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Studies on the effect of the lowered activity of the erythrocytic pyridoxal kinase on *Plasmodium falciparum* blood stages

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Table of Contents

Table of Contents 1
Abbrevations 4
1 Introduction **8**
1.1 Malaria in the past and present 8
1.2 The malaria parasite and its life cycle 10
1.3 Clinical aspects of malaria 13
1.4 Host genetics and malaria 15
1.4.1 Erythrocytic enzymopathies and their influence on malaria 18
1.6 Vitamin B_6, function and metabolism 22
1.6.1 Metabolism of vitamin B_6 23
1.6.2 Metabolism of vitamin B_6 in the malaria parasite 24
1.7 Polymorphism of the human erythrocytic pyridoxal kinase 27
1.8 Aim of Project **28**
2 Materials and Methods **29**
2.1 Materials **29**
2.1.1 Chemicals 29
2.1.2 Buffers, media and solutions 30
2.1.3 Kits 34
2.1.4 Equipments 35
2.1.5 Consumables 36
2.1.6 Enzymes, Antibodies and Ladders 37
2.1.7 *P. falciparum* laboratory strains 38
2.2 Methods **39**
2.2.1 Screening human genomic DNA for the 8 bp insertion in the promoter region of the pyridoxal kinase gene 39
2.2.1.1 Isolation of human genomic DNA 39
2.2.1.2 Whole genome amplification (Genomiphi) 40
2.2.1.3 Polymerase Chain Reaction (PCR) 40
2.2.1.5 PCR purification 42
2.2.1.6 Cloning of the 700bp PCR fragment 42
2.2.1.7 Nested PCR for screening the 700 bp PCR fragments for the presence of the 8 bp insertion 45
2.2.1.8 Alternative PCR Screen 48
2.2.2 Cell Culture 49
2.2.2.1 In vitro culture of *P. falciparum* 49
2.2.2.2 Preparation of human serum and erythrocytes 50
2.2.2.3 Staining of *P. falciparum* with giemsa 50
2.2.2.4 Determining parasitemia 50
2.2.2.5 Freezing parasites 50
2.2.2.6 Thawing parasites 51
2.2.2.7 Parasite synchronisation with sorbitol 51
2.2.2.8 Gelatine flotation 52
2.2.3 Growth experiments 52
2.2.3.2 ABO blood group test 53
2.2.3.3 Haemoglobin variant test 53
2.2.3.4 Growth experiment set up 53
2.2.3.5 Flow cytometry 54
2.2.3.6 Determining parasitemia with flow cytometry 56

2.2.3.7 Short term growth experiments 56
2.2.4 Erythrocyte deformability assay 57
2.2.4.2 Erythrocyte count 57
2.2.4.3 Erythrocyte filtration 58
2.2.4.4 Infected erythrocytes deformability assay 58
2.2.5 Western blot analysis 59
2.2.5.1 Erythrocyte preparation 59
2.2.5.2 Erythrocyte count and lysis 59
2.2.5.3 SDS Polyacrylamide gel electrophoresis 60
2.2.5.4 Blotting (Semi-dry) 61
2.2.5.5 Detection 62
2.2.5.6 Blot quantification 62
2.2.6 Erythrocytic pyridoxal kinase activity assay 63
2.2.6.1 Preparation of erythrocytes 63
2.2.6.3 Pyridoxal kinase assay 63
2.2.7.1 Erythrocyte and parasite lysis 65
2.2.8 Electron microscopy 67
2.2.8.1 Tokuyasu method 68
2.2.9 Quantitative real time PCR 71
2.2.9.1 Isolation of mRNA from *P.falciparum* 71
2.2.9.2 DNase treatment 71
2.2.9.3 Reverse Transcription (cDNA synthesis) 72
2.2.9.4 Real time PCR 72
3 Results **74**
3.1 Screening individuals for the 8 bp insertion in the promoter region of the pdxK gene 74
3.1.1 Screening Caucasians for the 8 bp insertion in the promoter region on the *pdxk* gene 76
3.1.2 Screening east Africans for the 8 bp insertion in the promoter region of the *pdxk* gene 76
3.1.3 Sequencing the promoter region of the *pdxk* gene 77
3.1.3.1 In/Del event in the promoter region of the *pdxk* gene 78
3.1.3.2 MatInsepector analysis of the 7 bp In/Del event 79
3.3 Pyridoxal kinase activity 85
3.4 Pyridoxal 5- phosphate content of the erythrocytes of the various genotypes under in vitro culture conditions 89
3.5 In vitro growth experiments 91
3.5.1 ABO Blood group and Hb variant test for growth experiments 92
3.5.2 Comparing parasitemia in pyridoxine depleted medium 94
3.5.3 The growth of *P. falciparum* in erythrocytes of two unrelated individuals bearing the same genotype 96
3.6 Expression analysis of *pfpdx1* and *pfpdxk* following growth in erythrocytes of the various genotypes 98
3.6.1 Expression analyses of *pfpdx1* and *pfpdxk* following culture in pyridoxine depleted medium 100
3.7 PLP content of parasites cultured in erythrocytes of the various genotypes 101
3.8 Erythrocyte deformability assay 102
3.8.1 Evaluating the method 102
3.8.2 Filtration rate of erythrocytes bearing the +/+, +/- and -/-genotype 103
3.9 Parasite morphology 104
4. Discussion **110**

4.1 Conclusions.....121
References **125**

Abbrevations

APS	Ammonium sulphate
ATP	Adenosine triphosphate
AP	alkaline phosphatase
BSA	Bovine serum albumin
Bp	Basepair
°C	degree celsius
DNA	Desoxynucleotide acid
cDNA	complementary DNA
gDNA	genomic desoxynucleotide acid
G6PD	glucose-6-phosphate dehydrogenase
EDTA	Ethylendiaminotetraacetic acid
h	Hour
Hb	Haemoglobin
In/del	Insertion/deletion
Kb	kilo basepair
MgCl	Magnesium chloride
Min	Minute
mm	millimeter
mRNA	messenger RNA
ng	nanogram
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PdxK	Pyridoxal kinase
Pf	*Plasmodium falciparum*
PL	Pyridoxal
PN	Pyridoxine
PM	Pyridoxamine
PLP	Pyridoxal 5-phosphate
PVM	parasitophorous vacuole membrane
PV	parasitophorous vacuole
RNA	Ribonucleic acid
RT	Room temperature
SDS	Dodecylsulfat-Sodium salt
Vit B_6	Vitamin B_6
TAE	Tris/Acetic acid/EDTA

TE	Tris/EDTA
UV	ultraviolet
V	Volt
µ	micro (1 x 10-6)
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
+/+	homozygote insertion
+/-	heterozygote
-/-	homozygote deletion

Summary

Population studies in humans have long detected the enormous selective pressure placed by the malaria parasite on its human host and the fundamental role of host genetics factor for the progression of the disease. The erythrocyte is the major site for genetic polymorphisms that offer protection against malaria. Genetic alterations that affect erythrocytic metabolic enzymes have been associated with reduced susceptibility to malaria.

The erythrocytic pyridoxal kinase (PdxK) plays an important part in the metabolism of vitamin B_6 and it has been proposed that genetic polymorphism reducing the activity of this enzyme in erythrocytes could also be a trait that offers protection against malaria. An early study attempted to correlate the lowered erythrocytic PdxK activity with reduced susceptibility to malaria. The genetic basis for the lowered erythrocytic PdxK activity has been resolved. An 8 bp insertion in the promoter of the *pdxk* gene leads to a higher activity of the enzyme in erythrocytes.The absence of the 8 bp insertion (-/-) leads to a lower activity.

In my study I investigated if the lowered activity of the erythrocytic Pdxk has an effect on the malaria parasite *Plasmodium falciparum* in an in vitro culture system.

Employing western blot analysis, I could show that the lowered erythrocytic PdxK activity is as a result of lower protein levels of the enzyme in erythrocytes of individuals without the 8 bp insertion in the *pdxk* promoter. I cultured *P.falciparum* blood stages with erythrocytes from individuals homozygous for the insertion event (+/+), heterozygotes (+/-) and homozygous for the deletion event (-/-). I observed that the growth of the parasite is impaired when cultured in erythrocytes with lowered erythrocytic PdxK activity and that the parasite responds to this trait by an upregulation of its own de novo and salvage pathway for vitamin B_6. I also developed a PCR based method for screening human genomic DNA for the presence or absence of the 8 bp insertion in the promoter of the *pdxk* gene. Screening individuals for this polymorphism showed that the absence of the 8 bp insertion which leads to the lower erythocytic PdxK activity is more prevalent among Africans as compared to Caucasians. My results provide additional evidence for the hypothesis that the lowered erythrocytic PdxK activity could indeed be another genetic trait offering protection against malaria

Zusammenfassung

Studien haben gezeigt, dass der Malaria-Erreger einen starken Selektionsdruck auf den menschlichen Wirt ausübt. Genetische Faktoren des menschlichen Wirts spielen eine wichtige Rolle im Verlauf der Malaria-Erkrankung. Der Malaria-Erreger infiziert und vermehrt sich in den roten Blutkörperchen des Menschen. Genetische Polymorphismen, die für ihre schützende Wirkung vor Malaria bekannt sind, kommen daher vor allem in den roten Blutkörperchen vor.

Die Pyridoxal kinase (PdxK) der Erythrozyten spielt eine wichtige Rolle in dem Metabolismus von Vitamin B_6. In 1976 wurde bereits versucht den Zusammenhang zwischen der niedrigeren Aktivität der erythrozytären PdxK und einer verminderten Anfälligkeit für Malaria herzustellen. Mittlerweile ist der genetische Hintergrund für die niedrigere Aktivität der PdxK in Erythrozyten bekannt. Es konnte gezeigt werden, dass eine 8 bp Insertion im Promoter des *pdxk* Gens zu einer höheren Aktivität des Enzyms in den roten Blutkörperchen führt. Bei Abwesenheit dieser 8 bp Insertion hingegen ist die Aktivität von PdxK verringert.

In meiner Doktorarbeit untersuchte ich, ob die niedrige PdxK Aktivität einen Einfluss auf dem Malaria-Erreger, *Plasmodium falciparum*, in vitro hat.

Meine Daten haben gezeigt, dass Individuen ohne die 8 bp Insertion im Promoter des *pdxk* Gens, in ihren Erythrozyten eine geringere Menge des Proteins vorhanden ist und dadurch auch eine niedrigere Aktivität des Enzyms aufweisen. Ich habe darüber hinaus beobachtet, dass das Wachstum des Parasiten gehemmt ist, wenn er in Erythrozyten von Individuen mit einer niedrigen PdxK Aktivität kultiviert wird. Der Parasit reagiert auf dieses genetische Merkmal mit einer Hochregulierung der eigenen Enzyme für die Erhaltung des Vitamin B_6 Haushalts. Meine Daten haben auch gezeigt, dass die Abwesenheit des 8 bp Insertion in dem *pdxk* Promoter, die zu einer niedrigeren PdxK Aktivität führt, häufiger bei Afrikaner als bei Europäer auftritt.

Meine Ergebnisse weisen darauf hin, dass die niedrigere PdxK-Aktivität in Erythrozyten ein genetisches Merkmal ist, das wahrscheinlich gegen Malaria schützen kann.

1 Introduction

1.1 Malaria in the past and present

Malaria is, and has always been, a major plague of mankind. Malaria is an ancient disease and references to this disease occur in historical documents from China as early as about 2700 BC and from Mesopotamia about 2000 BC. Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC mention a disease that is most certainly malaria. In ancient Greek, Hippocrates was the first to describe the manifestations of the disease and relate them to the time of year and to the marshy places where people lived. In the 18 century the people related these typical fevers to the foul air near swamps and therefore it was widely named as malaria from the Italian “mala aria”

In 1880 the army physician A. Laveran (1845-1922) first described malaria parasites in human blood. With the accidental discovery of a methylene blueeosin stain by D. L Romanowsky in 1891, the parasites could be stained in blood smears and this facilitated the search for other malaria parasites in birds, reptiles and mammals. Over the centuries, circumstantial evidence had accumulated that suggested that mosquitoes might somehow be connected with malaria and by 1883 the American physician, Albert King, had assembled the mass of evidence that was to become known as the mosquito-malaria doctrine. In 1884 Sir Patrick Manson proposed a transmission from person to person by mosquitoes. He had earlier shown that the parasite of filariasis was taken up by female mosquitoes during their blood meal, and that the parasites continued their development in the abdomen of the mosquito. He considered that this could as well be the case for the malaria parasite and his idea was supported by Sir Ronald Ross. Ross who was working in India found the pigmented cysts on the stomach wall of the Anopheles mosquito in 1897. A year later Ross worked out the complete life cycle of bird malaria and was able to show that the route of infection was via the bite of an infected mosquito.

The life cycle of the parasite in humans remained incompletely understood and exoerythrocytic development of the parasite remained a mystery. In 1898, MacCullum observed developmental stages of *P. relictum* in the liver and spleen of infected birds. The question of the exoerythrocytic development of the human malaria

parasite however remained unresolved until 1947 when H. Shortt and C. Garnham showed that a phase of division in the liver preceded the development of parasites in the blood (Cox 2010).

In 1900, the area of human malaria risk was about 53% of the earth's land surface. From the middle Ages until last century parts of Europe and North America were endemic areas. Malaria in the more northern parts of Europe had begun to decline in the last century. Denmark and Sweden became free of malaria transmission in the 1920s and 1930s whereas countries in southern Europe, Italy, Turkey and Greece were freed of malaria transmission in the 1960s. The percentage of the global population at risk has decreased from 77% at the turn of the 20th century to as low as 48% in 2002.

Today, malaria remains in poor areas of the world with climate suitable for its transmission. More than 120 Plasmodium species have been identified of which *P. falciparum*, *P. vivax, P. knowlesi, P. ovale* and *P. malariae* are able to infect human hosts. 2.2 billion People are exposed to the threat of *P. falciparum* malaria which is the most lethal form of this disease. With 70% and 25%, most clinical events attributed to *P .falciparum* are found in Africa and in South East Asia (Snow, Guerra et al. 2005). Between 700,000 and 2.5 million persons die yearly from malaria, over 75% of them are children in Sub-Saharan Africa (Breman 2001). Figure 1.1 shows the global distribution of *P. falciparum* malaria today.

The health- and socio-economic impact of *P. falciparum* malaria is highest in Sub-Saharan Africa. Malaria is also strongly associated with poverty. Taking into consideration factors such as tropical location, colonial history, and geographical isolation, it has been shown that countries with intensive malaria had income levels of only 33% of that of countries without malaria, whether or not the countries were in Africa. The impact of malaria on the economic growth of countries is large. The loss in growth of countries with endemic malaria is estimated to be as high as 1.3% per year and the annual loss in productivity in Africa due to malaria is calculated to be up to 12 billion US$ (Gallup and Sachs 2001; Samba 2001).

In the on-going fight against malaria, an important event took place in April 2000, when a large numbers of African leaders gathered to attend the "Summit on malaria" and discussed ways to fight malaria and thereby ease the physical and economic suffering of its people (Samba 2001).

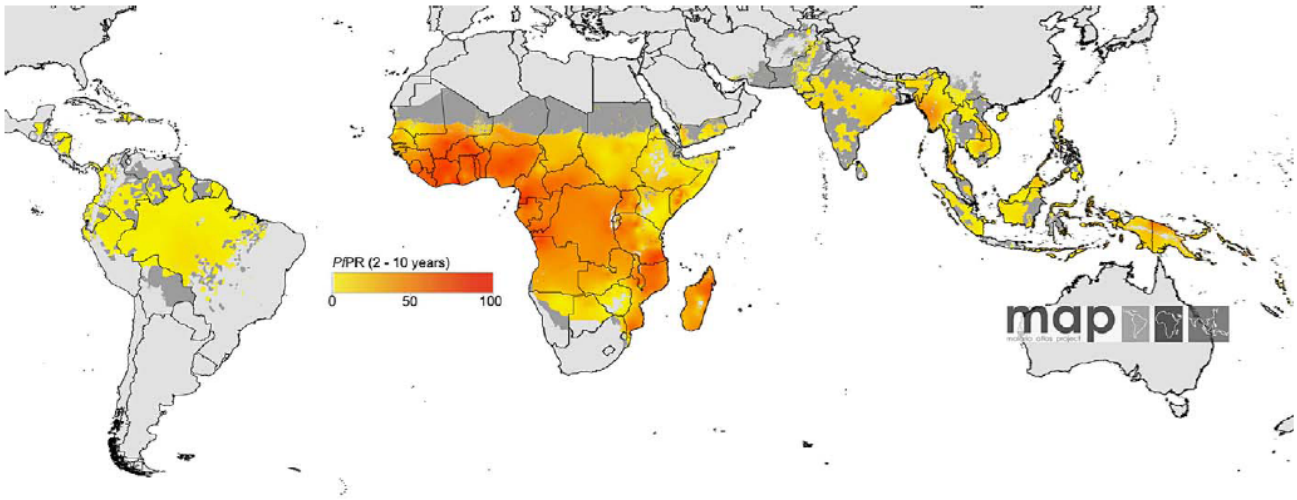

Figure 1.1: The spatial distribution of P. falciparum malaria endemicity
The global distribution of the annual *P. falciparum* parasite rates (PfPR) in children from 2-10 years (yellow to red, 0%–100%) is shown. The rest of the land area was defined as unstable risk (dark grey) (*Hay, Guerra et al. 2009)*.

1.2 The malaria parasite and its life cycle

Malaria parasites are protozoa of the genus *Plasmodium.* Genealogically they are organized in the phylum of *Apicomplexa*, suborder of *Haemosporidiae* and family of *Plasmodiiae*. The malaria parasites are transmitted by the female *Anopheles* mosquito and possess a very complex life cycle. The natural ecology of malaria involves the malaria parasites infecting successfully two different hosts, humans and the female Anopheles mosquito. The parasite must therefore adapt to very different environmental and host specific conditions such as cellular metabolism, temperature etc. All *Plasmodium* species have a common ancestor which evolved as early as 130 million years ago with a two host life cycle in Dipterans and vertebrates suggesting a strong co-evolution of the parasite and its host (Carter and Mendis 2002).

The complex life cycle of the parasite includes three stages of two distinct proliferation forms with massive intracellular asexual multiplication, the sporogony (mosquito midgut) and schizogony (liver and blood).

During a blood meal, a malaria infected female *Anopheles* mosquito inoculates the parasite in its sporozoite form into the skin of the human host. The sporozoites migrate in the skin of individuals, eventually enter the blood stream and travel to the liver. Sporozoites enter the liver sinusoids either via the hepatic arteriole or the portal venule. Kupffer cells are the resident macrophages of the liver and are strategically positioned on the sinusoidal lumen of the liver. Sporozoites actively invade these cells, safely transverse them before finally invading hepatocytes (Frevert 2004). During invasion the parasitophorous vacuole membrane (PVM) forms around the sporozoite. The liver stage undergoes nuclear replication and matures to hepatic schizonts followed by the release of tens of thousands of merozoites into the blood stream (Amino, Thiberge et al. 2006; Vaughan, Aly et al. 2008). The liver stage infection in *P. falciparum* takes a period of seven days (VanBuskirk, O'Neill et al. 2009). Intensive studies on the development of the parasites in hepatocytes have been carried out on the rodent malaria model. It has been shown that in the case of the rodent malaria, *Plasmodium berghe*i merozoites are released directly into the bloodstream. The parasite induces the death and detachment of their host hepatocytes, followed by the budding of parasite-filled vesicles (merosomes) into the sinusoid lumen of the liver (Sturm, Amino et al. 2006).

In the case of *P. vivax* and *P. ovale*, some of the sporozoites entering hepatocytes (the proportions vary, depending on the strain) do not develop into exo-erythrocytic schizonts directly, but instead form hypnozoites. These small parasite forms (4-5 µm in diameter) can remain dormant in the liver for years. At a given point in time, although the triggering signal is still unknown, the hypnozoites develop into exo-erythrocytic schizonts, producing thousands of merozoites, thereby causing relapses of the disease (Gilles and Warrel 1993).

Merozoites released into the bloodstream, invade erythrocytes und become enclosed in a parasitophorous vacuole, thus beginning the asexual blood stage of the life cycle. In the erythrocytes the parasite undergoes another asexual reproduction, the erythrocytic schizogony. The first stage of the intraerythrocytic development is the ring stage, which is followed by the metabolically very active trophozoite stage. Parasite development is finally completed with the fully developed schizont stage

harbouring 16 – 32 daughter merozoites (Gilles and Warrel 1993). The length of the intraerythrocytic cycle differs between different *Plasmodium* species and strains, typically 48 hours (*P. falciparum, vivax* and *ovale*) or 72 hours (*P. malariae*), which explains the periodicity of the fever paroxysms experienced by the patient. Upon primary rupture of the parasitophorous vacuole followed by rupture of the erythrocyte membrane, the merozoites are released into the blood stream where they invade new erythrocytes (Wickham, Culvenor et al. 2003).

A few merozoites do not develop into schizont but mature to sexually differentiated gametocytes. When an *Anopheles* mosquito ingests blood from an infected person, the red blood cells infected with asexual parasites are digested with the uninfected, but it is the only gametocytes, which undergo further development. The gametocytes differentiate into male microgametes and the female macrogamete within the midgut lumen of the mosquito. The male microgametes fertilize the female macrogamete and the zygote develops into an ookinet, which penetrates the midgut epithelium and forms an oocyst on the basal membrane. After rupture of the mature oocyst more than 1,000 sporozoites are released and some invade the mosquito's salivary glands via the haemolymph to be injected into a new human host when the female mosquito feeds again (Han, Thompson et al. 2000).

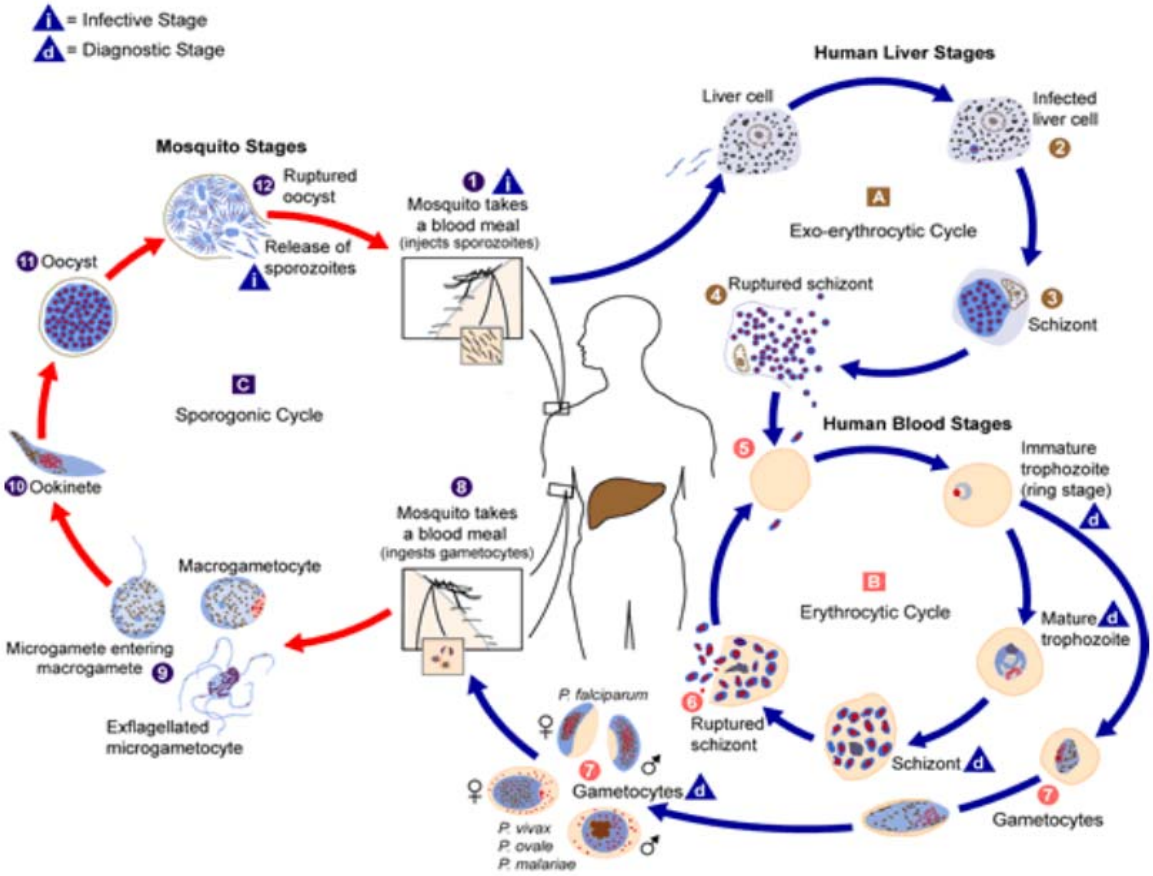

Figure 1.2: Life cycle of the malaria parasite. Details described in text at www.dpd.cdc.gov

1.3 Clinical aspects of malaria

The clinical manifestations of malaria vary with geography, epidemiology, immunity and age. In the highly endemic regions of Africa, malaria-attributable death rates have been reported as high as 25–30 percent for children. Another group highly at risk are pregnant women, who are at risk of delivering low birth newborns.

Malaria is an acute febrile illness and the severity of disease outcome depends on factors attributed to both the parasite and its host. The emergence of natural acquired immunity (NAI) against malaria is a slow process in which factors like exposure, age and intervention play a vital role. The severity outcome of the disease

depends largely on the transmission dynamics in endemic areas. In regions where endemic transmission is low, severe malaria occurs mostly among children but also adults are susceptible. In areas of stable transmission older children and adults develop partial immunity after repeated infections and are at relatively low risk for severe disease (Baird, Masbar et al. 1998; Breman 2001; Doolan, Dobano et al. 2009).

The first clinical sign of malaria is fever followed by febrile paroxysms. 2 to 3 days before the first paroxysm patients often complain of malaise, headache, dizziness, fatigue, pain, anorexia and nausea. The paroxysms are divided into three stages: the cold stage (shivering), the hot stage (fever) and the sweating stage with subsequent declining fever. The paroxysms periods repeat every 48 or 72 hours in dependence of the *Plasmodium* species. Due to these patterns malaria was given its other names: Benign tertian malaria (*P. vivax*), malignant tertian (*P. falciparum*), tertian (*P. vivax*, *P. ovale*) and quartana malaria (*P. malaria*). It only occurs with a synchronized parasite population in the patient. In *P. falciparum* malaria the periodicity is often not clearly defined resulting in continuous or remittent fever in patients.

The majority of malaria cases present as these febrile episodes that can be easily terminated either by anti-malarial treatment or finally by host responses. However a minority of cases of about 1% progress to a life threatening form of disease which is referred to as severe or complicated malaria. The development of severe malaria is caused by adhesion and sequestration of parasites in the vasculature, release of bioactive molecules as well as host immune responses. Severe malaria is a multi complex disorder which features as cerebral malaria, severe anaemia, metabolic acidosis, hypoglycaemia, renal failure, respiratory distress, hepatic dysfunction, circulatory collapse and coma (Miller, Good et al. 1994; Mackintosh, Beeson et al. 2004; Kirchgatter and Del Portillo 2005).

Pregnant women comprise the main adult risk group of malaria. The outcome of malaria in pregnancy is severe maternal anaemia, low birth weight and fetal loss (Mackintosh, Beeson et al. 2004). Pregnancy associated malaria is frequent in first time pregnancies and the risk declines with following pregnancies (Salanti, Dahlback et al. 2004).

1.4 Host genetics and malaria

The malaria parasite and its human host have been closely linked for over a million years (Carter and Mendis 2002). The *Plasmodium* parasite successfully infects and replicates in both its human and insect host. During its whole life cycle, the parasite depends on specific interactions with the host for to gain access to nutrients, to overcome physical barriers and to avoid immune responses. Today, the parasite is highly adapted to its host and an expert in survival. Studies on human populations in endemic regions show that the parasite exerts enormous selective pressure on its human host, revealing the trails of coevolution. Until now, several human genetic traits have been identified that affect susceptibility to and/ or pathogenesis of *P. falciparum* malaria and these traits are widely distributed in regions where malaria is endemic (Cooke and Hill 2001).

Any inherited genetic variant, that affects the replication of the parasites in its human host (erythrocytes, hepatocytes), in the mosquito midgut and/or alters the recognition of the parasite by the host immune system, has as a great impact on the outcome of the disease (Bongfen, Laroque et al. 2009). Figure 1.3 show different host factors that interact with the parasite during its life cycle which when genetically altered lead to protection against the disease.

In the human host the erythrocyte is the major site of parasite infection and replication and is therefore the main spot for genetic alterations affecting erythrocytic proteins and enzymes that confer resistance against malaria (Min-Oo and Gros 2005). The best described example for genetic polymorphisms affecting the erythrocyte are the haemoglobinopathies (Roberts and Williams 2003; Richer and Chudley 2005). The most common haemoglobinopathies are the α- and β-thalassaemia where there is a deficient synthesis of the globin protein and three structural mutations of the β-globin chain, haemoglobin S HbS (β6 Glu → Val), Haemoglobin C HbC (β6 Glu → Lysin), haemoglobin E HbE (β26 Glu → Lys).

