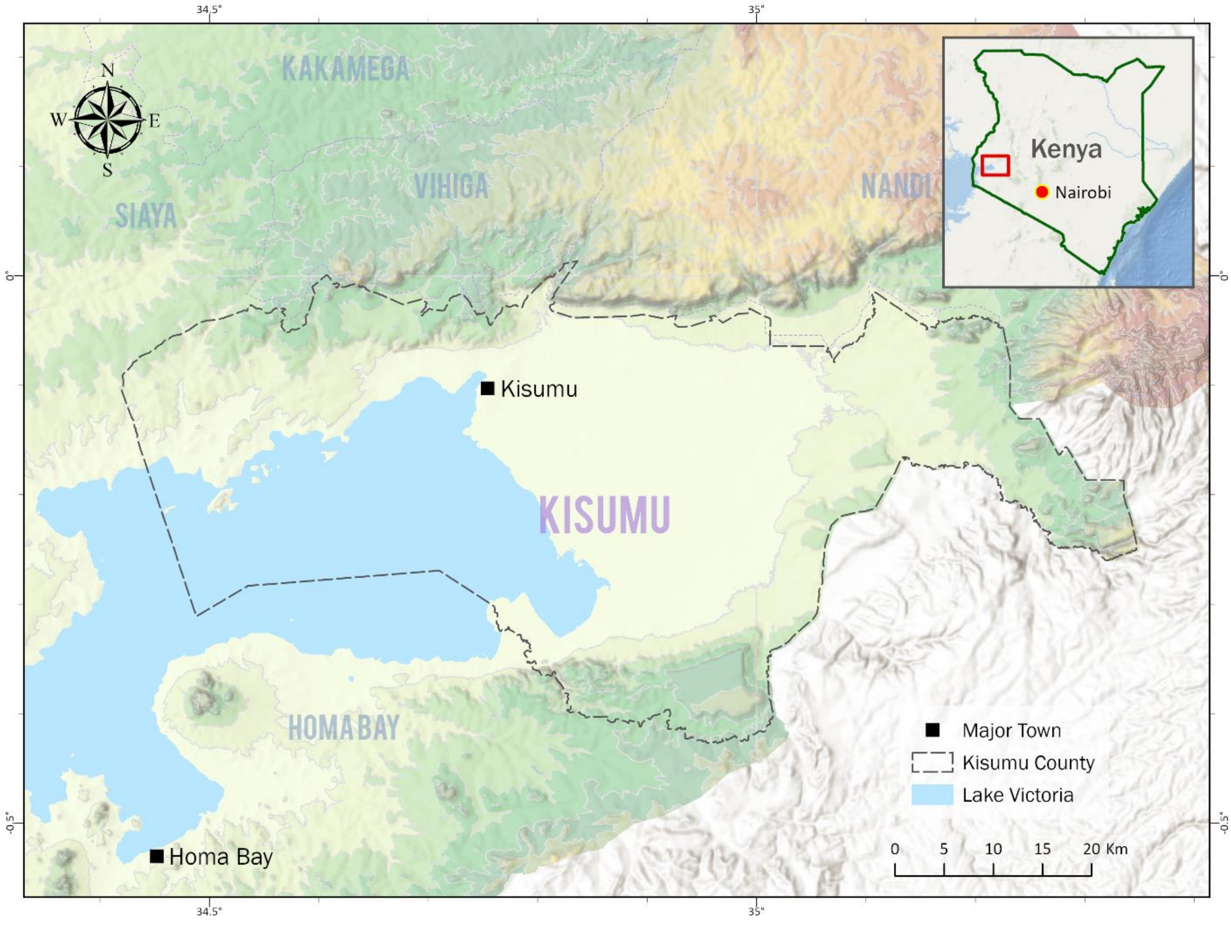


Figure 3. Map of Kisumu County showing the study location

Study procedure

Enrollment of study particpants: Passive case detection (PCD) of malaria cases was conducted between November 2019 and March 2020 in three hospitals in Kisumu County, two public hospitals, and one private hospital frequently attended by communities. A technician stationed at each of the three hospitals recruited study participants and collected malaria-related data from consenting adults and assenting children out-patients as they were being referred for laboratory diagnosis. The antimalarial treatment regimen of these patients was recorded and blood smears were subsequently re-examined (stained using 10% Giemsa for 15 minutes then examined) to check if they had malaria parasites. A total of 1131 febrile cases consented to participate in the study. Malaria diagnosis and treatment in these cases was assessed. Of the febrile cases, 936 were assessed for slide positivity rates and antimalarial treatment, 387 re-examined for accuracy in slide reading and antimalarial treatment, 126 diagnosed by RDT, and 55 were examined for presumptive treatment prescribed by clinicians. In these febrile cases, disease symptoms other than malaria and the prescribed medications, and hospital admission cases were also recorded.

Slide positivity rate: To determine slide positivity rate, the technicians at each of the three hospitals enrolled out-patients referred to the laboratory for malaria diagnosis. Finger prick blood smears were taken from 936 of the febrile cases and examined by microscopy and the treatment prescribed to these patients recorded.

Discrepancy in slide reading: To assess the accuracy of hospital diagnosis, two samples of thick and thin blood smears and Dry Blood Spot on filter paper were taken from the 387 of the febrile cases. One slide sample was read at the hospital laboratory, while the other slide was taken to the International Center of Excellence for Malaria Research (ICEMR) laboratory in Homa Bay where they were examined by independent microscopists where thick and thin blood films were stained using 10% Giemsa for 15 minutes then examined. Two certified microscopists examined the slides using oil-immersion under magnification x 1,000 to identify and count the parasite species. A slide was considered positive if at least one asexual blood-stage *P. falciparum* parasite was identified. Parasite density was determined by counting the number of parasites per 200 white blood cells and if each subject will have 8000 white blood cells/μL of blood. For quality control, slides were checked at random by another technician at ICEMR. The slide readings were compared between the ICEMR and hospital laboratory results. The antimalarial and antibiotic drugs given were also recorded.

Rapid diagnosis Test: To assess malaria parasite positivity by RDT and microscopy, a finger-prick blood sample was taken from 126 of the febrile cases for diagnosis by RDT and for thick and thin blood smears. The RDT samples were examined at the hospital while the slide smears were taken to the ICEMR laboratory in Homa Bay and examined by project microscopists. The RDT and microscopy results were compared and discrepancies were recorded.

Presumptive clinical diagnosis: The outpatient attendees who had fever (axillary temperature $\geq 37.5^{o}C$) at the time of examination and complaints of fever and other nonspecific constitutional symptoms such as shivering/ shaking, sweating, joint pains, headache, and vomiting were clinically diagnosed by a clinician and prescribed either antimalarial and/or antibiotic treatment without a laboratory diagnosis of their illnesses. The main reasons for presumptively treated cases were recorded, which included, clinical observation, affordability of the laboratory tests, and the absence of a laboratory technician. Blood smears from the 55 presumptively treated cases were prepared by our technician at the health facility and taken to the ICEMR laboratory for examination.

