

Network: Infection and Inflammation: from Pathogen-induced Signatures to Therapeutic Target Genes**Project: Genetic Association Approach to Identify Endothelial Receptors for *Plasmodium falciparum*****Rolf Horstmann - Bernhard Nocht Institute for Tropical Medicine, Hamburg - horstmann@bni.uni-hamburg.de****Introduction**

Among the four malaria species affecting man, *Plasmodium falciparum* is by far the most important one. It shows highest transmission intensities, causes most infections and is the only human malaria parasite responsible for life-threatening complications, which occur in 1-2% of infected individuals but result in over 1 million fatalities annually.

It has long been known that human genetic factors influence malaria susceptibility. These include red-blood-cell factors like the sickle-cell trait (HbS), α -thalassaemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency, which belong to the most common monogenic disorders of humans and reach prevalences of up to 20% in West Africa, but also tumor-necrosis factor (TNF) and other components of the immune response¹. It is also agreed upon, however, that malaria due to high childhood mortality may have selected for many more variants of the human genome, whereby life-threatening malaria complications may be most relevant to natural selection.

The occurrence of life-threatening disease complications is considered to primarily result from the ability of *P. falciparum* to make parasitized red blood cells adhere to deep-organ vascular endothelium thereby escaping the clearance function of the spleen². The process of adherence not only allows forceful multiplication, it also results in micro-circulatory disturbances or dysregulation. *P. falciparum* adhesins are products of the large family of highly variant *var* genes, which appear to be sequentially expressed in the course of infection. The *P. falciparum* genome sequenced contains 50 gene copies whereby the total number of alleles present in the parasite population is unknown. Most importantly, antibody binding to *var* products appears to be a major component of natural immunity.

So far, eleven human endothelial surface moieties have been identified which function as adherence receptors². Most of them have physiological roles as leukocyte receptors. Since *var*-product adhesion is receptor-specific, organ-specificity of certain endothelial receptors may explain organ-specificity of malaria complications such as cerebral malaria. Using cell biology, it took fifteen years to find the eleven *P. falciparum* endothelial receptors known to date, whereby the last one was reported only a year ago. The time course indicates that many receptors might not have been discovered yet and that methods of cell biology are not readily successful in identifying these molecules. This may be explained by the non-controllable expression of *var* copies by cultured parasite isolates and the rare accessibility of deep organ endothelial cells for *in-vitro* studies.

Therefore, we have started a genetic "systems approach" to search for novel endothelial cell receptors for *P. falciparum* and to investigate the role of established candidates. The genetic approach has the advantage that stratification of patient groups might provide insight into possible organ-specific functions of receptors.

The classic forms of complicated malaria are severe anaemia and cerebral malaria, defined by coma or repeated convulsions³. Cerebral malaria is considered to primarily result from the adherence of parasitized erythrocytes to brain endothelium, but may also involve immunopathology. For instance, high TNF responses were found to increase the risk of developing cerebral malaria. Severe malaria anaemia, defined as a blood haemoglobin concentration of ≤ 5 g/dL in the presence of *P. falciparum* parasitaemia³, is the

commonest presentation of severe malaria in sub-Saharan Africa. The underlying mechanisms are complex and may include destruction of infected and uninfected red blood cells and bone-marrow dysfunction. When severe anaemia was found together with respiratory distress, case fatality was significantly enhanced. This observation raises the question as to whether severe malaria anaemia is a homogeneous syndrome.

Recently, it has become increasingly recognized that complicated malaria is a more complex disease of a number of distinct, although partly overlapping syndromes including respiratory distress and metabolic acidosis, which may well require a wider range of approaches to management.

Results

In NGFN-1, a matched-pair case-control study on severe and complicated malaria was initiated in the University Hospital of Kumasi, Ghana (Fig. 1). Phenotyping strictly followed the definition of the syndrome by WHO³. Paediatric patients with malaria parasitaemia and a coma score of < 3 , blood haemoglobin of < 5 g/dL, or lactate of > 5 mmol/L were enrolled and further examined as to asexual *P. falciparum* parasite density, prostration (age-dependent incapacity of the child to suck, sit, stand, or walk), respiratory distress (deep, acidotic breathing), total blood cell count, venous blood gas analysis, and acid base status. Patients with parasite densities of $> 200,000/\mu\text{L}$ were classified as hyperparasitaemic, those with a base excess of less than -5 as acidotic, and those with a blood glucose of < 2.2 mmol/L as hypoglycaemic. Controls were matched for age, sex, and residence, i. e. the same town quarter or village. As of July 2005, approximately 3200 index cases, 1700 matched controls and 1000 pairs of parents were recruited.