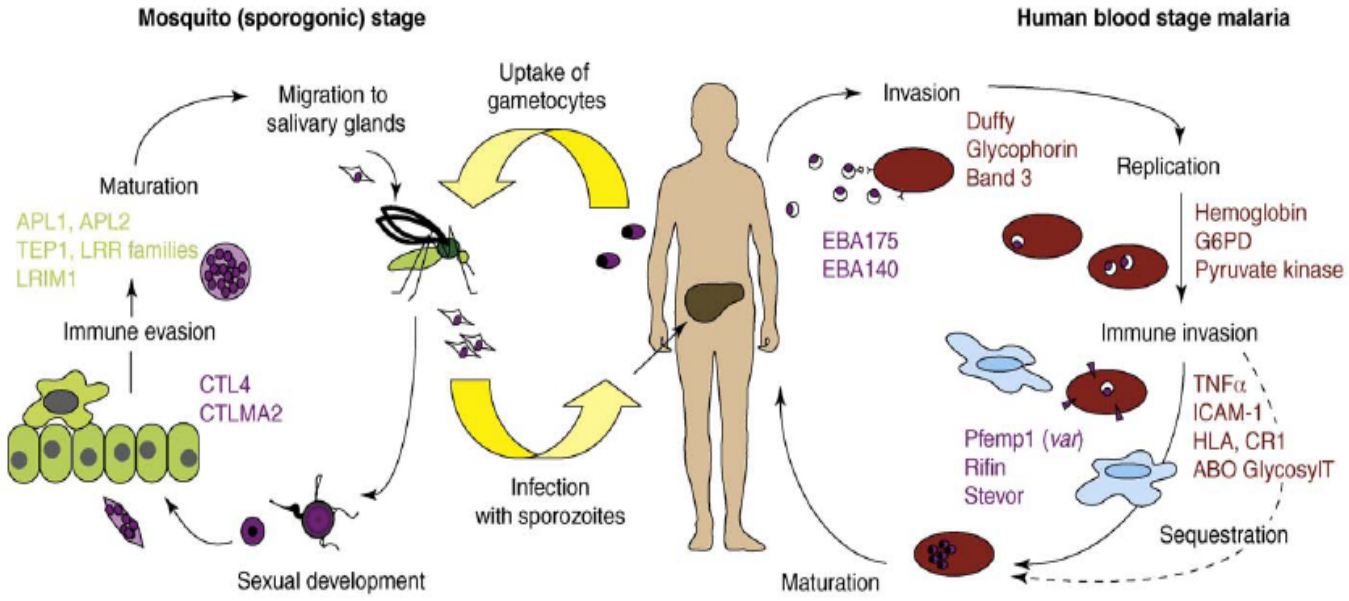

Figure 1.3: Genetic interactions between host and parasite during the malaria life cycle. Both human and mosquito have evolved a series of genetic mechanisms that offer protection against *Plasmodium* parasite infection. Bongfen et al., 2009

The haemoglonopathies are widely distributed in malarious regions of the world. Figure 1.4 shows the global distribution of erythrocytes polymorphism.

HbS reaches its highest frequencies in tropical and sub-tropical Africa, Saudi Arabia and India, HbC is very frequent in West Africa, HbE in Southeast Asia, β thalassaemia in the Mediterranean and Asia and α thalassaemia in India (Flint, Harding et al. 1993).

The advantage of the HbS gene against fatal malaria is due to its heterozygote state HbAS. The sickle cell disease which is caused by the homozygous state of the sickle gene (HbSS) is lethal in early childhood. The protective effect of the HbAS trait has been intensively studied. It has been shown that HbAS trait offer protection against all causes of mortality, severe malaria anaemia and high density parasitemia. The HbAS trait contributes to a 90% reduced risk of severe malaria (Aidoo, Terlouw et al. 2002; Williams, Mwangi et al. 2005).

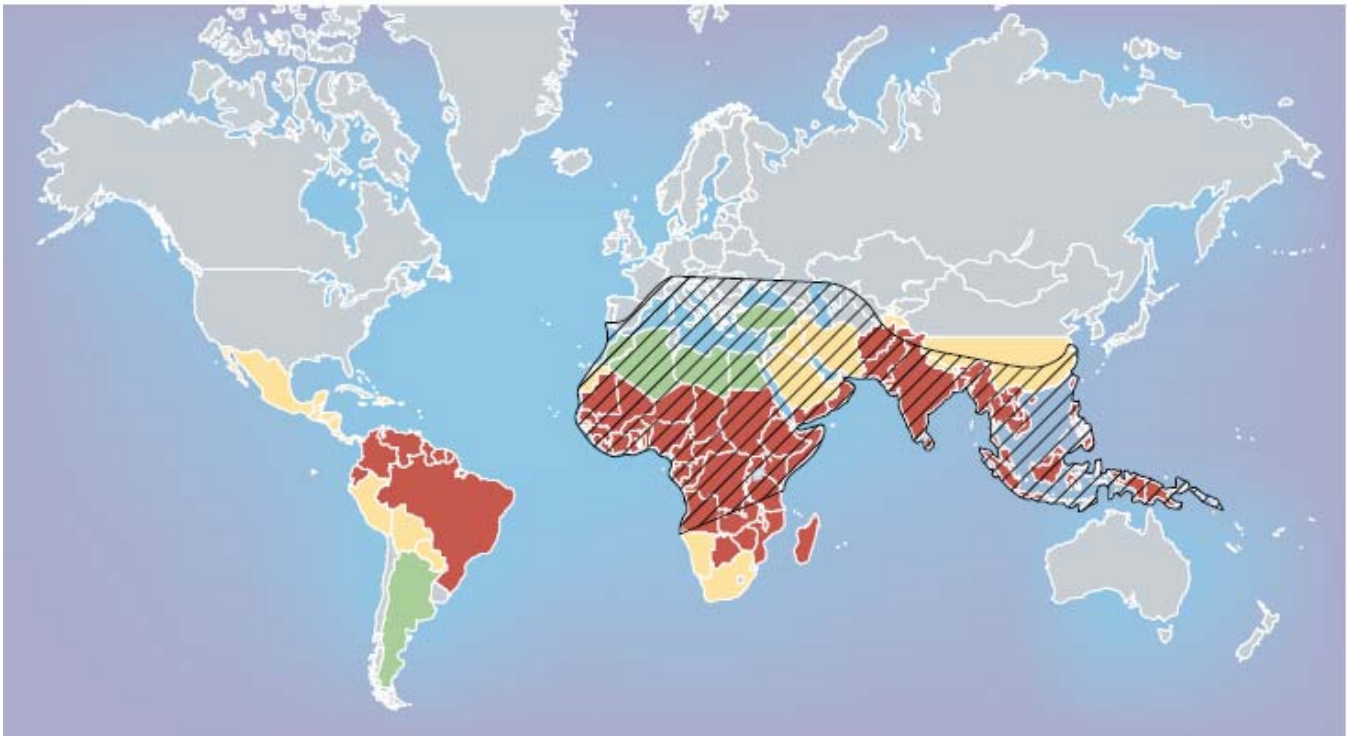

Figure 1.4: Global distribution of malaria and red blood cell disorders. Green indicates areas where malaria is only present in a few remote locations, yellow indicates areas with intermediate malaria risk and red indicates areas with high malaria risk. The hatched area shows the distribution of red-blood-cell disorders. *Cooke et a*l., 2001

The HbC and HbE trait have also been shown to have an influence on malaria. These genetic traits are known to have only very mild clinical effects in their homozygous state. Case control studies have shown that HbC is associated with a 29% reduction in risk of clinical malaria in HbAC heterozygotes and with a 93% reduction in risk in HbCC homozygotes. A homozygous advantage for HbC is consistent with observations from a smaller study which reported no episodes of severe malaria in HbCC subjects (Modiano, Luoni et al. 2001; Mockenhaupt, Ehrhardt et al. 2004). HbE has been shown to protect against high levels of parasitemia which is associated with severe malaria (Chotivanich, Udomsangpetch et al. 2002).

The protective mechanism of the thalassaemia trait for malaria remains unclear. Although this trait has no effect on parasite densities, studies have provided sufficient evidence to show its protective effect against severe malaria (Williams, Maitland et al. 1996; Mockenhaupt, Ehrhardt et al. 2004; Williams, Mwangi et al. 2005; Williams, Wambua et al. 2005).

Erythrocyte polymorphisms that affect the invasion of the parasite have been shown to have an impact on malaria. These red blood cell variants include the Duffy

negativity and the Gerbich erythrocyte variant. The invasion pathway of *Plasmodium vivax* involves the Duffy antigen on the surface of the erythrocyte. The absence of its expression in the Duffy negative blood group leads to complete resistance to *P. vivax* invasion. *P. vivax* malaria is therefore nearly absent in Sub-Saharan African where the Duffy negative blood group is close to fixation (Miller, Mason et al. 1975; Miller, Mason et al. 1976; Langhi and Bordin 2006). A family of glycophorin (GYP) proteins (GYPA, GYPB, and GYPC) binds to *Plasmodium* erythrocyte binding antigen (EBA) 175 (GYPA/B) and EBA140 (GYPC) and are required for invasion by *P. falciparum.*
It has been shown that the GYPC-non expressing Gerbich-negative blood group found in malaria-endemic coastal areas of Papua New Guinea [PNG] (45% frequency) shows reduced invasion of erythrocytes by *P. falciparum* (Maier, Duraisingh et al. 2003).

One genetic polymorphism that affects the cytoskeleton of the erythrocyte is the Melanesian ovalocytosis. This occurs due a mutation in the band 3 protein (an anion exchanger) of the red blood cell and is lethal in a homozygous state. Case control studies have shown that this polymorphism is highly protective against severe malaria (Genton, al-Yaman et al. 1995; Allen, O'Donnell et al. 1999).

1.4.1 Erythrocytic enzymopathies and their influence on malaria

Today a number of genetic polymorphisms are known that affect enzymes of the erythrocytic metabolism. These enzymopathies have been shown to have malaria protective effect. The most common of these enyzymopathies is the glucose -6-phosphate dehyrogenase (G6PD) deficiency. The geographical distribution of G6PD deficiency and its high frequency in areas which are holo-endemic for malaria, suggest that it is protective against *P. falciparum* malaria. The G6PD deficiency is an X chromosome linked disorder and approximately 400 variants are reported. The highest prevalence rate are found in tropical Africa and tropical/ subtropical Asia and have been shown to protect against severe malaria (Gilles, Fletcher et al. 1967; Roth, Raventos-Suarez et al. 1983; Ruwende, Khoo et al. 1995; Guindo, Fairhurst et al. 2007).

Recent in vitro studies have shown that erythrocytic pyruvate kinase deficiency also protects against malaria. The protective mechanism was associated with lower replication rates of *P. falciparum* in erythrocytes bearing the deficient pyruvate kinase

and enhanced phagocytosis by macrophages (Min-Oo, Fortin et al. 2003; Ayi, Min-Oo et al. 2008)

Another current in vitro study revealed that deficiency of the erythrocytic glutathione reductase (GR) also protects against malaria. It was shown that GR deficiency of red blood cells however had no effect on the replication rates of the parasites but increased the rate of phagocytosis of infected erythrocytes by macrophages (*Gallo*, *Schwarzer* et al. 2009).

The human inducible nitric oxide synthase (iNOS) is another enzyme that has been proposed to be genetically altered due to the selective pressure of malaria. This enzyme is not associated with the erythrocyte but is found in the peripheral blood mononucleated cells (PBMC). The iNOS generates nitric oxide, which is a free radical with antiparasitic properties (Balmer, Phillips et al. 2000). iNOS is a product of the *NOS2A* gene and several polymorphic variants of the gene are present. The NSO2A-954C allele of this gene has been shown to protect against severe malaria (Kun, Mordmuller et al. 1998). However there are human population studies that provide contradictory evidence illustrating no association between polymorphisms of the iNOS and malaria (Levesque, Hobbs et al. 1999).

An overview of various host factors associated to malaria and their characteristics are listed in table 1

Table 1: Host factors associated with malaria.

Different human factors are listed affecting resistance (a), polymorphisms of host receptors for cytoadherence by *P. falciparum* infected erythrocytes influencing susceptibility or resistance (b) and immune gene associations with resistance and susceptibility to malaria (c) (adapted from *Kwiatkowski, 2005)*

a) **Common Erythrocyte Variants That Affect Resistance to Malaria**

Gene	Protein	Function	Reported Genetic Associations with Malaria
FY	Duffy antigen	Chemokine receptor	FY*O allele completely protects against *P. vivax* infection.
G6PD	Glucose-6-phosphatase dehydogenase	Enzyme that protects against oxidative stress	G6PD deficiency protects against severe malaria.
GYPA	Glycophorin A	Sialoglycoprotein	GYPA-deficient erythrocytes are resistant to invasion by *P. falciparum*.
GYPB	Glycophorin B	Sialoglycoprotein	GYPB-deficient erythrocytes are resistant to invasion by *P. falciparum*.
GYPC	Glycophorin C	Sialoglycoprotein	GYPC-deficient erythrocytes are resistant to invasion by *P. falciparum*.
HBA	α-Globin	Component of hemoglobin	α^+ Thalassemia protects against severe malaria but appears to enhance mild malaria episodes in some environments.
HBB	β-Globin	Component of hemoglobin	HbS and HbC alleles protect against severe malaria. HbE allele reduces parasite invasion.
HP	Haptoglobin	Hemoglobin-binding protein present in plasma (not erythrocyte)	Haptoglobin 1-1 genotype is associated with susceptibility to severe malaria in Sudan and Ghana.
SCL4A1	CD233, erythrocyte band 3 protein	Chloride/bicarbonate exchanger	Deletion causes ovalocytosis but protects against cerebral malaria.

b) **Host Molecules That Mediate Cytoadherence by *P. falciparum*–Infected Erythrocytes and That Have Been Reported to Show Association with Resistance or Susceptibility to Malaria**

Gene	Protein	Interaction with Parasitized Erythrocyte[a]	Reported Genetic Associations with Malaria
CD36	CD36 antigen, thrombospondin receptor	PE-binding receptor on endothelium and dendritic cells	*CD36* polymorphisms show variable associations with severe malaria in the Gambia, Kenya, and Thailand.
CR1	CR1, complement receptor 1	PE-binding receptor on erythrocytes	*CR1* polymorphisms show variable associations with severe malaria in the Gambia, Thailand, and Papua New Guinea.
ICAM1	CD54, intercellular adhesion molecule-1	PE-binding receptor on endothelium	*ICAM1* polymorphisms show variable associations with severe malaria in Kenya, Gabon, and the Gambia.
PECAM1	CD31, platelet-endothelial cell–adhesion molecule	PE-binding receptor on endothelium	*PECAM1* polymorphisms show variable associations with severe malaria in Thailand, Kenya, and Papua New Guinea.

[a] PE = parasitized erythrocyte.

c) **Immune Genes Reported to Be Associated with Different Malaria Phenotypes**

Gene	Protein	Function	Reported Genetic Associations with Malaria
FCGR2A	CD32, low affinity receptor for Fc fragment of IgG	Clearance of antigen-antibody complexes	Association with severe malaria in the Gambia
HLA-B	HLA-B, a component of MHC class I	Antigen presentation that leads to cytotoxic T cells	HLA-B53 association with severe malaria in the Gambia
HLA-DR	HLA-DR, a component of MHC class II	Antigen presentation that leads to antibody production	HLA-DRB1 association with severe malaria in the Gambia
IFNAR1	Interferon α receptor component	Cytokine receptor	Association with severe malaria in the Gambia
IFNG	Interferon γ	Cytokine with antiparasitic and proinflammatory properties	Weak associations with severe malaria in the Gambia
IFNGR1	Interferon γ receptor component	Cytokine receptor	Association with severe malaria in Mandinka people of the Gambia
IL1A/IL1B	Interleukin-1α and -1β	Proinflammatory cytokines	Marginal associations with severe malaria in the Gambia
IL10	Interleukin-10	Anti-inflammatory cytokine	Haplotypic association with severe malaria in the Gambia
IL12B	Interleukin-12 β subunit	Promotes development of Th1 cells	Association with severe malaria in Tanzania
IL4	Interleukin-4	Promotes antibody-producing B cells	Association with antimalarial antibody levels in Fulani people of Burkina Faso
MBL2	Mannose-binding protein	Activates classic complement	Association with severe malaria in Gabon
NOS2A	Inducible NO synthase	Generates NO, a free radical	Various associations with severe malaria in Gabon, the Gambia, and Tanzania
TNF	Tumor necrosis factor	Cytokine with antiparasitic and proinflammatory properties	Various associations with severe malaria and reinfection risk in the Gambia, Kenya, Gabon, and Sri Lanka
TNFSF5	CD40 ligand	T cell–B cell interactions leading to immunoglobulin class switching	Association with severe malaria in the Gambia

1.5 Vitamins and their influence on malaria

Vitamins are essential nutrients and are usually required in small amounts. Vitamins are classified as either water soluble or fat soluble. In humans there are 13 vitamins, 4 fat soluble (A,D, E and K) and 9 water soluble (8 B vitamins and vitamin C). Vitamins have diverse biochemical functions. Some have hormone-like functions as regulators of mineral metabolism (e.g. vitamin D), or regulators of cell and tissue growth and differentiation (e.g. some forms of vitamin A). Others function as antioxidants (e.g. vitamin E and sometimes vitamin C). The largest number of vitamins (e.g. B complex vitamins) functions as precursors for enzyme cofactors, which help enzymes in their work as catalysts in metabolism.

Today it is clear that nutrition strongly influences the disease burden of malaria. However, the relationship between malaria and nutritional status remains complex. It has been shown that clinical manifestations and mortality from malaria is more severe in malnourished children (Shankar 2000; Caulfield, Richard et al. 2004; Ehrhardt, Burchard et al. 2006; Muller and Kappes 2007).

In this perspective, some vitamins have demonstrated to play an important role. Vitamin A is essential for normal immune function (Semba 1998). Several studies suggest that it could play a role in potentiating resistance to malaria. It has been shown that vitamin A supplementation can enhance host resistance to malaria leading to a reduction in *P.falciparum* febrile episodes and lower parasite densities (Shankar, Genton et al. 1999; Serghides and Kain 2002; Muller and Kappes 2007).

Riboflavin also known as vitamin B_2 has an influence on malaria morbidity. It has been demonstrated that riboflavin deficiency confers a certain degree of protection against malaria (Thurnham, Oppenheimer et al. 1983; Dutta, Pinto et al. 1985; Das, Das et al. 1988). Riboflavin is an essential factor for the glutathione reductase which is an antioxidative enzyme and it has been proposed that deficiency promotes an oxidative environment in the erythrocyte which is not conducive for the parasite's survival (Barraviera, Machado et al. 1988; Das, Thurnham et al. 1990). On the other hand recent in vitro studies have shown that a high dose of riboflavin suppresses the growth of the parasite by preventing the oxidation of haemoglobin which is required for its digestion by the parasite (Akompong, Ghori et al. 2000). This finding is in paradox to previous studies that have shown riboflavin deficiency protects against

malaria and highlights the complex mechanisms in which nutrients can affect malaria parasites and the outcome of the disease.

Several studies on human and rodent malaria have shown that deficiency of Vitamin E tend to protect against malaria. Vitamin E is an antioxidant and its deficiency could make the parasite more vulnerable to oxygen radicals in the erythrocyte (Eaton, Eckman et al. 1976; Eckman, Eaton et al. 1976; Levander, Ager et al. 1989; Davis, Binh et al. 1994).

The role of B vitamins other than B_2 on the severity of malaria is not clear. It has been shown thiamine (Vitamin B_1) deficiency in individuals can complicate *P. falciparum* malaria suggesting that deficiency in thiamine is associated with greater risk of severe malaria (Krishna, Taylor et al. 1999).

In summary, the precise role of vitamins on the outcome of a malaria infection is not always very clear. Moreover, the deficiency of some vitamins reduces the risk of malaria infection while other vitamins are required in sufficient amount to reduce malaria infection.

1.6 Vitamin B_6, function and metabolism

Vitamin B_6 is a water soluble vitamin and is a collective term for pyridoxal, pyridoxamine and pyridoxine and their respective phosphate easters. Pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM) are phosphorylated by the pyridoxal kinase (PdxK) to their phosphate ester derivates with ATP being the phosphate donor. The phosphate easter derivate pyridoxal 5-phosphate (PLP) is the principle coenzyme form and plays a vital role in the function of more than 100 enzymes that catalyze essential chemical reactions in the human body. The majority of enzymes dependent on PLP are involved in the metabolism of amino acids. These enzymes are usually aminotransferases and aminodecarboxylases (Alexander, Sandmeier et al. 1994). PLP also functions as a co-enzyme for glycogen phosphorylase which is an enzyme that catalyzes the release of glucose from stored glycogen. PLP is usually found

bound to glycogen in muscles (Vul'fson 1986; Chang, Scott et al. 1987). PLP is also an important co-enzyme in reactions involved in the release of glucose from amino acids, a process known as gluconeogenesis (Leklem and Shultz 1983).

In the human brain, the synthesis of neurotransmitters such as dopamin, serotonin and histamin are carried out by enzymes dependent on PLP. Failure to maintain the right level of PLP can lead to neurological disorders such as epileptic encephalopathy (Waymire, Mahuren et al. 1995; Mills, Surtees et al. 2005). PLP also plays a vital role in the synthesis of haemoglobin in reticulocytes. The enzyme δ-aminolaevulinic acid synthase is dependent on PLP and involved in the synthesis of the haeme precursor δ-aminolaevulinic acid. The X-linked siederoblastic anaemia is a disease which is due to a deficient variant of the δ-aminolaevelinic acid synthase and results in insufficient synthesis of the globin chains. It has been shown that 90% of sideroblastic anaemia cases respond positiviely to PLP treatment (Clayton 2006; Hunter and Ferreira 2009).

There have been studies showing the effect of PLP on hormone function. PLP binds to steroid receptors in a manner that inhibits the binding of steroid hormones, thus decreasing their effects (Cake, DiSorbo et al. 1978; Muldoon and Cidlowski 1980; Tully, Allgood et al. 1994).

Deficiency in vitamin B_6 has been associated with weak immune responses characterized by impaired interleukin 2 production and lymphocyte proliferation in adults (Talbott, Miller et al. 1987; Meydani, Ribaya-Mercado et al. 1991).

1.6.1 Metabolism of vitamin B_6 in humans

Vitamin B_6 exists as pyridoxine, pyridoxamine and pyridoxal as well as their phosphorylated forms in food. Certain plant food contain a unique form of vitamin B_6 called pyridoxine glucoside. The phosphorylated forms pyridoxal 5-phosphate (PLP), pyridoxine 5-phosphate (PNP) and pyridoxamine 5-phosphate (PMP) are dephosphorylated in the intestines by an intestinal alkaline phosphatase before absorption (Hamm, Mehansho et al. 1979; Mehansho, Hamm et al. 1979). PN, PM and PL are absorbed by passive diffusion and enter the circulation. They are rapidly taken up in the liver by passive diffusion where they are phosphorylated by the

pyridoxal kinase. PMP and PNP are converted to PLP by the PMP- and PNP oxidase respectively. PLP is then bound to apo-proteins and transported to the blood or dephosphorylated by alkaline phosphotase (AP) (Merrill, Henderson et al. 1984). Although other tissues contribute to the metabolism of vitamin B_6, the liver is thought to be responsible for the PLP found in plasma (Lumeng, Lui et al. 1980). It has been shown that PLP binds more tightly to serum albumin than pyridoxal. When bound to proteins, PLP cannot be hydrolysed by phosphatases and is therefore not readily available to cells. It has been proposed that pyridoxal released from the liver into the bloodstream is the major source of vitamin B_6 for tissues (Leklem J et al., 1991)

The erythrocyte contains the required enzymes to convert all three forms of vitamin B_6 (PL, PN and PM) to the active coenzyme PLP. Pyridoxal, pyridoxine and pyridoxamine are rapidly taken up by the erythrocytes via passive diffusion and converted to their phosphorylated forms by the pyridoxal kinase. PNP and PMP have to be converted to PLP by an oxidase. PLP, PNP and PMP can be metabolized to their non phosphorylated forms by alkaline phosphotase and then exit the cell (Mehansho and Henderson 1980; Ink and Henderson 1984; Anderson, Perry et al. 1989).

In erythrocytes PLP binds tightly to the β globin chain of haemoglobin. Pyridoxal also binds to the α globin chain of haemoglobin but not as tightly as PLP. The binding of PLP and PL to haemoglobin affects the oxygen affinity. PLP binding to hemoglobin decreases the oxygen affinity of haemoglobin, whereas PL binding results in an increase on oxygen affinity (Benesch, Yung et al. 1973; Ink and Henderson 1984; Natta and Reynolds 1984).

1.6.2 Metabolism of vitamin B_6 in the malaria parasite

Humans need to take up vitamin B_6 through their diet. Other organisms such as plants, bacteria and fungi are capable of synthesizing this vitamin de novo (Tambasco-Studart, Titiz et al. 2005; Fitzpatrick, Amrhein et al. 2007). It has also been confirmed that the malaria parasite *P. falciparum* possesses a functional de novo synthesis pathway for vitamin B_6. The parasite synthesizes PLP employing the two proteins Pdx1 and Pdx2. It has been shown that Pdx1 and Pdx2 form a complex

that functions as a glutamine amidotransferase with Pdx2 as the glutaminase and Pdx1 as pyridoxal 5-phosphate synthase domain. Ribose 5-phosphate and ribulose 5-phosphate, as well as glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) are substrates for the plasmodial PLP synthase. In vitro studies have shown that glutamine and ammonium sulphate can serve as ammonium donor for the PLP synthase (Gengenbacher, Fitzpatrick et al. 2006)

There is also evidence that *P. falciparum* has a pyridoxal kinase that is required for the phosphorylation of vitamin B_6 upon uptake and for the parasitic salvage pathway of vitamin B_6 (Wrenger, Eschbach et al. 2005). The parasite also possesses phosphatases (Muller, Knockel et al.; Bozdech, Zhu et al. 2003). It is therefore likely that PLP can also be dephosphorylated to PL which could then exit the parasite (Muller, Hyde et al.). Homologues of the human pyridoxine 5-phosphate and pyridoxamine 5-phosphate oxidase have so far not been found in the parasite suggesting that the parasite is not capable of converting the phosphorylated forms of pyridoxine and pyridoxamine to PLP.

Since a de novo synthesis pathway for vitamin B_6 is absent in the human host, the parasite specific biosythesis pathway for this vitamin has been considered a powerful potential drug target (Muller, Hyde et al.; Muller and Kappes 2007)

Figure 1.5 shows a model illustrating a potential cross talk between the parasite and the erythrocyte regarding vitamin B_6.

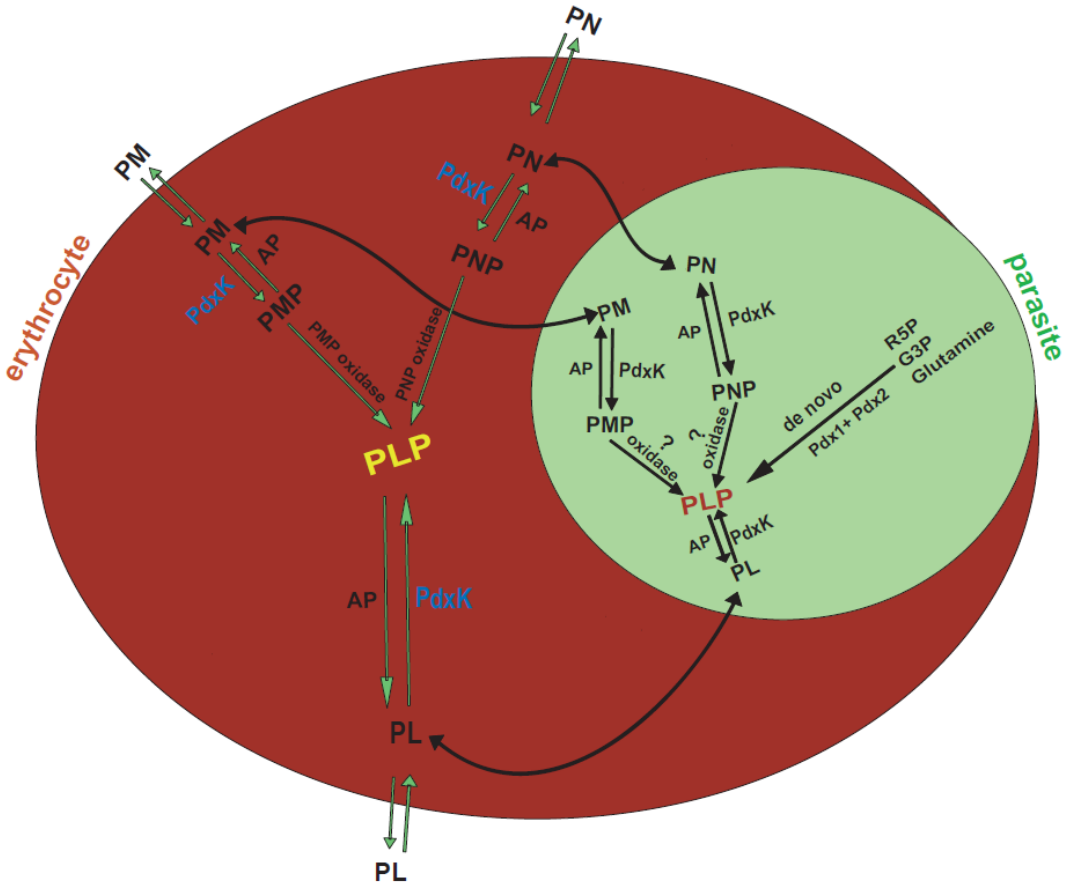

Figure 1.5: A hypothetical model for a potential cross talk between the parasite and erythrocyte regarding vitamin B_6. Pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) are taken up into the erythrocyte by passive diffusion. In the erythrocyte, they are phosphorylated by the pyridoxal kinase (PdxK) to their phosphate ester forms. The phosphorylated vitamers can be dephosphorylated by the alkaline phosphotase (AP) upon which they can exit the erythrocyte. Pyridoxamine 5-phosphate (PMP) and pyridoxine 5-phosphate (PNP) are converted to the co enzyme form pyridoxal 5-phosphate (PLP) by the respective oxidase. PL, PN, and PM in the erythrocyte can be passively taken up by the parasite. The parasitic pyridoxal kinase converts them into their phosphorylated forms. It is not clear if PMP and PNP can be converted to PLP in the parasite. The respective oxidases have not yet been identified. PMP, PNP and PLP can also be dephosphorylated in the parasite by the phosphotase. The parasite is able to synthesize pyridoxal 5-phosphate de novo utilizing the Pdx1/Pdx2 complex and the substrates ribose 5-phosphate (R5P), glyceraldehyde 3-phosphate (G3P) and glutamine.