Protection of Human Subjects

Ethical approval for the study was obtained from Maseno University Ethics Review Committee, reference number: MSU/DRPI/MUERC/00778/19 and Institutional Review Board of the University of California, Irvine, USA (HS# 2017-3512). Individuals seeking treatment at the three hospitals were requested to participate in the study and those consenting were recruited. Written informed consent of the respondents was sought before the study was conducted and as for the minors, consent was provided by the parents/guardians. Patients unwilling to participate in the study or changed their willingness to participate were excluded.

Data analysis

The data collected were analyzed using SPSS. The Chi-square test was used to test for differences in malaria prevalence, frequencies of presumptive treatment, and frequencies of monthly malaria diagnosis. Cohen's kappa statistic was used to measure adjusted agreement between hospital microscopy and ICEMR microscopy. Categorical variables were described using frequency tables (counts and percentages).

Results

A total of 1131 individuals visiting the outpatient department at the three hospitals consented to participate in the study. Table 1 summarizes descriptive demographic information of the study participants.

Descriptive demographic information of study participants

The three hospitals were found to have similar demographics in age structure, gender, education, and occupation. There was a significant difference in sex distribution with the females being higher in number in all the three hospitals ($\chi^2 = 12.266$; df = 2 $P <0.002$). There was a significant difference in age distribution amongst the hospitals ($\chi^2 = 103.113$; df = 2 $P <0.0001$) with individuals aged ≥15 years old comprising about 57.4% of the study population (Table 1). There was significant difference in the education level of the patients ($\chi^2 = 449.719$; df = 2 $P <0.0001$). The literacy rate was high with attainment of primary and secondary education at 40.1% and 30.9% respectively. In public hospital 1, about 31.5% of the patients had secondary education; in public hospital 2, primary education was higher at 63.3%, and about 39.6% of the patients who sought treatment in the private hospital had a college education and above (Table 1). The occupation of the patients who attended the three hospitals showed significant difference ($\chi^2 = 212.562$; df = 12 P <0.0001) with most of the patients 20.1% having business as their main income-generating occupation (Table 1).

Table 3. Demographic information of study participants in Kisumu County

Parameters	Details	Enrollment (n, %)	Hospitals			P-value
			Public 1. (n,%)	Public 2. (n,%)	Private (n,%)	
Total Enrollment		1131	317	488	326	
Blood smear slide examination		936	317	307	312	
Malaria rapid diagnosis test		126	0	126	0	
Clinical diagnosis		69	0	55	14	
Sex	Male	464 (41.0)	127 (40.1)	195 (40.0)	142 (43.6)	0.002
	Female	687 (59.0)	190 (59.9)	293 (60.0)	184 (56.4)	
Age	<5	138 (12.2)	66 (20.8)	60 (12.3)	12 (3.7)	p<0.0001
	5 ~ 15	344 (30.4)	96 (30.3)	190 (38.9)	58 (17.8)	
	≥15	649 (57.4)	155 (48.9)	238 (48.8)	256 (78.5)	
Education	illiterate	68 (6.0)	6 (1.9)	41 (8.5)	21 (6.4)	p<0.0001
	Pre-primary	85 (7.5)	68 (21.5)	9 (1.8%)	8 (2.5)	
	Primary	454 (40.1)	99 (31.2)	309 (63.3)	46 (14.1)	
	Secondary	349 (30.9)	107 (33.8)	120 (24.6)	122 (37.4)	

	College & above	175 (14.8)	37 (11.7)	9 (1.8)	129 (39.6)	
Occupation	Farmer	135 (11.9)	39 (12.3)	59 (12.1)	37 (11.3)	p<0.0001
	Business person	227 (20.1)	42 (13.2)	120 (24.6)	65 (19.9)	
	Office worker	48 (4.2)	15 (4.7)	4 (0.8)	29 (8.9)	
	Unemployed	71 (6.3)	15 (4.7)	3 (0.6)	53 (16.3)	
	Student	529 (46.8)	138 (43.5)	266 (54.5)	125 (38.3)	
	Non-school child	103 (9.1)	66 (20.8)	31 (6.4)	6 (1.8)	
	Others	18 (1.6)	2 (0.6)	5 (1.0)	11 (3.4)	

Health facilities slide positivity rate

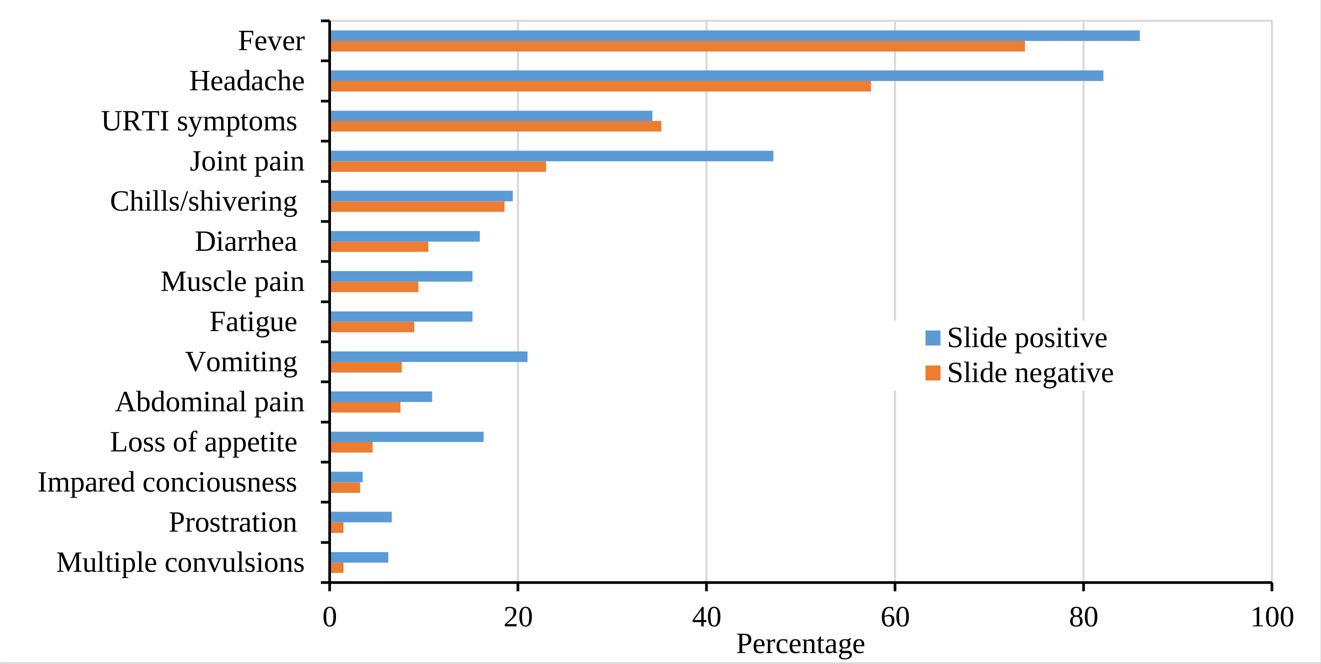
Out of the 1131 outpatients', 936 febrile cases were assessed for slide positivity rates (317 from public hospital 1, 307 from public hospital 2, and 312 from private hospital). Among the study participants, the overall slide positivity rate in the three hospitals was 27.5% (257/936) with public hospital 1 at 30.9% (98/317), public hospital 2 at 28.7% (88/307) and private hospital at 22.8%(71/312) though there was no significant difference in the slide positivity within the hospitals(χ^2 = 5.589, df. = 2, P = 0.061). There was no significant difference in slide positivity rates between the males and the female within the hospitals (χ^2 = 4.263, df. = 2, P = 0.1197) (Table 2). However, there was a significant difference in the slide positivity rate within the age groups with the school-going children aged between 5-15 years old from the three hospitals having the highest slide positivity rates (χ^2 = 45.818, df. = 4, P < 0.001) (Table 2).