Fig 1: Enrolment of children with complicated malaria on paediatric wards of the 1000-bed Komfo Anokye Teaching Hospital of Kumasi University, Ghana.

Studies on phenotype definitions

Aim of the first study was to determine the role of bacteraemia in children presenting with clinical signs and symptoms of severe malaria as defined by WHO and applied in our study. Bacterial blood cultures were performed in addition to the standard phenotyping procedure on 251 patients meeting the inclusion criteria but, for the period of this study, in addition including those without malaria

parasitaemia. Evaluation of clinical signs and symptoms in relation to the presence of parasitaemia and bacteraemia revealed that, on the basis of clinical signs alone, the 182 malaria-film positive and 69 negative patients were indistinguishable⁴. Forty percent of film-negative patients were bacteraemic compared to 12% of film-positive ones. The small overlap indicated that severe malaria and bacteraemia were not positively associated, as previously suggested. Film-negative, bacteraemic patients had a fatality of 39% primarily affecting the age group of below 30 months. Bacteraemias were predominantly caused by non-typhoid salmonellae. In summary, the study showed that, in our setting of a tertiary medical referral centre in sub-Saharan Africa, the clinical syndrome of severe and complicated malaria to approximately 12% comprises children with bacteraemia. The fatality of 39% of children with bacteraemia makes early diagnosis or preventive antibiotic treatment mandatory.



Fig 2: Child with severe malaria anaemia and no other malaria complication.

The second study aimed at determining the role of prior ineffective antimalaria medications on the presentation of children meeting the inclusion criteria. To this end, 189 consecutive patients enrolled were, in addition to the standard phenotyping procedure, studied for the presence of chloroquine (CQ)-resistant parasites as indicated by mutations in the CQ resistance transporter gene *pfcr1*, plasma CQ levels and prior antimalaria medications by obtaining medical histories through structured interviews with parents or guardians⁴. Of the patients studied, 77% had CQ present in plasma, and 88% were carrying *P. falciparum* of the CQ-resistance genotype. Significant associations were found (i) between the CQ-resistance genotype of parasites and plasma CQ levels, (ii) between the presence of CQ in plasma and the reported duration of illness, and (iii) between the reported duration of illness and the occurrence of severe anaemia in the absence of other complications. The findings suggest that ineffective malaria treatment may support the development of a form of severe malaria anaemia occurring in the absence of other complications. This form of severe anaemia appears to develop over a prolonged period of time and, therefore, may result from a slow decline in haemoglobin (Fig. 2).

Association studies

The first series of association studies applied to the large study group comprised the classic malaria protection factors of HbS, α -thalassaemia, and G6PD deficiency. In particular stratifications for the various forms of severe and complicated malaria revealed several novel and unexpected associations, which have been described and submitted for publication or presently are under more detailed study. So far, seven candidate genes for endothelial adherence receptors of parasitized red-blood cells have been re-sequenced in 23 cases with selected forms of complicated

malaria (Fig. 3) and 23 control children. Substantial numbers of novel variants have been identified. Some of them have been typed in more than 1500 case-control pairs. Whereas selected variants of the CD36, ICAM-1, Fractalkine, and P-selectin genes so far showed trends of associations only, others yielded significant results. At present, the wealth of genotyping data shifts the workload further to medical insight and statistics in order to for each candidate select and analyse the appropriate patient groups and sub-groups.

Outlook

The value of what we believe is the largest well characterized patient group of severe and complicated malaria is greater than we initially thought because, first, our initial studies on phenotype definitions have shown that the syndrome of severe and complicated malaria as defined by WHO, which also serves as gold standard for research, includes more than 10% admixture from other etiologies such as bacteraemias. More importantly, our first genetic analyses have clearly indicated a significant heterogeneity of the genetic influences on the individual clinical forms of the complex syndrome, implying that the relevant sample size is not that of the entire study group but that of the smallest relevant sub-group. Accordingly, we have been seeking continued funding, mostly through intervention studies, in order to further increase the sample size.

However, analysing the study group as it stands has already yielded exciting results, simply due to the fact that the group presently is so large and well characterized. Therefore, we expect that our approach will in the near future continue to allow important new insights into the pathogenesis of life-threatening malaria complications and, thereby, the design of new intervention strategies.



Fig 3: Child with severe malaria anaemia in conjunction with acidosis and respiratory distress.

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Members of the study team include Tsiri Agbenyega, Christa Ehmen, Jennifer Evans, Jürgen May and many more.

Photography: Mika Väisänen (www.mika-photography.de)