1.7 Polymorphism of the human erythrocytic pyridoxal kinase

The human pyridoxal kinase (PdxK) is a polypeptide of 312 amino acid residues and is present in various human tissues such as liver, heart, kidney, skeletal muscles, brain, small intestine, spleen etc. It is also present in blood cells such as leukocytes and erythrocytes (Hanna, Turner et al. 1997).

Thirty years ago it was reported that the pyridoxal kinase activity in erythrocytes of African – Americans was strikingly lower than that of individuals with European ancestry. This racial difference in activity was found to be tissue specific, with leukocyte and fibroblast PdxK activity being the same in both racial group (Chern and Beutler 1975). These findings were confirmed and it was suggested that the selective pressure of malaria was the cause of the lowered erythrocyte PdxK activity among individuals of African ancestry (Martin, Miller et al. 1978)

The mechanism by which evolution has selectively lowered the PdxK activity in erythrocytes but not in the other cell types has also been elucidated. It was proposed that an 8 bp insertion in the promoter region of the *pdxk* gene leads to an increase in activity of the erythrocytic PdxK. It has also been demonstrated that the 8 bp insertion introduces a binding site for a putative core promoter binding protein (CPBP) which is known to increase transcriptional activity at least four-fold. Further investigations revealed that the putative CPBP binding site was adjacent to a binding site for an erythrocyte specific transcription factor, the erythroid Krüppel like factor (EKLF). These findings allow the hypothesis that the presence of the 8 bp insertion would lead to an increased transcription of *pdxk* in erythrocytes only and thereby leading to enhanced activity. It also means that the absence of the 8 bp insertion would lead to lower erythrocytic PdxK activity (Flanagan and Beutler 2006)

1.8 Aim of Project

The severity of malaria infection depends a great deal on host factors. Although factors such as natural acquired immunity play a key role in shaping the clinical outcome of the disease, genetic factors that have evolved due to the selective pressure of malaria on the human genome are known also to a play an important part on this aspect. Today, host genetic polymorphisms that are known to reduce the susceptibility to malaria provide a foundation for the investigation of host-parasite interactions.

The erythrocyte is the main spot for genetic polymorphisms that are known to offer protection against malaria and thus are predominant in endemic regions.

An early study reported that the erythrocytic pyridoxal kinase activity is lowered in American blacks as opposed to American whites. Another study attempted to associate this lowered activity with reduced susceptibility to malaria. Recently, the genetic basis for the lowered erythrocytic PdxK activity was resolved and it was shown that an 8 bp deletion in the promoter region of the *pdxk* gene led to reduce activity of the enzyme in erythrocytes. Although the lowered erythrocytic PdxK activity has been associated with protection against malaria, its effect on the parasite has never been studied.

Therefore, the aim of this project was to test the hypothesis that the lowered erythrocytic pyridoxal kinase activity could be a genetic trait that offers protection against malaria. It was my goal to provide further evidence for this hypothesis by investigating the effect of the lowered erythrocytic Pdxk activity on *P.falciparum* in an in vitro culture system and to disclose the mechanism of protection of this genetic trait.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetic acid	J.T. Baker, Deventer, NL
Agarose	invitrogen Gmbh Karlsruhe,
Acrylamide/Bis solution 30%	Biorad, Munich, DE
Albumax II	Gibco Invitrogen, Karlsruhe, DE
Ampicillin	Sigma Aldrich, Taufkirchen, DE
Ammouniumpersulphate (APS)	Sigma Aldrich, Taufkirchen, DE
BactoTM Agar	BD, Heidelberg, DE
BactoTM	BD, Heidelberg, DE
D-(+) glucose	Sigma Aldrich, Taufkirche, DE
D-sorbitol	Fluka, Sigma Aldrich
Dimethylsulfoxide (DMSO)	Sigma Aldrich, Taufkirchen, DE
Dodecylsulfat-Na-salt (SDS)	Serva, Heidelberg, DE
Ethylendiamintetraacetic acid (EDTA)	Acros
Ethanol absolute 100 %	J.T. Baker, Denventer, NL
Ethanol 96 %	JT Baker, Deventer, NL
Ethidiumbromide 1 % (10mg/ml)	Carl Roth GmbH, Karlsruhe
Ethidiumbromide	Sigma Aldrich, Taufkirchen, DE
Gentamicin 50 mg/ml	Gibco Invitrogen, Karlsruhe, DE
Giemsa solution	Merck, Darmstadt, DE
Glycerol	Carl Roth GmbH, Karlsruhe, DE
HEPES (N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid)	Carl Roth GmbH, Karlsruhe
Human AB serum	Blood bank, Uniclinic Heidelberg
Hydrogenchloride	Merck, Darmstadt, DE
Hypoxanthine (10 mM)	ccpro, Oberdorla, DE
Potassiumacetate	VWR, Darmstadt, DE
Potassiumchloride	AppliChem GmbH, Darmstadt, DE

Methanol	JT. Baker, Deventer, NL
Sodiumacetate	Grüssing, Filsum, DE
Sodiumchloride	Riedel, Hannover, DE
Tri-sodiumcitrate-dihydrate	Carl Roth GmbH, Karlsruhe
Sodiumhydrogencarbonate	Grüssing, Filsum, DE
Sodiumhydroxide	Merck, Darmstadt, DE
Sodium-dodecylsulphate (SDS)	Applichem, DE
Phosphate buffered saline (PBS)	Sigma Aldrich, Taufkirchen, DE
Pyrimethamine	Sigma Aldrich, Taufkirchen, DE
RNA DNA Stabilization reagent for blood	Roche, Mannheim, DE
and bone marrow	Roche Mannheim, DE
RPMI1640 with L- Glutamine	Gibco, Invitrogen, Karlsruhe, DE
RPMI 1640 Medium without glutamine	Sigma Aldrich, Taufkirchen, DE
RPMI 1640 Medium without pyridoxine	cc pro,Oberdolar,DE
Dodecylsulfat-sodium salt (SDS)	Serva, Heidelberg, DE
Sybr Safe DNA gel stain 10.000 x in DMSO	Invitrogen GmbH, Karlsruhe, DE
Hydrochloride acid	Merck, Darmstadt, DE
Saponin	Sigma Aldrich, Taufkirchen, DE
Tris	Carl Roth GmbH, Karlsruhe, DE
Trishydrochlorid	Carl Roth GmbH, Karlsruhe, DE
Triton x 100	Carl Roth GmbH, Karlsruhe, DE
Tween 20	Gerbu Biochemicals GmbH, Gaiberg, DE

2.1.2 Buffers, media and solutions

Buffer

10 x SDS running buffer	30.3 g Tris base 144 g glycine 10 g SDS fill to 1 litre with H_2O
10 x TBS buffer:	500 mM Tris pH 8 1,5 M NaCl

	fill to 1 litre with H_2O
1 x TBST buffer	1 x TBS buffer 0.1% Tween
Transfer buffer	48 mM Tris 39 mM Glycine 20 % Methanol
2 x SDS sample buffer	2 ml 0.5M Tris HCL pH 6.8 1.6 ml Glycerol 1.6 ml 20 % SDS 1.4 ml dH_2O 0.4 ml 0.05 % bromphenol blue 7 µl β-mercaptoethanol
0.4 M PHEM buffer	240 mM 1,4 Piperazinediethanesulfonic Acid (Pipes) 100 mM N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (Hepes) 8 mM MgCl 40 mM EDTA pH 6.9
Fix 1	0.2 M PHEM Buffer 8 % PFA 0.2% glutaraldehyde
Fix 2	0.1 M PHEM buffer 4 % PFA 0.1% glutaraldehyde
Cellpack buffer	Sysmex, Germany

0,5 M Ethylenediaminetetraacetic acid EDTA (1 L)	186,1g Na_2EDTA, pH 8 (with NaOH)
Lysis buffer	12mM triethanolamine hydrochloride NaOH, pH 7.4
50 x TAE buffer (1 L)	2 M Tris base (242 g) 1 M glacial acetic acid (57,1 ml) 0,05 M EDTA, pH 8
10 x TBE buffer (1 L)	890 mM Tris base (108 g) 890 mM boric acid (55 g) 20 mM EDTA, pH 8
RIPA buffer (500 ml)	50 mM Tris pH 8 150 mM NaCl 5 mM EDTA 50 mM NaF 0.5 % NaDOC 0.1 % SDS 1 % Triton x 100

Medium

LB medium for bacterial culture (1 L)	5 g yeast extract 5 g NaCl 10 g trypton (15 g agar for plates) ampicillin 0,1 mg/ml for selection

Complete cell culture medium (500 ml)	RPMI1640 with L-Glutamine (Gibco) 12 ml AB serum (heat inactivated 56°C) 0,1 mM hypoxanthine 250 µl gentamicin (Stock 50mg/ml) 2,5 g albumax (filtered sterile)
RPMI basic medium (500ml)	RPMI 1640 with L-Glutamine (Gibco)

Solutions

Thawing solution 1	12 % NaCl Filtered steril
Thawing solution 2	1.6 % NaCl Filtered steril
Thawing solution 3	0.9 % NaCl 0.2 % Glucose Filtered steril
Freezing solution (50 ml)	3 % sorbitol (1,5 g) 0.65 % NaCl (0,325 g) 28 % glycerol (14 ml) Filtered
Commassie solution	20 % Methanol 72.5 % H_2O 7.5 % Acetic acid 0.04 g Commassie

Destain solution	20 % Methanol 72.5 % H_2O 7.5 % Acetic acid
Ringer solution (1 L)	122.5 mM NaCl 5.4 mM KCl 1.2 mM $CaCl_2$ x $2H_2O$ 0.8 mM $MgCl_2$ x $6H_2O$ 10 mM Hepes 11 mM D-Glucose 1 mM $NaH_2PO_4.H_2O$ p.H 7.4

2.1.3 Kits

Fast Sybr Master Mix	Applied bioystems (ABI),DE
mRNA Isolation Kit for blood and bone	Roche, Mannheim, DE
QIAamp DNA Blood Kit	QIAGEN, Hilden, DE
QIAgen PCR Purification Kit	QIAGEN, Hilden, DE
QIAquick Gel extraction Kit	QIAGEN, Hilden, DE
RETROscript	Ambion, Huntingdon, UK
RNeasy Mini Kit	QIAGEN, Hilden, DE
RNase-Free DNase Set	QIAGEN, Hilden, DE
Turbo DNA free Kit	Ambion, Huntingdon, UK

2.1.4 Equipments

Adventurer ProTM balance	Ohaus
Computer hardware	iMac
Computer software	
7500 system software	Applied biosystems
Bioedit	Ibis Therapeutics, Carlsbad
Endnote	Thomson Reuters
EnzymeX3.1	Mekentosj.cin
FACS DIVA	BD, DE
ImageJ	NIH, USA
Microsoft office 2008	Microsoft,
Quantity one®	BIO-RAD
Primer Express	Applied biosystems
Prism 5 GrahpPad Software	
Centrifuge	
Microcentrifuge GALAXY mini	vWR
Biofuge pico	Heraeus, Kendro, Langensolbold, DE
Fresco 21	Heraeus Instruments, Hanau, DE
Megafuge 1.0 R	Heraeus Instruments, Hanau, DE
Vi-Cell^{TM} XR Cell Viability Analyzer	Beckman Coulter, Krefeld, DE
DNA Workstation (DNA/RNA UV cleaner)	G. Kisker, Steinfurt, DE
Eclipse E 200 Microscope	Nikon
Electrophoresis System	BIO-RAD, Munich, DE
Freezer	
-80°C	Heraeus GmbH, Hanau, DE
Premium no frost (-20°C)	Liebherr, Biberach, DE
Premium (4°C)	Liebherr, Biberach, DE
Flow cytometer BD LSR II	Becton Dickenson, DE
Geldoc XR	Biorad, Munich, DE

Herasafe bench	Heraeus GmbH, Hanau, DE
Incubator Heracell 150	Heraeus / Thermo, Hanau, DE
Isotherm System 3880	Eppendorf, Hamburg, DE
Microwave LG.	
Mini PROTEAN 3	BIO-RAD, Munich, DE
Mini Sub cell	BIO-RAD, Munich, DE
Multipipette, 10 µl, 100 µl, 300 µl	Eppendorf, Hamburg, DE
Mastercycler epgradient	Eppendorf, Hamburg, DE
Mini-PROTEAN® 3 CELL	BIO-RAD, Munich, DE
Nanodrop	Peqlab, Erlangen, DE
Ph Meter	Schott, DE
Pipette controller accu jet pro	Brand GmbH, Wertheim, DE
Pipette controller accu jet pro	Brand GmbH, Wertheim, DE
Pipettes, 0,1-2,5 µl, 2-20 µl,	
20 µl-200 µl, 100 µl-1000 µl	Eppendorf, Hamburg, DE
PowerPacTM Basic Power Supply	BIO-RAD, Munich, DE
PowerPacTM HC Power Supply	BIO-RAD, Munich, DE
Printer hpdeskjet 6122	Hewlett Packard, Heidelberg, DE
7500 Real time PCR system	Applied biosystems, Foster City, USA
Databases:	
PlasmoDB	www.plasmobd.org
NCBI	www.ncbi.nlm.nih.gov/pubmed/
GeneDB	www.genedb.org

2.1.5 Consumables

ABgene PCR plates	Thermo Scientific, Waltham,USA
Amersham HyperfilmTM ECL	GE Healthcare, Buckinghamshire, DE
Cryotobes	Nunc, Langenselbold, DE
Cover for 96 well plate	Greiner Bio-one, Firckenhausen, DE
FIA-Plate, black 96 well plate	Greiner Bio-one, Frickenhausen, DE
Filter Tip 10E, 20, 100, 200, 1000	Greiner Bio-one, Frickenhausen, DE
Filter cap cell culture flask, 25cm^2, 175cm^2	Greiner Bio-one, Firckenhausen, DE
Kodak BioMax MR film	Sigma Aldrich, Taufkirchen, DE

Universal Tips, yellow, blue	Greiner bio-one, Firckenhausen, DE
PP test tube, 15 ml, 50 ml	Greiner bio-one, Firckenhausen, DE
Pipette, 1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Greiner bio-one, Firckenhausen, DE
Safe lock tubes 1,5 ml	Eppendorf, Hamburg, DE
RNase free Biopure 1,5 ml	Eppendorf, Hamburg, DE
PCR softstrips 0,2 ml, colour	Biozym Scientific GmbH Oldendorf, DE
epT.I.P.S. Reloads 2-200 µl	Eppendorf, Hamburg, DE
epT.I.P.S. Reloads 20-300 µl	Eppendorf, Hamburg, DE
96 well plate (black and clear)	Greiner Bio-one, Firckenhausen, DE
Ultrafree-MC Centrifugal filter device	Millipore, DE
Millipore Centrifugal filter units	Millipore, DE
Immobilon Millipore transfer membranes PVDF Filtertype	Millipore, DE

2.1.6 Enzymes, Antibodies and Ladders

Enzymes

All restriction enzymes and corresponding buffers were purchased from New England Biolabs.

DNase was purchased from Ambion, Huntingdon, UK, Taq poylermase from Fermentas, St Leon Rot, DE.

DNA Ladder

GenerulerTM 1 kb DNA Lader	Fermentas, St Leon-Rot, DE
100 bp DNA Ladder	Invitrogen, Karlsruhe, DE
DNA Molecular Weight Marker III (Digoxigenin-labeled)	Roche, Mannheim, DE

Antibodies

Anti human pyridoxal kinase	Santa Cruz, Heidelberg, DE
Secondary antibody goat anti rabbit	Dianova, Hamburg, DE

2.1.7 *P. falciparum* laboratory strains

Table 2.1.1 lists the *P. falciparum* laboratory strains that were used in this work

P. falciparum strain	Country of origin	Donated from
Dd2	Indochina	Prof. Lanzer
HB3	Honduras	Prof. Lanzer
3D7		Prof. Lanzer
FCR3	The Gambia	Prof. Lanzer

2.1.8 Oligonucleotides

Primer	Forward primer	Reverse primer	Accession number
pdxk	5' TAATAACTCGAGGGCCCTGCA GGCACTTGA GAG 3'	5' TAATAAGGATCCGCG GATGACGTGGCTCTGTATG 3'	NCBI AP001752
+ insertion	5'GGCCACCTGGCGCGGCG3'	5'TGCCGGGCCTGGCCGCG3'	NCBI AP001752
- insertion	5'GGCCACCTGGCGCGGCC3'	5'TGCCGGGCCTGGCCGCG3'	NCBI AP001752
***pdxk* 80 bp**	5'CCCAGCGTTTCGGGCGGC3'	5'GCTGGGAGGAAGCGAAACC G3'	NCBI AP001752
pfpdx1	5'TACTCCAGCTGATGCAGCCAT3'	5'AAAACTCCATCCATTCCTAA TTGC3'	PlasmoDB PFF0775w
pfpdxk	5'TATGTGATCCCGTTATGGGAGA T3'	5'CACGTTCATCAACATATAGT CTTCCG3'	PlasmoDB PF14_0570

2.2 Methods

2.2.1 Screening human genomic DNA for the 8 bp insertion in the promoter region of the pyridoxal kinase gene

A PCR based method was developed for screening human genomic DNA of various individuals for the 8 bp insertion. The sequence for the human pyrdoxal kinase gene was obtained from the NCBI data base.

2.2.1.1 Isolation of human genomic DNA

9 ml venous blood from individuals was collected into EDTA tubes. The blood was transferred to a 15 ml falcon tube and centrifuged at 4000 rpm for 10 min at 25°C. After centrifugation, the plasma fraction was discarded. The fraction with the white blood cells (buffy coat) was carefully collected with a pipette and transferred into a fresh 15 ml falcon tube. The buffy coat was washed twice with 2 ml PBS and each time carefully transferred into a fresh 15 ml falcon tube. 200 µl of the buffy coat was used for the isolation of gDNA.

gDNA was isolated using the QIAamp DNA blood kit following the kit instructions, eluted in 80 µl elution buffer and stored at 4°C. The DNA concentration of each sample was determined using the Nanodrop.

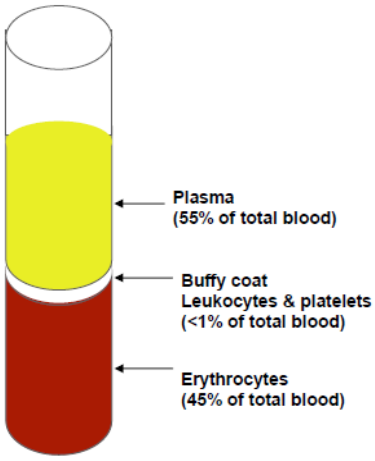

Figure 2.2.1: Schematic drawing of the different blood fractions. The drawing shows the 3 main fractions obtained after blood is centrifuged at 4000 rpm for 10 min. The red fraction shows the erythrocytes, the thin white fraction the leukocytes (buffy coat) and the upper yellow fraction is the plasma.

2.2.1.2 Whole genome amplification (Genomiphi)

Whole genome amplification (Genomiphi, GE Healthcare, UK) was used to amplify gDNA samples from kilifi Kenya to increase the amount of DNA. 35 gDNA samples from Kilifi were amplified using the following procedure:
1 µl of template DNA was added to an eppendorf tube and mixed with 9 µl of sample buffer. The mix was heated for 3 min at 95°C and then cooled to 4°C on ice. 9 µl of reaction buffer was mixed with 1 µl of enzyme mix and then added to the cooled sample. The samples were incubated at 30°C for 2 h and then heat inactivated by incubating for 10 min at 65°C. Samples were then cooled to 4°C on ice.

2.2.1.2.1 Purification of genomiphi amplified samples

The whole genome amplified samples were purified prior to PCR reactions. 20 µl of water was added to each sample. 4 µl of sodium acetate /EDTA buffer was added to the samples and mixed. 100 µl of 100 % ethanol was added to each sample to a concentration of 70 % ethanol and kept at 4°C overnight. The next day the samples were centrifuged for 15 min at 12000 rpm. The supernatant was discarded and the DNA pellet was washed with 70 % ethanol by centrifugation for 1 min at 12000 rpm. The supernatant was discarded and the pellets were air dried for 2-5 min. The pellet was resuspended in an appropriate amount of TE or water.

2.2.1.3 Polymerase Chain Reaction (PCR)

PCR was used to amplify fragments at the region of interest in the promoter of the *pdxk* gene using gDNA from different individuals as template. The platinum *Pfx* polymerase which possesses a 3' to 5' proof reading function was used for the amplification of a 700 bp fragment.
The PCR reaction was optimized by testing different annealing temperatures and varying the Magnesium concentration. Since the region of interest is very GC rich, PCR enhancer solution was added to the reaction mix.

A standard reaction mix was prepared composed of:

3 µl gDNA

5 µl 10 x PCR buffer

5 µl Enhancer

5 µl 2 mM dNTPs mix

1 µl 50 mM $MgSO_4$

0,5 µl 5' Primer (25 pmol)

0,5 µl 3' Primer (25 pmol)

0,5 µl Pfx DNA polymerase

H_2O to a total volumen of 50 µl

The annealing temperatures (AT) of the Primers were approximated:

$T_m \approx 4(C_n + G_n) + 2 (A_n + T_n)$ $AT \approx T_m - 5$

T_m is the melting temperature and n the number of nucleotides in the annealing part of the primer. For the reaction, the AT of the Primer with the lower AT was used. The 5' primer possesses a *Xho*I restriction site and the 3' primer a *BamH*I site. The PCR was run under standard conditions:

94°C	6 min	
94°C	45 s	⎫
56.7°C	30 s	⎬ 35 x
72°C	1 min	⎭
72°C	4 min	
4°C	∞	

2.2.1.4 Agarose gel electrophoresis

The PCR fragments were separated on different percentages of agarose ranging from 0.8 to 1.5 % depending on the size of the fragment. The appropriate quantity of agarose was added to 1 x TAE buffer and the mixture was boiled in a microwave until the agarose was completely dissolved. Ethidium bromide was added to a final

concentration of 1 µg/ml. Ethidium bromide intercalates in DNA allowing visualisation of DNA by UV irradiation. 10 µl of the PCR sample was mixed with loading buffer (1:5, v/v) and loaded on the gel. As a size marker the 1 Kbp ladder (Fermentas) was used. Electrophoresis was performed at a constant voltage of 120 V in TAE. The result was photographed and documented with the BIO-RAD Gel DOC^{TM} XR imaging system.

2.2.1.5 PCR purification

After a successful PCR reaction, the remaining PCR product was purified using the QIAgen PCR Purification kit following the kit instructions. The system is based on DNA absorbing to a silica membrane in the presence of a high salt concentration while contaminants pass through the column. Impurities are efficiently washed away and the pure DNA is eluted with 30 µl of water.

2.2.1.6 Cloning of the 700bp PCR fragment

2.2.1.6.1 Competent cells

XL1 blue cells were made competent under sterile conditions by a method according to Hanahan (Hanahan, 1983). The method is based on the permeabilisation of the cell membranes. A 5 ml pre-culture (in LB medium) of non competent XL1 blue cells was grown in a shaker at 37°C overnight. The next day 50 ml of LB and 2 ml of pre-culture was incubated again in a shaker at 37°C till an OD of 0.3-0.4 was reached (optical density at 600 nm measured in an eppendorf photometer). The culture was transferred into a 50 ml falcon and centrifuged at 2400 rpm for 10 min at 4°C. The supernatant was discarded and the tube was placed upside down on paper for one minute to dry. The cell pellet was re-suspended in 15 ml cold TBF1 buffer and incubated on ice for 60 min. The solution is centrifuged at 2400 rpm for 10 min at

4°C. The supernatant was again discarded and the cell pellet was resuspended in 900 µl TBF2 buffer. Aliquots of 100 µl cell suspension were made on ice and frozen away at -80°C.

2.2.1.6.2 Restriction analysis and digestion

The PCR fragments and the pBluescript plasmid were digested for subsequent cloning. A standard reaction mix was prepared with the following content:

30 µl DNA

2 µl *Xho*I

2 µl *BamH*I

4 µl reaction buffer 2

2 µl H_2O

Total volumen 40 µl

The reaction is incubated for 2 h at 37°C.

2.2.1.6.2.1 Dephosphorylation of the plasmid

For the ligation of two DNA ends, at least one 5´ end must still have its phosphate group. The phosphate groups can be removed by an alkaline phophatase to prevent religation of the plasmid vector. 1 µl of the calf intestinal alkaline phosphotase (CIP) was therefore added to the reaction mix with the vector 30 min prior to end of incubation.

2.2.1.6.3 DNA extraction from agarose gel

After restriction the reaction mix was loaded on a 1% agarose gel. To increase the purity of the DNA to be cloned, the PCR and plasmid fragment were cut out of the gel and extracted from agarose gel with the QIAquick Gel Extraction Kit (Qiagen). The protocol using a micro centrifuge included in the Kit was followed. The System is based on that DNA absorbing to a silica membrane in the presence of high salt while contaminants or impurities pass through.

2.2.1.6.4 Ligation

For the ligation of the digested DNA fragment and plasmid, a standard reaction mix with the following component was prepared:

1 µl plasmid
8 µl PCR fragment
1 µl ligation buffer (10x)
The mix was incubated for 3 hours at room temperature.

2.2.1.6.5 Transformation of XL1 blue competent cells

100 µl of the competent cells were used for the transformation of the total ligation mix. The competent cells were removed from the -80°C freezer and thawed on iced. The cells were then added to the tube with the ligation mix, incubated on ice for 20 min, heat shocked for 40 seconds at 42°C and immediately transferred to ice. 1 ml LB media was added and the cells were incubated horizontally in a shaker (37°C, 240 rpm) for 1 hour. The mixture was then evenly spread on LB_{amp} plate and incubated overnight at 37°C.

2.2.1.6.6 Plasmid preparation from bacteria

Small – to large- scale preparations based on alkaline lysis of bacteria cells were performed to isolate plasmids from bacteria**.**

2.2.1.6.7 Small scale preparation (Miniprep)

Individual bacteria colonies from LB_{amp} plates were selected to inoculate 1 ml LB_{amp} medium in culture test tubes. The cultures were incubated overnight at 37°C in a shaker (240 rpm). The following day the cultures were transferred to eppendorf tubes and centrifuged at 13000 rpm. Plasmids were then isolated from the cell pellet using the commercially available QIAprep Spin Plasmid Miniprep Kit following the kit instructions.

2.2.1.6.8 Large scale preparation (Maxiprep)

Plasmid large-scale preparation was carried out using the commercially available high Pure plasmid maxi kit by following the kit instructions. 50 ml culture was used.

2.2.1.6.9 Restriction analysis of isolated plasmids

To determine if the cloning was successful, 2 µl of the plasmid was digested in the following reaction:

2 µl plasmid
1 µl *BamH*I
1 µl *Xho*I
2 µl reaction buffer 2
14 µl H_20
The reaction mix was incubated for 2 h at 37°C.

After incubation the reaction mix was loaded on a 1 % agarose gel and all plasmids with the 700 bp fragments were considered positive clones.

2.2.1.7 Nested PCR for screening the 700 bp PCR fragments for the presence of the 8 bp insertion

2.2.1.7.1 Screening clones for the presence or absence of the 8 bp insertion

Positive clones were screened for the presence or absence of the 8 bp insertion in the 700 bp fragment using PCR with allele specific primers. Each clone was screened in two parallel PCR reactions using allele specific 5´ primers with the 8 bp insertion or without the 8 bp insertion and the fermentas Taq polymerase.

The reaction mix for the nested PCR with allele specific 5´primers consisted of the following components:

1 – 2 µl plasmid/PCR product
5 µl 10x PCR buffer
5 µl Enhancer
5 µl dNTPs mix (2 mM)
1 µl $MgSO_4$ (50 mM)
0.5 µl 5´primer +insert/-insert (25 pmol)
0.5 µl 3´primer (25 pmol)
0.5 µl fermentas taq polymerase
H_2O to a total volume of 50 µl.

The PCR was run under standard conditions with the following programm:

94°C	6 min	
94°C	45 s	30 x
59°C	30 s	
72°C	1 min	
72°C	4 min	
4°C	∞	

One positive clone with the 8 bp insertion and one without the 8 bp insertion were sent for sequencing.