Table 4. The slide positivity rate of febrile cases in three hospitals in Kisumu

Hospitals		Public Hosp. 1	Public hosp. 2	Private Hos.	P-value
Total tested (microscopy)		317	307	312	
Positive		98	88	71	0.061
Positivity rate (%)		30.9	28.7	22.8	
Sex(n/N, %)	Male	41/127 (32.3)	41/118 (34.7)	41/136 (30.1)	0.119
	Female	57/190 (30.0)	47/189 (24.9)	30/176 (17.0)	
Age(n/N, %)	< 5	28/66 (42.4)	11/41 (26.8)	2/11 (18.2)	0.000
	5 ~ 15	40/96 (41.7)	54/114 (47.4)	22/58 (37.9)	
	≥15	30/155 (19.4)	23/152 (15.1)	47/243 (19.3)	

Symptoms presented by slide positive and negative patients

There was a significant difference in symptoms between slide positive and negative groups (chi = 2165.42, d.f. = 13, $P < 0.0001$. More symptoms were recorded in the slide positive patients compared to the slide negative patients. The most common symptoms were fever, headache, upper respiratory tract infection (URTI), joint pains, and shivering. The severe symptoms were common among slide positive residents, which were multiple convulsions, prostration, and impaired consciousness (Fig 2).



Note. Significance levels in the difference between slide positive and negative groups for the same symptom: * P<0.01, ** P<0.01, *** P<0.001, and n.s. not significant at the level of 0.05.

Figure 2. Percentage of symptoms between slide positive and slide negative cases

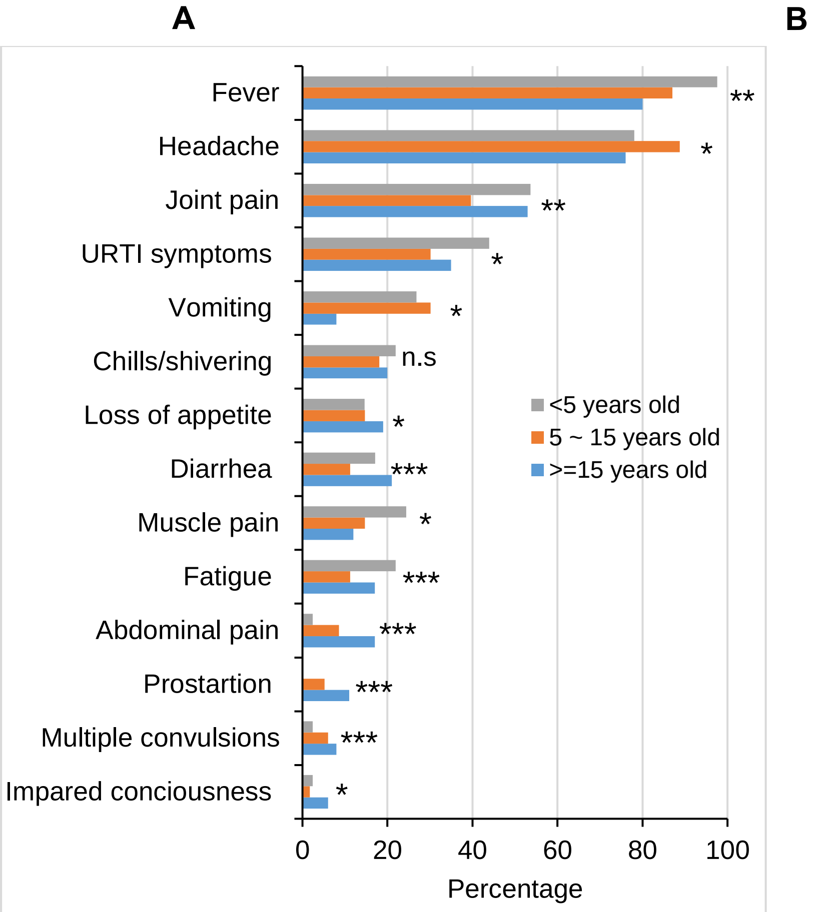
Symptoms presented by blood smear positive and negative patients within age groups

Among the 257 slide positive patients, there was significant difference in symptoms among age group (χ^2 = 44.769, df. = 26, P =0.0031)). The most common symptoms among the 41 children aged <5 years old was fever 97.6% (40/41), and joint pains 53.7% (22/41), Of the 116 school-going children aged between 5 – 15 years old, the common symptoms were headache 88.8% (103/116), While of the 100 individuals aged above 15 years old, the common symptoms were: Loss of appetite 19.0% (19/100), and abdominal pain 17.0% (17/100). Figure 3.

Of the 679 slide negative patients, there was a significant difference in symptoms within the age groups (χ^2 = 95.173, df. = 1, P <0.0001) with common symptoms among 77 children less than 5 years being fever 85.7% (66/77), among the 152 school-going children aged between 5 – 15 years old, the common symptoms was headache 74.3% (113/152), and chills/ shivering

29.6%(45/152). While of the 450 individuals aged above 15 years old, the common symptoms were: Joint pains 28.2% (127/450), abdominal pain 8.9% (40/450). Figure 3.

Severe malaria symptoms were common among individuals above 15 years old (Prostration 6.0% (6/100), Multiple convulsions 8% (8/100) & Impaired consciousness 6.0% (6/100) Figure 3.



Note. Significance levels in difference between slide positive and negative within groups for the same symptom: * P<0.01, ** P<0.01, *** P<0.001, and n.s. not significant at level of 0.05.

