Human genetic determinants of severe malaria in Burkina Faso (BF)

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About this study

Severe malaria is a life-threatening disease that kills over a million individuals every year, with 90% of the deaths occurring in sub-Sahara African children under the age of five (Greenwood B and Mutabingwa T, 2002). People living in an endemic area often become infected with *Plasmodium falciparum* malaria during childhood, but a small proportion of children experience severe complications, the clinical outcome depending on many factors, including the genetic make-up of the human host (Verra F, Mangano VD and Modiano D, 2009).

Genetic association studies can inform us on mechanisms of protective immunity against malaria at every stage of infection as well as on mechanisms of pathogenesis. The understanding of which is crucial for the development of effective vaccines and therapeutic measures.

Summary

In order to investigate the human genetic determinants of severe malaria in Burkina Faso, we set up a retrospective case-control study including children experiencing severe malaria (cases), children with an attack of uncomplicated malaria (mild malaria controls) and children with no signs and symptoms of malaria or other diseases (healthy controls).

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic

countries. As part of the sample handling process by the MalariaGEN Resource Centre, baseline genotyping data has was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis. Candidate genes include loci encoding erythrocyte factors (e.g. HBB, ABO, G6PD) as well as immunological factors (e.g. TNFA, TLR4, NOS2A) and factors involved in cyto-adherence (e.g. CD36, ICAM1).

Study site description

The University of Rome together with Centre National de Recherche et de Formation sur le Paludisme and Centre Médicale St. Camille recruited malaria patients and healthy children from the capital of Burkina Faso, Ouagadougou, and surrounding rural zones in the province of Bazega, district of Sapone.

The study area lies in a plateau characterised by shrubby savannah vegetation. While in the city the type of occupation is diverse, in rural zones the main occupation is subsistence farming. Most people live in compounds housing multiple nuclear families often closely related. The healthcare system at the district level consists of 14 community clinics (CSPS) with a dispensary and a maternity unit, which among other activities provide EPI vaccination and malaria treatment. A medical centre with a surgical unit (CMA) is located in Sapone; the first line of referral. The highest levels of referral are sent to the national hospital (CHN) Yalgado Ouédraogo in Ouagadougou. The population of Ouagadougou is estimated around 1 million and that of the district of Sapone around 90,000 people. The great majority of the population belongs to the Mossi ethnic group.

The climate in this area is characteristic of the Sudanese savannah, with a dry season from November to May and a rainy season from June to October. Malaria is endemic with a marked seasonal pattern characterised by high transmission during the rainy season and low transmission during the dry season. Differences exist in malaria transmission levels between urban and rural areas: entomological inoculation rates vary from 1 to 10 per person per year in urban areas of Ouagadougou, and from 50 to 500 in the surrounding rural zones. *P. falciparum* is the predominant malaria parasite, accounting for more than 95% of infections in children under 5 years of age. The main malaria vectors are *Anopheles gambiae*, *A. arabiensis*, and *A. funestus*. The use of insecticide-treated nets was uncommon at the time of the study (about 1%) and the use of indoor residual spraying was non-existent with malaria control relying mainly on the treatment of clinical cases (Nebie I *et al*, 2008).

Methods

An unmatched case-control study was conducted. Severe malaria cases and mild malaria controls were children (aged 1 month – 15 years) of Mossi ethnicity, admitted to hospital with signs of severe and uncomplicated malaria, respectively. The children were recruited from patients admitted to the paediatric ward of three hospitals in Ouagadougou (Centre Hospitalier Universitaire Yalgado Ouédraogo, Centre Médical Paul VI and Centre Médical Saint Camille) during the high malaria transmission seasons of 1993-94.

The criteria for inclusion followed the definitions stated by the World Health Organization (WHO). Severe malaria was defined by the presence of *P. falciparum* in the thick blood film associated with at least one of the following conditions: prostration (incapacity of child to sit without help in absence of coma), unrousable coma (Blantyre coma score ≤ 2), repeated generalised convulsions (more than two episodes in the preceding 24 hr), severe anaemia (haemoglobin <5 g/dl), hypoglycemia (<40 mg/dl), pulmonary edema/respiratory distress, spontaneous bleeding, and renal failure (plasma creatinine >3 mg/dl). Children with other detectable infections were not included in the study. Uncomplicated malaria was defined as a clinical illness characterised by an axillary temperature >37.5°C associated with a *P. falciparum*-positive thick blood film. Patients were treated according to WHO guidelines with a complete regimen of drugs that were provided free of charge as part of the study (Modiano D *et al*, 1998; Modiano D *et al*, 2001).

Healthy controls were children (aged 0-6 years) of Mossi ethnicity and no signs of severe or uncomplicated malaria or other diseases. They were recruited from rural villages 50 km south-west of Ouagadougou (district of Sapone, province of Bazega) during a cross-sectional survey conducted in August 2004. Children presenting with any clinical sign of malaria following direct physical examination were excluded from the study.

A standardised case report form (CRF) was created for the MalariaGEN Consortial Project 1 (CP1) and used by all sites to collect standardised clinical data. The data collected in Burkina Faso (and all other sites) was uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood at the University of Rome La Sapienza using the Qiagen DNeasy Blood kits (http://www.qiagen.com/) [Qiagen, Crawley, UK] and Nucleon™ BACC2 Genomic DNA extraction kit (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1. Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 983 420 severe malaria 563 uncomplicated malaria	Male: 532 (54) Female: 415 (42) Not recorded: 34 (4)	<1: 68 (7) 1-2: 189 (19) 2-5: 447 (45) 5-15: 273 (28) Not recorded: 6 (1)	Mossi: 983 (100)
Healthy controls: 816	Male: 407 (50) Female: 391 (48) Not recorded: 18 (2)	<1: 161 (20) 1-2: 197 (24) 2-5: 453 (56) 5-15: 5 (<1)	Mossi: 816 (100)

Ethics

The study was approved by the Health Research Ethical Committee of the Ministry of Health of Burkina Faso (proposal number: ID No 2007-048) and by the Ethical Committee of the University of Oxford.

For the recruitment of children with severe and uncomplicated malaria, informed consent was obtained from parents or legal guardians after admission to hospital. For the recruitment of healthy children informed consent was obtained prior to enrolment from a parent or legal guardian of each participating child.

Additional contributors

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The recruitment of children with severe and uncomplicated malaria was sponsored by the Italian Cooperation Programme in Burkina Faso and was supported by the World Health Organization, Division of Control of Tropical Diseases, and by the Fondazione Pasteur-Istituto Cenci Bolognetti of the University of Rome La Sapienza. The recruitment of healthy children was supported by Centre National de Recherche et de Formation sur le Paludisme (CNRFP).

Human genetic determinants of severe malaria in three regions of Cameroon (CM)

Key People

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About this study

Malaria remains one of the most widespread infectious diseases of humankind, causing debilitating illness in hundreds of millions of people, particularly in sub-Saharan African children under five years of age. The susceptibility of human populations to severe *Plasmodium falciparum* malaria has been associated with variations in more than 30 genes (Sinha S *et al*, 2008), some of which have exhibited differential association in distinct populations of the world (Serghides L *et al*, 2003).

Carriers of the sickle-cell trait (haemoglobin [Hb] type AS), have been shown to be less likely to experience severe, potentially fatal, malaria (Aidoo M *et al*, 2002). Studies by Mackinnon and colleagues in Kenya suggest that human genetics explain 25% of the variation in susceptibility to and manifestation of malaria but that HbS explains only 2% of the total variation (Mackinnon MJ *et al*, 2005). This implies the existence of many unknown protective genes, notably single nucleotide polymorphisms (SNPs), each individually having small population effects.

Summary

In order to understand the mechanisms of pathogenesis and protective immunity against malaria and inform future vaccine and anti-malaria therapies, we designed large case-control studies of severe malaria in children living in the Centre, Littoral and South West regions of Cameroon. Two studies were conducted; the first study between 2003/05 and the second in 2007/08. These studies aim to identify human genetic determinants of severe malaria and the sub-phenotypes cerebral malaria and/or severe malarial anaemia, hyperparasitaemia and respiratory distress as well as mild/uncomplicated malaria and anaemia.

Severe malaria cases were recruited from the paediatric wards of eight health facilities in Buea, Douala, Limbe and Yaounde. Most of the chosen health facilities were the main government institutions in the selected towns, also receiving patients from surrounding areas. Cases consisted of children (aged 1 month - 17 years) with cerebral malaria (CM) and/or severe malarial anaemia (SMA) or uncomplicated/mild malaria recruited according

to WHO criteria. Two control categories were used; school children (afebrile and free from any obvious illness) and blood bank donors (asymptomatic, from the community).

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis.

Single- and multi-locus analysis will be conducted using various multivariate logistic regression models to assess the relationship between these polymorphisms and well defined clinical phenotypes. Phenotypes that could potentially be tested include severe malaria and sub-phenotypes such as cerebral malaria, severe malarial anaemia, hyperparasitaemia and respiratory distress.

Study site description

This study was conducted in four towns across three regions of Cameroon, namely: Yaounde in the Central region, Douala in the Littoral region and Buea and Limbe in the South Western region. The study sites included hospitals (Bota District Hospital - Limbe, Laquintinie Hospital - Douala, Mother and Child Hospital - Yaounde, Regional Hospital - Limbe and Regional Hospital Annex - Buea) and health centres (Bokova Health Centre, Mount Mary Health Centre - Buea and PMI Down Beach - Limbe). Except for Mount Mary, the chosen health facilities were the main government institutions in the selected towns, also receiving patients from surrounding areas. Controls were recruited from primary schools which included: Catholic School (CS) Buea Station, CS Great Soppo, CS Muea, Government School (GS) Bolifamba, GS Bonduma, Government Practising School (GPS) Molyko I and II, GPS Muea I and II, HOTPEC Primary School Mile 15 Buea, Oxford Primary School Muea and Government Bilingual Primary School Muea.

Although malaria is endemic throughout Cameroon, the country has very different geographical and epidemiologic strata that may alter the course of the infection. In general, malaria transmission is intense and perennial in the Central, Littoral (Coastal) and South Western regions, with peak periods corresponding to the rainy seasons (Mackinnon MJ *et al*, 2005). Furthermore, Bolifamba, Molyko and Muea are located at low altitude (400–650 m above sea level) while Bonduma, Great Soppo and Buea Town are situated at high altitude (900–1000 m above sea level).