2.2.1.7.2 DNA sequencing

DNA sequencing was done by GATC (Konstanz, Germany), using the Sanger dideoxynucleotide method (Sanger et al., 1977). PCR fragments were purified, solved in water and sent with the respective oligonucleotide.
The following concentrations and volumes were sent in for sequencing:

PCR Product: 10-50 ng/µl in 30 µl
Plasmid: 30-100 ng/µl in 30 µl
Oligonucleotide: 10 pmol/µl in 30 µl

The sequences were analysed with the BioEdit software.

2.2.1.7.3 Work flow for the established PCR screen

A 700 bp PCR fragment was amplified from gDNA samples from different individuals. The PCR fragment was then used as a template in a nested PCR. The control clon for the insertion (C+) and the control clone without the insertion (C–) were always used as a control for each nested PCR. Two parallel reactions were carried out for each template with an allele specific forward primer for the insertion or without the insertion in each reaction respectively.

42 Caucasians from Heidelberg and 33 Africans from Kilifi Kenya were screened for the 8 bp insertion in the promoter region of the pyridoxal kinase gene. Individuals that were homozygous for the insertion event, were referred to as bearing the insertion genotype (+/+), heterozygous genotype (+/-) and individuals without the insertion event as bearing the deletion genotype (-/-)

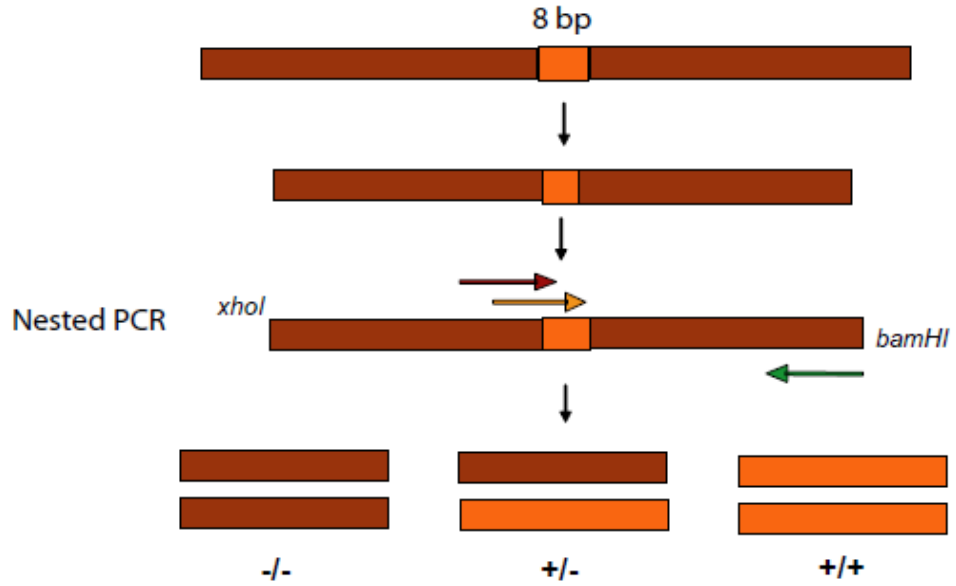

Figure 2.2.2: Schematic drawing of the PCR based method for screening human gDNA for the 8bp insertion. The drawing shows the PCR set up used for screening for the 8bp insertion. The Light orange bars represent the 8 bp insertion allele and the dark orange bars represent allele without the insertion. -/- stands for individuals homozygous for the 8 bp deletion event, +/- for heterozygote and +/+ for individual homozygous for the 8 bp insertion event.

2.2.1.8 Alternative PCR Screen

A small fragment of 80 bp around the region of interest in the promoter area of the *hpdxk* gene was amplified. The 700 bp PCR fragment amplified from the genomic DNA from various individuals was used as template and the control clons for the insertion (C+) and deletion (C-) were used as controls for the PCR. A PCR reaction mix with the following components was prepared:

3 µl gDNA
5 µl 10x reaction buffer (10 x)
5 µl Enhancer
5 µl dNTPs (2mM)
1 µl MgSO4 (50mM)
0.5 µl forward primer (25 pmol)
0.5 µl reverse primer (25 pmol)
0.5 µl Pfx Taq polymerase
H_2O to a total volume of 50µl.

The PCR was run with the following program:

94°C	6 min	
94°C	45sec	35 x
58°C	30sec	
72°C	30sec	
72°C	4 min	
4°C	∞	

2.2.1.8.1 Metaphor gel electrophoresis

The difference in size of fragments with and without the 8 bp was analysed on a metaphor gel. A 4% metaphor gel was prepared by adding 4 g of metaphor to 100 ml of TAE buffer. The suspension was stirred for 20 min and then heated gently in a microwave until the gel was completely dissolved.

The gel was run at 80 V for 1 h. The result was photographed and documented with the BIO-RAD Gel DOC™ XR imaging system.

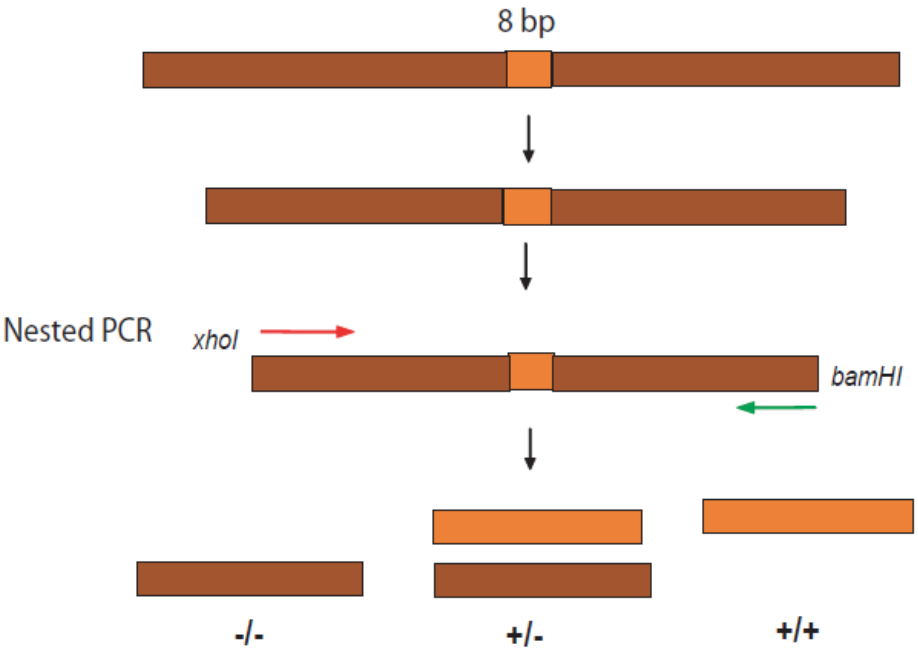

Figure 2.2.3: Schematic drawing for the 80/88 bp fragment PCR

2.2.2 Cell Culture

2.2.2.1 In vitro culture of *P. falciparum*

P. falciparum parasites of the strain 3D7, FCR3, Dd2 and HB3 were cultured by an adaptation of the method of Trager and Jensen (Trager and Jensen 1976). The erythrocytic forms of *P. falciparum* were cultured in T 25 or T 75 cell culture flasks with RPMI1640 medium supplemented with 200 mM hypoxanthine, 20 µg/ml Gentamicin , 10 % A+ serum and albumax at a hematocrit of approxiamately 5 %. The parasitemia was checked and the medium changed every two or three days. At a parasitemia of 5-10 % the culture was diluted in a new culture flask to avoid death of parasites due to high levels of degraded toxic products in the medium. The parasite cultures were incubated in an atmosphere of 5 % CO_2, 5 % O_2 and 90 % of N_2 at 37°C.

2.2.2.2 Preparation of human serum and erythrocytes

Human group A^+ or O^+ erythrocytes concentrate and A^+ human serum was obtained from the blood bank of the German Red Cross. The human serum was prepared as follows. It was incubated at 60°C in a water bath for 30 min to inactivate the serum and then aliquoted into 10 ml falcon tubes. The tubes were then centrifuged at 4000 rpm for 1 hour to pellet the fibrin. Thereafter the serum was kept at -20°C.
The erythrocyte concentrate was aliquoted into 50 ml Falcon tubes and stored at 4°C.

2.2.2.3 Staining of *P. falciparum* with giemsa

To enable determination of parasitaemia and parasite developmental stage, thin smears of the culture were made as follows: 50 µl of culture was spread evenlyonto the surface of a clean microscope slide, air dried, fixed in 100 % methanolfor 1 min, and air dried. Fixed cells were stained for 10-20 min in a solution of 10 % Giemsa, washed with water and allowed to dry. Slides were examinedmicroscopically on a light microscope using a 100 X objective under oilimmersion.

2.2.2.4 Determining parasitemia

The parasitemia is the percentage of *P. falciparum* infected erythrocytes. A single layer of stained cells was used. Infected and uninfected erythrocytes were counted to a total number of 1000. The parasitemia was then calculated according to the following formula: (Number of infected erythrocytes/number of uninfected erythrocytes) x 100 = parasitemia in %

2.2.2.5 Freezing parasites

Only the parasites from the ring stage can be recovered after freezing. To guarantee that the parasites will appear quickly after thawing the parasitemia should be higher than 5 %. Infected erythrocytes were pelleted at 2000 rpm for 3 min. The supernatant was then removed, and the sediment re-suspended in 1 volume of sterile freezing

solution (28 % Glycerol, 3 % Sorbitol, and 0.65 % NaCl). The suspension was gently mixed, filled into a cryotube and snap frozen in an ethanol/dry ice slurry for 15 min. The tube was then transferred to –80°Cfor not less than one day, before being stored at –196°C in liquid nitrogen.

2.2.2.6 Thawing parasites

The cryotube was removed from the liquid nitrogen tank and thawed at 37°C in a water bath for 2 min before being transferred to a 15 ml falcon tube. Per 1 ml of blood 200 µl of thawing solution 1 was added at a rate of 2 drops per second with constant mixing. After a 5 min incubation period at RT, a further 9 ml of thawing solution 2 was then gently added drop wise and the suspension was centrifuged at 2000 rpm for 3 min. The supernatant was discarded. 7 ml of thawing solution 3 was added drop wise and the suspension was again centrifuged at 2000 rpm for 3 min. The supernatant was removed and the parasite pellet washed in 3ml ofcomplete RPMI medium, resuspended in 1 ml of complete RPMI medium and transferred to a T25 flask containing fresh 10 ml RPMI medium. 0.5 ml of fresh erythrocytes was then added and the flask transferred to the incubator. All solutions were pre-warmed to 37°C before starting the thawing procedure.

2.2.2.7 Parasite synchronisation with sorbitol

Sorbitol treatment causes osmotic lysis of late stage trophozoites (Lambros and Vanderberg 1979). This selective lysis by osmotic shock is possible due to the presence of an induced transport pathway in the red cell membrane that is permeable to sorbitol, and absent in ring stage parasites. The parasite culture was transferred to a 15 ml falcon tube for a T25 flask culture or to a 50 ml falcon tube for a T75 culture. The parasite culture was centrifuged at 2000 rpm for 3 min and Culture media was removed. The parasite pellet was resuspended in 9 ml (or 30 ml for a T75 culture) of sterile, pre-warmed 5% D-sorbitol solution and incubated for 5 min at 37°C in a water bath. The suspension was then centrifuged (2000 rpm, 3min, RT), the pellet was washed once in 3 ml culture medium, resupended in 1 ml medium and transferred to a new flask with 10 ml fresh medium. For a tightly synchronised culture, this procedure was repeated in the next cycle at the transition from schizonts

to ring stage, were only the first few rings survived with an internal age difference of only a few hours.

2.2.2.8 Gelatine flotation

There are different methods for the enrichment of trophozoite and schizont stages. One of them used in this study,is the enrichment by gelatine flotation which is based on different sedimentation rates depending on the presence of knobs on the surface of the infected erythrocytes (Jensen 1978). The parasite culture is centrifuged at 2000 rpm for 3 min at RT, the medium is discarded and pellet resuspended in 4.5 ml (T25 flask) or in 9 ml (T75 flask) of preheated 0.5 % sterile gelatine in RPMI. Parasites are incubated at 37°C for 1 hour, and the supernatant is transferred to a new falcon tube, centrifuged at 2000 rpm for 3 min, washed once in medium or binding buffer (if followed by panning or adhesion assay) and finally resuspended in the amount of medium or binding buffer wanted, usually 10 ml.

2.2.3 Growth experiments

2.2.3 1 Blood collection and preparation of erythrocytes

21 ml venous blood was collected from individuals who were homozygous for the 8 bp insertion (+/+), heterozygous (+/-) and homozygous for the deletion event (-/-). The blood was collected into Li-heparin tubes. The blood was transferred into 50 ml falcon tubes and centrifuged at 4000 rpm for 10 min. The plasma fraction and buffy coat were discarded. The erythrocytes were washed 2-3 times with RPMI 1640 basic medium and each time the buffy coat was carefully removed. The erythrocytes were stored as 50 % packed cells in pyridoxine depleted RPMI 1640 medium at 4°C for 1 week.
Blood was collected from blood donors on a weekly basis.

2.2.3.2 ABO blood group test

The blood group of all blood donors was determined with anti-A, anti-B and anti- AB monoclonal test reagents (SD-nostik Diagnostik, Germany). The human ABO blood group system is defined by the presence or absence of antigens A and B on the erythrocytes. The test reagents are used to identify the AB antigens on the red cells. The specific monoclonal antibodies of the test reagents agglutinate red cells possessing the relevant antigen or antigens. The non-occurrence of agglutination indicates the absence of the relevant antigen.
One drop of test reagent anti-A, anti-B and anti-AB was placed on a clean glass slide. To each drop of test reagent, 1 drop of blood is added and carefully mixed. After 5 minutes of incubation agglutination can be read.

2.2.3.3 Haemoglobin variant test

Blood donors for the growth experiments were also tested for the haemoglobin variants HbC, HbS and HbF. 9 ml of venous blood was collected into EDTA tube and sent to the central lab of the University Hospital Heidelberg for analysis.

2.2.3.4 Growth experiment set up

One week before the growth experiment a *P. falciparum* culture was tightly synchronized by treating the culture 2 times with sorbitol. Infected erythrocytes were added to uninfected erythrocytes from individuals bearing the +/+, +/- and -/- genotype to give an initial parasitemia of ca. 0,1 %. The experiment was started with ring stages and the culture was set up in 6 well plates with triplicates for each genotype. The parasites were cultivated with RPMI 1640 complete medium and medium depleted of pyridoxine for a period of 15-20 days. Medium was changed every second day and the culture was splitted at a parasitemia of 3-6 %. Parasitemia was determined by Giemsa stained slides and flow cytometry.

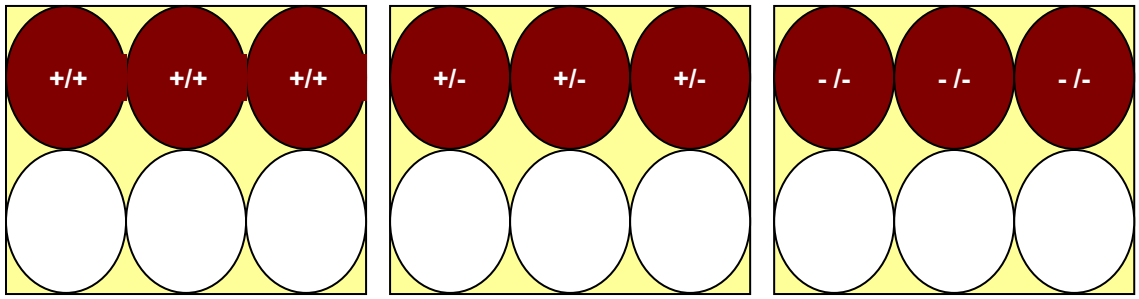

Figure 2.2.4: Schematic drawing showing the set up of the growth experiments. The drawing shows the set up for the in vitro growth experiments. The parasites were grown in 6 well plates with 3 wells of each plate containing cultures with erythrocytes of the same genotype. Pyridoxine containing and pyridoxine depleted medium was used for culure.

2.2.3.5 Flow cytometry

Flow cytometry is a laser based technology that is used to measure characteristics of biological particles. The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as light from the excitation source strikes the moving particle. Flow cytometry allows qualitative and quantitative examination of whole cells and cellular constituents that have been labelled with a range of commercially available reagents such as dyes and monoclonal antibodies. Cells or particles are prepared as single-cell suspensions for flow cytometric analysis. This allows them to flow single in a liquid stream past a laser beam. As the laser beam strikes the individual cells, two types of physical phenomena occur that yield information about the cells. First, light scattering occurs that is directly related to structural and morphological cell features. Second, fluorescence occurs if the cells are labelled with a fluorescent probe. The fluorescent probes are typically monoclonal antibodies that have been conjugated to fluorochromes or fluorescent stains or reagents that are not conjugated to antibodies. There are two distinct types of flow cytometers that can be used to acquire data from particles. One type can perform acquisition of light scattering and fluorescence only. The other type is capable of acquiring scattering and fluorescence data but also has the ability to sort particles. Thus cytometers that perform acquisition without sorting are the more common of the two types.

2.2.3.5.1 Principles of flow cytometry instrumentation

Flow cytometers can be described as a four interrelated system. There is a fluidic system that transports particles from a sample through the instrumentation for analysis. The primary component of this system is a flow chamber. Typically a diluent such as phosphate buffered saline or FACS flow (BD, Germany) is directed by air pressure into the flow chamber. This fluid is referred to as sheath fluid. The sample under analysis in the form of a single particle suspension is directed into the sheath fluid stream prior to sample interrogation. The pressure of the sheath flow against the suspended particles aligns the particles in a single-file fashion.

The second system is an illumination system that is used for particle interrogation. This consists of a laser beam that intercepts a cell or particle that has been hydro dynamically focused by the fluidic system. Light and fluorescence are generated when the focused laser beam strikes a particle within the sample stream. These light signals are then quantified by the optical and electronic system to yield data.

The third system is an optical and electronic system for the direction, collection and translation of scattered and fluorescent light signals that result when the particles are illuminated. The optical and electronic system of a typical flow chamber is responsible for collecting and quantifying at least five types of parameters from the scattered light and emitted fluorescence. Two of these parameters are light scattering properties. Light that is scattered in the forward direction (FSC) is analysed as one parameter. Forward scattered light is a result of diffraction. It provides basic morphological information such as relative cell size. Light that is scattered at 90° to the incident beam is refracted and reflected light – side angle light scatter (SSC). This parameter is an indicator of granularity within the cytoplasm of cells as well as surface/membrane irregularities. Fluorescence emission is detected simultaneously along with FSC and SSC data. Fluorescence is detected using networks of mirrors, optics, and beam splitters that direct the emitted fluorescence light toward highly specific filters. The filters collect light within the range of wavelength associated with each of the three fluorescent channels (FL1, FL2 and FL3).

The fourth system in flow cytometry instrumentation is data storage and computer control system that interprets translated light and electrical signals and collates them into meaningful data for storage and subsequent analysis.

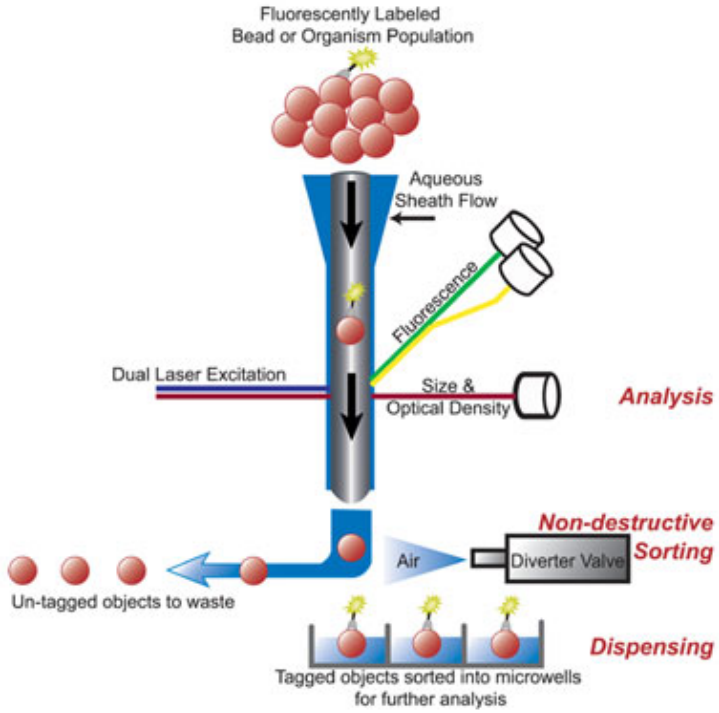

Figure 2.2.5: Diagram showing the instrumentation and principles of a flow cytometer with cell sorting

2.2.3.6 Determining parasitemia with flow cytometry

Parasitemia was determined daily or every second day during the growth experiment by flow cytometry. The cultures were resuspended by shaking the flask thoroughly per hand. 10 µl from each culture was added to a FACS tube containing 1 ml of ringer solution with ethidium bromide (1 µg/µl). The suspension was mixed and incubated for 1-1.5 hours in the dark. The samples were analysed on a BD LSR II system and the parameters FSC and SSC were collected. Fluorescence was collected in the FL 2 channel.

2.2.3.7 Short term growth experiments

P. falciparum strains 3D7, FCR3, Dd2 and HB3 were cultivated in erythrocytes from individuals bearing the +/+, +/- and -/- genotypes for at least 3 weeks before the short term growth experiment. The cultures were cultivated in T25 flasks with pyridoxine containing RPMI medium. The cultures were then synchronised by treating two times

with sorbitol. Infected erythrocytes with ring stages were then added to fresh erythrocytes with the respective genotypes to an initial parasitemia of 0.1 %. The cultures were incubated at 37°C for 3 days and parasitemia was determined daily using flow cytometry.

2.2.4 Erythrocyte deformability assay

The deformability of erythrocytes was assessed according to the methods of Beder et al., 2003. The deformability of erythrocytes was determined by means of filtration combined with centrifugation. Diluted erythrocytes suspensions were filtered by centrifugation through membrane filters with pores of 5 µm in diameter.

2.2.4.1 Preparation of erythrocytes

7 ml venous blood was collected from an individual homozygous for the insertion genotype +/+ and from an individual homozygous for the deletion genotype -/- into heparin tubes and then transferred to 15 ml falcon tubes. The tubes were then centrifuged at 4000 rpm for 10 min at room temperature. The plasma fraction and buffy coat was discarded and the erythrocytes were washed twice with ringer solution.

2.2.4.2 Erythrocyte count

Erythrocytes were counted with the Neubauer improved cell chamber. The Neubauer improved cell chamber consists of 3 x 3 big squares. The central big square is divided into 5 x 5 small groups of squares. The erythrocytes were diluted 1:2500 with cell pack buffer and 7 µl of the suspension was pippetted onto the cell chamber for counting. All cells on the central square were counted; cells on the boardering lines were not counted.

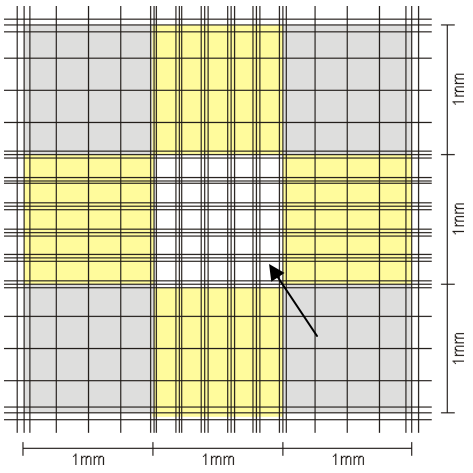

Figure 2.2.6: Neubauer cell chamber. The chamber consists of 3 x 3 big squares. The central big square consists of 5 x 5 smaller square groups. The cells on the central big squares were counted with the exception of cells on boarding lines.

2.2.4.3 Erythrocyte filtration

Erythrocytes were diluted 1:250 with cell pack buffer. 30 µl of the cell suspension was added to a Millipore centrifugal unit onto a membrane with pores of 5 µM in diameter. The centrifugal unit was centrifuged at 1400 rpm for 5 min. The number of erythrocytes before and after filtration was counted and the filtration rate was calculated as follows:

Number of erythrocytes after filtration/number of erythrocytes before filtration x 100 = filtration rate in %

2.2.4.4 Infected erythrocytes deformability assay

The deformability of infected erythrocytes was also determined. The *P. falciparum* strain FCR3 was used to infect erythrocytes from an individual bearing the insertion genotype +/+ and erythrocytes from an individual with the deletion genotype -/-. The cultures were cultivated for at least three weeks and then synchronized by treating the culture two times with sorbitol.

At a parasitemia of at least 3%, the trophozoite stages were enriched by treating the culture with gelatine as described (2.2.2.6). The enriched infected erythrocytes were then diluted 1:250 with the cell pack buffer and 30 µl of the suspension was added to the centrifugal unit and filtered as described above. The number of erythrocytes before and after filtration was determined with a cell viability analyser and the number

of infected erythrocytes before and after filtration was determined by flow cytometry as described in 2.2.3.6. The filtration rate of infected erythrocytes was then calculated: number of infected erythrocytes after centrifugation/ number of infected erythrocytes before centrifugation x 100

2.2.5 Western blot analysis

Western blot analysis was used to determine the protein level of PdxK in erythrocytes. Erythrocytic spectrin was used as a loading control.

2.2.5.1 Erythrocyte preparation

For the Western blot analysis of the human erythrocytic pyridoxal kinase, 7 ml venous blood from individuals bearing the various genotypes: +/+, +/- and -/- was collected into heparin tubes. The blood was transferred into 15 ml falcon tubes and centrifuged at 4000 rpm for 10 min at room temperature. The plasma fraction and buffy coat was discarded. The erythrocytes were then washed twice with ringer solution and stored in an equal volume of Ringer solution at 4°C.

2.2.5.2 Erythrocyte count and lysis

The erythrocytes were centrifuged at 2000 rpm for 5 min and the ringer supernatant was discarded. 5 µl of erythrocytes was added to 10 ml ringer solution (1:2000), the suspension was analyzed with a cell viability analyzer (Vi-Cell, Beckman coulter) and the number of erythrocytes per ml was determined. Equal amounts of erythrocytes were lysed with 6 x equal volume of water (1:6 dilution). The lysates were stored at -80°C.

2.2.5.2.1 Cell viability analyzer (Vi-Cell, Beckman coulter)

The cell viability analyzer automates the widely accepted trypane blue dye exclusion method to assess cell viability. This is based on the fact that when cells die, their membranes become permeable, thereby allowing the uptake of the dye. As a result the dead cells become darker than the viable cells. 500 µl of the cellular suspension is aspirated, mixed with trypane blue and is pumped to the flow cell for imaging. The cells are imaged during measurement. The total number of cells per ml, the number of viable cells per ml and the percentage of viable cells are automatically determined.
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2.2.5.3 SDS Polyacrylamide gel electrophoresis

The erythrocyte proteins were resolved on an SDS polyacryamide gel. A 10% resolving SDS gel was prepared with the following components:
3.3 ml 30% acrylamide/bis solution
2.5 ml 1.5 M Tris-HCL, pH 8.8
4 ml H_2O
100 µl 10 % SDS
100 µl 10 % APS
10 µl TEMED → total volume of 10 ml, was used for casting two gels.

The gels were quickly cast between two glass plates of the Mini-Protean 3 Cell with a spacing of 1mm. The gel was allowed to polymerize for 2 hours. After polymerization of the resolving gel, a 5 % stacking gel was prepared with the following components:
830 µl 30 % acrylamide/Bis solution
630 µl 1 M Tris pH 6.8
50 µl 10 % SDS
50 µl 10 % APS
10 µl TEMED
3.4 ml H_2O → total volume of 5 ml was used for casting two gels above the resolving gel.
The gel was allowed to polymerize for 30 min.

2.2.5.3.1 Denaturation of protein samples

10 µl of the erythrocyte lysate sample was mixed with an equal volume of SDS sample buffer (2x) and the sample was heated for 5 min at 90°C. The protein marker was also heated for 5 min.

10 µl of each sample and 7 µl of the marker were loaded onto the gel. The electrophoresis was run at 200 V for 1 h in 1 x SDS running buffer.

Two gels were run parallel, one of the gels was blotted and the other was Commassie stained.

2.2.5.3.2 Commassie blue staining

At the end of electrophoresis one of the gels was immediately stained in a Commassie blue solution by incubating for at least 2-3 hours on a shaker. The gel was then destained by incubating the gel in a destain solution for at least 1 hour and changing the solution every 20 min.

2.2.5.4 Blotting (Semi-dry)

The gel was blotted on a PVDF transfer membrane with a pore size of 0.45 µM. Before blotting, the membrane was cut according to the gel size, shortly soaked in methanol and then in transfer buffer for at least 20 min. 8 layers of Whatman paper were also cut according to the gel size and soaked in the transfer buffer for at least 20 min. 4 layers of Whatman paper were carefully placed on the plate of the blotting machine (Bio-Rad Trans-Blot Semi dry) and air bubbles were rolled out. The membrane was then placed on the Whatman paper stack, the gel was carefully placed on top of the membrane and then 4 layers of Whatman were placed on top and air bubbles were again rolled out. The gel was blotted onto the membrane at 24 V for 50 min. After blotting the membrane was incubated overnight in 5 % milk at 4°C.