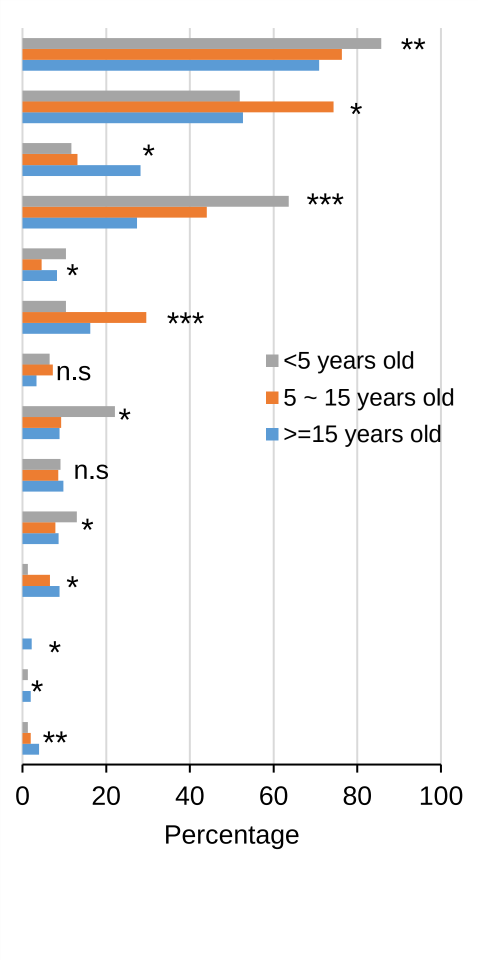


Figure 4. A. Percentage of symptoms of slide positive cases within the age groups. **B** Percentage of symptoms of slide negative cases within the age groups

Slide positivity hospitalization, severe symptoms, and antimalarial treatment

There was a significant difference in the hospitalization (χ^2 = 31.000, df. = 3, P <0.0001) with hospitalization level being higher in the private hospital among the slide positive patients at 40.8% (29/71). Most of the hospitalized patients from the private hospital had severe malaria symptoms such as impaired consciousness, prostration, and multiple convulsions (Table 3).

Antimalarial treatment among slide negative patients was high in the public hospitals1 at 18.7%.

There was a significant difference in the malaria treatment among the three hospitals with the private hospital having the lowest antimalarial treatment among the slide positive individuals at 81.7% (58/71) (χ^2 = 10.151, df. = 2, P=0.006) (Table 3). The patients who missed on the antimalarial were linked to the affordability especially in the private hospitals.

Table 5. Slide positivity hospitalization, severe symptoms, and antimalarial treatment

Hospitals		**Public Hospital. 1**		**Private Hospital**		**P-value**
Blood smear (n)		**Positive (n=98)**	**Negative (n=219)**	**Positive (n=71)**	**Negative (n=241)**	
Hospitalized (%, n)		**7.1 (7) a**	**0.9 (2) b**	**40.8 (29) a**	**7.5 (18) b**	**0.000**
Severe symptoms (%, n)	**Total**					
	Impaired consciousness	**1.0 (1)**	**0.5 (1)**	**11.3 (8)**	**7.5 (18)**	
	Prostration	**2.0 (2)**	**0.5 (1)**	**11.3 (8)**	**3.7 (9)**	
	Multiple convulsions	**4.1 (4)**	**0.5 (1)**	**12.3 (9)**	**2.9 (7)**	
Coartem treatment (%, n)		**90.8 (89)**	**18.7 (41)**	**81.7 (58)**	**3.7 (9)**	**0.006**

Treatment of slide positive and negative cases in public and private hospital

The treatment of the febrile cases for both the slide positive and the slide negative patients in the public and the private hospital was with Coartem, antibiotics, and analgesics (Table 4). Antimalarial treatment was high in the public hospital at 94.6%. Antimalarial treatment among the slide negative patients in public hospitals was 14.6%. About 8.8% of slide positive patients could not Ford antimalarial drugs. Most of the analgesics were given to slide negative patients (38.8%, 59/241) in the private hospital. The treatment with antimalarial plus antibiotics among the slide positive patients in the public hospitals was 53.2% (100/186) (Table 4).

Table 6. Treatment of slide positive and negative cases in public and private hospitals

Parameters	Public Hospitals		Private hospital	
Blood smear	+Ve	-Ve	+Ve	-Ve
Treatment	n=186	n=438	n=71	n=241
Coartem + analgesic	77(41.4%)	42(9.6%)	50(70.4%)	15(9.9%)
Antibiotics + analgesic	5(2.7%)	273(62.3%)	2(2.8%)	76(50.6%)
Coartem + antibiotics + analgesic	100(53.2%)	22(5.0%)	6(17.2%)	2(1.3%)
Analgesic only	4(2.4%) *	101(23.0%)	7(6.0%) *	59(38.8%)

Note: 1. Antibiotics: Amoxicillin, ciprofloxacin, metronidazole, clotrimazole, * referred to buy antimalarial in the local chemist due to stock-out in Public hospital and patients' affordability in a private hospital

Treatment among the of slide positive and slide negative patients within age groups

To determine the relationship between age and treatment, the treatment with antimalarial was observed to be high among children as compared to the adults in both the public and private hospitals. Among the slide positives, treatment with coartem was high among the 5-15 years old at 75.9% (88/116). Antibiotic treatment among children <5 years at 22.0% (9/41). Treatment with analgesics alone among individuals >15 years old was 11.0% (11/100)(Table 5). Of the slide negative patients, antimalarial treatment among the 5-15 years old was 9.9% (15/152). Antimalarial plus antibiotic treatment among the >=15 years old was 3.6% (16/450) (Table 5).

Table 7. Treatment within the age groups among the slide positive and slide negative patients

Parameters	<5		5 ~ 15		>=15	
Blood smear	+Ve	-Ve	+Ve	-Ve	+Ve	-Ve

Treatment	n=41	n=77	n=116	n=152	n=100	n=450
Coartem + analgesic	30(73.2%)	4(5.2%)	88(75.9%)	15(9.9%)	70(70.0%)	26(5.8%)
Antibiotics + analgesic	1(2.4%)	47(61.0%)	1(0.9%)	76(50.6%)	1(1.0%)	188(41.8%)
Coartem + antibiotics + analgesic	9(22.0%)	2(2.6%)	20(17.2%)	2(1.3%)	18(18.0%)	16(3.6%)
Analgesic only	1(2.4%)*	24(31.2%)	7(6.0%)*	59(38.8%)	11(11.0%)*	220(48.9%)

Note: 2. Antibiotics: Amoxicillin, ciprofloxacin, metronidazole, clotrimazole, * referred to buy antimalarial in the local chemist due to stock-out in the hospital

Sensitivity and specificity of hospital microscopy using PCR and independent microcopy as standards

To determine the sensitivity and specificity of hospital microscopy, independent microscopy and PCR were used as standards on 276 samples out of 936 samples. The slide positivity rate for hospital microscopy was 26.1%, and 37.3% for independent microscopy. PCR positivity rate was 58.7%. Using qPCR as standard, the hospital microscopy sensitivity was at 38.3% and specificity at 91.2%. Using independent microscopy as standard, the hospital microscopy sensitivity was at 47.6% and specificity at 86.7%. The inter-reliability reading between qPCR and hospital microscopy showed a low level of agreement (Kappa = 0.26) while the inter-reliability reading between hospital microscopy and independent microscopy showed a fair level of agreement (Kappa = 0.41; $P < 0.0001$) (Table 6). The hospital diagnosis was very specific but sensitivity was not very high