The central region (Yaounde) is located within the rainforest belt of central Africa and has the Guinea-type equatorial climate. This is characterised by fairly constant temperatures (ranging from 17- 30oC [mean = 23.1oC]) (Manga L *et al*, 1997), abundant rainfall (1,500–2,000 mm), an average relative humidity index ranging from 85% to 90%, and four distinct seasons: two rainy seasons (March–May/June and September–November) and two dry seasons (December–February and June/July–August). Maximal transmission of malaria occurs during and immediately following the two rainy seasons (Quakyi IA *et al*, 2000). The

Mother and Child Hospital is a referral hospital for children and mothers, located in the heart of the city of Yaounde. It also attracts patients from neighbouring villages such as Simbok and Etoa that are stable, rural, farming communities with fields irrigated by water from the Mefou and Biyeme Rivers (Quakyi IA *et al*, 2000). Inhabitants of this region are of the Ewondo tribe and part of the Bantu ethnic group.

The South Western and Littoral regions have a Cameroonian-type equatorial climate characterized by fairly constant temperatures and two seasons: a short dry season (November–March) and a long rainy season (March-November) with abundant precipitation (2,000–10,000 mm) (Manga L *et al*, 1997). In the Mt Cameroon region of the South West, the mean annual rainfall is 2625 mm, relative humidity is constantly high (75%–80%) and the temperature varies from 18oC in August to 35oC in March (Wanji, S *et al*, 2003). Human malaria is meso-endemic during the dry season but becomes hyper-endemic in the rainy season, with incidence peaking in July–October (Achidi EA *et al*, 2008). The prevalence of malaria parasitaemia in the low-altitude areas range from 30% in the dry season to 65% in the rainy season. *P. falciparum* accounts for up to 96% of malaria infections in this area, with Anopheles gambiae s. s. the dominant vector (Wanji, S *et al*, 2003). The great majority of the population in these regions belong to the Bantu and Semi-Bantu ethnic groups.

Methods

An unmatched case-control study was conducted between 2003/05 and 2007/08. The children were recruited from nine hospitals and health centres in Cameroon. Seven were in the South-West Region (e.g. the Mt Mary Hospital Buea and Bota district Hospital Limbe), one in the Littoral Region (Hospital Laquintinie de Douala) and one in the Cental Region (Mother and Child Hospital - Yaounde).

Cases included febrile children (aged 1 month-17 years) with an axillary temperature ≥ 37.5oC (measured within 24hrs of admission), with symptomatic malaria parasitaemia infection and without any other disease with signs/symptoms similar to malaria.

The criteria for diagnosis of severe malaria were those included in the standard WHO definition for 2000 i.e. the presence of asexual parasitaemia and at least one of the following: cerebral malaria (impaired consciousness or unarousable coma [(Blantyre coma score ≤2, corrected for hypoglycaemia (blood glucose <2.2 mmol/l or <40 mg/dl)] and no record of recent severe head trauma, neurological disease or any other cause of coma), severe malarial anaemia (haemoglobin <5g/dl (or haematocrit <15%), be fully conscious, no cases of severe bleeding or observed convulsions), convulsions before/during admission, respiratory distress (presence of alar flaring, intercostals or subcostal chest recession, use of accessory muscles of respiration, or abnormally deep respiration), hypoglycaemia (blood glucose <2.2mmol/l); hyperpyrexia (axillary temperature ≥40oC), hyperparasitaemia (>250,000 parasites/µl) as well as uncomplicated malaria (fully conscious with haemoglobin ≥8g/dl and no signs of severity and/or evidence of vital organ dysfunction).

Controls consist of apparently healthy children (aged 1-14 years, afebrile and free from any obvious illness, though a fraction had asymptomatic parasitaemia) and asymptomatic adults (aged 17-52 years from the community). Children were recruited from primary schools

located in the South-West Region of Cameroon (Buea Metropolis) between 2004-2005 and 2007-2008. Children with parasitaemia and a temperature of 37.5°C or above were not recruited as controls. Adults were identified from a blood bank in the Centre region of Cameroon (Mother and Child Hospital - Yaounde) between July and August 2007.

A standard case report form (CRF), created by MalariaGEN was used to collect standardised clinical data. The relevant data fields as per the MalariaGEN CP1 CRF were extracted from CRFs of the studies conducted prior to MalariaGEN (ie 2003/05 study conducted in south western region of Cameroon). The data collected in Cameroon (and all other sites) were uploaded onto the MalariaGEN central repository via secure web-based software, Topheno, developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood or packed cells at the Malaria Research Laboratory, University of Buea using the Promega Wizard (Promega Corporation, Madison, USA) or Nucleon™ BACC Genomic DNA Extraction (Gen-Probe Life Sciences, Manchester, UK) kits using manufacturer's instructions and quantified. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 914	Male: 430 (47) Female: 389 (42) Not recorded: 97 (11)	<1: 179 (19) 1-2: 220 (24) 2-5: 280 (31) 5-15: 135 (15) >15: 1 (<1) Not recorded: 99 (11)	Bantu: 348 (38) Semi-Bantu: 384 (42) Other: 102 (11) Not recorded: 80 (9)

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Healthy controls: 914	Male: 592 (65) Female: 305 (33) Not recorded: 17 (2)	<1: 0 (0) 1-2: 2 (>1) 2-5: 66 (7) 5-15: 391 (43) >15: 372 (41) Not recorded: 83 (9)	Bantu: 377 (41) Semi-Bantu: 395 (42) Other: 62 (7) Not recorded: 96 (10)

Ethics

This study was reviewed by the scientific and ethical review board of the University of Buea (proposal number: ID D7.1.A/MPH/SWP/PDPH/PS.CH/2340/811) and the South West Regional Delegation of Public Health. Authorization to conduct the surveys in primary schools was obtained from the Regional Delegation of Basic Education or the Catholic Education Secretariat.

For the cases and blood bank donors, informed consent was obtained from each case or their caregiver following a clear explanation of the content of the information sheet. Only subjects/caregivers who returned signed consent forms were enrolled into the study.

Written informed consent was obtained from the parents/guardians of healthy school children. Information sheets and consent forms were sent to parents/guardians of each child through head teachers in the schools and only those who returned signed consent forms were enrolled into the study.

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Human genetic determinants of severe malaria in the Kassena-Nankana District of Northern Ghana (GH)

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About this study

Malaria, caused by the parasite Plasmodium falciparum, remains the most important disease in sub-Saharan Africa and a leading cause of global morbidity and mortality. As part of an effort to characterise the Kassena-Nankana District (KND) of Northern Ghana for future vaccine trials, we set up a large, frequency-matched, case-control study of severe malaria.

A number of malaria candidate genes, and more recently genome-wide association studies, have identified genomic loci associated with severe P. falciparum malaria disease. Notable among these is the protection conferred by the sickle-cell locus (HbS) in the beta-globin gene (Jallow M et al, 2009). Though Mackinnon and colleagues in Kenya estimated that host genetic factors contribute about a quarter of total malaria protection, only 2% of the total can be attributed to HbS (Mackinnon MJ et al, 2005), the ability of these genotypes to censor severe disease remains a strong component of the molecular mechanisms underpinning host response to malaria disease.

Summary

A frequency-matched and an individually-matched case-control study were conducted in the Kassena-Nankana District between 2002/04, and 2007/08, respectively, with the aim of identifying human genetic determinants of resistance to severe malaria in Northern Ghana. Severe malaria cases were recruited from the paediatric ward of the Navrongo War Memorial Hospital (NWMH), which serves the health needs of the Kassena-Nankana District population. Cases consisted of children (aged 6-60 months) who were diagnosed with severe malaria (SM). Criteria for diagnosis of SM were those included in the standard WHO

definition. Cases were matched to controls on age category, sex, ethnicity and location. Two control categories were used; mild malaria and healthy (asymptomatic, community) controls. Two controls were recruited per case for each category of controls. The Navrongo Health and Demographic Surveillance System keeps vital records of the KND population and played a central role in the location and recruitment of community controls. Mild malaria controls were recruited from four out-patient clinics, including the out-patient department of the NWMH.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 10 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis. Single- and multi-locus analysis will be conducted using various multivariate logistic regression models to assess the relationship between these polymorphisms and well defined clinical phenotypes. Phenotypes that could potentially be tested include severe malaria and sub-phenotypes such as severe malarial anaemia, hyperlactatemia and respiratory distress.

Study site description

Kassena-Nankana District, Ghana

The studies were conducted in the Kassena-Nankana District of the Upper East region of Northern Ghana. The district covers an area of 1675Km2 of Sahelian savannah along the Ghana-Burkina Faso border. The population is estimated at 152,000 inhabitants, the majority of whom are engaged in subsistence farming. The major ethnic groups in the area are the Kassem and the Nankam with a minority of Buli. Most people live in traditional family housing units called compounds that typically house multi-families (different households) of common ancestry.

Annual rainfall averages 850mm, almost all of which occurs in the wet months of July - November with the rest of the year being relatively dry. One of the main features of the area is a large reservoir in the district, which provides water for irrigation throughout the year. Malaria epidemiology has been extensively characterized in this part of Northern Ghana (Owusu-Agyei S et al, 2001; Owusu-Agyei S et al, 2002; Koram KA et al, 2000; Koram KA et al, 2003; Oduro AR et al, 2007). Malaria transmission in the KND is stable but with a marked seasonality in intensity that coincides with the months of rainfall. The overall entomological inoculation rate at the time of these studies was estimated to be >400 infective bites/person-year (Appawu M et al, 2004), but current estimates are around 150

infective bites/person-year. The main malaria vectors are Anopheles gambiae s.l and A. funestus, constituting about 94.3% of the vector population.

One hospital, the Navrongo War Memorial Hospital (NWMH) serves as a referral facility for the KND residence while about six public Health Centres are strategically located across the districts to support the NWMH. In addition, most communities have a Community Health Planning Services (CHPS) compound that is run by community health officers and serves as the first line of contact for basic health services that includes door-to-door health services and early identification of referral cases.

The Navrongo Health Research Centre maintains the Navrongo Health and Demographic Surveillance System (NHDSS) in the KND. The NHDSS collects and updates vital records of the KND population and was a key resource for these studies. The NHDSS database was used to identify potential participants to be recruited as healthy controls based on the matching criteria.

Methods

A frequency-matched and an individually-matched case-control study were conducted in the Kassena-Nankana District between 2002/04, and 2007/09, respectively. Severe malaria cases were recruited from the paediatric ward of the Navrongo War Memorial Hospital (NWMH) which serves the Kassena-Nankana District.