2.2.5.5 Detection

The following day the membrane was washed for 10 min in TBST buffer. The polyclonal anti hPdxK antibody and anti α-spectrin was diluted 1:1000 in 10 ml 1 % milk. The upper part of the membrane was carefully cut and incubated with the anti spectrin antibody for 2 h. The rest of the membrane was incubated with the anti-hPdxK solution also for 2 h. After incubation the membranes were washed for 10 min (3 times) with TBST buffer. The secondary antibody anti-rabbit was diluted 1:2500 in 10 ml 1 % milk. The membranes were then incubated with the secondary antibody for 1 h. At the end of the incubation, the membranes were washed 3 times for 10 min with TBST buffer.

Bound antibodies were detected by incubating the membrane with the ECL detection reagent which comprises of detection reagent 1 and 2 mixed in ratio of 1:1, for 5 min. The membranes were quickly transferred with its protein side facing up into a development folder in a plastic bag.

In the dark room an Amersham Hyperfilm™ ECL, a high performance chemiluminescene film was cut to the appropriate size and exposed to the membrane for 10 s, 30 s, 1 min and 5 min. The exposure time was sometimes increased depending on the strength of the signal. The film was then passed through a developing machine.

2.2.5.6 Blot quantification

The western blots were quantified with the ImageJ software. The integrated densities of each band was analyzed and correlated with the protein level of the sample.

2.2.6 Erythrocytic pyridoxal kinase activity assay

The erythrocytic pyridoxal kinase activity of individuals homozygous for the insertion (+/+), heterozygous for the insertion (+/-) and individuals homozygous for the deletion (-/-) was determined.

2.2.6.1 Preparation of erythrocytes

To determine the activity of the erythrocytic pyridoxal kinase, 7 ml venous blood was collected in lithium heparin tubes from individuals bearing the various genotype +/+,+/- and -/-. The blood from each individual was transferred into a 15 ml falcon tube and centrifuged for 10 min at 4000 rpm. The plasma and the white blood cell fraction were discarded and the erythrocytes were washed twice with an equal volume of Ringer solution. The erythrocytes were stored in an equal volume of Ringer solution over night at 4°C.

2.2.6.2 Erythrocyte count

The erythrocyte solution was centrifuged at 2000 rpm for 5 min and the supernatant was discarded. To determine the numbers of erythrocytes per ml for each individual, 5 µl of erythrocytes were added to 10 ml of Ringer solution (1:2000 dilutions) and this suspension was analyzed with the cell viability analyzer.

2.2.6.3 Pyridoxal kinase assay

8 x 10^9 erythrocytes/ml from each individual were lysed by diluting the appropriate volume of erythrocytes with 10 volumes of lysis buffer in a 50 ml falcon tube and incubation on ice for 20 min. To obtain a membrane free cytoplasmic fraction,
The erythrocyte lysate was centrifuged at 10000rpm for 3 0min at 4°C.
The reaction mix was added to a 15 ml falcon tube. A master mix for 6 samples was prepared.
Pyridoxal kinase reaction mix:
1, 35 ml reaction buffer
50 µl 40 mM $MgCl_2$

100 µl 160 µM pyridoxal hydrochloride
100 µl 200 mM Na_2ATP
400 µl erythrocyte lysate
1 ml of the reaction mix was added to a dark eppendorf tube and incubated at 37°C under constant shaking at 400 rpm. To ascertain the amount of PLP that is formed per min, the samples were measured after 5, 15, 30, 60 and 90 min of incubation. It is important for the detection of the product pyridoxal 5-phosphate (PLP) to deprive the samples of haemoglobin. Therefore, 1 ml of the PdxK reaction mix was immediately transferred into a Millipore centrifugal filter unit and centrifuged for 15 min at 3100 rpm and 10°C (T_0). The membrane of the Millipore centrifugal unit had a cut off of 30 KDa and therefore all proteins with a molecular weight above 30 KDa remained in the filter. The flow through was a clear solution and was transferred into a dark eppendorf tube and stored at -21°C.
After the incubation time the reaction mix was deprived of haemoglobine by centrifugation at 3100 rpm for 15 min at 10°C using a Millipore centrifugal unit. The flow through was transferred to a dark eppendorf tube and stored at -21°C till further analysis. The PLP concentration after 3 h of incubation was also measured.

2.2.6.4 Detection and quantification of pyridoxal 5- phosphate (PLP)

The amount of pyridoxal 5-phosphate at T_0 and the various incubation times was quantified for each sample. The samples were analysed at the central lab of the University Hospital Heidelberg.

The method is based on High –Performance-Liquid-Chromatography (HPLC) with fluorescence detection. Before the analysis the samples were treated to convert pyridoxal 5-phosphate into a strong fluorescence derivate, 4-pyridoxic acid 5′-phosphate. After the HPLC separation the samples were then analysed with a fluorescence detector for quantification.

2.2.6.4.1 Workflow

50 µl of cyanide containing derivatisation reagent (Chromsystems) was added to 100 µl of the sample and shortly mixed. The samples were incubated for 20 min at 60°C in a water bath and then cooled in the refrigerator for 10 min. After centrifugation at 13000 rpm for 5 min, the samples were ready for HPLC analysis.

50 µl of the sample was injected into HPLC system consisting of a RP 18 column (Chromsystems) and a fluorescence detector. The PLP derivate was excited at 320 nm and its fluorescence was detected at 415 nm.

2.2.7 Pyridoxal 5-phosphate content of parasites and erythrocytes in culture

To determine the parasitic content of pyridoxal 5-phosphate, a 3D7 strain of *P. falciparum* was cultivated in erythrocytes of the +/+, +/- and -/- genotypes for a period of at least four weeks. Parallel cultures were also set up with pyridoxine depleted medium. The cultures were synchronized by treating two times with sorbitol as described in 2.2.2.7.

To determine the PLP content of the parasite after culturing in erythrocytes from individuals bearing the various genotypes, trophozoite stages at a parasitemia of 4 % were used.

2.2.7.1 Erythrocyte and parasite lysis

The cultures with trophozoites were transferred into 15 ml falcon tubes and centrifuged at 2000 rpm for 3 min. The supernatant was discarded and the

erythrocyte pellet was adjusted to the same parasitemia by diluting with uninfected erythrocytes bearing the respective genotype.

The number of erythrocytes/ml from each culture was determined with the cell viability analyser. Equal amounts of erythrocytes were lysed.

2.2.7.1.1 Saponin lysis of erythrocytes

Erythrocytes were lysed by adding 13 volumes (14 ml) of a 0.2% saponin solution. The suspension was vortexed and shaked vigorously and then incubated on ice for 20 min. The suspension was centrifuged at 4000 rpm for 5 min. 1 ml of the erythrocytic lysate supernatant was transferred to an eppendorf tube and stored at -21°C. The rest of the supernatant was discarded.

2.2.7.1.2 Parasite lysis

The parasitic pellet was washed twice with PBS by centrifugation at 14000 rpm for 1 min to remove all traces of the erythrocyte lysate. The parasite pellet was then resuspended in 500 µl of cold RIPA buffer, vortexed and stored at -21°C.

2.2.7.2 Detection of pyridoxal 5-phosphate

For the detection of PLP, the parasitic and erythrocytic lysate were deprived of haemoglobin and other high molecular weight proteins by centrifugation with the Millipore centrifugal filter unit (cut off 30KDa) at 3100 rpm for 15 min. The clear flow through was transferred into dark eppendorf tubes and sent to the central lab of the University Hospital Heidelberg. The PLP content of each sample was analyzed by HPLC and fluorescence detection as described in 2.2.6.4.

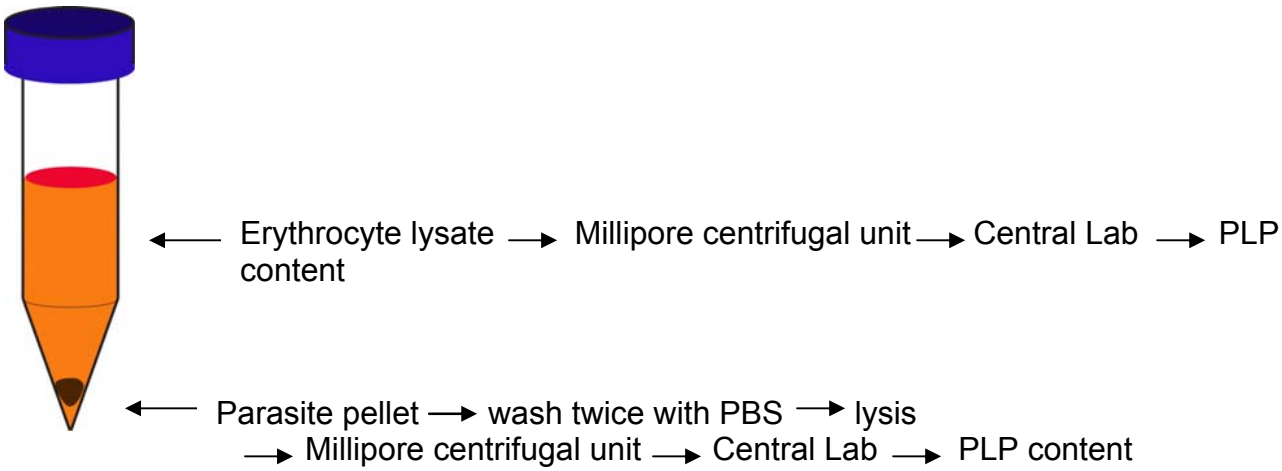

Figure 2.2.7: Workflow for determining the PLP content of the parasite and erythrocytes in culture.
The diagram shows the result of saponin lysis of infected erythrocytes after centrifugation at 4000 rpm for 5 min. The dark parasite pellet is at the bottom of the tube and the erythrocyte lysate is the supernatant. The erythrocyte lysate and the parasite lysate were sent to the Central lab for the quantification of PLP

2.2.8 Electron microscopy

To investigate the morphology of the parasite when cultured in erythrocytes bearing the deletion genotype (-/-) and insertion genotype (+/+), we employed the techniques of electron microscopy.

The electron microscope (EM) uses a particle beam of electrons to illuminate the specimen and create a magnified image of it. The electron microscope has a greater resolving power (magnification) than a light-powered optical microscope, because it uses electrons that have wavelengths about 100,000 times shorter than visible light (photons), and can achieve magnifications of up to 1,000,000 x, whereas light microscopes are limited to 1000 x magnification. The electron microscope uses electrostatic and electromagnetic "lenses" to control the electron beam and focus it to form an image. These lenses are analogous to, but different from the glass lenses of an optical microscope that forms a magnified image by focusing light on or through the specimen.

2.2.8.1 Tokuyasu method

It is often important to section cells and tissues before microscopic examination. However, biological materials are usually fragile and optimal sections cannot be obtained without embedding the sample in a supporting medium

Ultra thin sections of the samples were therefore prepared according to the method proposed by Tokuyasu, 1980.

2.2.8.1.1 Selection of trophozoites using magnetic cell sorting (MACS)

P. falciparum strain 3D7 was cultured in T 75 flasks using erythrocytes from individuals bearing the +/+ and -/- genotype. The parasites were cultivated for a period of at least 4 weeks. The cultures were synchronized by treating 2 times with sorbitol. At a parasitemia of at least 4 % the trophozoite stages were enriched using the MACS column (Ahn, Shin et al. 2008). This method is based on the principle that haemozoin which is accumulated in late blood stages, posses paramagnetic properties. Trophozoites and later blood stages therefore remain in the MACS column while uninfected erythrocytes and ring stages flow through.

The column is connected with a 3-way stopcock, with which the column can be opened or closed. A 10 ml syringe was filled with 2 % BSA/PBS and connected to the left opening of the 3-way stopcock. The column is loaded with 2 % BSA/PBS by carefully pushing the syringe and filling up to 10 mm above the column matrix. The 3-way stopcock was closed and the column incubated for at least 5 min. The syringe was refilled with 2 % BSA/PBS and reconnected to the column.

The parasite cultures were then centrifuged at 2000 rpm for 3 min. The pellet was resuspended in 4 ml of 2 % BSA/PBS. The column was added to the magnet block .After eluting the BSA/PBS solution on the column, the parasites were then loaded onto the column and allowed to migrate into the column. Some 2 % BSA/PBS was added and the cells were allowed to sediment by quick opening of the stopcock, allowing a flow rate of 1 drop every 3 seconds. The column was washed with BSA/PBS. The bound parasites were then eluted by taking the column out of the magnet, opening the 3 way stopcock and collecting the parasites in a falcon tube.

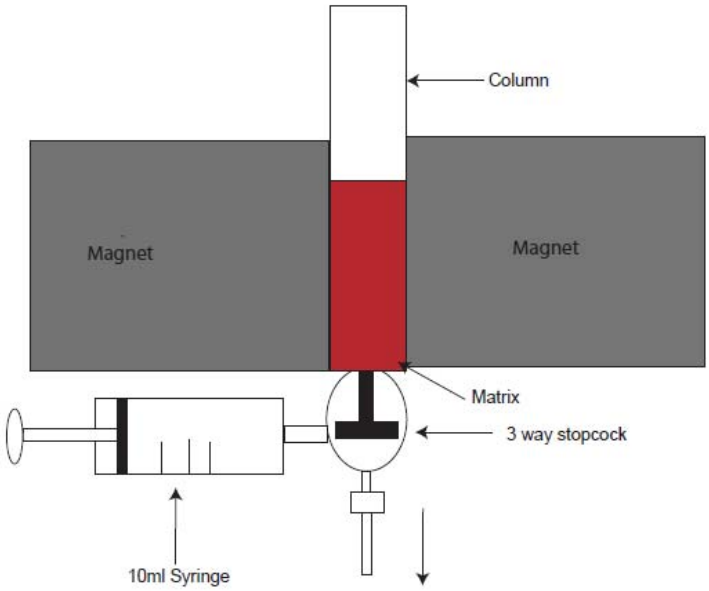

Figure 2.2.8: Schematic drawing of the MACS column.

2.2.8.1.2 Cell fixation and embedding

The parasite elute was centrifuged at 2000 rpm for 3 min and the pellet was resuspended in 2 ml of PBS. 2 ml of Fix 1 was added to the cell suspension and allowed to incubate for 5 min. The suspension was centrifuged at 2000 rpm for 3 min and the supernatant was discarded. The pellet was resuspended in 2 ml of Fix 2 and allowed to incubate for 1h.

The suspension was centrifuged and the supernatant was discarded. The pellet was washed twice with 10 ml PBS and rinsed with 2 ml of 50 mM glycine for 10 min. The cell suspension was transferred to a 2 ml eppendorf tube and centrifuged. The supernatant was discarded and the pellet was suspended in 1 ml of 10-12 % gelatine.

The suspension was incubated for 5 min at 37°C, then centrifuged for 30 s and the supernatant was discarded. The pellet was incubated on ice for 30 min. With a flattened tooth pick the pellet was pushed out of the tube, put in a drop of sucrose and cut into tiny cubes of appropriate size on ice. The cubes were put into a suitable container with 2.3 M sucrose and put on a turning wheel in the cold room over night. Sucrose prevents the formation of ice crystals when freezing the samples. After the

infiltration with sucrose the cubes were put on a specimen holder (small metal pins) and frozen immediately in liquid nitrogen.

2.2.8.1.3 Cryo-sectioning

The instructions of the microtome manual were followed to install the knife and cooling down the microtome to the desired temperature of -90 to -100°C. When the microtome had been adjusted to the desired temperature, the pin (sample holder) with the frozen sample was transferred to the cryo-chamber and fixed properly in the microtome arm. The sample block was trimmed with a glass knife by cutting semi thin sections. After trimming, the glass knife was removed and replaced with a diamond knife which was then used for sectioning. Thin sections of 60nm and semi thin sections of 100 nm were made.

The sections were retrieved with a droplet of 2.3 M Sucrose, the pick-up droplet in a stainless steel loop mounted on a 15 cm long bamboo sticks. The sections were put on grids and the grids were transferred to 2 % solid gelatine plates and stored at 4°C.

2.2.8.1.4 Contrast staining

The sections were stained for contrast and supported by polymers in order to prevent drying artefacts. The grids were incubated in uranyl acetate/methyl cellulose (UA/MC) mixture which is composed of 4 % uranyl acetate and 2 % methyl cellulose in a ratio of 1:9 for 5-10 min. The grid was picked with a wire loop. The loop and the grid were tilted to an angle of 45-60° and the side of the loop was touched to an absorbing filter paper to remove excess UA/MC. The grids were then allowed to dry at room temperature.

2.2.8.1.5 Microscopy

The samples were analysed with the TECNAI F30, 300kv FEG, FEI at the electron microscope core facility of the EMBL in Heidelberg. This microscope is a tomography

microscope and is mostly devoted to acquiring tilt series from embedded samples. It is also a cryoEM microscope that allows the analysis of frozen hydrated sections.

2.2.9 Quantitative real time PCR

Quantitative real time PCR was employed to investigate the fold change in mRNA levels of two enzymes PfPdx1 and PfPdxK, involved in the parasitic metabolism of vitamin B_6.

2.2.9.1 Isolation of mRNA from *P.falciparum*

P. falciparum was cultivated in erythrocytes from individuals bearing the +/+ genotype, +/- genotype and -/- genotype. The parasites were cultured with complete RPMI medium and also in medium depleted of pyridoxine using T75 flasks. The cultures were cultivated for a period of at least 4 weeks. Cultures were synchronized by treating 2 times with sorbitol as described in 2.2.2.7. Trophozoite stages at a parasitemia of about 3-5 % were used fro the isolation of mRNA. The cultures were transferred to 50 ml falcon tubes and centrifuged at 2000 rpm for 3min. The supernatant was discarded and 10 ml of Roche mRNA stabilization solution was added to the pellet. The samples were stored at -21°C until mRNA purification. The isolation of the mRNA was then performed with the mRNA Isolation Kit for blood and bone marrow (Roche, Mannheim, DE). The instructions were followed as indicated in the protocol of the manufacturer.

2.2.9.2 DNase treatment

The mRNA isolation kit does not include a DNase digestion step during purification. To avoid contamination of mRNA with genomic DNA which could lead to false results, the samples were therefore treated with Dnase. The DNase treatment was carried out with the Turbo DNA free kit and the instructions of the manufacturer were followed.

2.2.9.3 Reverse Transcription (cDNA synthesis)

mRNA was transcribed into cDNA with the RETROscript Kit from Ambion. Oligo d'T primers were used to initiate cDNA synthesis and the instructions were followed as indicated in the protocol by the manufacturer.

2.2.9.4 Real time PCR

The changes in mRNA levels of *pfpdx1* and *pfpdxk* when the parasite was cultured in erythrocytes bearing the various genotypes and in medium depleted of pyridoxine was analyzed with real time PCR using a Sybr green based assay.

Primers were designed for both genes using the primer express 3.0 software from ABI.

The reaction mix with a total volume of 25 µl was prepared as follow:

3 µl	cDNA
12.5 µl	2 x Sybr power master mix
0.025 µl	forward primer
0.025 µl	reverse primer
9.45 µl	H_2O

A negative control containing only H_2O and mRNA to exclude gDNA contamination was run on each plate.

All measurements were run as duplicates on each plate. The PCR was run on an ABI 7500 cylcer machine with the following conditions:

Stage	Repetitions	Temperature	Time
1	1	95°C	5 min
2	40	95°C	15 sec
		58°C	15 sec
		60°C	45sec
3 (dissociation)	1	95°C	15 sec
		60°C	1 min
		95°C	15 sec

3 Results

3.1 Screening individuals for the 8 bp insertion in the promoter region of the *pdxK* gene

To investigate the effect of lowered erythrocytic pyridoxal kinase activity on *P. falciparum* in an in vitro culture system, I had to screen individuals for the polymorphism in the promoter region of the *pdxk* gene (Flanagan and Beutler 2006). I established two PCR based methods for this purpose. The first being a PCR using allele specific primers to detect the 8 bp insertion (Figure 3.1) and the second a PCR amplifying a small fragment of 80 bp and detecting the difference in size on a metaphor gel (Figure 3.2).

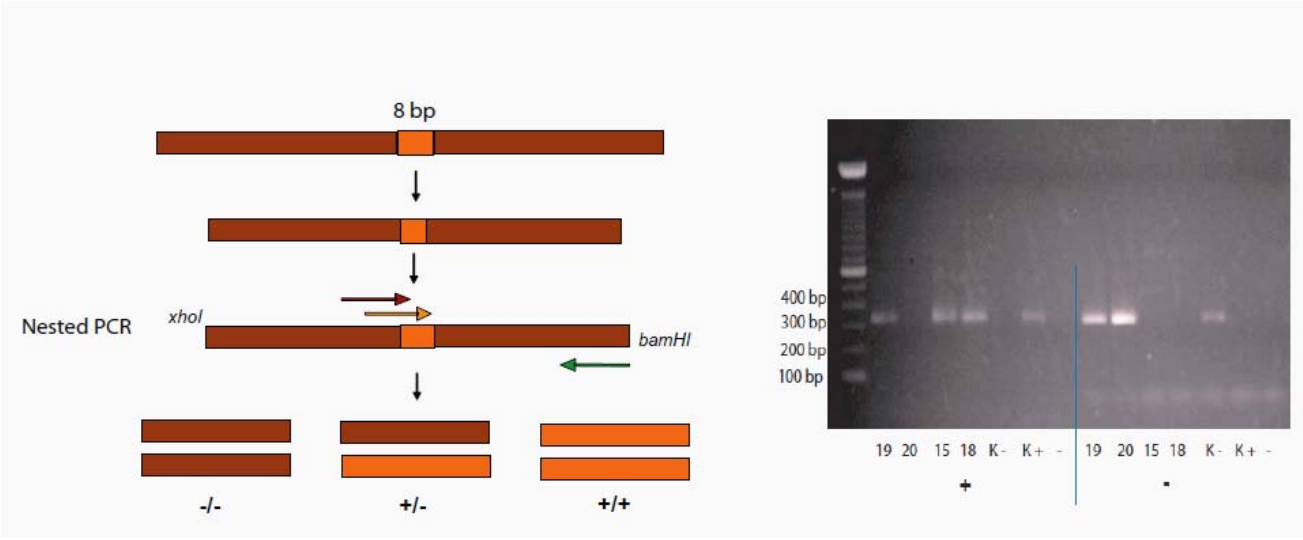

Figure 3.1: Allele specific PCR. A: Schematic drawing of the allele specific PCR. **B:** A nested PCR with allele specific primers was carried out using a 700 bp amplified fragment as template. The PCR was carried out in two separate reactions using a forward primer with the insertion (+) and without the insertion (-) respectively.

Figure 3.1B shows the result of a nested PCR. + represents the reaction with the insertion forward primer and – the reaction with the forward primer without the insertion. Sample 19 represents a heterozygote since a fragment was amplified in both reactions. Sample 20 is an individual who is homozygous for the deletion as I could only observe a band in the - reaction. Sample 15 and 18 are individuals who

are homozygous for the insertion event as the reaction was only positive with the insertion forward primer. The positive clones for the insertion (K+) and deletion event (K-) were always used for controls in each PCR reaction and a H_20 sample (-) as an absolute negative control for the PCR reaction

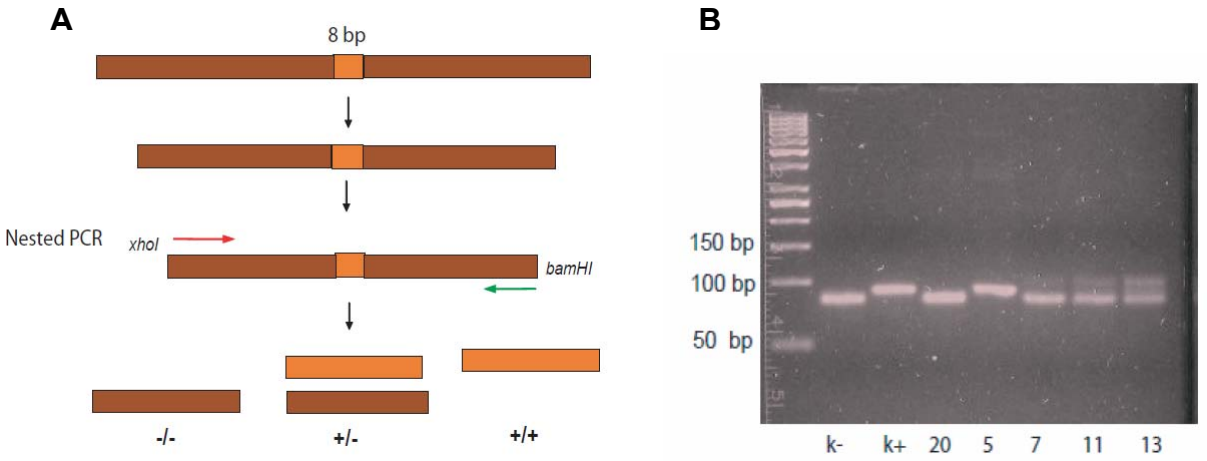

Figure 3.2: Metaphor gel analysis of PCR fragments, A. Schematic drawing of the PCR for the small 80 bp fragment. **B.** A small fragment of 80/88 bp was amplified around the region of interest in the promoter region of the *pdxk* gene in a nested PCR using the amplified 700 bp as template. The small fragments were separated on a 4% metaphor gel. K- is the control clone for the 8 bp deletion event, K+ the control clone for the 8 bp insertion event. Sample 20 represents an individual homozygous for the deletion event, 5 an individual homozygous for the insertion event and samples 7, 11 and 13 represent individuals that are heterozygotes.

Figure 3.2 shows the result of metaphor gel analysis of the 80/88 bp PCR fragments. This PCR method was not used for screening individuals due to the fact that it was difficult detecting the heterozygotes. Although the size difference between the homozygotes could be clearly seen, I observed that the upper band of the heterozygotes was not as strongly amplified as the lower band. In some cases (data not shown) the upper band could not be detected on the gel and this could lead to a misinterpretation of the PCR results.

3.1.1 Screening Caucasians for the 8 bp insertion in the promoter region on the *pdxk* gene

Applying the PCR with allele specific primers (Figure 3.2), I screened 42 individuals resident in Heidelberg, Germany, for the 8 bp insertion in the promoter of the *pdxk* gene. These individuals were all Caucasians. 10 individuals were homozygous for the insertion event (+/+), 30 were heterozygous (+/-) and only 2 individuals were homozygous for the deletion event (-/-) (Table 3.1).

This gave an allele frequency of 66.2% for the insertion allele (+) and 33.8 % for the deletion allele (-). In all, I found a genotype distribution among these individuals of 22 % for insertion event (+/+), 73 % heterozygotes (+/-) and 5 % for the deletion event (-/-) (Fig.3.3)

3.1.2 Screening east Africans for the 8 bp insertion in the promoter region of the *pdxk* gene

Genomic DNA samples from 33 individuals resident in Kilifi, Kenya were also screened for the 8 bp insertion using the allele specific PCR. All individuals were Africans. 2 individuals were homozygous for the 8 bp insertion event (+/+), 22 were heterozygotes (+/-) and 9 individuals were homozygous for the deletion event (-/-) (Table 3.1). I calculated an allele distribution of 34.09% for the insertion allele (+) and 65.9% for the deletion allele (-). We had a genotype distribution of 6.06% for the insertion event (+/+), 66.6% for the heterozygotes (+/-) and 27.2% for the deletion event (-/-) (Fig 3.3)

Table 3.1: Genotype distribution among Caucasians and Africans

Race	number of individuals screened	+/+	+/-	-/-
Caucasians	42	**10**	**30**	**2**
East Africans	33	**2**	**22**	**9**

The results of my PCR screen showed clearly that more individuals were homozygous for the deletion event (-/-) among the African population as compared to the Caucasians from Heidelberg. In both cases heterozygotes were the highest numbers. Only two individuals were homozygous for the deletion event -/- among 42 Caucasians from Heidelberg whereas 9 had the -/- genotype among 33 individuals from Kenya. The genotype distribution in percent among the two races is shown in figure 3.3

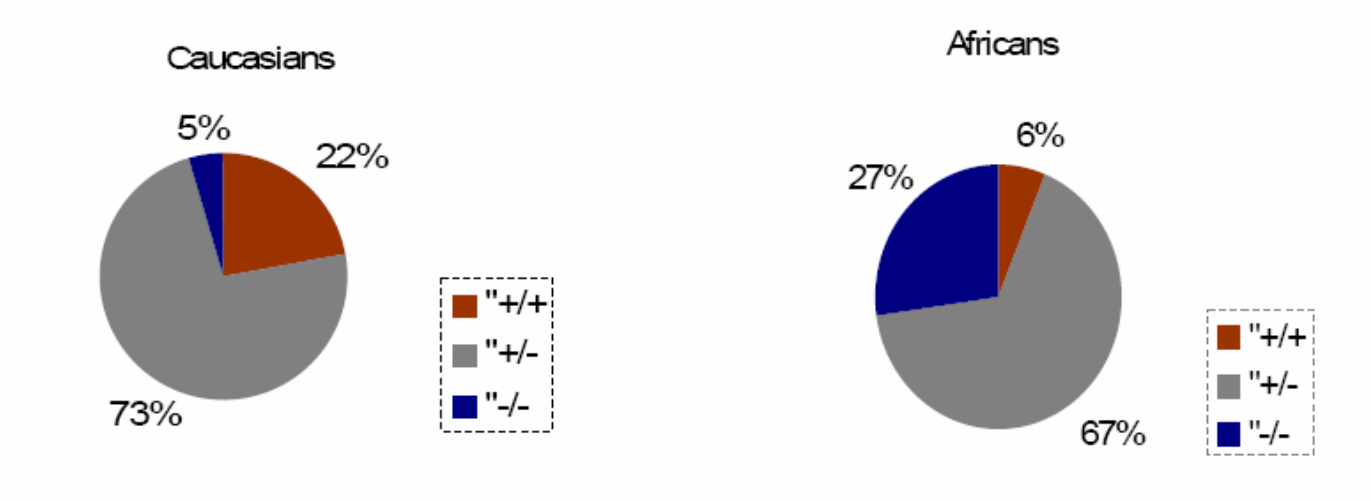

Figure 3.3: Genotype distribution of the 8 bp insertion allele among Caucasians and Africans. 45 Caucasians and 33 Africans were screened for the 8 bp insertion in the promoter region of the *pdxk* gene.