Table 8. Sensitivity and Specificity of Hospital microscopy with Independent microscopy and PCR as standards

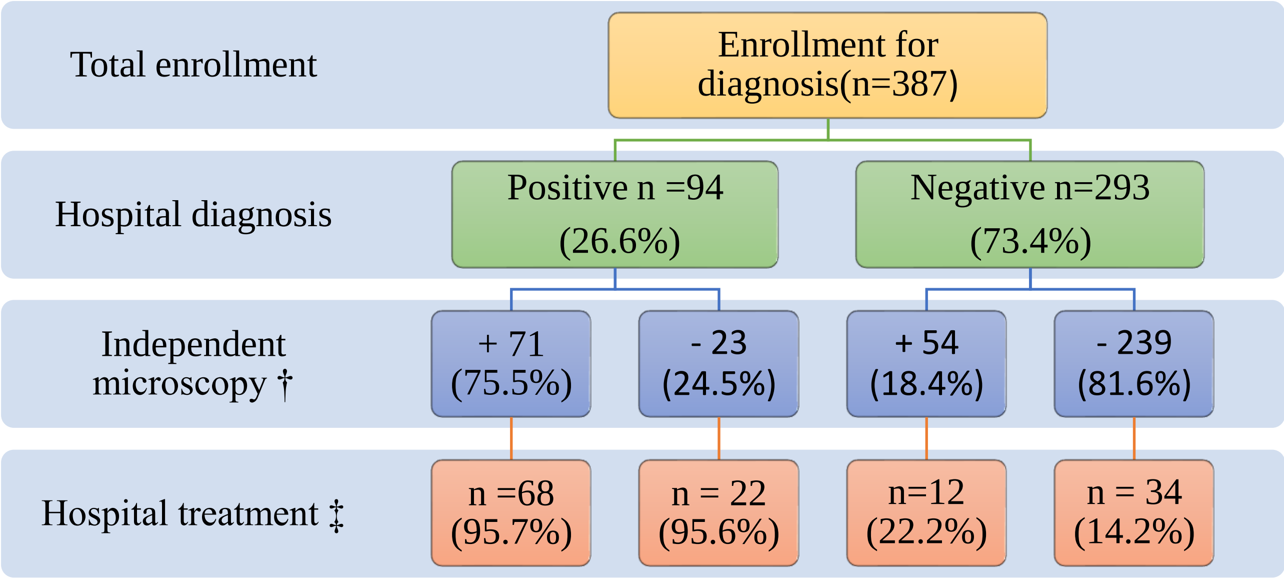
Hospital Microscopy	Independent Microscopy	qPCR

	Positive	Negative	Positive	Negative
Positive	49	23	62	10
Negative	54	150	100	104
Sensitivity (%, 95%CI)	47.6 (38.2, 57.1)		38.3 (31.1, 45.9)	
Specificity (%, 95% CI)	86.7 (80.8, 91.0)		91.2 (84.6, 95.2)	
Positive Predictive Value (%, 95%CI)	68.1 (56.6, 77.7)		86.1 (76.3, 92.3)	
Negative Predictive Value (%, 95%CI)	73.5 (67.1, 79.1)		51.0 (44.2, 57.8)	
Diagnostic Accuracy (%, 95%CI)	72.1 (66.4, 77.3)		60.1 (54.1, 66.0)	
Agreement (Cohen's kappa)	0.37 (0.25, 0.48)		26.0 (0.18, 0.35)	

Note: 3. Total sample size: N=276; 2. The slide positivity rate for hospital microscopy was 26.1%, and 37.3% for independent microscopy. PCR positivity rate was 58.7%.

The discrepancy in slide reading and malaria treatment

Out of the 936 blood smears taken from febrile cases, follow-up analysis was done on 387 samples to determine the misdiagnosis of hospital microscopy and antimalarial treatment (Figure 4). The misdiagnosis was at 19.9% (77/387). Of the 77 misdiagnosed cases, PCR confirmed 4/23 as false positive and 37/54 as false negative. Among the 94 slide positive patients, 4.5% did not receive treatment due to stock-out of drugs and were referred to buy in chemists and affordability especially in the private hospitals. Among the slide negative patients, 15.7% (46/293) were treated with Coartem. Of the 293 true negative patients, 34 were treated with an antimalarial. However, PCR confirmed that 19 out of the 34 treated with antimalarial had malaria parasite. Therefore, about 5.1% (15/293) were over treated.



† + positive, - negative,

‡ All treatments were coartem

Figure 5. The discrepancy in slide reading and misdiagnosis at the health facilities

Malaria parasite positivity by RDT and microscopy

Out of the 1131 febrile cases, 126 samples were examined with malaria Rapid Diagnostic Test in Public hospital 2, and slides examined by independent microscopists: The RDT parasite positivity was at 48.4% (61/126) while independent microscopy was 41.3% (52/126). Using RDT as standard, independent microscopy sensitivity was at 73.8% and specificity at 89.2%. The inter-reliability reading between RDT and project microscopy showed a moderate level of agreement (Kappa = 0.633; *P*=0.00). All the 61 RDT positive patients were treated with antimalarial treatment. Power outage at the health facility and absence of lab technician during the survey resulted in 126 cases being diagnosed with RDTs.

Presumptive clinical diagnosis

Out of the 1131 febrile cases, 69 patients were clinically diagnosed. The presumptive treatment happened during a period of labor disputes and strikes that resulted in absence of laboratory technicians on duty. The clinical diagnosis was based on active fever and the malaria symptoms the patients were presenting; headache, fever, joint pains, and vomiting.

Out of the total 69 presumptive clinical diagnoses, 55 blood slides samples were taken and examined by independent microscopy to determine whether these presumptively treated patients had malaria infection and whether they were prescribed antimalarial. The presumptive treatment was at 11.3% (55/488) with about 63.6% of the cases treated with antimalarial without a confirmed diagnostic test. The clinician's decision to treat with antimalarial was based on active fever and malaria-like symptoms the patients had. About 14/55 patients were slide positive with only 9/14 of them correctly treated with antimalarial. Of the 55 clinically diagnosed patients 74.5% (41/55) were slide negative. Of the 41 slide negative patients, 63.4% were over treated with Coartem. Antibiotics treatment among the slide positive and slide negative patients was at 35.7% (5/14) and 15.0% (36.6%) respectively (Figure 5).