Cases consisted of children (aged 6-60 months) who were diagnosed with severe malaria at the NWMH. Criteria for diagnosis of severe malaria followed the definitions stated by the World Health Organization i.e. the presence of asexual parasitaemia and at least one of the following: cerebral malaria (Blantyre coma score of 3 or below and coma persists for more than 30 minutes after fits have ceased); repeated or prolonged generalized convulsions; severe malarial anaemia (haemoglobin <5g/dl); respiratory distress (presence of alar flaring, intercostals or subcostal chest recession, use of accessory muscles of respiration, or abnormally deep respiration); hypoglycaemia (blood glucose <2.2mM/l); circulatory collapse (systolic blood pressure <50mmHg); renal failure (urine output less than 12 ml/kg/24 hours or serum creatine >3.0mg/dl); hyperpyrexia (anxillary temperature >39oC, hyperlactataemia (blood lactate >2.0mmol/l) and impaired consciousness). Further details may be reviewed (Oduro AR et al, 2007; Osafo-Addo AD et al, 2008).

Using the case as an index, the NHDSS was used to recruit two groups of population controls; mild malaria and community ('healthy') controls (a fraction of whom had asymptomatic parasitaemia). Two controls were recruited per case in each control category. These were matched by age category (6-24 months and 25-60 months), gender, location (areas of residence) and ethnicity prior to 2007, after which they were individually matched to severe malaria cases using the same criteria.

A standardised case report form (CRF) was created by MalariaGEN and used by all sites to collect standardised clinical data. The relevant data fields as per the CP1 CRF were extracted from CRFs of the studies conducted prior to MalariaGEN (ie 2002/04 study conducted in Ghana and all other sites). Data were uploaded onto the MalariaGEN central repository via

secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood, at the Noguchi Memorial Institute for Medical Research, using the Chelexmethod or Nucleon™ BACC2 Genomic DNA extraction kit® (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K et al, 1993; Zhang L et al, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 2685	Male: 1369 (51) Female: 1059 (39) Not recorded: 263 (10)	<1: 830 (31) 1-2: 1176 (44) 2-5: 677(25) Not recorded: 2(<1)	Kasem: 1526 (57) Nankam: 792 (30) Other: 360 (13) Not recorded: 6 (<1)
Healthy controls: 2378	Male: 1126 (47) Female: 957 (40) Not recorded: 301 (13)	<1: 573 (24) 1-2: 872 (37) 2-5: 503 (21) Not recorded: 430 (18)	Kasem: 1302 (54) Kasem mixed: 103 (4) Namkam: 102 (4) Nankam: 706 (29) Other: 197 (8)

Ethics

These studies were reviewed and approved by scientific and Institutional review boards of the Noguchi Memorial Institute for Medical research, the Navrongo Health Research Centre, The Ghana Ministry of Health Ethics review committee, and the U.S. Naval Medical Research Unit # 3 (proposal number: NMIMR-IRB CPN 016/01-02; ID NMIMR-IRB CPN 029/05-06).

At recruitment, written informed consent was obtained from participants (cases and controls) and witnessed by an individual who was not part of the research team. Individual informed consent was obtained from parents or legal guardians of children.

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Human genetic determinants of severe malaria in Kumasi, Ghana (GH)

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About this study

Severe malaria, caused by infection with *Plasmodium falciparum*, remains one of the major health problems in sub-Saharan Africa (World Health Organization, 2000).
Severe *falciparum* malaria is a complex clinical syndrome comprising a number of life threatening conditions including severe malarial anaemia, cerebral malaria, metabolic acidosis, respiratory distress and other, less frequent complications (Marsh K *et al*, 1995). In previous studies, carriers of the sickle cell haemoglobin locus (HbS) were found to be protected from all major forms of severe malaria, whereas protection for carriers of the haemoglobin C (HbC) variant was restricted to cerebral malaria (May J *et al*, 2007). This underscores the role of genetics in determining the severity of malaria.

Summary

The aim of this prospective study is to identify human genetic determinants of severe malaria and the sub-phenotypes severe malarial anaemia, respiratory distress and hyperlactataemia.

Two studies (case-control and family trio) were conducted simultaneously between August 2006 and December 2008. Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis.

Study site description

The studies were conducted in the Kumasi Metropolitan District of the Ashanti Region of Ghana where Kumasi is the capital city. Kumasi is located in the transitional forest zone. It is between 6.35°–6.4° latitude and 1.3° –1.35° longitude, at an elevation which ranges between 250–300 metres above sea level, with an area of about 254km^2. The unique centrality of the city as a traversing point from all parts of the country makes it particular attractive to migrants.

It has been projected to have a population of 1,625,180 based on a growth rate of 5.4% pa and this accounts for just under a third (32.4%) of the region's population with the majority engaged in small businesses. The major ethnic groups are the Asantes but almost all other tribes are present due to the location of the Region. Houses in the metropolis can be classified into about five types. These include: single storey, traditional compound houses, multi-storey compound houses, government–built detached or semi-detached low-income households, large single household houses built on relatively large plots and blocks of flats. The rich cultural heritage of the people of Kumasi is visible in Akwasidae festival, funerals, child-naming ceremonies, communal spirit and religion. The traditional religious practices are still upheld through the pouring of libation, marriage rites and rites of passage.

Komfo Anokye Teaching Hospital (KATH), which is one of the two national autonomous hospitals, is located within the district. With over 1000 beds, it is the second largest hospital in Ghana. It is a major referral centre for the North and Middle belt of Ghana. The Paediatric Directorate is divided into neonatal and children's ward. The Paediatric Emergency Unit is the first point of call for all childhood illnesses reporting to the hospital.

The Metropolis falls within the wet sub-equatorial climatic zone. The average minimum temperature is about 21.5oC and a maximum average temperature of 30.7°C. The average humidity is about 84.2% at 09:00 GMT and 60% at 15:00 GMT. Rainfall is highest in June (214.3mm) and September (165.2mm). The city falls within the moist semi-deciduous South-East Ecological Zone.

Transmission of malaria in the area is intense and perennial with some seasonal variations. Most of the transmission (91.4%) occurs during bedtime hours of 21;00 to 06:00h. Annual Biting Rates (ABRs) and Annual Entomological Inoculation Rates (AEIRs) in one study were reported to be 11,643 and 866, respectively. The main malaria vectors are *Anopheles gambiae* and *A. funestus*, and the main parasite species are *Plasmodium falciparum*, *P. malariae*, and *P. ovale* (Abonuusum A *et al*, 2011).

Methods

A prospective, unmatched case-control study was conducted between August 2006 and December 2008 recruiting cases admitted to the Paediatric Emergency Unit of Komfo Anokye Teaching Hospital.

Cases consist of children (aged 3 months - 12 years) with severe malaria. Criteria for diagnosis of severe malaria were those included in the standard WHO definition i.e. the presence of asexual parasitaemia and at least one of the following: cerebral malaria (Blantyre coma score of 3 or below and coma persists for more than 30 minutes after fits

have ceased); repeated or prolonged generalized convulsions; severe malarial anaemia (haemoglobin <5g/dl); respiratory distress (presence of alar flaring, intercostals or subcostal chest recession, use of accessory muscles of respiration, or abnormally deep respiration); hypoglycaemia (blood glucose <2.2mM/l); circulatory collapse (systolic blood pressure<50mmHg); renal failure (urine output less than 12ml/kg/24 hours or serum creatinine >3.0mg/dl); hyperpyrexia (anxillary temperature >39°C , hyperlactataemia (blood lactate >2.0mmol/l and impaired consciousness.

Controls consisted of cord blood samples collected from the labour ward of the same hospital at the same time as cases were collected. Blood samples were also collected from the biological parents of children with severe malaria.

A standardised case report form (CRF) was created by MalariaGEN and used by all sites to collect standardised clinical data. Data was uploaded onto the MalariaGEN central repository via secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardized and amalgamated.

Genomic DNA was extracted from the whole blood samples in Kumasi using the Nucleon™ BACC2 Genomic DNA extraction kit® (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 1923	Male: 954 (50) Female: 846 (44) Not recorded: 125 (6)	<1: 434 (23) 1-2: 476 (25) 2-5: 713 (37) 5-15: 296 (15) Not recorded: 4 (<1)	Akans (Ashanti Eastern): 1038 (54) Frafra Nankana Grushie Kusasi: 188 (10) Notherner: 186 (10) Other: 511 (27)
Healthy controls: 2326	Male: 1160 (50)	<1: 2326 (100)	Akans (Ashanti Eastern): 1526 (67)

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
	Female: 1079 (46) Not recorded: 88 (4)		Akans (Ashanti Eastern) Mixed: 140 (6) Frafra Nankana Grushie Kusasi: 108 (5) Other: 520 (23)

Ethics

Ethical clearance was obtained from the Committee on Human Research, Publications and Ethics of KATH / Kwame Nkrumah University of Science and Technology, Kumasi (proposal numbers: ID GHS-ERC-03/9/06, CHRPF/07/01/06, CHRPE SMS UST/24/05/2007).

For the recruitment of children with severe and uncomplicated malaria, written informed consent was obtained from the parents or legal guardians after admission to hospital. For the recruitment of healthy controls informed consent was obtained prior to enrolment from a parent or legal guardian of each participating child.

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The main source of funding was from MalariaGEN Consortial sub-grant award and additional downstream laboratory support by MalariaGEN.

A case-control approach to the identification of polymorphisms associated with severe malaria in Kilifi, Kenya (KE)

Key People

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About this study

Malaria is a leading cause of death in much of sub-Saharan Africa (World Health Organization, 2008). Host genetic factors are thought to be important modifiers of severe and fatal malaria (Mackinnon MJ *et al*, 2005), yet little is known about which genes are involved or the degree of protection that they might confer.

Summary

This study was undertaken a case–control study of severe *P. falciparum* malaria in a rural area on the coast of Kenya to investigate the genetics of malaria resistance. Cases were children younger than 14 years who were admitted from within a defined study area to Kilifi District Hospital between June 1995 and February 2008. Controls consisted of children born consecutively within the same study area as cases who were recruited at 3-12 months of age into a genetic cohort study (the Kilifi Genetic Birth Cohort Study) between August 2006 and July 2008.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs

at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis and genome-wide SNP typing is planned for the future.

Single- and multi-locus analysis will be conducted using multivariate logistic regression, accounting for the effects of a range of confounders, to assess the relationship between genetic markers and well-defined severe malaria including sub-phenotypes such as severe malarial anaemia, cerebral malaria and respiratory distress.