3.1.3 Sequencing the promoter region of the *pdxk* gene

To verify the results of my PCR screen, I sent in some of the 700 bp PCR fragments for sequencing. Sequencing confirmed the results of my PCR screen which showed that the allele specific PCR was reliable.

3.1.3.1 In/Del event in the promoter region of the *pdxk* gene

Sequencing the 700 bp fragment amplified from genomic DNA of 23 individuals from Heidelberg and 9 from Kilifi followed by aligning the sequences with the Bioedit software revealed that there is another In/Del event of 7 bp in the promoter region of the *pdxK* gene. It was a 4 times repeat of the sequence 5´GGGGCGC 3´ which is located at -442 of the ATG and about -130 bp of the known 8 bp insertion (figure 3.5) It was not possible to get clean sequence readouts for heterozygotes for both In/Del events (figure 3.4). However when the chromatogram is studied carefully, it is possible to read the single sequences

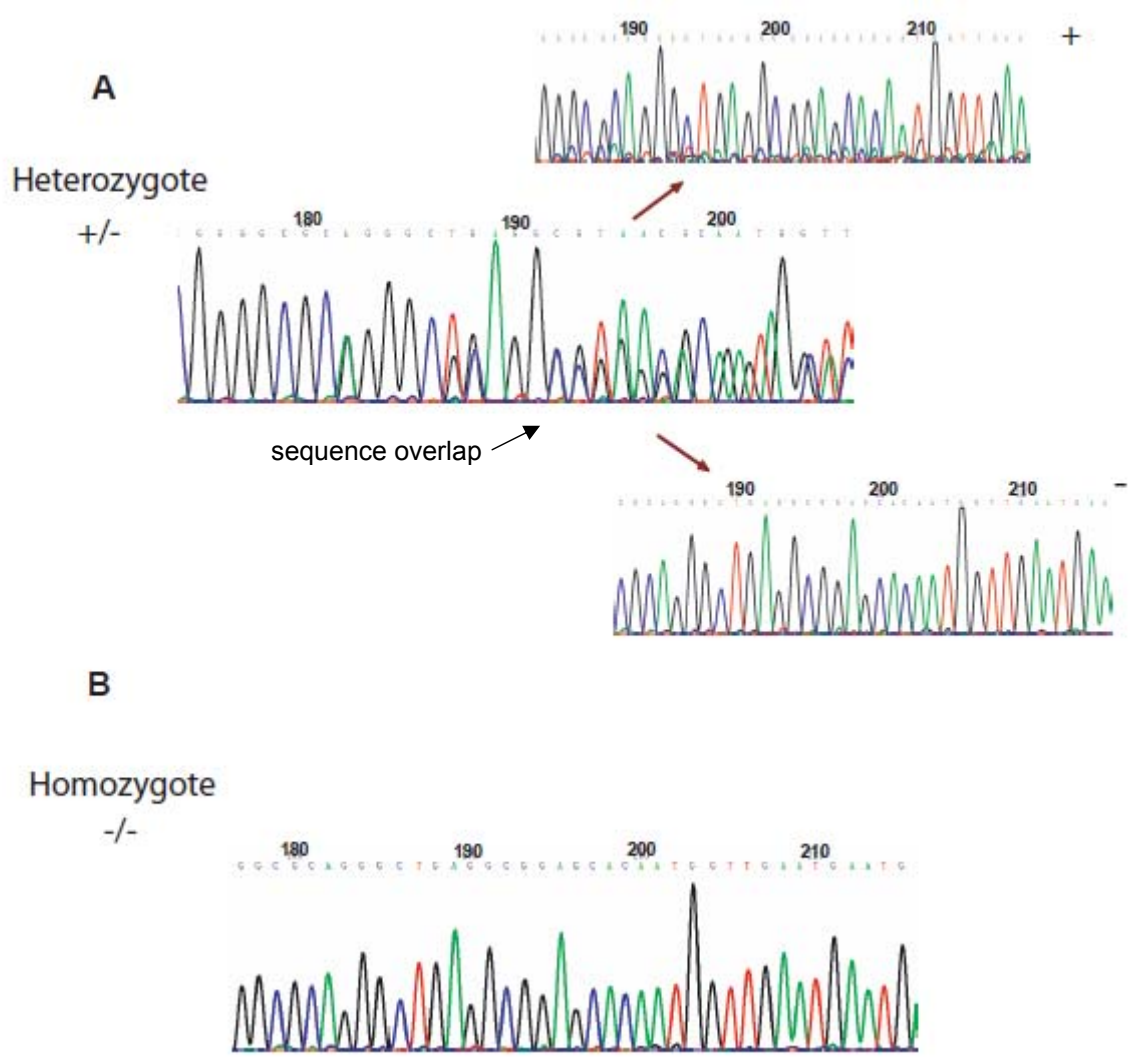

Figure 3.4: Sequence chromatograph in the region of the 7 bp In/Del event. A: heterozygote sequence. **B:** homozygote sequence (-/-).

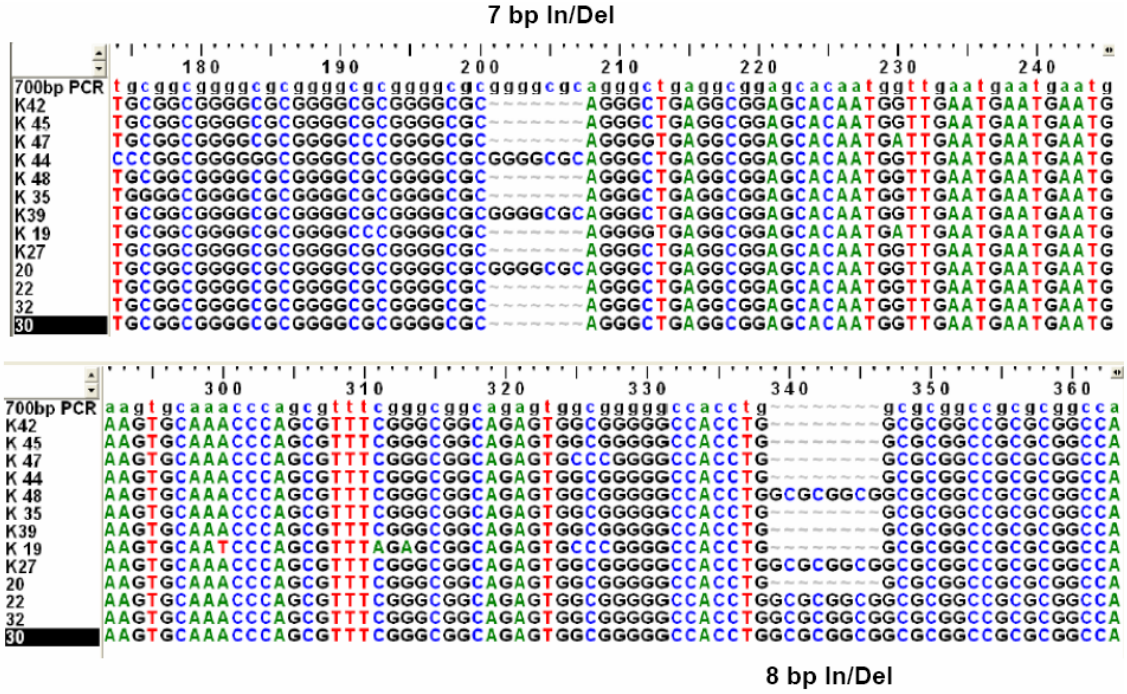

Figure 3.5: Sequence alignments. Sequences of homozygotes were aligned with the Bioedit software. Aligning sequences displayed two In/Del events in the promoter region of the *pdxk* gene. It shows the 8 bp insertion which has already been reported and the 7 bp insertion which is at - 442 bp of ATG.

3.1.3.2 MatInsepector analysis of the 7 bp In/Del event

As the 7 bp In/Del event in the promoter region has not been reported before, I decided to explore if this polymorphism could also have an effect on the transcription of the *pdxk* gene. Using the MatInspector software, I examined if the 7 bp sequence 5´GGGGCGC3´ is a binding site for transcription factors. Feeding the sequence around the region of interest into the program showed that the repeat GGCGCGG which is CCGCGCC on the minus strand is a recognition motif of the RNA polymerase II transcription factor IIB (Table 3.2).

The transcription factor IIB (TFIIB) plays a critical role in the assembly of the RNA polymerase II pre-initiation complex. TFIIB makes sequence specific DNA contacts both upstream and down stream of the TATA box. These specific sequences have been defined as BREs, one upstream BRE (BRE^u) and one down stream BRE (BRE^d) of the TATA box. The BRE^u consensus sequence is 5´-G/C-G/C-G/A-C-G-C-C-3´. The BRE^u may act as a positive or negative element in transcriptional regulation (Deng and Roberts 2006). BRE sequences are found in bidirectional and non-bidirectional promoters (Yang and Elnitski 2008)

Table 3.2: MatInspector analysis of the 7 bp repeat. Analysis of the 7 bp sequence GGCGCGG (CCGCGCC, minus strand) gave the highest hit score of 1 for the RNA polymerase II transcription factor II B.

V$ZBPF	Zinc binding protein factors	V$ZF9.01	603	625	614	(-)	0.888	ccgcgccCCGCgccccgccgcac
V$SP1F	GC-Box factors SP1/GC	V$SP1.02	610	624	617	(+)	0.851	gggGCGCggggcgcg
V$PLAG	Pleomorphic adenoma gene	V$PLAG1.01	610	630	620	(+)	0.894	GGGGcgcggggcgcggggcgc
V$ZBPF	Zinc binding protein factors	V$ZF9.01	610	632	621	(-)	0.888	ccgcgccCCGCgccccgcgcccc
V$EBOX	E-box binding factors	V$MYCMAX.03	611	623	617	(-)	0.919	gcgcccCGCGccc
V$MAZF	Myc associated zinc fingers	V$MAZ.01	611	623	617	(+)	0.911	gggcGCGGggcgc
O$TF2B	RNA polymerase II transcription factor II B	O$BRE.01	612	618	615	(-)	1.000	ccgCGCC
V$SP1F	GC-Box factors SP1/GC	V$SP1.02	617	631	624	(+)	0.851	gggGCGCggggcgcg
V$PLAG	Pleomorphic adenoma gene	V$PLAG1.01	617	637	627	(+)	0.894	GGGGcgcggggcgcggggcgc
V$ZBPF	Zinc binding protein factors	V$ZF9.01	617	639	628	(-)	0.888	ctgcgccCCGCgccccgcgcccc
V$EBOX	E-box binding factors	V$MYCMAX.03	618	630	624	(-)	0.919	gcgcccCGCGccc
V$MAZF	Myc associated zinc fingers	V$MAZ.01	618	630	624	(+)	0.911	gggcGCGGggcgc
O$TF2B	RNA polymerase II transcription factor II B	O$BRE.01	619	625	622	(-)	1.000	ccgCGCC
V$SP1F	GC-Box factors SP1/GC	V$SP1.02	624	638	631	(+)	0.851	gggGCGCggggcgca
V$EBOX	E-box binding factors	V$MYCMAX.03	625	637	631	(-)	0.919	gcgcccCGCGccc
V$MAZF	Myc associated zinc fingers	V$MAZ.01	625	637	631	(+)	0.911	gggcGCGGggcgc
O$TF2B	RNA polymerase II transcription factor II B	O$BRE.01	626	632	629	(-)	1.000	ccgCGCC
V$MTF1	Metal induced transcription factor	V$MTF-1.01	627	641	634	(-)	0.896	ccctGCGCcccgcgc
V$PAX5	PAX-5 B-cell-specific activator protein	V$PAX5.01	629	657	643	(+)	0.845	gcggggCGCAgggctgaggcggagcacaa
V$PRDM	PRDI-BF1 and RIZ homologous (PR) domain proteins (PRDM)	V$PRDM5.01	629	657	643	(+)	0.797	gcggggcgcAGGGctgaggcggagcacaa

I could only detect the presence of the 7 bp In/Del event by sequencing the 700 bp amplified PCR fragment. It was not possible to apply allele specific PCR to screen for this polymorphism. The 7 bp sequence CCGCGCC (- strand) is a binding sequence for the TFII B. It is not clear whether/how the presence or absence of this sequence could affect the transcription of the *pdxk* gene. I sequenced 22 individuals from Heidelberg, Germany and 9 from Kilifi, Kenya. I observed that among the individuals from Heidelberg, those bearing the +/+ genotype for the 8 bp insertion event were homozygous for the deletion genotype (-/-) for the 7 bp event, heterozygotes for the 8 bp insertion event (+/-) were also heterozygous for the 7 bp event and one individual bearing the deletion genotype (-/-) for the 8 bp event had the insertion genotype (+/+) for the 7 bp event.The second individual bearing the deletion genotype (-/-) for the 8 bp event was heterozygous for the 7 bp event.

Among the Africans, 3 individuals with the deletion genotype (-/-) for the 8 bp event also bore the -/- genotype for the 7 bp event. 2 individuals bearing the +/+ genotype

for the 8 bp event had the -/- genotype for the 7 bp event and 1 individual with the +/- genotype for the 8 bp event bore the -/- genotype for the 7 bp event.
This result is presented in table 3.3.

Table 3.3: Genotype distribution for both In/Del events in the promoter region of the *pdxK* gene

Individuals		8bp insertion	7bp insertion
Caucasians	1	+/+	-/-
	2	+/+	-/-
	3	+/+	-/-
	4	+/+	-/-
	5	+/+	-/-
	6	+/+	-/-
	7	+/+	-/-
	8	+/+	-/-
	9	+/-	+/-
	10	+/-	+/-
	11	+/-	+/-
	12	+/-	+/-
	13	+/-	+/-
	14	+/-	+/-
	15	+/-	+/-
	16	+/-	+/-
	17	+/-	+/-
	18	+/-	+/-
	19	+/-	+/-
	20	+/-	+/-
	21	-/-	+/+
	22	-/-	+/-
Africans	1	+/+	-/-
	2	+/+	-/-
	3	+/+	-/-
	4	+/-	+/-
	5	+/-	-/-
	6	-/-	-/-
	7	-/-	+/+
	8	-/-	-/-
	9	-/-	-/-

The presence or absence of the 7 bp In/Del event did not correlate with the PdxK protein level in erythrocytes or the erythrocytic activity of the enzyme.

3.2 Western blot analysis

It has been proposed that the presence of an 8 bp insertion in the promoter region of the *pdxk* gene leads to enhanced transcription of the gene. It is proposed that there is a 4 fold increase in transcription of the gene (Flanagan and Beutler 2006). It is not possible to detect mRNA levels in matured erythrocytes. Since an increased level of the transcripts should correlate with an increased amount of the protein, I decided to compare the protein level of PdxK in erythrocytes of individuals bearing the genotypes +/+, +/- and -/-. The number of erythrocytes/ml was determined for each individual (Table 3.4) and equal amounts were lysed for Western blot analysis. Samples were resolved on an SDS gel and stained with Commassie blue (Figure 3.6) for visual inspection. The Commassie blue stained gel showed that the samples had equal amount of proteins.

Table 3.4: Erythrocyte counts obtained with the cell viability analyser. The number of erythrocyte per ml of individuals with the various genotypes was determined. The washed erythrocytes were diluted 1:2000 and analysed with the cell viability analyser. Equal amounts of erythrocytes per individual were lysed for western blot analysis.

Individual	Genotype	Total cells/ml	x dilution	Volume lysed (µl)
1	+/+	3.6×10^6	7.2×10^9	140
2	+/+	1.82×10^6	3.64×10^9	276.9
3	+/+	1.36×10^6	2.72×10^9	370
4	+/+	2.10×10^6	4.20×10^9	120
5	+/+	1.60×10^6	3.30×10^9	315
6	+/-	5.04×10^6	10.08×10^9	100
7	+/-	2.98×10^6	5.96×10^9	169
8	+/-	2.02×10^6	4.40×10^9	249.5
9	+/-	3.14×10^6	6.28×10^9	160
10	+/-	3.86×10^6	7.72×10^9	130.5
11	+/-	4.7×10^6	9.40×10^9	107.2
12	+/-	4.9×10^6	9.80×10^9	102.8
13	-/-	5.02×10^6	10.04×10^9	100.3
14	-/-	3.36×10^6	6.72×10^9	150

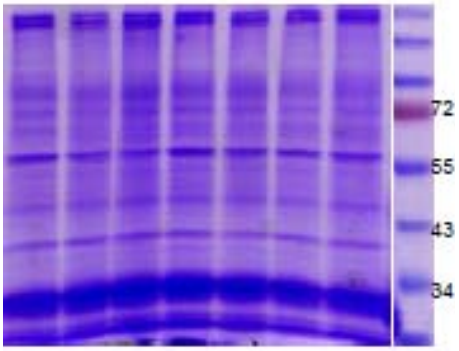

Figure 3.6: Commassie stained gel of erythrocyte lysates. Some of the erythrocyte lysate samples of various genotypes were stained with Commassie to detect the protein levels of the samples. The staining revealed that all samples had roughly the same amount of protein.

Using Western blot analysis I could detect the erythrocytic PdxK by probing with a polyclonal antibody specific for the protein. Densitometric analysis of the blots revealed that the protein level of PdxK was significantly lower in erythrocytes from individuals bearing the deletion genotype (-/-) as compared to those of individuals bearing the +/- and +/+ genotypes (t test p value ++/-- :0.0097, +-/--: 0.024)

I used erythrocytic α-spectrin as a loading control. Densitometric analysis of the blot showed that the protein level of spectrin was roughly the same in erythrocyte lysate of the various genotypes. The polyclonal rabbit anti-α-spectrin antibody used in the Western blot analysis of the erythrocytic α- spectrin, cross reacted with ß-spectrin band, which appears as the lower of the two bands in the western blot analysis.

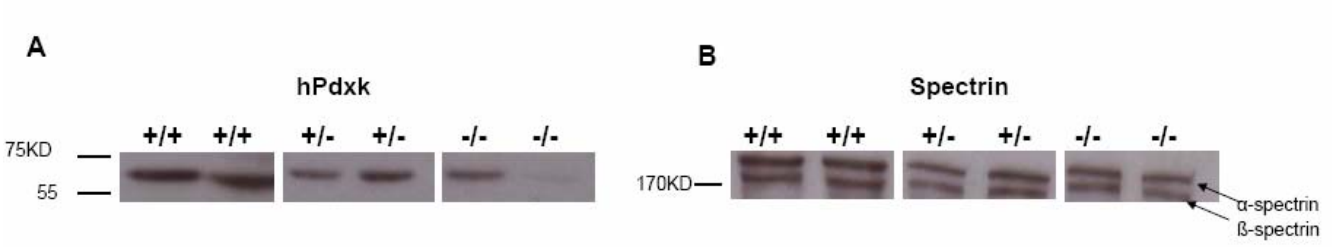

Figure 3.7: Western blot analyses of the erythrocytic PdxK and α-spectrin. Equal amounts of erythrocytes from each individual were lysed for Western blot analysis **A:** hPdxK probing: Western blot of erythrocyte lysate of the various genotypes. The PdxK has a molecular weight of 40 KDa but is detected at a size between 55 and 72 KDa on an SDS gel (Kerry J.A 1986). Individuals bearing the deletion genotype had weaker bands as compared to those with the +/+ and +/- genotype. **B:** The α-spectrin band was detected at above 170 KDa. The anti α-spectrin polyclonal antibody cross reacted with the ß-spectrin subunit (lower band).

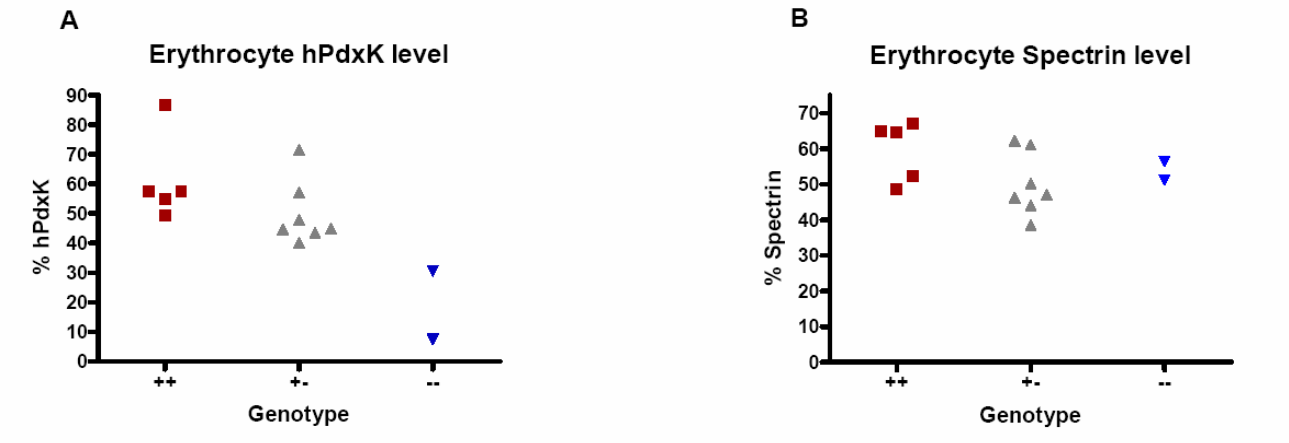

Figure 3.8: Densitometric analysis of the individuals' bands obtained by Western blot analyses. **A:** The hPdxk Western blots were analysed with the imageJ software and the integrated densities of the bands were compared. Erythrocyte with the -/- genotype had lower levels of hPdxK as compared to levels in erythrocyte of the +/+ and +/- genotypes. **B:** The α-spectrin levels in erythrocytes of various genotypes were also compared and the levels were roughly the same in the various samples.

3.3 Pyridoxal kinase activity

After observing that the protein level of erythrocytic PdxK is lower in erythrocytes of individuals bearing the -/- genotype, I investigated if this observation correlates with the activity of the enzyme. It has been previously shown that the presence of the deletion event in the promoter of the *pdxk* gene leads to a lower activity of the enzyme in erythrocytes (Flanagan and Beutler 2006). I adapted the method as proposed by Kark J et al for the determination of PdxK enzymatic activity (Kark, Haut et al. 1982). Equal amounts of erythrocytes were lysed (Table 3.5) and equal volumes of the lysate were added to the reaction mix (see material and methods). The erythrocytes used in this experiment were fresh and not older than a day to avoid an inhibited activity of the enzyme due to cell age. The amount of PLP (nmol/l) was analysed and quantified in the Central Lab of the University Hospital Heidelberg.

Firstly, I measured the amount of PLP that is formed by the pyridoxal kinase of erythrocytes from individuals bearing the various genotypes after an incubation period of 3 hours. The individuals had different amounts of PLP at the onset of the experiment (T_0). At the end of the 3 hour incubation time, I could measure a significant increase in the amount of PLP present in the reaction mix with lysate of erythrocytes with the various genotypes (Table 3.6)

Table 3.5: Erythrocyte count with the cell viability analyser. The amount of erythrocytes per ml of individuals bearing the various genotypes was determined. The erythrocytes from the individuals were highly viable. Equal volumes of erythrocytes were lysed to determine pyridoxal kinase activity.

Individual	Genotype	Total cells/ml	Viable cells/ml	Viability %	x dilution factor cells/ml	Volume lysed (ml)
1	+/+	2.57×10^6	2.57×10^6	99,8%	5.14×10^9	1.55
2	+/+	2.88×10^6	2.88×10^6	97.9%	5.76×10^9	1.38
3	+/+	2.58×10^6	2.50×10^6	96.8%	5.10×10^9	1.55
4	+/+	3.32×10^6	3.17×10^6	95.5%	6.64×10^9	1.20
5	+/+	3.06×10^6	2.34×10^6	76.5%	6.12×10^9	1.30
6	+/-	2.23×10^6	2.19×10^6	98.8%	4.46×10^9	1.79
7	+/-	2.71×10^6	2.61×10^6	96.1%	5.40×10^9	1.47
8	+/-	3.33×10^6	3.21×10^6	96.4%	6.60×10^9	1.20
9	+/-	3.06×10^6	2.99×10^6	97.7%	6.12×10^9	1.30
10	+/-	3.53×10^6	3.46×10^6	98.0%	7.06×10^9	1.13
11	+/-	2.78×10^6	2.73×10^6	98.4%	5.56×10^9	1.43
12	-/-	2.90×10^6	2.81×10^6	97.0%	5.16×10^9	1.37
13	-/-	1.82×10^6	1.82×10^6	99.7%	3.64×10^9	2.19

Table 3.6: Amount of PLP formed after 3 hours of incubation. The amount of PLP formed after 3 hours of incubation was measured for the various genotypes. There was a high amount of PLP formed after 3 hours of incubation (T_1). The experiment was conducted once.

Individual	Genotype	T_0 PLP (nmol/L)	T_1 PLP (nmol/L)	$T_1 - T_0$ PLP (nmol/L)
1	+/+	15.4	753.5	738.1
2	+/+	9.4	391.7	382.3
3	+/+	5.1	648.6	643.5
4	+/+	3.1	518.4	515.3
5	+/-	11.2	257.9	246.7
6	+/-	5.7	420.4	414.7
7	+/-	3.7	366.0	362.3
8	+/-	8.3	529.3	521.0
9	+/-	8.2	511.4	503.2
10	+/-	24.4	533.7	509.3
11	-/-	23.5	625.0	601.5
12	-/-	9.0	505.9	496.9

There was only a weak correlation between the amount of PLP formed after 3 hours of incubation and the various genotypes. The amount of PLP formed by the PdxK in lysates of erythrocytes of the four tested individuals bearing the +/+ genotype varied significantly. It ranged from 382.3 nmol/L to 738.1 nmol/L with an average value of 569.8nmol. The amount of PLP formed after incubation of lysates of the +/- genotypes also varied but was intermediate to that of the +/+ and -/- genotypes. Here in average 462.2nmol/L PLP was generated. The amount of PLP formed by the enzyme in lysate of erythrocytes of the two individuals with the -/- genotypes was with an average value of 459.2 nmol/L not significantly lower than that of +/+ genotype (Figure 3.9).

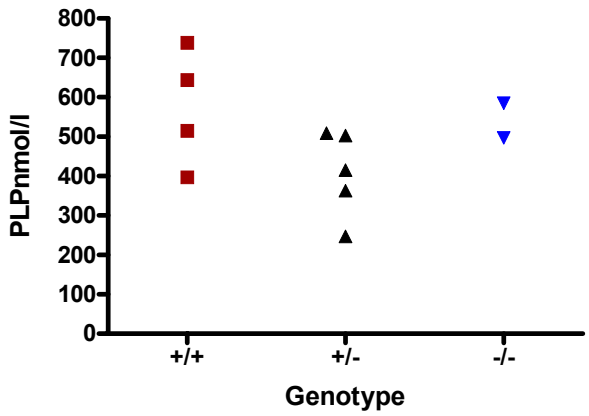

Figure 3.9: Amount of PLP formed after 3 hours of incubation. The amount of PLP formed after 3 hours incubation of the reaction mix with erythrocyte lysate of the various genotypes.

3.3.1 Determining the amount of PLP formed per minute

Since the amount of PLP formed after 3 hours of incubation did not correlate with the expected hPdxK activity of the individual genotypes, I decided to measure the quantity of PLP that is formed per minute when lysate of the +/+ and -/- genotype were added to the reaction mix.

For this experiment, equal amounts of fresh erythrocytes from an individual bearing the +/+ genotype and from an individual of the -/- genotype were lysed and added to

the reaction mix. The amount of PLP formed after 5, 15, 30, 60 and 90 min was determined.

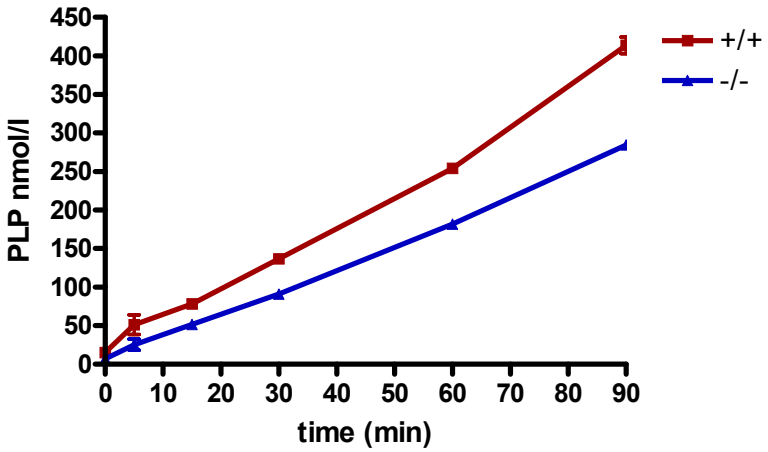

Figure 3.10: PLP (nmol/L) formed over time. The amount of PLP was determined at different time points as plotted on the x axis for the +/+ genotype and -/- genotype. The amount of PLP formed over time was higher for the +/+ genotype. The data is representative for two independent experiments.