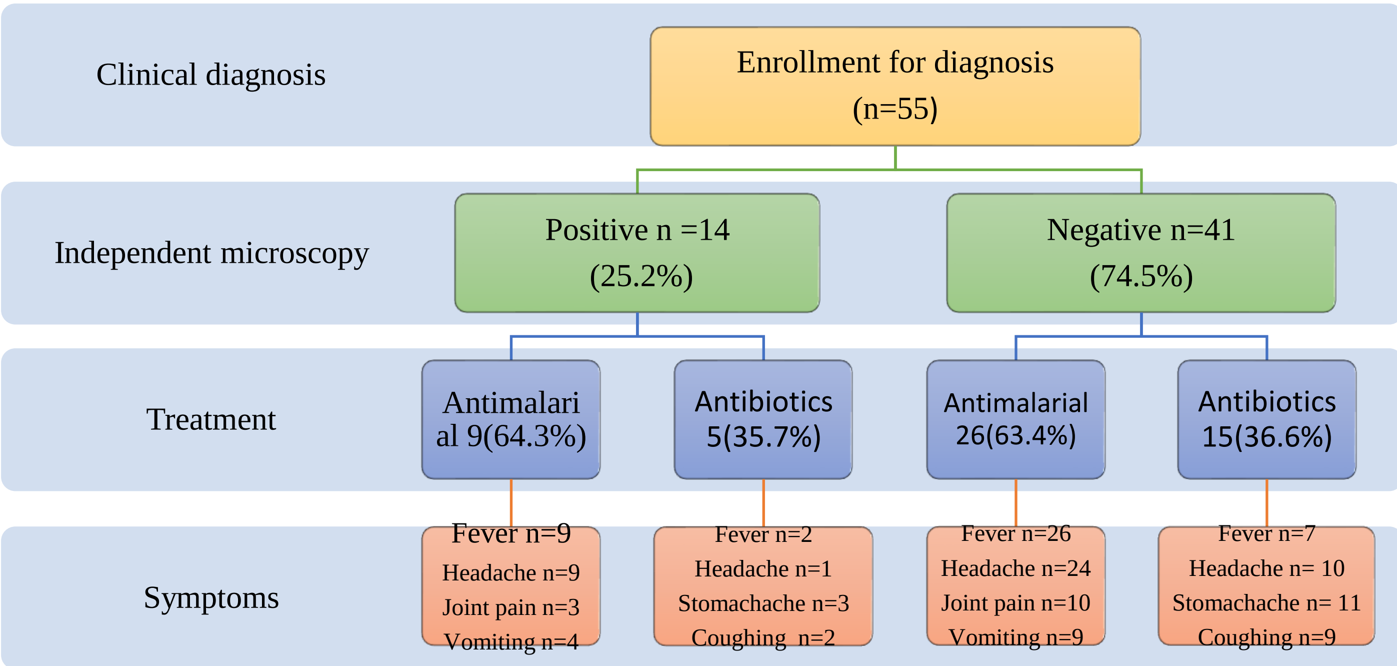


Figure 6. Presumptive treatment and symptoms

Discussion

The malaria burden in Kenya remains high despite increased efforts by the Ministry of Health to scale up intervention strategies(16–18). For effective malaria control, symptomatic cases should always be diagnosed accurately and appropriately treated as per the Kenya National Malaria Treatment Guidelines(13). The current study assessed the healthcare providers' practices in diagnosis and treatment of malaria in rural community. The overall hospital based malaria prevalence in the study area was 27.5% (N=936) with public hospitals having high slide positivity 29.8% (N=624) compared to the private hospital 22.8% (N=312). The public hospitals are government hospitals where antimalarial treatment is free and is accessible and accommodates any patients irrespective of their socio-economic status, unlike the private hospitals where patients from low socio-economic status cannot afford treatment. The study has highlighted that most of the patients who sought treatment in the private hospital 39.6% had college diploma and above. The higher slide positivity in the study areas is attributed to the hospital's catchment population mostly living near Lake Victoria. High malaria transmission along the lake shoreline has been reported in Malawi among residents living along the shorelines of Lake Malawi(4). The economic activity of the residents such as fishing at night and staying outdoor at night without exposes the residents to mosquito bites.

In this study, there was no significant difference in malaria prevalence between sexes and could be linked to the socio-economic activities that the males and the females are involved exposing themselves to equal exposure to mosquito bites. In contrast to the current study, previous studies have shown a significant difference in malaria infection by gender with contributing factor linked to bed net usage and gender-based differences in immune responses to malaria parasites infection where the males are prone to malaria infection due to the existing predisposing factors, hence more susceptible to malaria infection (19–23).The study showed a high infection at 43.3% among school-going children. The results are consistent with a study done in Malawi that found a prevalence of 31% among school-going children who may act as reservoirs of parasites that maintain malaria transmission (6). Similarly, a study in China found high malaria infection in symptomatic school-going children (19). A cross-sectional survey data extracted from the Kenya Malaria Indicator Survey (KMIS), 2015 observed malaria prevalence of 10.8% among the school-going children (24). Another study on the assessment of malaria in Africa found malaria prevalence to be higher among children aged 5–18 years (25). The high infection among the 5-15 years old children may be due to the low bednet usage among this age group(26). Among the adults, the fewer fever cases may be linked to protective immunity acquired with increasing age(27).

Malaria symptoms always mimic other disease conditions such as respiratory infections, typhoid, diarrhea, and HIV that occur in the same area. The study showed a significant difference in symptoms presented by the slide positive and slide negative patients ($P < 0.0001$) with 86.0% reporting fever, 82.1% headache, 47.1% joint pains, 34.2% URTI, and about 19.4% reporting chills/shivering (18.6%) among the slide positives and while 73.8% reporting fever, 57.3% headache, 23.0% joint pains, 35.2% URTI, and about 18.6% chills/shivering (18.6%) among slide negative patients. With other infection symptoms mimicking malaria symptoms, it is more likely for patients to be overtreatment or under treatment with antimalarial thereby increasing consumption of ACT. An understanding of the range of non-malarial infections is important to improve the management of infections of non-malarial illnesses. In Kenya, the parasitological diagnosis of malaria is recommended for all patients suspected of malaria and the government policy of treatment not be delayed or denied due to the inability to test for malaria(28,29).

Health-care providers in the current study were more likely to give antimalarial to slide negative children compared to adults. This is partly because children can easily develop severe malaria and become very sick compared to adults. At the time of the hospital visit, the parasitemia might still be below detection threshold levels hence undetected. The study area being malaria-endemic zone, about 10.0% of the slide negative patients were treated with antimalarial based on clinician assessment of febrile cases. Consistent with the present study, other studies have reported overtreatment with antimalarial among the slide negative patients (8,30,31). A study conducted in Homa Bay and Kisii counties in Kenya linked overtreatment with antimalarial to severe illness and high fever cases especially among children prompting treatment with antimalarial drugs despite the patients being malaria negative (32). Presumptive treatment is considered lifesaving in such circumstances (33). Under normal circumstances, the management of fever cases includes a diagnostic test for confirmation of the malaria infection followed by recommended treatment. Under the exceptional situations that may arise in the context of the COVID-19 pandemic, the need for presumptive diagnosis in the in the wake of the COVID-19 pandemic has been recommended by WHO(34).