Study site description

The KEMRI-Wellcome Trust Research Programme recruited severe malaria cases and healthy controls as part of on-going epidemiological studies of severe malaria at Kilifi District Hospital, Kenya. Kilifi District Hospital is situated in a rural area on the Kenyan Coast and has a catchment population of roughly of 500,000 people. The local economy is predominantly rural, being based on subsistence farming of maize, cassava, cashew nuts, and coconuts as well as dairy cows and goats. Two large agricultural estates, two research institutes and several tourist hotels contribute to local employment. The majority of the population fall within the Chonyi, Giriama and Kauma sub-divisions of the Mijikenda ethno-linguistic group.

Malaria transmission in Kilifi is seasonal, generally coinciding with the long and short rains in October and May, respectively. Recent years have seen a significant decline in the rate of transmission from meso-endemic in the 1990's to hypo-endemic transmission today. *P. falciparum* is the cause of the vast majority of malaria infections. Malaria is predominantly transmitted by mosquitoes of the species *Anopheles gambiae* (Mbogo CN *et al*, 1993). The overall annual entomological inoculation rate (EIR) has been estimated at 1-100 (Mbogo CN *et al*, 1993; Mbogo CM *et al*, 2003).

Methods

An unmatched case-control study was conducted in Kilifi District. Cases of severe malaria, resident within a defined study area, were recruited through a process of systematic surveillance of the paediatric wards at Kilifi District Hospital between June 1995 and February 2008. Cases consisted of children less than 14 years of age who were admitted to the high dependency ward with a primary diagnosis of severe malaria (defined by the presence of P. falciparum parasitaemia complicated by one or more of the following features: severe anaemia (Hb <5g/dl), convulsions before/during admission, cerebral malaria (Blantyre Coma Score ≤3) or respiratory distress).

Controls consisted of children born consecutively within the same study area as cases who are recruited at 3-12 months of age into a genetic cohort study (the Kilifi Genetic Birth Cohort Study) between August 2006 and July 2008.

A standardised case report form (CRF) was created for the Consortial Project and used by all sites to collect standardised clinical data. The data collected in Kenya (and all other sites) were uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardized and amalgamated.

Genomic DNA was extracted from whole blood, in Kilifi, using Qiagen DNeasy Blood kits (http://www.qiagen.com/) [Qiagen, Crawley, UK]. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 2740	Male: 1358 (49) Female: 1235 (45) Not recorded: 175 (6)	<1: 526 (19) 1-2: 609 (22) 2-5: 1144 (42) 5-15: 297 (11) Not recorded: 1 (<1)	Chonyi: 621 (23) Giriama: 1622 (59) Kambe: 101 (4) Kauma: 197 (7) Other: 183 (7) Not recorded: 16 (<1)
Healthy controls: 4183	Male: 2058 (49) Female: 2022 (49) Not recorded: 103 (2)	<1: 4078 (97) 1-2: 105 (3)	Chonyi: 1508 (36) Giriama: 1930 (46) Kauma: 465 (11) Other: 273 (7)

Ethics

The study was approved by KEMRI Research Ethics Committee, Kilifi (proposal number: SCC1192).

Informed consent was obtained from parents or guardians of cases and mothers for the population controls. Population controls were collected by consenting women attending maternity units to give birth. DNA was extracted from cord blood samples.

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Human genetic determinants of severe malaria in Malawi (MW)

Key People

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About this study

Plasmodium falciparum malaria has been identified as the cause of 30-40% of paediatric hospital attendances and up to 30% of in-hospital child deaths in Malawi. Parasitaemia is common in the population and is frequently asymptomatic, so that attributing an illness to malaria may often be erroneous. Nevertheless, responses to treatment and the findings of autopsy studies confirm that malaria imposes a heavy burden of both morbidity and mortality upon children in Malawi.

Asymptomatic parasitaemia and uncomplicated malarial febrile illness greatly outnumber episodes of severe and life-threatening malaria. The factors determining progression to severe disease remain unknown. Failure to obtain prompt treatment increases the risk of disease progression, but severe and complicated *P. falciparum* malaria may develop rapidly and cannot always be attributed to delayed treatment. The virulence of an infecting strain of *P. falciparum*, the frequency and inoculum-size of infective mosquito bites and the strength of the host's existing specific anti-malarial immunity may each play a part in determining the severity of a malarial illness. It is also likely that host genetic factors contribute to determining the outcome of the parasite-host interactions that lead to disease.

Summary

As part of the Malaria Genomic Epidemiology Network, we set out to accurately identify children with severe *P. falciparum* malaria, to obtain samples of their DNA and to analyse these for genetic variants that might be associated with susceptibility or resistance to severe malaria.

A total of 1,815 children (aged 2 months - 14 years) admitted to the national hospital in Blantyre, Malawi, were enrolled into an unmatched case-control study during 1994-2007. All cases (children diagnosed with severe malaria) were followed-up according to a detailed protocol providing information on the clinical phenotype and outcome of the disease. Information on the presence or absence of retinopathy, a clinical feature that strengthens

the diagnosis of cerebral malaria in a patient with coma and parasitaemia, was also collected. DNA was extracted from the buffy coat of venous blood collected from cases on admission to hospital. As a background comparator population, DNA from cord bloods was collected from 3,777 infants born in the same hospital during 2006-2007. DNA was also obtained from blood samples collected from the parents of a number of cases.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data were generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data have been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis.

These malaria associated SNPs will be analysed for an association with severe malaria and the sub-phenotypes cerebral malaria and severe malarial anaemia. We will also investigate the association of various haplotypes with these phenotypes. This will identify any genetic factors that are over- or under- represented among children with severe malaria, and that may therefore play a part in affecting a child's susceptibility to severe *P. falciparum* infection.

Study site description

The Malawi-Liverpool Wellcome Clinical Research Programme (MLW) together with the Blantyre Malaria Project (BMP) recruited severe malaria cases, healthy controls and parents from the Queen Elizabeth Central Hospital (QECH) in Blantyre, Malawi. Blantyre, a compact, densely populated city of 500,000 people, is situated at 1000m above sea level. Daytime temperatures average around 25°C, with considerable variation throughout the year. The climate is characterised by a cool, dry season (May - September) and a hotter rainy season (October - April). Malaria transmission is year-round, with considerable seasonal fluctuation and most new infections occur during the hot and wet season. Over 90% of human malaria infections are due to *P. falciparum*, while 5-10% are *P. malariae* and 1-2% *P. ovale*. *P. vivax* is not encountered, owing to the almost universal presence of Duffy-negativity among the Malawian population. The entomological inoculation rate (EIR) for *P. falciparum* in the city of Blantyre is estimated to be around one infective bite/person/year, but a high proportion of families make regular visits to nearby rural areas where the EIR is estimated to be greater than 100.

Methods

An unmatched case-control study, including parents of cases, was conducted. Cases and controls were recruited from the Queen Elizabeth Central Hospital in Blantyre, between 1994 and 2007.

Cases consist of children (aged 2 months - 14 years) admitted to the national teaching hospital with signs of severe malaria, during the period of January to June each year. Severe

malaria was defined by the presence of *P. falciparum* parasitaemia in the thick blood film and either severe anaemia (packed cell volume (PCV) of 15% at any time during admission) or cerebral malaria (Blantyre Coma Score of 2 on admission and for at least 2 hours thereafter, no evidence of meningitis, and no improvement after correction of hypoglycaemia or within 2 hours of a witnessed convulsion) (World Health Organization, 2008). From 1998 onwards, the presence or absence of malarial retinopathy was recorded for each patient. For those with cerebral malaria, this will allow more accurate classification.

Controls consist of cord blood samples taken from the Queen Elizabeth Central Hospital in Blantyre, between September 2006 and September 2007. Blood samples from the parents of a number of severe malaria cases were also collected.

A standardised case report form (CRF) was created for MalariaGEN Consortial Project 1 and used by all sites to collect standardised clinical data. The data collected in Malawi (and all other sites) were uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data were standardised and amalgamated.

Genomic DNA was extracted from whole blood, at the MLW Laboratory in Blantyre, Malawi, using the Chelex®method according to manufacturer's guidelines. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming that appropriate clinical data were available. Baseline genotype data for 69 malaria-associated SNPs were generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 1815	Male: 907 (49) Female: 844 (46) Not recorded: 86 (5)	<1: 213 (12) 1-2: 350 (19) 2-5: 922 (51) 5-15: 321 (18) Not recorded: 9 (<1)	Malawi: 1815 (100)
Healthy controls: 3777	Male: 1781 (47)	<1: 3777 (100)	Malawi: 3777 (100)

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
	Female: 1613 (43) Not recorded: 385 (10)		

Ethics

The study was approved by the College of Medicine Research and Ethics Committee (COMREC), University of Malawi (proposal number: P.05/06/442).

All sample collections were subject to the informed consent of the parent or guardian of the child, and in the case of cord blood samples to the informed consent of the mother obtained prior to parturition.

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- Paul Pensulo, Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Queen Elizabeth Central Hospital, and Blantyre Malaria Project, University of Malawi, Malawi

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The Malawi-Liverpool-Wellcome Trust Programme and the Blantyre Malaria Project together provided clinical and laboratory support that made the study possible. These have been funded by The Wellcome Trust, UK, and the National Institutes of Health, USA, respectively.

Human genetic determinants of severe malaria in Mali (ML)

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About this study

Malaria is a life-threatening parasitic disease transmitted by *Anopheles* mosquitoes. Despite major efforts to control the disease, it still persists as a major health burden, and caused over 780,000 deaths in 2009, mainly among children in sub-Sahara Africa. In Mali, there are over 800,000 recorded cases of malaria among its ~12 million people every year, and it accounts for 17% of child deaths (World Health Organization, accessed 12 Dec 2011). Malaria is a complex disease with many genetic and environmental determinants influencing the observed variation in response to infection, progression and severity. It has been estimated that 25% of the total variation in mild and severe malaria in a Kenyan cohort was explained by host genes (Campino S, Kwiatkowski D and Dessein A, 2006). The different geographical distributions of sickle-cell disease, α-thalassemia, glucose-6-phosphatase dehydrogenase (G6PD) deficiency, ovalocytosis, and the Duffy-negative blood group are examples of the general principle that different populations have evolved different genetic variants to protect against malaria (Mackinnon MJ *et al*, 2005).

Summary

A case-control study, including parents of cases, was conducted in Mali with the aim of identifying human genetic determinants associated with susceptibility or resistance to severe malaria and its sub-phenotypes: severe malarial anaemia, respiratory distress and cerebral malaria. We consider a cohort of over 800 individuals recruited in Bamako, predominantly from the Bambara ethnicity, which is under-represented in other genetic epidemiological studies in Western Africa.