Figure 3.10 shows the result of the experiment in which I investigated the amount of PLP that is formed over time for the +/+ and -/- genotype. I observed that over time more PLP was formed in the reaction mix with the +/+ lysate than in the -/- reaction.
I calculated a formation rate of 3.9 nmol/min for the +/+ reaction and 2.6 nmol/min for the -/- reaction.

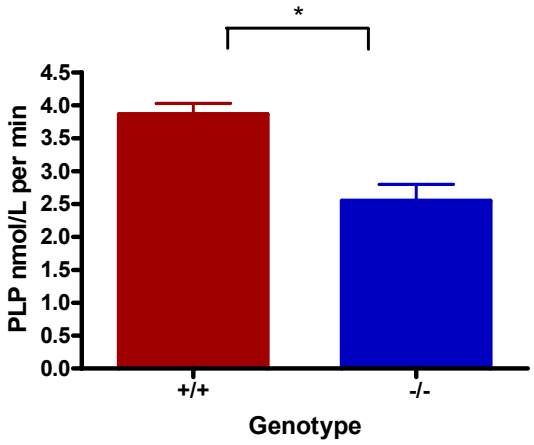

Figure 3.11: PLP nmol/L formed per minute. The formation of PLP was followed over time. There was a significant difference between the amount of PLP formed per minute in the +/+ reaction and the -/- reaction.

3.4 Pyridoxal 5- phosphate content of the erythrocytes of the various genotypes under in vitro culture conditions

I investigated whether there were variations in the PLP content of erythrocytes with the various genotypes in an in vitro culture system. To clarify if the presence or absence of pyridoxine in the culture medium has an effect on the PLP content of erythrocytes and parasites in culture, a parallel culture was held in the absence of pyridoxine in the medium. Erythrocytes were lysed with a saponin solution and parasites with RIPA buffer (see material and methods). The PLP content of the erythrocyte and parasite lysate was then measured in the central lab of the University Hospital Heidelberg.

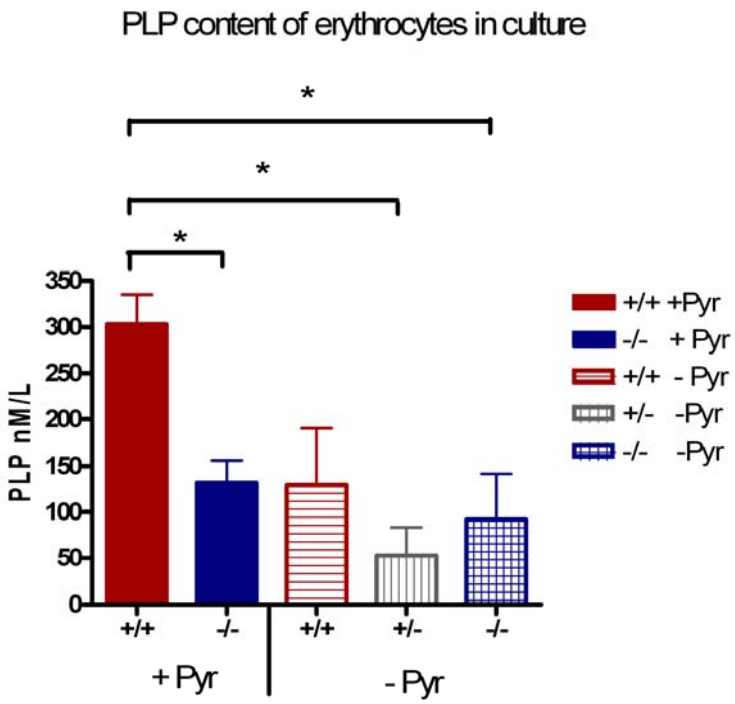

Figure 3.12: Erythrocytic PLP content. The PLP content of erythrocytes in culture was determined. Erythrocytes of the different genotype were maintained in RPMI medium containing pyridoxine (+ Pyr) or depleted of pyridoxine (-Pyr). The error bars stand for the standard error of 3 independent measurements. The Y axis shows the amount of PLP in nmol/l and is plotted against the various genotypes in + Pyr and –Pyr medium on the x axis.

Figure 3.12 shows the PLP content of erythrocytes of the different genotypes used in culture. The amount of PLP in erythrocytes of the +/+ and -/- genotype in RPMI medium containing pyridoxine was determined as well as that of erythrocytes of the different genotypes used in culture in pyridoxine depleted medium.

I observed that the amount of PLP was highest in erythrocytes of the +/+ genotype and in the presence of pyridoxine in the medium. I could not detect any significant difference in the amount of PLP in erythrocytes of the various genotypes in pyridoxine depleted medium. The PLP content in erythrocytes of the +/+ genotype in pyridoxine containing medium was significantly higher than that of erythrocytes of the +/- and -/- genotype. In contrast the PLP content of +/+ erythrocytes cultured in pyridoxine depleted medium was in similar range as that of erythrocytes of the +/- and -/- genotype.

3.5 In vitro growth experiments

It has been shown that red blood cell polymorphisms that offer protection against malaria also affect the growth of the parasite in an in vitro culture system (Friedman 1978; Roth, Raventos-Suarez et al. 1983; Fairhurst, Fujioka et al. 2003). To explore if the lowered erythrocytic pyridoxal kinase activity has an effect on the development of *P. falciparum* in an in vitro culture system, I conducted some growth experiments.

The 3D7 strain of *P. falciparum* was cultivated in erythrocytes from individuals bearing the +/+, +/- and -/- genotype for the 8 bp insertion using medium with pyridoxine and depleted of pyridoxine. Parasitemia was determined by FACS analysis (Fig. 3.13) and Giemsa staining.

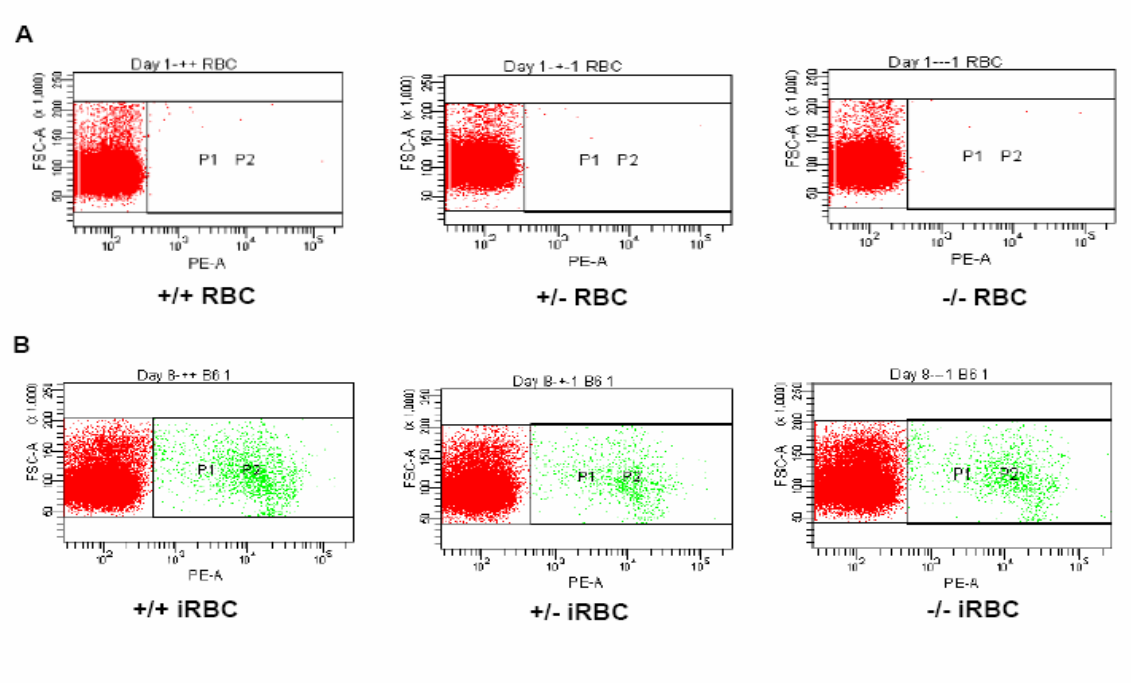

Figure 3.13: Dot plots of FACS analysis during growth experiments. A upper panel shows the dot plots of the control samples. Erythrocytes of each genotype were stained with ethidium bromide. There was no fluorescence signal detected on the FL2 channel for each genotype which is represented as PE-A on the X axis. The forward scatter (FCS-A) is plotted on Y-axis. B, lower panel displays the dot plots of infected erythrocytes (iRBC) bearing the different genotypes on day 8 of the growth experiments. Fluorescent signals were detected on the FL2 channel after staining with ethidium bromide.

3.5.1 ABO Blood group and Hb variant test for growth experiments

The outcome of malaria has been associated with ABO blood group. The blood group O has been suggested to protect against severe malaria (Fischer and Boone 1998). It was therefore essential for the in vitro growth experiments to determine the blood group of the blood donors. Most individuals therefore that were screened for the 8 bp insertion were also tested for their blood group type. The majority of the individuals had the blood group A or O (Table 3.7). All blood donors, who gave blood for the in vitro growth experiments, were Caucasians and had the same ABO blood group A with the exception of one donor who had the blood group type O.

To exclude the effect of Haemoglobin variants such as HbAS, HbSS, and HbCC on the growth of the parasite, my blood donors were also tested for the different Haemoglobin variants. Each blood donor tested negative for the different Haemoglobin variants which was expected as these Hb variants are not common among Caucasians (Table 3.8)

Table 3.7: ABO blood group of some of the individuals screened for the 8 bp insertion

Individuals	Blood group
1	**O**
2	**A**
3	**A**
4	**A**
5	**A**
6	**A**
7	**B**
8	**A**
9	**A**
10	**A**
11	**B**
12	**O**
13	**A**
14	**O**
15	**A**
16	**O**
17	**O**
18	**A**
19	**A**
20	**A**
21	**A**
22	**A**
23	**AB**

Table 3.8: Percentage of haemoglobin variants. The percentage of the various haemoglobin variant was determined for the various blood donors. The results are in accordance with what is expected of adult Caucasians. Hb AO 95%, HbA2 1.8 -3.2% and less than 1% for HbF (fetal Hb), HbS (sickle Hb) and HbC

Individual	Genotype	Hb A0	Hb A2	Hb F	Hb S	Hb C
1	**+/+**	95.5%	2.3%	< 1%	< 1%	<1%
2	**+/-**	94.7%	2.8%	< 1%	< 1%	< 1%
3	**-/-**	94.7%	2.8%	< 1%	< 1%	< 1%

Under standard in vitro cell culture conditions, parasites were able to grow in erythrocytes from individuals bearing the deletion genotype (-/-)**.**

In a set of three representative growth experiments, the mean parasitemia in erythrocytes of the -/- genotype was about half of that in erythrocytes of the +/+ and +/- genotype after 8 days of culture (figure 3.14 A). The impaired growth phenotype was first observed after 8 days of culture. At the beginning of the experiement, infected erythrocytes (genotype unknown) were added to uninfected erythrocytes of the respective genotype in a ratio of 1:10. Thus 1/10 of the erythrocytes in culture were not of the respective genotype. Eventually since the cultures were splitted every third day, only erythrocytes of the respective genotype were present in the culture.

On day 16 *e.g.* we measured a parasitemia of 3.58% for the +/+, 3.45% for the +/- and 1.75% for the -/- culture (t test, p value ++/-- :0.0001, p value: +-/--: 0.0001) We estimated that the cumulative increase in parasitemia over time and the parasitemia in erythrocytes of individuals bearing the +/+ and +/- genotype was approximately 10^1 higher than in erythrocytes of the -/- genotype (Figure 3.14 B).

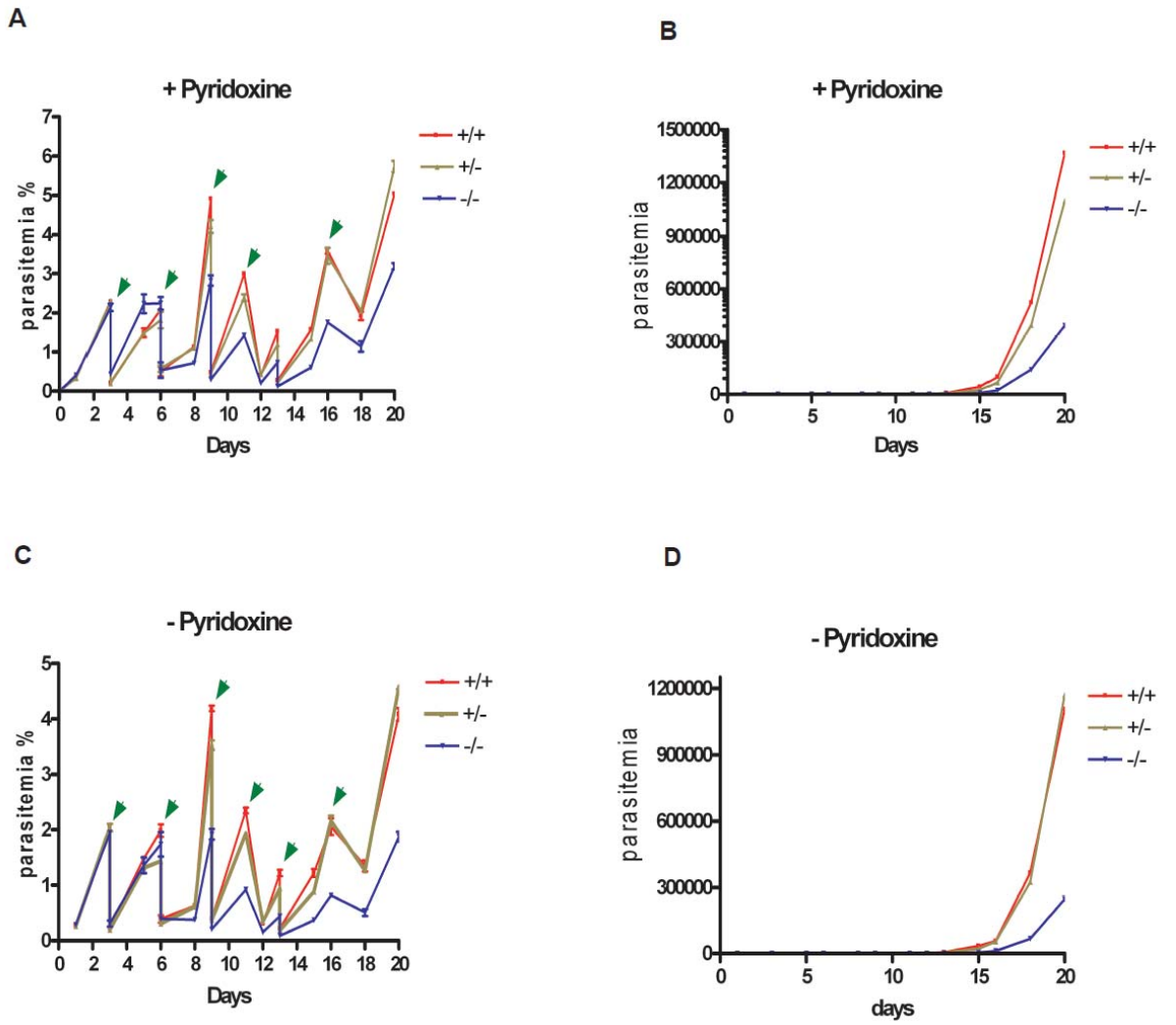

Figure 3.14: In vitro growth experiments using erythrocytes of the various genotypes. **A,** shows the growth of the parasite in erythrocytes bearing the various genotype and in medium containing pyridoxine, **B** shows the cumulative increase in parasitaemia over time. **C** and **D** portray the growth of the parasite in pyridoxine depleted medium and correspond to A and B respectively. The parasites were cultured for 20 days as represented on the X axis. The course of the parasitemia curves are due to the splitting of the cultures. The arrows indicate when the culture was splitted. The data is representaitive for 3 independent experiments. Each experiment was conducted with triplicates. The error bars stand for the standard error with values of < 0.5%.

3.5.2 Comparing parasitemia in pyridoxine depleted medium

I observed the same growth phenotype when the parasite was cultivated in pyridoxine depleted medium (Figure 3.14 C and D). The mean parasitemia in erythrocytes of individuals bearing -/- genotype was also about half of that in erythrocytes erythroytes of the +/+ and +/- genotype after 8 days of culture. On day

16 for example, the parasitemia was significantly lower (t test, p value: 0.0001) in erythrocytes of the -/- genotype as compared to that of the +/- and -/- genotype.
However, comparing the growth of the parasite when cultivated in erythrocytes bearing the same genotype using pyridoxine containing and pyridoxine depleted medium, revealed that the growth of the parasite is partially affected when cultured in pyridoxine depleted medium. The growth of the parasite was hindered in the absence of pyridoxine in the medium. On day 16, I measured parasitemias that were significantly lower in the pyridoxine depleted medium than in complete medium (t test, p value; +/+ +Pyr/-Pyr: 0.0008, +/- +Pyr/-Pyr: 0.0001, -/- + Pyr/-Pyr: 0.0001)
This effect was most clearly seen when the parasite is cultivated in erythrocytes bearing the -/- genotype (Figure 3.15).

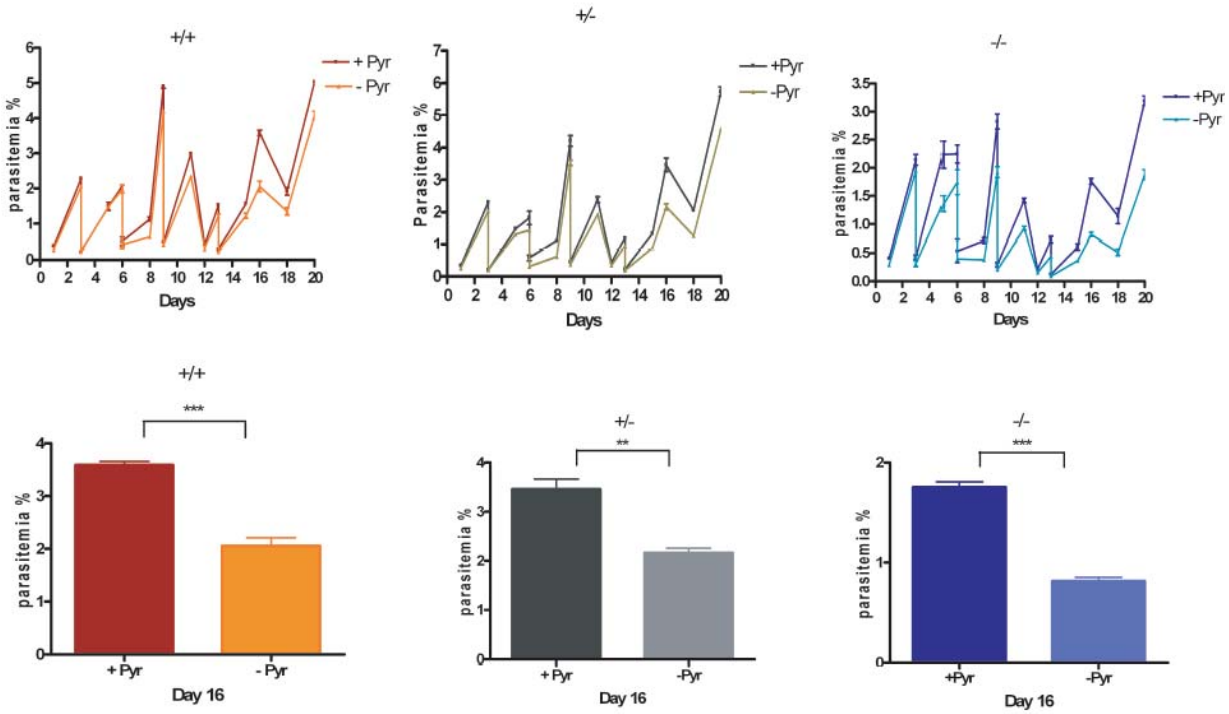

Figure 3.15: In vitro growth experiments in pyridoxine depleted medium. The growth of the parasite when cultured in complete medium was compared to that in pyridoxine depleted medium. The erythrocytes were of the same genotype and the parasites were cultured for a period of 20 days as represented on the X axis (upper panel). On the lower panel the parasitemia on day 16 after culture in complete medium was compared to that of pyridoxine depleted medium. This data is representative for two independent experiments.

3.5.3 The growth of *P. falciparum* in erythrocytes of two unrelated individuals bearing the same genotype

I also conducted the growth experiments with erythrocytes from two unrelated individuals bearing the +/+ and the -/- genotype. All individuals were Caucasians and had the blood group A with the exception of the second individual bearing the -/- genotype who had the blood group O. In each case the parasites showed the same impaired growth phenotype when cultivated in erythrocytes bearing the -/- genotype. I observed the same effect when the parasites were cultured in pyridoxine depleted medium (Figure 3.16).

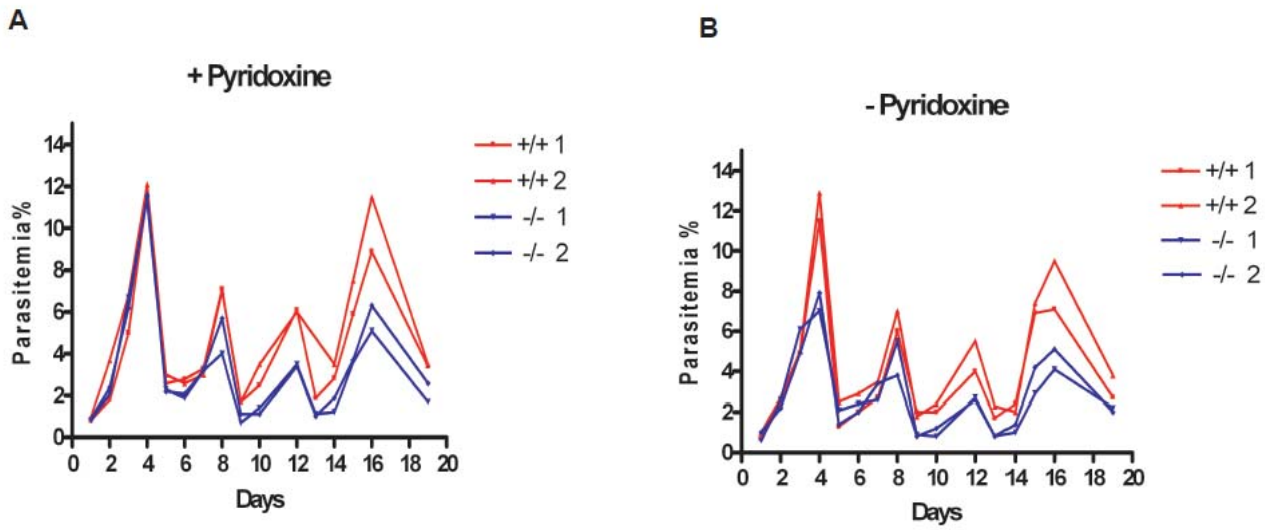

Figure 3.16: In vitro growth experiments using erythrocytes of unrelated two individuals bearing the same genotype. Parasites were cultured fro a period of 20 days and the parasitemia was determined by counting giemsa stained slides. The growth of the parasite was impaired in erythrocytes from both individuals bearing the deletion type as compared to those with +/+ genotype. The data is representative for 3 independent experiments.

3.5.4 Short term growth experiments

In a short term growth assay, I followed up the growth of 4 different *P. falciparum* strains 3D7, FCR3, HB3 and Dd2 cultured in erythrocytes with the different genotypes. The parasites were cultured in RPMI complete medium. I determined parasitemia every day with FACS analysis for a period of 3 days. I could confirm my observations of the retarded growth phenotype in -/- erythrocytes for each *P. falciparum* strain. I also observed an intermediate growth phenotype for most strains with the exception of Dd2 when cultured in erythrocytes of the +/- genotype.

The growth of the parasite was already impaired on day 1 of the experiment in erythrocytes of the -/- genotype. This can be explained by the fact that the parasite strains were precultivated for four weeks in erythrocytes of the various genotypes respectively. Therefore, at the onset of the short term growth experiment, only erythrocytes of the respective genotypes were present in the culture (see Material and Methods 2.2.3.7)

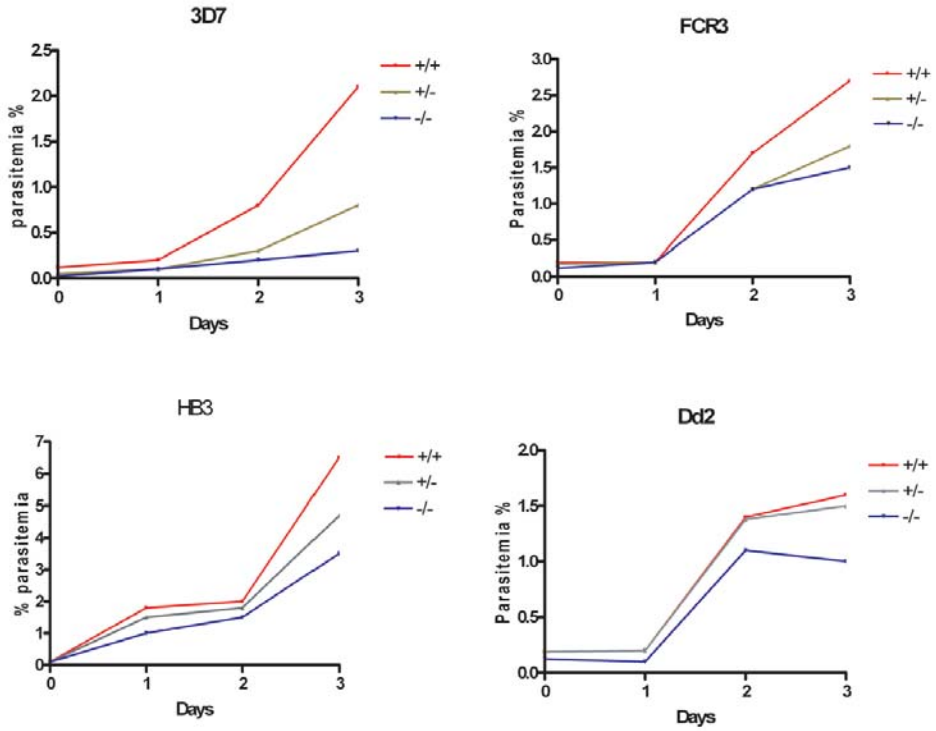

Figure 3.17: Short term growth assays with different *P. falciparum* strains. Different *P. falciparum* strains 3D7, FCR3, HB3 and Dd2 were cultured in erythrocytes of the various genotypes +/+, +/- and -/- for a short period of 3 days. Each strain exhibited the impaired growth phenotype when cultured in erythrocytes of the -/- genotype. The data is representative for 2 independent experiments.

3.6 Expression analysis of *pfpdx1* and *pfpdxk* following growth in erythrocytes of the various genotypes

It has been shown that *P. falciparum* possesses a de novo synthesis pathway for Vitamin B_6 (Fitzpatrick, Amrhein et al. 2007). For this purpose, it utilizes the two proteins PfPdx1 and PfPdx2. In order to synthesize PLP, Pdx1 and Pdx2 form a complex that acts as a glutamine amidotransferase with Pdx2 as the glutaminase and Pdx1 as the PLP synthase domain (Gengenbacher, Fitzpatrick et al. 2006). The parasite also has a functional pyridoxal kinase for the phosphorylation of B_6 vitamers upon uptake and for the salvage of vitamin B_6 (Wrenger, Eschbach *et a*l. 2005)

Since individuals homozygous for the deletion event in the promoter region of the *pdxk* gene have a lower level of the PdxK protein in their erythrocytes which is accompanied by a lower activity of the enzyme, it is therefore assumed that erythrocytes from individuals bearing this genotype would also have a lower level of PLP. It is possible that the parasite could respond to the low PLP levels in its erythrocytic environment by upregulating its own enzymes involved in vitamin B_6 metabolism.

To examine whether this is indeed the case, I analysed the fold change in mRNA levels of two of the enzymes involved in vitamin B_6 homeostasis of the parasite namely, PfPdx1 and PfPdxK, when the parasite was cultured in erythrocytes bearing the diverse hPdxK genotypes. For this experiment a 3D7 strain of *P. falciparum* was cultured in erythrocytes of the genotypes +/+, +/- and -/- genotypes for four weeks. Parasites were then synchronized, mRNA was isolated from trophozoite stages, reverses transcribed to cDNA and the mRNA levels of *pfpdx1* and *pfpdxk* was quantified by real time PCR (see material and methods 2.2.7). To exclude contaminiation with genomic DNA, a negative control with mRNA was always run in parallel. Contaminiation with genomic DNA was never detected. The parasites grown in +/+ erythrocytes in the presence of pyridoxine in the medium were used as reference and the ß-tubulin gene was used as internal control.