Antimalarial stock out at the health facilities may also contribute to slide positive patients missing out antimalarial treatment due to affordability of medication especially in private health facilities. The current study reported under treatment with antimalarial at the health facilities with about 5.0% of patients from public hospitals and 15.0% from a private hospital. The private health facilities charge about $5.0 for malaria treatment which is largely unaffordable forcing the patients to buy drugs from local pharmacies. The diagnosis laboratory fee charged at the health facilities may also be a challenge to the resource-poor patients who may end up in self-treatment by buying drugs from the local drug shops. The first-line treatment, Coartem (AL) is widely available across drug-outlets in most rural areas with the cost of malaria treatment approximately $1.10 (35). Most of the hospital admission was in private hospital which is well equipped. Availability of drugs, confidence in the treatment, and the faster treatment services were the main reasons for the patients to seek treatment at the public hospital despite being costly while the treatment are fee in the government hospitals.

The current study has shown that hospital microscopy is very specific but the sensitivity is not very high compared to independent microscopy and PCR as standard controls. Low sensitivity of hospital microscopy has been reported in Ethiopia (23), Tanzania (36), and Cameroon (10). The inconsistency of laboratory technicians' availability at the health facilities and inadequate laboratory reagents. Power outage at the health facility and the absence of a laboratory technician during the survey resulted in the patients being diagnosed with RDTs.

Misdiagnosis and prescription of antimalarial medication to patients who are diagnosed negative for malaria at the health facilities is on the rise(8,30,31). The present study showed discrepancy of about 20% between the hospital microscopy and independent microscopy with the misdiagnosis confirmed by PCR. Even though study patients were true negative, they were treated with antimalarial based on clinical symptoms. It is necessary to confirm the slide negative patients with RDT before treatment with antimalarial as the RDT has higher sensitivity as the current study indicated. Therefore, the clinicians based on the clinical symptoms proceeded to diagnose the patients based on the clinical symptoms despite being blood smear negative. An overtreatment of about 6.0% among the slide negative individuals were reported in the study. Overtreatment as a result of misdiagnosis may result in increased cost of care, and drug wastage (8).

Kenya National Malaria Treatment Guidelines recommends parasitological confirmation of diagnosis on all suspected malaria cases before treatment with antimalarial(13). However, presumptive treatment based on clinical observation without laboratory confirmation is ongoing. About 11.0% of the patients in the correct study were clinically diagnosed without a confirmed laboratory test. The trend in presumptive clinical diagnosis has also been reported in other health facilities (8,11,31,37). The accuracy of the clinical diagnosis from the current study was low where about 63.4% of the patient presumptively treated with antimalarial were confirmed as slide negative by independent microscopy. Consistent with our study, a study conducted in Vihiga and Kakamega counties in Western Kenya reported about 36% of the individuals who were presumed positive for malaria by the hospital laboratory did not have the parasite(38). In Kisumu County, there are always labour disputes where health workers are always out on strike, hence the health facilities' commitment to clinical diagnosis is relaxed. The clinical diagnosis of 65 patients may partially be due to laxity by the laboratory staff, industrial unrest that resulted in clinicians going on strike during the study period, inadequate diagnostic reagents where in some cases, the laboratories lacked blood smear staining reagents for days, and affordability of laboratory tests by some patients. Mismanagement of drugs observed during labour disputes that resulted in the presumptive treatment should be avoided in the future to ensure all the fever patients are tested before treatment with antimalarial.

The impact of malaria intervention strategies depends on whether individuals with malaria can easily access and receive the right diagnosis and treatment at an affordable cost. The WHO initiative of T3: Test, Treat, Track urges malaria-endemic countries to test every malaria suspected case, to treat every confirmed case, and to track the disease through timely and accurate surveillance system (39). Our findings indicated that there is a high malaria transmission in Kisumu County, Kenya but also a high rate of misdiagnosis and presumptive treatment. There is a need for the County MOH to strengthen the health facilities' capacities and supervision towards full implementation of the MoH malaria treatment guidelines.

Conclusion

The result of this study showed that there is a high rate of misdiagnosis and overtreatment in public and private hospitals in Kisumu County, Kenya. The hospital's diagnosis is very specific but the sensitivity is not very high. Clinical diagnosis and wrong treatment were due to lack of adequate personnel in the laboratory, lack of laboratory reagents, and affordability of diagnostic tests by the patients. Misdiagnosis and overtreatment remain a major concern in local health facilities which results in the over-use of antimalarial and antibiotics. It is recommended that the government invest more in technical training to strengthen malaria diagnosis capabilities and motivation of laboratory technicians for an effective malaria case management program.

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Conflict of interest

The authors declare that they have no competing interests

References

1. WHO. World malaria report (2020): 20 years of global progress and challenges. Vol. WHO/HTM/GM, World Health Organization. 2020.

2. National Malaria Control Programme (NMCP), Kenya National Bureau of Statistics

(KNBS) and II. Kenya Malaria Indicator Survey 2015. Nairobi, Kenya, and Rockville, Maryland, USA: NMCP, KNBS, and ICF International; 2016.

3. Guerra M, Sousa B De, Mabale NN, Berzosa P, Arez AP (2018). Malaria determining risk factors at the household level in two rural villages of mainland Equatorial Guinea. Malar J [Internet]. 2018;17:203. Available from: https://doi.org/10.1186/s12936-018-2354-x

4. Hajison PL, Feresu SA, Mwakikunga BW. (2018 Malaria in children under-five : A comparison of risk factors in lakeshore and highland areas , Zomba district , Malawi. PLoS One. 2018;13(11):e0207207.

5. Kwenti TE, Tayong Dizzle Bita Kwenti AL, Njunda LA, Theresa Nkuo-Akenji (2017). Epidemiological and clinical profile of paediatric malaria: A cross sectional study performed on febrile children in five epidemiological strata of malaria in Cameroon. BMC Infect Dis. 2017;17(1):1–13.

6. Walldorf JA, Cohee LM, Coalson JE, Bauleni A, Nkanaunena K, Kapito-Tembo A, et al. (2015) School-age children are a reservoir of malaria infection in Malawi. PLoS One. 2015;10(7):1–13.

7. WHO.(2015 Guidlines for treatment of malaria. Vol. 35, World Health Organization. 2015.

8. Nyaoke BA, Mureithi MW, Beynon C. (2019) Factors associated with treatment type of non-malarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya. PLoS One. 2019;14(6):1–16.

9. Siahaan L, Panggabean M, Panggabean YC (2018). The comparison of detection methods of asymptomatic malaria in hypoendemic areas. IOP Conf Ser Earth Environ

Sci. 2018;125(1):012019.

10. Mfuh KO, Achonduh-Atijegbe OA, Bekindaka ON, Esemu LF, Mbakop CD, Gandhi K, et al.(2019) A comparison of thick-film microscopy, rapid diagnostic test, and polymerase chain reaction for accurate diagnosis of Plasmodium falciparum malaria. Malar J. 2019;18(1):1–8.