Cases were children (aged 3 months-14 years) with signs of severe or uncomplicated malaria. Severe malaria cases were recruited from the paediatric ward at the reference hospital of Gabriel Touré in Bamako, the capital of Mali, between November 2006 and January 2008. A small number of uncomplicated malaria cases were also enrolled and matched to severe malaria cases by age (+/- 6 months), ethnicity and location and duration of residence. Severe malaria was defined by the presence of *Plasmodium falciparum* parasitaemia or positive dipstick of a rapid diagnostic test and one or more criteria included in the WHO 2002 standard definition for severe malaria. Controls were healthy children (aged 5 months-14 years) with no personal known history of severe malaria. Controls were individually matched to a severe malaria case by age (+/- 6 months), ethnicity, location and duration of residence.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) were included in our analysis.

Study site description

The Malaria Research and Training Centre (MRTC) in Mali recruited malaria cases, healthy controls and a small number of parents of index cases. Cases were enrolled at the Paediatric ward of the National Hospital Gabriel Toure in Bamako, the capital of Mali, where the MRTC team has been conducting studies on severe malaria since 1999. This hospital receives patients from across the country but the majority of patients come from the city of Bamako and the surrounding rural areas. This catchment has a population of 1.8 million inhabitants and covers an area of 252 km^2. Bamako had become a major market town. Economical activities are mainly trade, retailing and others services. The majority of people belong to the Bambara and Malinke ethnic groups.

The climate is tropical with a hot dry season and a wet rainy season that extends from June to October. The end of the wet season coincides with a peak in the incidence of severe malaria (Doumbo O, 1992). Most people live in traditional compounds with several nuclear households.

Five patterns of malaria transmission have been described in Mali:

1. An area of long seasonal and intense malaria transmission between June and November in the south of Mali (corresponding with the Soudano-Guinean area where plasmodic index (parasite prevalence in children aged 2-9 years old) is higher than 75%);
2. An area of short, seasonal, yet intense, malaria transmission from July to October (corresponding with the northern Savannah and the Sahel where plasmodic index varies from between 50 and 75%);
3. An area of bimodal or multi-model transmission in the inner delta of the river Niger and areas of irrigated rice cultivation (where plasmodic index is <40%);

4. Urban areas (where plasmodic index is <10%); and,
5. An area of sporadic or epidemic malaria transmission in the sub-Saharan region, in the north of the country (where plasmodic index is <5%).

P. falciparum is the dominant parasite species (93.5%) and *Anopheles gambiae s.l.* and *A. funestus* are the main vectors. Entomologic Inoculation Rate (EIR) can be higher than 300 infective bites/person/year in areas of intense transmission and drops to almost zero during the dry season, specifically in the Sahel (Dicko A *et al*, 2005).

Methods

A matched case-control study, including parents of cases, was conducted between November 2006 and January 2008. Cases were children (aged 3 months-14 years) with signs of severe or uncomplicated malaria recruited from the paediatric ward at the reference hospital of Gabriel Touré in Bamako. A small number of uncomplicated malaria cases were also collected and matched to severe malaria cases by age (+/- 6 months), ethnicity and location and duration of residence.

All cases were children admitted to hospital with *P. falciparum* positive blood films and clinical features of severe malaria (Marsh K *et al*, 1995, World Health Organization, 1990).Severe malaria was defined by the presence of *P. falciparum* parasitemia or OptiMAL® test positive and one of the following: coma (Blantyre coma score < 3) without other obvious explanations (e.g. no evidence of pyogenic meningitis), anaemia (haemoglobin <5 g/dl or hematocrit < 15%) without obvious evidence of other causes (e.g. kwashiorkor).

Controls were healthy children (aged 5 months-14 years) with no personal history of severe malaria. Controls were individually matched to index cases by age (+/- 6 months), ethnicity and location and duration of residence. To select controls, the research team visited the home of cases the day after enrollment and sought an unrelated child whose age, ethnicity and residence duration matched that of the index case. Blood samples from a small number of parents of severe malaria cases were also collected to aid with haplotype construction.

A standardised case report form (CRF) was created for Consortial Project 1 (CP1) and used by all sites to collect standardised clinical data. The data collected in Mali (and all other sites) was uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted at the MRTC main campus from whole blood using the Nucleon™ BACC2 Genomic DNA extraction kit® (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PI's for local analyses.

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 510	Male: 279 (54) Female: 221 (43) Not recorded: 13 (3)	<1: 60 (12) 1-2: 106 (21) 2-5: 234 (46) 5-15: 110 (21)	Bambara: 216 (42) Malinke: 59 (12) Other: 235 (46)
Healthy controls: 389	Male: 183 (47) Female: 186 (48) Not recorded: 21 (5)	<1: 25 (6) 1-2: 82 (21) 2-5: 192 (49) 5-15: 88 (23) Not recorded: 2 (1)	Bambara: 180 (46) Malinke: 46 (12) Peulh: 23 (6) Sarakole: 21 (5) Other: 119 (31)

Ethics

This study has been approved by the Ethical Committee of the Faculty of Medicine, Pharmacy and Odonto-Stomatology (FMPOS), University of Bamako, Mali (Proposal number: ID No/06-18bis/FMPOS).

Written informed consent was obtained from adults and the parents or legal guardians of all cases enrolled in this study. For the recruitment of healthy children, informed consent was obtained prior to enrolment from a parent/guardian. During the search for matched healthy controls, compensation in the form of provision of one time treatment of all medical conditions encountered in all children living in the same compound was offered.

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Human genetic determinants of severe malaria in Nigeria (NG)

Key People

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About this study

Malaria remains one of the leading causes of morbidity and mortality in malaria endemic regions of the world. The epidemiology of malaria in Nigeria is characterised by a spectrum of different malaria transmission patterns, ranging from unstable transmission in the North to stable intense transmission in the South. The main evidence for a major genetic component of disease susceptibility comes from the growing number of disease associations with specific SNP polymorphisms in recent years. A number of malaria candidate genes (May J *et al*, 2007; Clark TG et al, 2009; Lyke KE *et al*, 2011; Millimono TS *et al*, 2011; Schuldt K *et al*, 2011), and more recently genome-wide association studies (Jallow M *et al*, 2009), have identified genomic loci, which contribute to susceptibility and/or protection against severe malaria.

Summary

An unmatched case-control study was conducted during the May-September transmission season of 2007 with the aim of identifying human genetic determinants of susceptibility/resistance to severe malaria in Ibadan, Southwest Nigeria.

The inclusion criteria for recruitment were categorised according to the World Health Organization definitions. Severe malaria cases (including those with severe malarial anaemia and cerebral malaria) were recruited from three hospitals: the University College Hospital (a tertiary Institution) and two secondary level hospitals, Adeoyo Maternity Hospital and Oni Memorial Hospital in Ibadan, Nigeria. Asymptomatic controls comprised of clinically-well children recruited during a cross-sectional survey in September 2007 from communities in the areas surrounding the three hospitals from which cases were recruited.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis. Single- and multi-locus analysis will be conducted using various multivariate logistic regression models to assess the relationship between these polymorphisms and well-defined clinical phenotypes.

Study site description

Malaria is endemic in Nigeria with seasonal transmission patterns, the peak transmission of which is during the rainy season (April-October) followed by the dry season (November-March). Average annual rainfall in Ibadan is 80 inches and it has a relative humidity typically above 80% during the wet season. The predominant species of malaria parasite is *Plasmodium falciparum* accounting for about 96% of malaria infections. The two main vector species in Nigeria are *Anopheles gambiae* and *A. funestus*, with an EIR of 24.7 infective bite per person per rainy season (Okwa OO *et al*, 2009).

The case-control study was conducted at three hospitals: the University College Hospital (a tertiary Institution) and two secondary level hospitals, Adeoyo Maternity Hospital and Oni Memorial Hospital in Ibadan, Nigeria. Ibadan city is located in the Southwest of Nigeria in the rainforest belt at 7.38 latitude, 3.8 longitude and at an elevation of 239m. The population of Ibadan is about 4 million. The secondary government hospitals cater for the middle and lower social class, and the UCH is a tertiary health care facility which caters for all classes and attends to more complicated and severe cases. The communities within the catchment areas of the three hospitals, with a population of about 200,000, are mainly traders, civil servants, academic and artisans. There are private health facilities and Mission-based facilities within the city and in the outlying communities, villages and markets. Patent medicine sellers provide health services to the people at cheaper rates but with the risk of being provided with fake, expired and adulterated drugs. The great majority of the population belongs to the Yoruba ethnic group.

Methods

An unmatched case-control study was conducted. Cases consist of children (aged 4 months - 11 years) presenting with severe malaria, cerebral malaria and/or severe malarial anaemia. Children were recruited from three hospitals in the city of Ibadan during the May-September transmission season of 2007. The inclusion criteria for recruitment were defined according to the World health Organization definitions. Severe malaria was defined by the presence of *P. falciparum* in the thick blood film with at least one of the following conditions: unrousable coma, BCS ≤2, clear cerebro-spinal fluid from lumbar puncture, extreme weakness/prostration (inability to sit or stand without support), convulsions (more than one episode within 24 hours), severe anaemia (haematocrit <15% or Hb <5 g/dl). Children with co-existing bacterial meningitis and other encephalitis (especially the locally prevalent viral encephalitides) were excluded from the study.

Controls comprised of children (aged 6 months – 12 years) recruited during a cross-sectional survey in September 2007 from communities in the areas surrounding the three hospitals from which cases were recruited. Clinically well children who had not been ill two weeks preceding recruitment, were recruited into the study.

A standardised case report form (CRF) was created for MalariaGEN CP1 and used by all sites to collect standardised clinical data. The data collected in Nigeria (and all other sites) was uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood at the Institute of Child Health, using Nucleon™ BACC2 Genomic DNA extraction kits® by Tepnel (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions and quantified. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 114	Male: 66 (58) Female: 43 (38) Not recorded: 5(4)	<1: 12 (11) 1-2: 27 (24) 2-5: 60 (53) 5-15: 15 (13)	Yoruba: 110 (96) Other: 4 (4)
Healthy controls: 88	Male: 43 (49) Female: 36 (41) Not recorded: 9 (10)	<1: 15 (17) 1-2: 19 (22) 2-5: 32 (36) 5-15: 22 (25)	Yoruba: 86 (98) Other: 2 (2)

Ethics

Ethical approval was obtained from the Joint University of Ibadan/ University College Hospital Ethical Review Committee (proposal number: ID UI/IRC/06/0034) and the Oyo State Ministry of Health Ethical Committee.

After a thorough explanation of the study and the procedures involved, consent (both verbal and written) was obtained from the mothers/care givers of the children recruited. Consent was also obtained from the Community head for the cross-sectional study.

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Human genetic determinants of severe malaria in Papua New Guinea (PG)

Key People

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About this study

The populations of the South West Pacific are highly diverse and exhibit a range of red blood cell (RBC) polymorphisms. Within Papua New Guinea (PNG), a variety of red cell variants are found that have geographical patterns paralleling malaria endemicity (Muller I *et al*, 2003). In particular, Southeast Asian Ovalocytosis (band 3 deletion SLC4A1Δ27 [SAO]) is found in up to 35% of the population in some coastal areas and has been associated with complete protection against cerebral but not other forms of severe *Plasmodium falciparum* malaria in previous studies in PNG (Genton B *et al*, 1995; Allen SJ *et al*, 1999). Alpha+-thalassaemia is found in more than 90% of people and has been associated with protection from severe malaria (Mockenhaupt FP *et al*, 1997) and severe non-malarial disease (Allen SJ *et al*, 1997). Finally, polymorphisms in complement receptor 1 protect against severe malaria through reduced red cell rosette formation (Cockburn IA *et al*, 2004).

Taken together, the high prevalence of these known genetic variants suggests that malaria has exerted strong selective pressure in Melanesian populations. However, similar to African settings, known genetic variations may account for only a small proportion of the total variability in genetic susceptibility to severe malaria in the population as a whole (Mackinnon MJ *et al*, 2005). The epidemiology and clinical features of severe malaria in Melanesian children differ substantially to African children and the presence of *Plasmodium vivax* or specific genetic factors are thought to be responsible for a lower mortality observed in a number of studies from this area (Maitland K *et al*, 1997; Manning L *et al*, 2011).

Summary

As a prelude to a genome wide analysis (GWA), we outline three separate severe malaria case-control studies undertaken at the same field site in Madang province, Papua New Guinea examining the genetic association for 69 candidate single nucleotide polymorphisms (SNPs) (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) that were performed during the years 1993-6 (Study A; Allen SJ *et al*, 1996), 2003-4 (Study B; Karunajeewa HA *et al*, 2006) and 2006-9 (Study C; Manning L *et al*, 2011). In the present report we outline the characteristics of the study populations and the methodology for the clinical assessment of cases and controls recruited into each study.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated SNPs and the appropriate data has been returned to each site for site-specific analysis.

Study site description

The present study was conducted in Madang Province on the northern PNG coast where most of the population are subsistence farmers. Malaria transmission is perennial but with seasonal variations. The annual entomological inoculation rate (EIR) for Madang Province has recently been estimated at 37 for *P. falciparum* and 24 for *P. vivax*. Malaria is transmitted by a number of mosquito vectors including *Anopheles punctulatus* complex, *A. farauti* and *A. koliensis* (Michon P *et al*, 2007). During the recruitment period for Study C, healthy, asymptomatic Madang children aged 1-10 years had spleen rates of 13% and the prevalence of asymptomatic parasitaemia by microscopy was 8.2% for *P. falciparum* (median [interquartile range] parasite density 1360 [453-2881] /µl) and 14.1% (348 [226-727] /µl) for *P. vivax* (Manning L *et al*, 2011). These malariometric indices are lower than reported from the same area during the period that Study A was performed (Burkot TR *et al*, 1987). Approximately 90% of local children have alpha-thalassemia trait (Muller I *et al*, 2003). The current national human immunodeficiency virus (HIV) seroprevalence is 0.9% (UNAIDS, 2010).

Methods

A matched case-control study was conducted. Cases consist of children (aged 5 months-12 years) with signs of severe or uncomplicated malaria. The study participants with severe malaria in this study were recruited at Modilon Hospital, the main referral centre for Madang Province during three separate studies performed during the years 1993-6 (Study A; Allen SJ *et al*, 1996), 2003-4 (Study B; Karunajeewa HA *et al*, 2006) and 2006-9 (Study C; Michon P *et al*, 2007).

The criteria for inclusion of severe malaria cases varied slightly between the three studies. For those recruited into Study A the WHO 1990 (World Health Organization, 1990) definition for severe childhood malaria was used whereas for studies B and C the contemporary WHO

2000 (World Health Organization, 2000) definition was applied. For each study, clinicians or trained research nurses carried out clinical assessments on admission. This included details of immunizations, past medical history and recent treatment with antimalarial drugs and antibiotics, as documented in each child's hand-held medical record book. The assessment of coma (Blantyre Coma Score [BCS]; Molyneux ME et al, 1989), lactate, glucose and haemoglobin (Hb) concentrations was measured in a standardised manner. This allowed consistent and comparable clinical phenotypes to be derived from each study that could be uploaded to the MalariaGEN CP1 website. A BCS≤2 was considered deep coma and a BCS≤4 as impaired consciousness at 0.5, 1 or 6 hours after correction of hypoglycemia, a seizure or parenteral anticonvulsant therapy, respectively. Respiratory distress was considered present if the child had i) deep breathing, ii) inter-costal in-drawing, iii) sub-costal recession, iv) persistent alar flaring, v) tracheal tug, and/or vi) respiratory rate >60/minute. Other indicators of severe malaria were defined in accordance with the respective WHO definitions. Chest radiography and lumbar puncture were performed in a minority of children whilst blood culture was only available during Study C.

In the latter study, recruitments were restricted to children with severe malaria, a pre-defined parasitaemia threshold (>1000 *P. falciparum*/µl and >500 *P. vivax*/µl) and limited to children from Madang, Morobe and Sepik provinces.

For Study A, recruitments were restricted to children with *P. falciparum* only and were initially treated with intramuscular quinine, followed by oral quinine and a single dose of sulphadoxine/pyrimethamine (SP). In Study B, children with severe malaria were given rectal artesunate or intramuscular quinine as part of a safety and efficacy trial, whilst in Study C children were given intramuscular artemether, followed by SP on day three and oral artesunate. During all 3 studies antibiotics (chloramphenicol 25 mg/kg by intramuscular injection 6-hourly) and intravenous dextrose/saline were given concurrently with antimalarials in accordance with local protocols. Blood transfusion was done at the discretion of the attending physician. The PNG standard treatment guidelines recommend transfusing all children with Hb <40g/l (4g/dl) or at higher concentrations in the presence of cardiovascular compromise (Paediatrics Society of PNG, 2005).

Healthy community controls were matched to severe cases for all 3 studies. During Study A, controls were recruited from the community soon after the recruitment of a severe malaria case and individually matched as closely as possible to a case for ethnicity, age, gender and residence. Controls for Study B were recruited to the study 4 years after the original study had been performed and were matched by age and sex. During Study C, controls were recruited at immunization clinics in villages of severe malaria patients and were matched by age, sex and ethnicity. In the latter two studies (B and C) children matched by age were within 12 months of the index case, in reasonable health as defined by the absence of i) a history of malaria within the previous fortnight, ii) current fever (axillary temperature >37.5°C) plus a positive rapid diagnostic test for malaria, iii) respiratory distress (respiratory rate >40/minute plus in-drawing of chest wall or dyspnea), iv) impaired consciousness (Blantyre Coma Score ≤4), or v) a hemoglobin concentration <50g/L (<5g/dl). In this part of PNG, malarial parasitaemia without acute illness is common and therefore children with asymptomatic parasitaemia were included.

During Study C, when possible an uncomplicated malaria control was also recruited for each severe malaria case. Children with uncomplicated malaria were matched by age, sex and ethnicity and defined by a history of or current fever, either a positive rapid diagnostic test for malaria or plasmodium parasites by light microscopy and had none of the clinical signs indicating severe illness.

The clinical data was double-entered by PNG Institute of Medical Research's (PNGIMR) data management unit before being uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked, standardised and amalgamated.

For Study A, genomic DNA was extracted on site from whole blood by proteinase K digestion followed by phenol chloroform extraction and shipped frozen to the Institute of Molecular Medicine, Oxford for molecular analysis. Genomic DNA was extracted from whole blood at molecular laboratory of the PNGIMR in Goroka using QIAamp 96 DNA Blood Mini Kit, QIAGEN, Valencia, CA. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PI's for local analyses.

Table 1: Breakdown of samples

Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 805 526 *severe* 279 *mild*	Male: 442 (55) Female: 361 (45) Not recorded: 2 (<1)	<5: 526 (65) 5-15: 132 (16) Not recorded: 147 (18)	Madang: 440 (55) Other: 152 (19) Not recorded: 213 (26)
Healthy controls: 553	Male: 300 (54) Female: 253 (46)	<5: 435 (79) 5-15: 109 (20) Not recorded: 9 (1)	Madang: 348 (63) Other: 116 (21) Not recorded: 89 (16)

Ethics

The study was approved by the PNG Institute of Medical Research Institutional Review Board (proposal number: IMR IRB 0603) and the Medical Research Advisory Committee of the PNG Health Department (proposal number: MRAC No: 06.21) and conducted according to the principles of the Declaration of Helsinki.

Written informed consent was obtained from parent(s)/guardian(s) of both cases and controls before recruitment. All written materials were available in English and Melanesian Pidgin languages. Trained nursing officers who were fluent in Melanesian Pidgin recruited participants into the study using the language most comfortable for the patient and their family.

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Human genetic determinants of severe malaria in North East Tanzania (TZ)

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About this study

Malaria is known to be a strong factor for selection of human genes that give protection against the disease. A quarter of the risk of severe malaria is determined by host genetic factors, and although we already know a number of human genes that appear to influence the risk of severe malaria, this represents only a small fraction of the total genetic component and only 2% is attributed to HbS (Mackinnon MJ *et al*, 2005). SNPs identified by the human genome HapMap project and improved technology of genome-wide association analysis will help to identify malaria resistance genes in a systematic and comprehensive manner which may assist in understanding the role and molecular mechanism of genes in malaria immunity (International HapMap Consortium, 2005).

Summary

A matched case-control study (children aged 3 months-10 years) was carried out in Muheza, Tanzania between 2006-2008 with the aim of identifying the genes involved in protection against severe malaria, and the sub-phenotypes acidosis, severe malarial anaemia, cerebral malaria, shock, respiratory distress and bacteraemia.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis.

In addition, conventional PCR and ELISA tests were conducted for alpha thalassaemia and antibody analysis, respectively. A number of regression models will be used to assess the association between genetic polymorphisms and malaria outcome.

Study site description

The Joint Malaria Programme together with the London School of Hygiene and Tropical Medicine recruited severe malaria cases and healthy participants from Teule Hospital and community, respectively, in Muheza, North East Tanzania. This hospital serves a rural population of approximately 277,000 with child mortality of 165/1,000. The hospital receivespatients from all over the Tanga region.