11. Amir A, Cheong FW, De Silva JR, Lau YL.(2018) Diagnostic tools in childhood malaria. Parasites and Vectors. 2018;11(1):1–12.

12. Graz B, Willcox M, Szeless T, Rougemont A. (2011) “ Test and treat ” or presumptive treatment for malaria in high transmission situations ? A reflection on the latest WHO guidelines. Malar J. 2011;10:136.

13. National Malaria Control Programme Ministry of Health (2016). National Guidelines for the diagnosis, treatment and prevention of malaria in Kenya. 2016.

14. WHO.(2014) Technical consultation to update the WHO Malaria microscopy quality assurance manual. WHO. 2014;(3):1–32.

15. Kenya National Bureau of Statistics. (2019) 2019 Kenya Population and Housing Census Volume 1: Population by County and Sub-County. 2019 Kenya Popul Hous Census [Internet]. 2019;I(November):49. Available from: https://www.knbs.or.ke/?wpdmpro=2019-kenya-population-and-housing-census-volume-i-population-by-county-and-sub-county

16. National Malaria Control Programme Ministry of Health.(2016) The Kenya Malaria Communication Strategy 2016–2021. 2016.

17. National Malaria Control Programme Ministry of Health(2019. Kenya Malaria Programme Review 2018. 2019.

18. National Malaria Control Programme Ministry of Health.(2018) The Kenya Malaria Strategy 2019-2023. 2019.

19. Zhao Y, Zeng J, Zhao Y, Liu Q, He Y, Zhang J, et al.(2018) Risk factors for asymptomatic malaria infections from seasonal cross-sectional surveys along the China-Myanmar border. Malar J [Internet]. 2018;17(1):1–13. Available from: https://doi.org/10.1186/s12936-018-2398-y

20. Buchwald AG, Sixpence A, Chimenya M, Damson M, Sorkin JD, Wilson ML, et al. (2019) Clinical implications of asymptomatic plasmodium falciparum infections in Malawi. Clin Infect Dis. 2019;68(1):106–12.

21. Cohee L, Laufer M. Tackling malaria transmission in sub-Saharan Africa. (2018) Lancet Glob Heal [Internet]. 2018;6(6):e598–9. Available from: http://dx.doi.org/10.1016/S2214-109X(18)30197-9

22. Odhiambo F, Atieli H, Njoroge S, Sang D (2019). Assessment of Factors Influencing Adherence to Malaria Microscopy Diagnosis in the Treatment of Out-patients at Kisumu County Referral Hospital in Kenya. J Adv Med Med Res. 2019;29(12):1–10.

23. Assefa A, Ahmed AA, Deressa W, Wilson GG, Kebede A, Mohammed H, et al. (2020) Assessment of subpatent Plasmodium infection in northwestern Ethiopia. Malar J [Internet]. 2020;19:108. Available from: https://doi.org/10.1186/s12936-020-03177-w

24. Sultana M, Sheikh N, Mahumud RA, Jahir T, Islam Z, Sarker AR. Prevalence and associated determinants of malaria parasites among Kenyan children (2017). Trop Med Health. 2017;45(1):1–9.

25. Nankabirwa J, Brooker SJ, Clarke SE, Fernando D, Gitonga CW, Schellenberg D, et al. (2014) Malaria in school-age children in Africa: An increasingly important challenge.

Trop Med Int Heal. 2014;19(11):1294–309.

26. Coalson JE, Cohee LM, Buchwald AG, Nyambalo A, Kubale J, Seydel KB, et al. (2018) Simulation models predict that school-age children are responsible for most human-to-mosquito Plasmodium falciparum transmission in southern Malawi. Malar J [Internet]. 2018;17(1):1–12. Available from: https://doi.org/10.1186/s12936-018-2295-4

27. Jenkins R, Omollo R, Ongecha M, Sifuna P, Othieno C, Ongeri L, et al. (2015) Prevalence of malaria parasites in adults and its determinants in malaria endemic area of Kisumu County, Kenya. Malar J. 2015;14(1).

28. Maina JK, Macharia PM, Ouma PO. (2017)Coverage of routine reporting on malaria parasitological testing in Kenya , 2015 – 2016. Glob Health Action [Internet]. 2017;10(1). Available from: https://doi.org/10.1080/16549716.2017.1413266

29. Ministry of Health K. (2017) Monitoring the Quality of Antimalarial Medicines Circulating in Kenya : Round 6. 2017.

30. Smith S, Koech R, Nzorubara D, Otieno M, Wong L, Bhat G, et al. (2019) Connected diagnostics : linking digital rapid diagnostic tests and mobile health wallets to diagnose and treat brucellosis in Samburu , Kenya. 2019;4:1–12.

31. Kiemde F, Tahita MC, Lompo P, Rouamba T, Some AM, Tinto H, et al. (2018) Treatable causes of fever among children under five years in a seasonal malaria transmission area in Burkina Faso. Infect Dis Poverty. 2018;7(1):1–10.

32. Onchiri FM, Pavlinac PB, Singa BO, Naulikha JM, Odundo EA, Farquhar C, et al. (2015) Frequency and correlates of malaria over-treatment in areas of differing malaria transmission : a cross-sectional study in rural Western Kenya. Malar J. 2015;14:97.

33. Chukwuocha UM OC, Ukaga CN NG. (2015) Presumptive Diagnosis and Treatment of

Malaria in Febrile Children in Parts of South Eastern Nigeria. J Infect Dis Ther. 2015;03(05).

34. WHO. (2020) Tailoring malaria interventions in the COVID-19 response [Internet]. 2020. Available from: https://www.who.int/malaria/publications/atoz/tailoring-malaria-interventions-covid-19.pdf?ua=1

35. Kioko U, Riley C, Dellicour S, Were V, Ouma P, Gutman J, et al. (2016) A cross - sectional study of the availability and price of anti - malarial medicines and malaria rapid diagnostic tests in private sector retail drug outlets in rural Western Kenya , 2013. Malar J. 2016;15:359.

36. Mahende C, Ngasala B, Lusingu J, Yong TS, Lushino P, Lemnge M, et al. (2016) Performance of rapid diagnostic test, blood-film microscopy and PCR for the diagnosis of malaria infection among febrile children from Korogwe District, Tanzania. Malar J. 2016;15(1):1–7.

37. Aiyenigba B, Ojo A, Aisiri A, Uzim J, Adeusi O, Mwenesi H. (2017) Immediate assessment of performance of medical laboratory scientists following a 10-day malaria microscopy training programme in Nigeria. Glob Heal Res Policy. 2017;2:1–7.

38. Afrane Y, Zhou G, Githeko A, Yan G. (2013) Utility of Health Facility-based Malaria Data for Malaria Surveillance. PLoS One. 2013;8(2).

39. WHO. (2012) Test. Treat. Track. Scaling up diagnostic testing , treatment and surveillance for malaria. World Heal Organ. 2012;