The study villages lie at an altitude of 199-300 metres. The area is dominated by *Anopheles gambiae sensu stricto* and *Anopheles funestus* (Maxwell CA *et al*, 2003) as the main vectors. Transmission of *Plasmodium falciparum* is intense (50-700 infected bites/person/year) and perennial, with two seasonal peaks (Maxwell CA *et al*, 2003). The community prevalence of *P. falciparum* in children aged 2-5 years in the study area was recorded as 88.2% in 2002 (Maxwell CA *et al*, 2003). The dominant climate is warm and wet. In most cases, there is not a big variation in temperature at the coast due to the influence of the Indian Ocean. However, during the hot season (December to March) the average temperature is approximately 30-32◦C during the day and 26-29◦C at night. During the cool season (May to October) temperatures are approximately 23-28◦C in the day and 20-24◦C at night. Another characteristic of the coastal climate is the high atmospheric humidity, which often goes up to 100% maximum and 65-70% minimum. Mean annual rainfall ranges from 600-800mm. The outstanding feature of the vegetation is its complexity. The coastal area is dominated by bush land, palm gardens, village cultivations and estates (mainly sisal). The upland plateaus are covered with bush land and shrub thickets interrupted by swampy low-lands and river swamps as well as village cultivations, estates and palm gardens.

The economy of Muheza depends on subsistence agriculture, livestock keeping and fishing. Food production to a large extent is undertaken by small holders, while cash crop production is carried out by both small holders and large scale farmers (public and private institutions). The leading and prominent food crops in terms of area coverage are maize, cassava, banana, pulses (mainly beans) and rice. Important cash crops include sisal, cotton, coffee, tea, cardamon, coconuts, tobacco and cashewnuts. Livestock reared are cattle, goats and sheep. Modern dairy farming and poultry keeping is not very common in the rural areas.

There are health facilities in most villages in Muheza, and a large part of the population has access to primary health facilities within a distance of 6kms. The main problems remain to be "shortage of medicines", user charges and the poor state of health facilities. About half of all households use iron sheeting for roofing while the rest use grass, leaves or mud (National Bureau of Statistics - Tanzania, 2005). The majority of the population belongs to the Mzigua and Wasambaa ethnic groups.

Methods

A matched case-control study was conducted. The cases were retrospectively recruited from patients who had been hospitalized for severe febrile illness (SFI) at Teule hospital in Muheza, Tanga in 2005/2006. Cases were recruited as part of a study which was investigating reasons why some patients were suffering from severe illness due to common disease (Nadjm B *et al*, 2010). Cases consist of children (aged 3 months-10 years) who were

admitted to hospital with signs of severe malaria. Consecutive daytime admissions were triaged for the need for emergency treatment and then screened for study eligibility.

Cases were aged between 2 months and 13 years with a history of fever within the previous 48 hours, asexual *P. falciparum* parasitaemia and any of the following: more than 2 seizures in the previous 24hrs (information given by the mother); Blantyre coma score <3 (repeated if BCS<5 and convulsion within 1 hr or anticonvulsant given within 6 hrs); prostration (inability to sit unsupported or, if age<8months, inability to drink); respiratory distress (deep breathing or low chest wall indrawing or respiratory rate>70 bpm or O2 sat<90%); jaundice (identified by inspection of sclera); severe anaemia (haemoglobin <5g/dl), blood glucose <2.5mmol/l, blood lactate >5mmol/l.

Controls consist of children (aged 1-10 years), in good health (i.e. eating and drinking normally and playful), haemoglobin >8 g/dl and had no skin condition or other impediment to obtaining a venous blood sample. Controls were recruited between July 2007 and August 2008. These were individually matched to cases for tribal origin of at least one parent, electoral ward of residence and within 3 years of age to a case. Potentially eligible controls were initially identified by the health worker in the local primary care clinics. These children were later visited by research staff and the ethnicity match was confirmed on interview with one of the parents. Controls were excluded if they had been admitted to Teule hospital for severe malaria in the previous 12 months.

A standardised case report form (CRF) was created for CP1 and used by all sites to collect standardised clinical data. The data collected in Tanzania (and all other sites) was uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood using the Nucleon™ BACC2 Genomic DNA extraction kit® (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions and quantified using spectrophotometer at the KCMC Biotechnology laboratory in Moshi, Tanzania. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 501	Male: 260 (52) Female: 224 (45) Not recorded: 18 (3)	<1: 121 (24) 1-2: 172 (34) 2-5: 188 (38) 5-15: 20 (4)	Mzigua: 132 (26) Wasambaa: 100 (20) Other: 269 (54)
Healthy controls: 504	Male: 222 (44) Female: 267 (53) Not recorded: 15 (3)	<1: 7 (1) 1-2: 119 (24) 2-5: 337 (67) 5-15: 51 (8)	Mzigua: 142 (29) Wabondei: 61 (12) Wasambaa: 101 (20)

Ethics

Ethical clearance was obtained from the ethical review board of the London School of Hygiene and Tropical Medicine and the Tanzanian National Medical Research Institute (Proposal number: ID 4093).

Written informed consent was obtained from adults and the parents or legal guardians of all children enrolled in this study. The details of the consenting procedure for cases have been detailed in Nadjm B *et al* (2010). Study information were read to the parents or legal guardians of all children recruited as controls. They were given the opportunity to ask questions and when they voluntarily agreed to participate in the study, were consented.

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Human genetic determinants of severe malaria in The Gambia (GM)

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About this study

Genetic factors play an important role in resistance to malaria infection but many of the genes responsible remain unknown. It has been estimated that host genetic factors contribute 25% of total malaria protection (Mackinnon MJ *et al*, 2005) but only 2% of this total can be attributed to HbS; a polymorphism which confers a well-documented protective effect against severe malaria. This raises the question: What other genetic variants have evolved that contribute to malaria protection or risk? In The Gambia, we have been building a DNA resource since 1996 with the aim of investigating the human genetic determinants of severe malaria.

Summary

An unmatched case-control study was conducted between 1996 and 2009 with the aim of investigating the human genetic determinants of severe malaria in The Gambia.Severe malaria cases (children aged between 4 months and 15-years) were recruited from the Western Health Region of the Gambia, the main referral hospital in Banjul, the MRC wards in Fajara, and some governmental health facilities. Clinical data and DNA were collected from both cases and controls. In about 50% of the cases, we collected DNA samples from both parents to allow a family-based (TDT) analysis. Controls were collected to represent the population of the Gambia; blood was collected from umbilical cords at the maternity units of the main hospitals. As with the cases, we also collected parental samples (mothers only) in about 50% of the samples.

Clinical data and DNA samples were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated

for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis. We will investigate the association between each SNP and several malaria phenotypes including severe malaria, cerebral malaria, severe malarial anaemia, respiratory distress and hyperparasitaemia.

Study site description

The Medical Research Council (MRC) Unit in The Gambia recruited severe malaria cases, healthy controls and parents from the western part of the country, including the Kanifing Municipal Council (KMC) with a population of 397,327, and the West Coast Region, with a population of 527,753. This catchment had a total population of 956,396 in 2009 (2009 population projected from 2003 Population Census). Some cases and their parents were also referred from the North Bank West Region which has a total population of 92,822. The four largest ethnic groups in The Gambia are the Mandinka, Jola, Fula and Wollof. About half the inhabitants of the Western Health Region (WHR) are farmers, relying on their agricultural produce for subsistence. The GDP per capita for the country in 2009 was USD $436 (World Bank, accessed 12 Dec 2011).

The WHR is served by twelve government health facilities (in and out-patient) and four hospitals. In addition, there are 12 government-operated clinics (out-patient only) in the region. The MRC Clinical Services Department, Ahmadiyya Hospital and several private clinics in the study area also provide health care to the inhabitants in this region.

Transmission of malaria in The Gambia is seasonal. The long dry season lasts from November-June with a short rainy season from July-October with an average annual rainfall of 1,015 mm. Morbidity and mortality from malaria follows this pattern; both occur more frequently during the rainy season with a peak in October. *P. falciparum* is the dominant species, being responsible for all severe disease and for over 95% of clinical attacks. Few cases of clinical malaria are caused by *P. malariae*. The main vector species in the WHR are *Anopheles gambiae*, *A. melas* and *A. arabiensis*.

Methods

An unmatched case-control study, including parents of cases and mothers of controls was conducted between 1996 and 2009 recruiting patients admitted to the Edward Francis Small Teaching Hospital (EFSTH), formerly the Royal Victoria Teaching Hospital (RVTH), and the Medical Research Council (MRC) wards. The EFSTH is located in the capital, Banjul, and is The Gambia's main referral hospital. Patients come from all over the country, with the majority from the western region. The MRC wards are located in the main research site of the MRC in Fajara.

Cases consist of children (aged 4 months-15 years) with severe malaria. Severe malaria was defined by the presence of asexual parasitemia and any of the following: cerebral malaria (Blantyre coma score of ≤2), severe anaemia (haemoglobin <6 g/dl or hematocrit <18%),

respiratory distress (respiratory rate >40 with 2 of the following: nasal flaring, intercostal and subcostal recession, and grunting), repeated generalised convulsions, hypoglycaemia (blood glucose <2.2mmol/l or <40mg/dl), renal failure (urine output <12ml/kg/day), haemoglobinuria, jaundice, prostration, hyperparasitaemia (≥500 parasites/HPF), hyperpyrexia, circulatory collapse, cold extremities, rapid pulses, systolic blood pressure below 50mmHg.

Controls consist of cord blood samples recruited from various labour wards, primarily in the western division of The Gambia, between 1999 and 2007. Blood samples were collected from the parents of a number of severe malaria cases, and mouth swab samples from mothers of a number of controls

A standardised case report form (CRF), was created for the Consortial Project 1 and used by all sites to collect standardised clinical data. The data collected in Gambia (and all other sites) was uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted at the MRC Unit in The Gambia from whole blood using the Nucleon™ BACC2 Genomic DNA extraction kit® (Gen-Probe Life Sciences Ltd., Manchester, UK) using manufacturer's instructions. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available, prior to selection for the genome-wide experiment. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to The Gambia to allow local analyses.

Table 1: Breakdown of samples

Number	Gender: n (%)	Age in years: n (%)	Ethnicity: n (%)
Malaria cases: 2,801	Male: 1429 (51) Female: 1296 (46) Not recorded: 87 (3)	<1: 236 (8) 1-2: 405 (14) 2-5: 1336 (48) 5-15: 813 (29) Not recorded: 11 (<1)	Fula: 344 (12) Jola: 432 (15) Mandinka: 900 (32) Wollof: 335 (12) Other: 780 (28) Not recorded: 10 (<1)

Table 1: Breakdown of samples			
Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Healthy controls: 4472	Male: 1979 (44) Female: 2315 (51) Not recorded: 221 (5)	<1: 3801 (85) 1-2: 0 (0) 2-5: 2 (<1) 5-15: 17 (<1) >15: 211 (5) Not recorded: 441 (10)	Fula: 784 (18) Jola: 527 (12) Mandinka: 1325 (30) Wollof: 597 (13) Other: 519 (12) Not recorded: 359 (8)

Ethics

The study was approved by The Gambia Government/Medical Research Council Joint Ethics Committee (proposal numbers: 670/630 and 1029v2).

Informed consent was obtained from parents or guardians of cases and mothers for the population controls (cord bloods). Population controls were collected by consenting women attending maternity units to give birth. Further controls were collected from The Gambia Biobank with appropriate ethics permissions.

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Human genetic determinants of severe *Plasmodium falciparum* malaria in Vietnam (VN)

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About this study

The last decade has seen a decline in the number of reported malaria cases in Vietnam from 187,994 in 1991 to 54,297 in 2010, with the number of deaths dramatically reduced from 4,646 in 1991 to 21 in 2010 (National Institute of Malariology Paristology and Epidemiology, 2010). This is a great achievement for malaria control programs in Vietnam, with the availability of highly effective therapy, namely artemisinin derivatives, playing a key role in this success (Hung le Q *et al*, 2002; Barat LM, 2006). However, malaria still exists within Vietnam, and it is believed by some that the number of malaria cases is under-reported (Erhart A *et al*, 2007). Many of the provinces in the central region of Vietnam, which provide the forested areas that support the mosquito vector (*Anopheles dirus* and *A. minimus*) border Cambodia where it has been reported that there is a decline in the effectiveness of artemisinin-based combination therapy (ACT) and artesunate monotherapy (Dondorp AM *et al*, 2009).

Acquisition of *Plasmodium falciparum* with resistance to ACT poses a real threat to the successful control of malaria in Vietnam and within the South-East Asian region. To be adequately empowered to combat malaria we need to completely understand the molecular mechanisms of protective immunity to enable the development of an efficacious multi-stage malaria vaccine capable of providing life-long protection. As part of the MalariaGEN Consortial Project 1 (CP1), the aim of our study is to identify mechanisms of protective immunity against malaria using a human genetics approach, which may lead to novel vaccine development.

Summary

Over the past 20 years the Oxford University Clinical Research Unit (OUCRU) in Ho Chi Minh City (HCMC), Vietnam, in collaboration with the Hospital for Tropical Diseases, has been performing clinical studies of malaria. These diverse clinical studies have led to a large collection of clinically well-defined severe malaria patients that are available for large scale genetics studies. This valuable resource includes archived genetic material and clinical characterisation of over 1,100 severe malaria patients and over 2,500 Vietnamese population controls.

Clinical data and DNA samples from many of these Vietnamese severe malaria cases and controls were contributed to the MalariaGEN Consortial Project 1 (CP1) along with those of 11 other case-control studies from a total of 11 malaria-endemic countries. As part of the sample handling process, baseline genotyping data was generated for a number of malaria–associated single nucleotide polymorphisms (SNPs) and the appropriate data has been returned to each site for site-specific analysis. A total of 69 SNPs at candidate genes (selection based on previous reports of association with severe malaria or on their likely biological role in malaria infection/disease) will be included in our analysis.

Study site description

All studies were conducted in the southern provinces of Vietnam. The main study site was the Hospital for Tropical Disease in Ho Chi Minh City which is a 550 bed tertiary referral hospital for infectious diseases for all of southern Vietnam. Southern Vietnam is divided into coastal lowlands, extensive forests and highland regions in south central Vietnam (West Highland). The hospital is situated in a large urban city (population over 7 million), however a significant number of the severe malaria patients are referred from surrounding provinces. Malaria cases are generally found in the rural highland region of south central Vietnam that tend to be impoverished, forested and less accessible to effective health systems (Trung HD *et al*, 2004). Approximately 75% of patients recruited for these genetics studies were enrolled at the Hospital for Tropical Diseases. Secondary study sites in Binh Phuoc province (population of 874,961 in 2009) are in the low highland regions of south central Vietnam. Phuoc Long district hospital and Dong Xoai Provincial Hospital within Binh Phuoc province recruited the remaining 25% of severe malaria patients for these genetics studies.

The southern area of Vietnam has a monsoon tropical climate. The seasons are divided into wet (May to November) and dry (December to April). In the southern plains around Ho Chi Minh City the temperature does not vary significantly throughout the year with an average temperature of approximately 32oC, which is slightly lower in the elevated low highland regions of Binh Phuoc province. This area has low seasonal transmission of *P. falciparum* and *P. vivax* mainly in the forested rural areas and entomological inoculation rates are very low (in most areas <1).

Methods

Since 1991 the Oxford University Clinical Research Unit, in collaboration with the Hospital for Tropical Diseases in Ho Chi Minh City (HCMC) has been performing clinical studies of

malaria. During this time the collection of severe malaria patients for genetics studies has been part of 4 distinct clinical studies. The first two studies were randomised controlled trials for the treatment of adults with severe *P. falciparum* malaria undertaken at the Hospital for Tropical Diseases during the 1990s (1991-1995 Artemether versus Quinine (Phu NH *et al*, 2010) and 1996-2001 Artesunate versus Artemether (Tran TH *et al*, 1996)). The third study was a matched case-control epidemiological study in adults and children conducted between 2000-2005 at the Hospital of Tropical Diseases and two provincial hospitals in Binh Phuoc province, Phuoc Long and Dong Xoai district hospitals. The forth study started in 2006 is an ongoing collection of adult severe malaria patients specifically for genetics studies based at the Hospital for Tropical Diseases, HCMC.

Severe malaria cases were defined as those who had asexual forms of *P. falciparum* in their peripheral blood smear and had at least 1 of the following; impaired consciousness (Glasgow coma score <11 or Blantyre coma score <5), pulmonary oedema, acute renal failure (oliguria and serum creatinine >265 µmol/L), jaundice (serum bilirubin >51 µmol/L with parasite count >100,000/µL or with serum creatinine >250 µmol/L), hypoglycaemia (blood glucose <2.2 mmol/L), anaemia (haematocrit <20% with parasite count >100,000/µL), hyperparasitaemia (parasite count >500,000/µL), hyperlactataemia (plasma lactate > 4 mmol/L), metabolic acidosis (standard base excess > - 5 mmol/L, base deficit <10 mmol/L), pigmented neutrophil count (>4/100) and shock (SBP< 80mmHg with cool extremities); or had parasitemia ^{3} 5% but none of the above features. Patients with cerebral malaria were defined as those with a Glasgow coma score <9.

The population control individuals were either cord blood controls or community controls. Cord blood control samples were collected from babies born in 2003 and between 2006-2007 at Hung Vuong Obstetric Hospital in HCMC and from babies born in 2003 at Dong Thap Hospital in Dong Thap province. In addition, community controls were recruited as part of the epidemiological study which were individually matched to a proportion of the severe malaria cases by age, gender, ethnicity and location. Potential community controls were questioned about any possible history of severe malaria or time spent in hospital. Any who had spent more than 48 hours in hospital other than for an operation, injury or known non-malaria diagnosis were excluded.

Case report forms (CRF) were specific to each clinical study. Extensive clinical data was collected on patients within the randomised controlled treatment trials, including demographic information, clinical history, clinical evaluation, laboratory testing and clinical outcome. The CRFs for the matched case-control epidemiological study included these parameters as well as information related to environmental exposures and risk factors. The forth study, specifically for human genetics, used a standardised CRF that was created by MalariaGEN CP1. It was used by all sites to collect standardised clinical data in prospective studies. The data collected on all types of CRF were uploaded onto secure web-based software developed by MalariaGEN. Here, the integrity of the data was checked and data was standardised and amalgamated.

Genomic DNA was extracted from whole blood using either the blood midi kit or maxi kit from Qiagen (http://www.qiagen.com/) [Qiagen, Crawley, UK] using manufacturer's instructions in the laboratories of the Oxford University Clinical Research Unit, HCMC,

Vietnam. Aliquots of the DNA samples were shipped to the MalariaGEN Resource Centre in Oxford for further processing and quality control for quantity, quality (by genotyping) and confirming appropriate clinical data was available. Baseline genotype data for 69 malaria-associated SNPs was generated for all contributing samples; briefly, samples underwent a primer-extension pre-amplification (PEP) step (Xu K *et al*, 1993; Zhang L *et al*, 1992) prior to genotyping on the Sequenom® MassArray® platform. Following curation, the genotype data were returned to the PIs for local analyses.

Table 1: Breakdown of samples

Number	**Gender: n (%)**	**Age in years: n (%)**	**Ethnicity: n (%)**
Malaria cases: 597	Male: 452 (75) Female: 140 (23) Not recorded: 9 (2)	5-15: 10 (2) >15: 587 (98)	Kinh: 568 (95) Other: 8 (1) Not recorded: 21 (4)
Healthy controls: 2219	Male: 1091 (49) Female: 1035 (46) Not recorded: 114 (5)	<1: 2219 (100)	Kinh: 2219 (100)

Ethics

Ethical approvals were granted by the scientific and ethical committees at either the Hospital for Tropical Diseases HCMC (proposal number: ID SECHTD 20/04/2006), Hung Vuong Hospital HCMC, Dong Thap Hospital Dong Thap Province, and the People's Committee of Ho Chi Minh City, Department of Health. Protocols were also approved by the Oxford Tropical Research Ethics Committee, UK.

Over the previous 20 years the Hospital for Tropical Disease, in collaboration with OUCRU, performed a number of clinical trials investigating the treatment of malaria. Over this time a large number of blood samples have been archived from severe malaria patients. Ethical approval was granted to use DNA prepared from these archived samples of severe malaria patients in an anonymised fashion. Additionally, ethical approval was granted to collect new

DNA samples (from cases and controls) in order to become a part of large-scale genetic studies.

Prior to study recruitment patients were informed of the risks and benefits of being in these studies. Written informed consent was obtained from each volunteer, however in one early study verbal informed consent was obtained. Where the patient was unable to consent (e.g. if unconscious), the consent of a relative was obtained. For cord blood control samples informed consent was obtained from the mother.

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