Figure 3.18 shows the results of the real time analysis of mRNA levels for the *pfpdx1* and *pfpdxK* genes. I observed an increase in mRNA levels of both genes when the parasites were cultured in erythrocytes of the -/- genotypes. For the *pfpdx1* gene which is involved in de novo synthesis of vitamin B_6, there was a slight but significant

increase in the mRNA levels when cultured in +/- and -/- erythrocytes as compared to that of the +/+ genotype. I detected a 2.7 fold change in *pfpdx1* mRNA levels after cultivation in -/- erythrocytes and 1.8 fold change after cultivation in +/- erythrocytes (t test, p value -/-: 0.029, +/-: 0.029).

There was also slight increase in mRNA levels of the *pfpdxk* gene when the parasite was cultured in erythrocytes of the +- and -/- genotype. I detected a 2.1 fold change in *pfpdxk* mRNA levels when parasites were cultured in -/- erythrocytes and a 1.58 fold change when cultured in +/- erythrocytes (t test, p value -/-: 0.031). The increase in mRNA levels of this gene following culturing in +/- erythrocytes was not significant. However, the increase in mRNA levels following culturing in -/- erythrocytes was slightly significant.

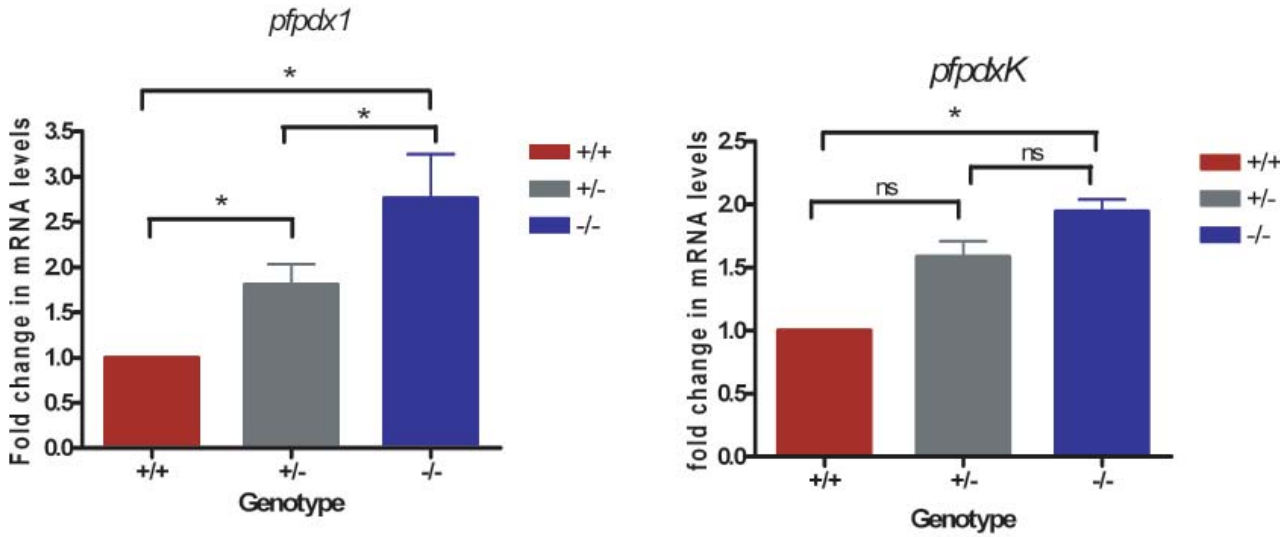

Figure 3.18: Expression analyses of *pfpdx1* and *pfpdxk* following growth in erythrocytes of the various genotypes. The fold change in mRNA levels of *pfpdx1* is shown on the left and that of *pfpdxk* on the right. The fold change in mRNA levels of both genes (Y axis) is plotted against the genotype of the erythrocytes used for culturing the parasites. Each measurement was carried out in duplicates (see material and methods 2.9). The mean and standard deviation was calculated as well as the p values. The data is representative of at least 3 independent experiments. Stars indicate a p value, ns stands for not significant.

3.6.1 Expression analyses of *pfpdx1* and *pfpdxk* following culture in pyridoxine depleted medium

I also investigated, if the absence of pyridoxine in the culture medium has an effect on the expression of the *pfpdx1* and *pfpdxk* genes when the parasite is cultured in erythrocytes of the various genotypes. A 3D7 strain was cultured in erythrocytes of the various genotypes using pyridoxine depleted medium. I compared the mRNA levels of both genes of the parasite cultured in erythrocytes of the various genotype in pyridoxine depleted medium to levels following culturing in +/+ erythrocytes in the presence of pyridoxine (Fig 3.19)

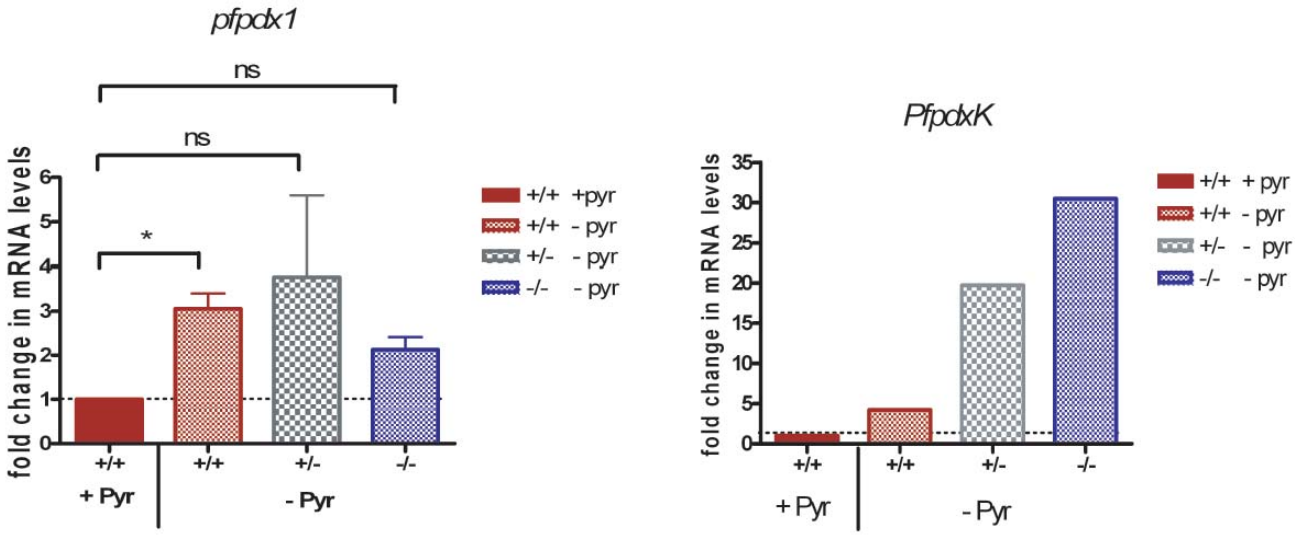

Figure 3.19: Expression analysis of *pfpdx1* and *pfpdxk* after culturing parasites in pyridoxine depleted medium. The fold change in mRNA levels is shown for each genotype in pyridoxine depleted medium (-Pyr) and compared to that of the reference strain cultured in +/+ erythrocytes in the presence of pyridoxine (+Pyr). The measurements were carried out in duplicates (see material and methods 2.9) .The data for the *pfpdx1* gene is representative for three independent experiments and the bars show the standard error. The mRNA level of the pfpdxk gene in pyridoxine depleted medium was measured only once and the columns stand for the mean of the duplicate measurements.

I discovered an increase in mRNA levels for the *pfpdx1* gene after the parasite was cultured in pyridoxine depleted medium using erythrocytes of the various genotypes. However it was not significant. The fold change in mRNA levels for the *pfpdx*k gene was relatively high with the highest increase in the -/- erythrocytes (30 fold) as compared to the mRNA levels of the reference strain which was cultured in +/+ erythrocytes in the presence of pyridoxine.

There was also an increase in the mRNA levels of the *pfpdx1* gene accompanying culture in pyridoxine depleted medium with erythrocytes of all various genotypes. I observed that this increase was highest when cultured in erythrocytes of the +/- genotype but this fold change was not significant. However, I observed a significant increase in the mRNA levels of *pfpdx1* after culturing in erythrocytes of the +/+ genotype in pyridoxine depleted medium.

3.7 PLP content of parasites cultured in erythrocytes of the various genotypes

To determine the PLP content of the parasites when cultured in erythrocytes of the various genotype, a 3D7 strain was cultured in erythrocytes of the respective genotype for at least 4 weeks. I measured the PLP content of parasites that were cultured in +/+ and -/- erythrocytes in complete medium and of parasites that were cultured in +/+, +/- and -/- using pyridoxine depleted medium. Figure 3.20 shows the result of this experiment. I could not detect any difference in the PLP content of parasites after culturing in erythrocytes of the various genotypes. The absence of pyridoxine in the culture medium had no effect on the PLP content of the parasites.

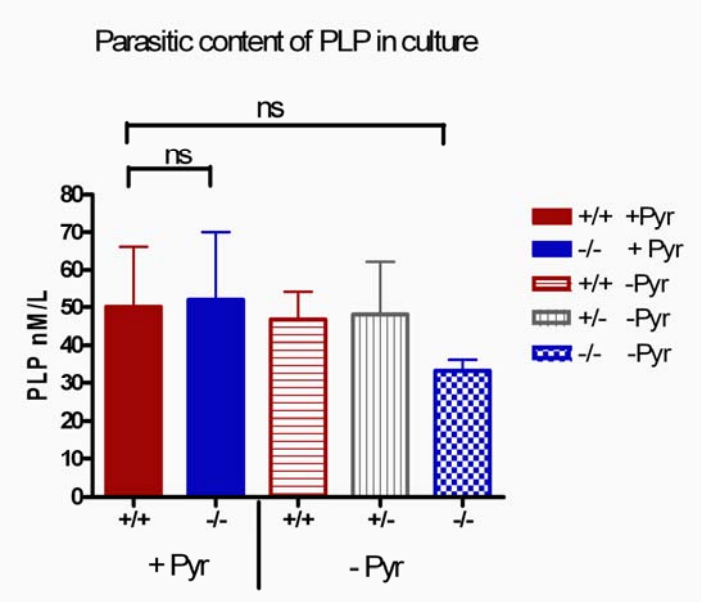

Figure 3.20: Parasitic PLP content. The PLP content of parasites (Y-axis) cultured in erythrocytes of the different genotypes was determined. The parasites were cultured in RPMI 1640 complete medium (+Pyr) or depleted of pyridoxine medium (-Pyr). There was no significant difference in the PLP content of the parasites. The error bars stand for the standard error of 3 independent measurements.

3.8 Erythrocyte deformability assay

It has been shown that certain substances that act as antioxidant can affect the elasticity of erythrocytes. The elasticity of erythrocytes reflects their deformability. Substances like aminoguanidine, pyridoxyliden-aminoguanidine and pyridoxal had a positive influence on the elasticity of erythrocytes. This was assessed by the erythrocyte filterability in which pyridoxal exhibited the highest positive effect on the filterability of erythrocytes (Beder, Kittova et al. 2003; Mataseje, Beder et al. 2003). To elucidate if the lowered erythrocytic pyridoxal kinase activity has an effect on the deformability of erythrocytes bearing this polymorphism, I analysed the filterability of erythrocytes bearing the +/+, +/- and -/- genotypes as proposed by Mataseje et al., 2003 (see material and methods 2.2.4).

The hypothesis behind this experiment is that individuals bearing the +/+ genotype and therefore having normal erythrocytic PdxK activity should have a higher level of pyridoxal 5-phosphate which positively influences the deformability of the erythrocytes. It is consequently expected that erythrocytes of individuals bearing the +/+ genotype should have a higher elasticity reflected by a higher filtration rate than erythrocytes of the -/- genotype.

3.8.1 Evaluating the method

To demonstrate the feasibility of the method for this purpose, I decided to compare the filterability of freshly drawn uninfected erythrocytes to that of infected erythrocytes (figure 3.21). As expected the filtration rate of uninfected fresh erythrocytes (50%) was significantly higher that of the infected erythrocytes (30%) (t test, p value: 0.009)

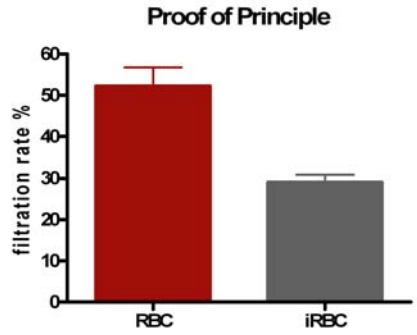

Figure 3.21: Proof of principle for the filterability method. The filtration rate, plotted on the Y axis of infected erythrocytes was lower than that of uninfected fresh erythrocytes. The data is representative for 3 independent experiments and proves that the method is sufficient.

3.8.2 Filtration rate of erythrocytes bearing the +/+, +/- and -/- genotype

The filtration rates of freshly drawn erythrocytes bearing the +/+, +/- and -/- was determined. I could not detect any significant differences in filtration rates among the various genotypes. I also determined the filtration rates of only infected erythrocytes bearing the various genotypes at a parasitemia of about 3% (see materials and methods) and I found no difference in filtration rates.

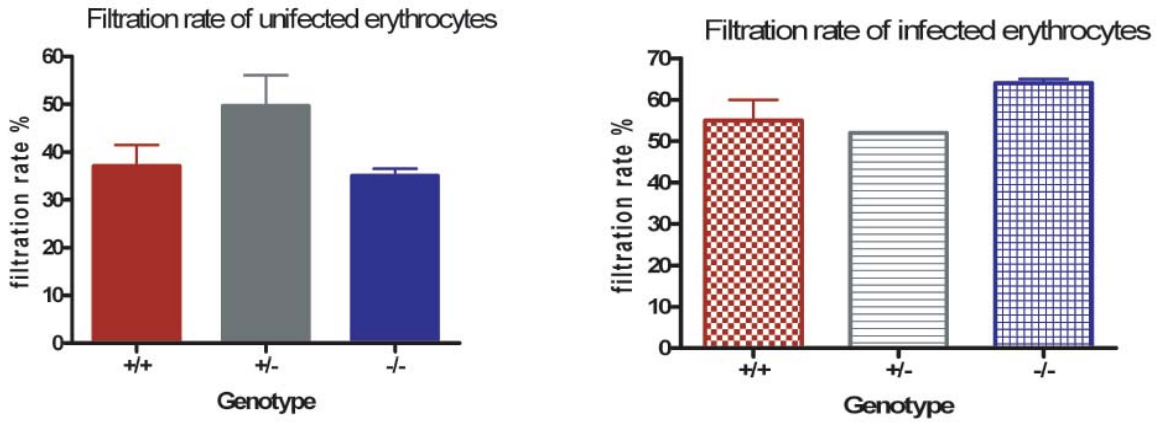

Figure 3.22: filtration rates of infected and uninfected erythrocytes. The filtration rates of freshly drawn erythrocytes bearing the various genotypes were compared (left panel) and there was no significant difference found. The filtration rates of infected erythrocytes of the +/+, +/- and -/- genotypes was also compared by comparing the parasitemia after filtration to the parasitemia before filtration. Filtration rate: no of infected erythrocytes after centrifugation / no of infected erythrocytes before centrifugation x 100. There was no difference detected.

Using the method as described by Mataseje A et al, I could not detect any difference in filtration rates of erythrocytes bearing the various genotypes which implies that the polymorphism has no effect on the deformability of the erythrocytes. It is also possible that no difference was found due to the limitation of the method. The method could detect differences in filtration rates when comparing infected erythrocytes from culture to freshly drawn erythrocytes. In this case a clear difference is expected. However, for the differences between the different genotypes, which are expected to be less pronounced, it is likely that the method is not sensitive enough. Other sophisticated more sensitive methods have been employed to measure the deformability of red blood cells (Park, 2008) and such methods might be a more appropriate approach for this analysis.

3.9 Parasite morphology

The in vitro growth assay experiment showed clearly that the growth of the parasite is impaired when cultured in erythrocytes from individuals bearing the -/- genotype. It is therefore possible that the morphology of the parasite is affected when cultivated in -/- erythrocytes. To elucidate if this is the case, the morphology of the parasites were scrutinized by light microscopy as well as electron microscopy.
Giemsa stained slides of infected erythrocytes with the +/+ and -/- genotype were examined with a light microscopy. The erythrocytes of a given gentotype were infected with the FCR3 strain and the culture was held for at least 20 days.

Light microscopical inspection of infected erythrocytes of the +/+ and -/- genotype did not result in the detection of any morphological alterations of the intracellular parasite. It appeared that the trophozoite stages of the parasite in +/+ erythrocytes in some cases looked slightly better than in -/- erythrocytes (Fig.3.23)

To have a closer look at the morphology of the parasite when cultured in erythrocytes of the +/+ and -/- genotype, I also examined the parasites with electron microscopy. A 3D7 strain which has been cultivated in erythrocytes of the various genotypes for a period of at least 3 months was used for this experiment. The trophozoite stages were enriched with the MACS column, fixed, embedded, cryo-sectioned and stained according to the method proposed by Tokuyasu for electron microscopy (Tokuyasu, 1980)

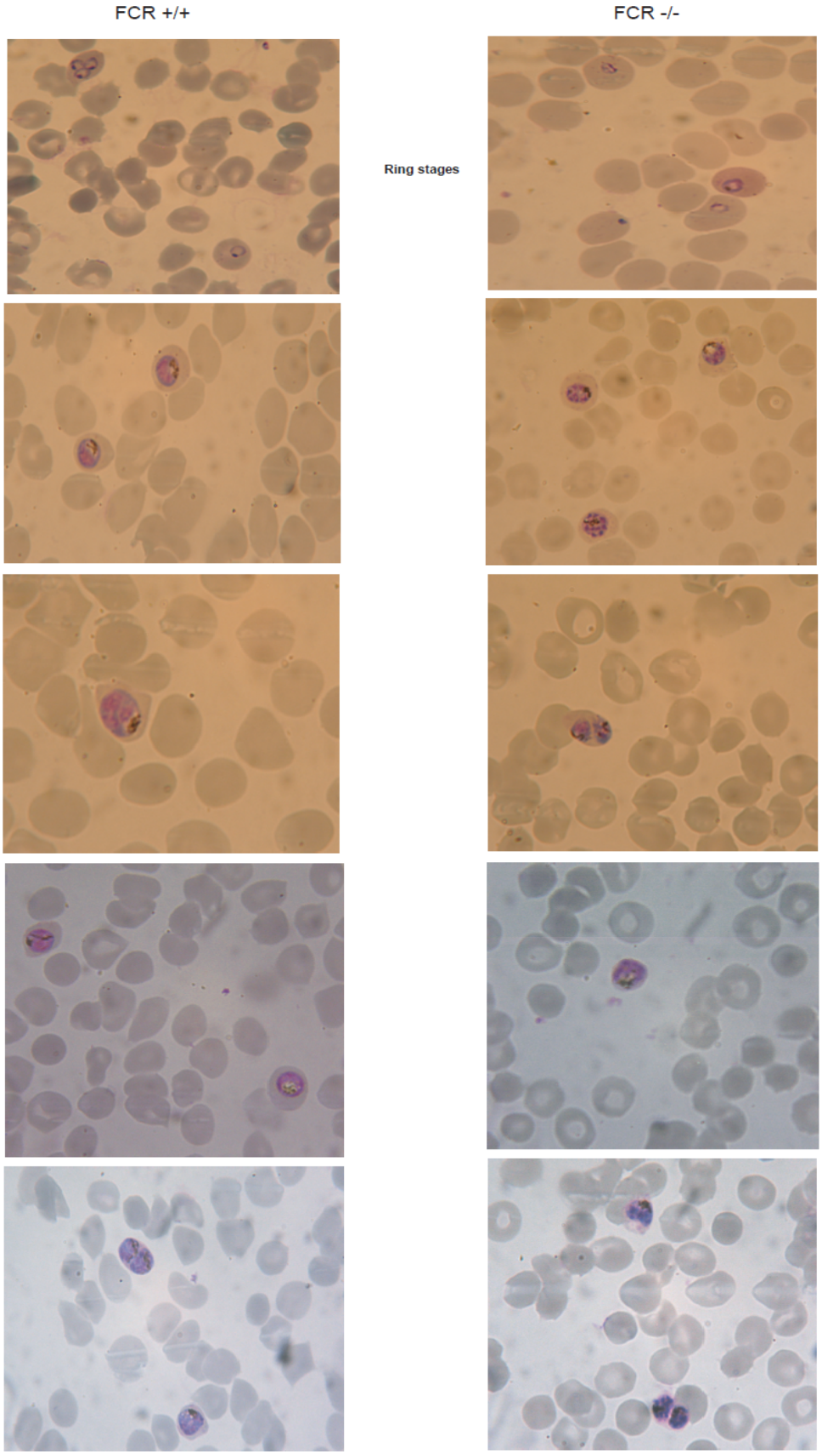

Figure 3.23: FCR3 strain in +/+ and -/- erythrocytes. Blood smears of +/+ and -/- erythrocytes infected with the FCR3 strain were stained with Giemsa and examined by light microscopy. Left panel: FCR3 in +/+ erythrocytes, right panel: FCR 3 in -/- erythrocytes.

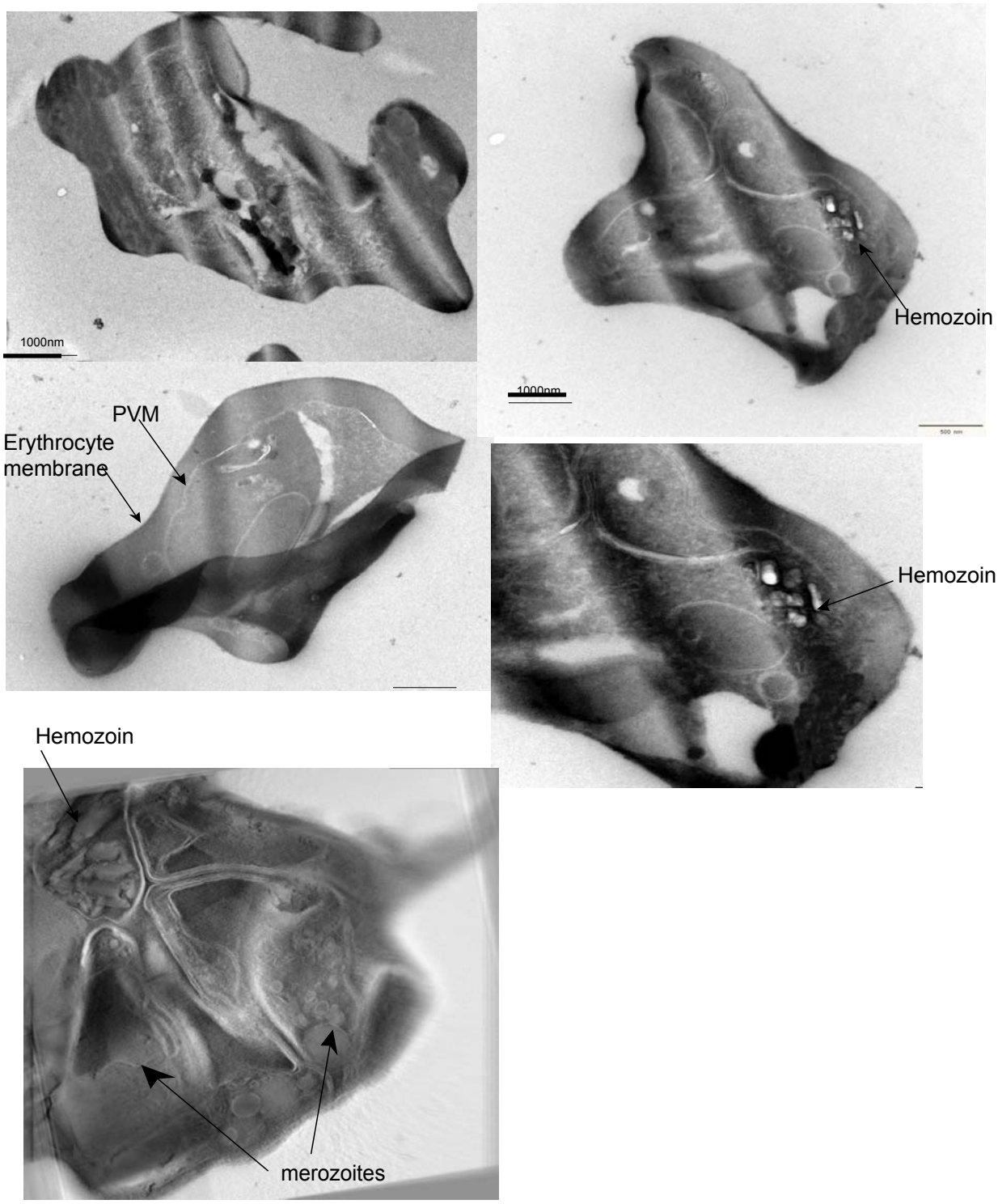

Figure 3.25: EM pictures of infected +/+ erythrocytes. Erythrocytes of the +/+ genotype infected with the 3D7 strain was examined by electron microscopy. The bar represents 1 µm.

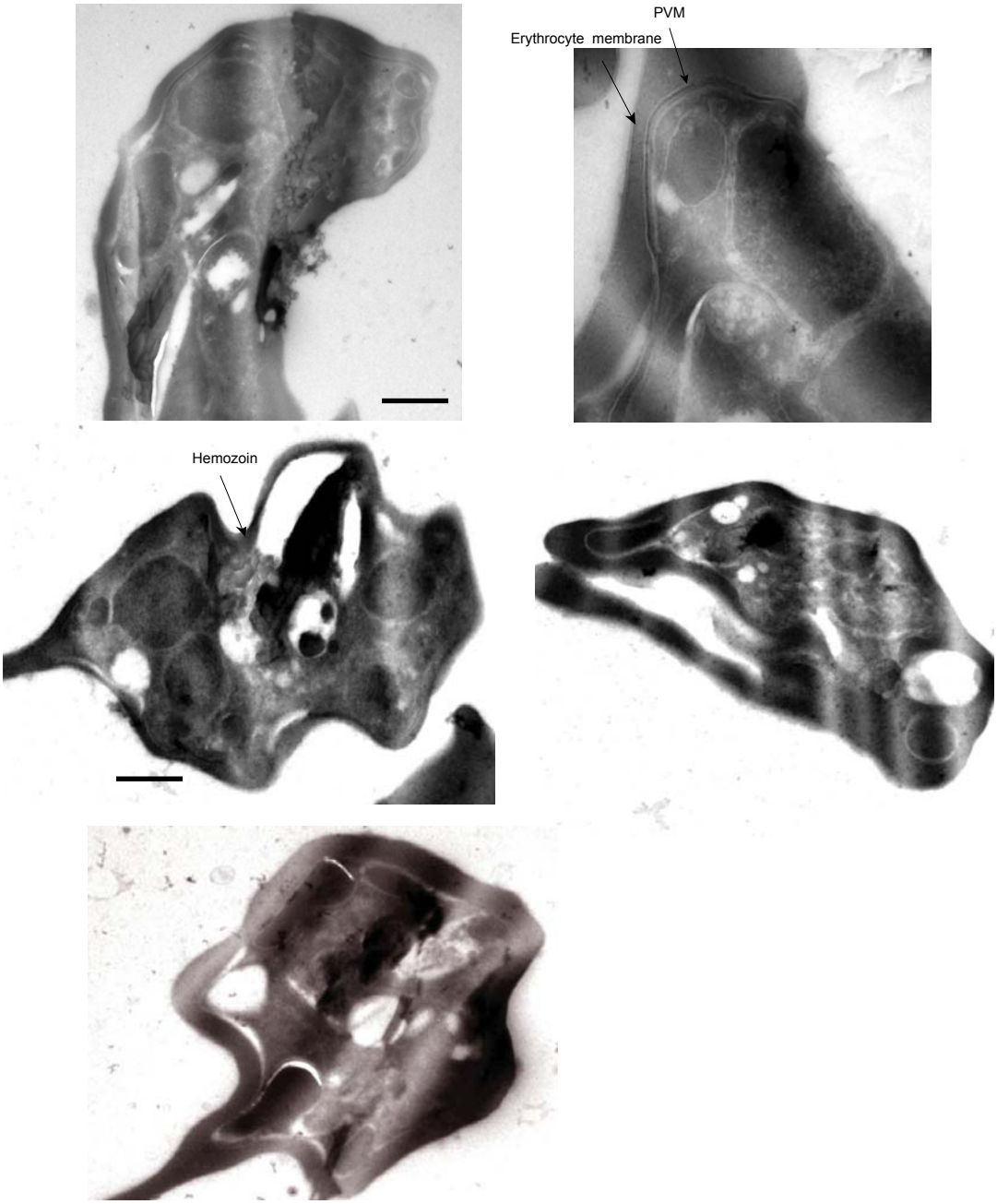

Figure 3.26: EM pictures of infected -/- erythrocytes. -/- erythrocytes infected with a 3D7 strain were imaged by electron microscopy. The bars represent 1 µm.

Figure 3.25 shows EM pictures of sections of infected +/+ erythrocytes. The erythrocytes are infected with trophozoite and schizont stages of the parasite. The last picture shows merozoites of a schizont stage. Hemozoin, the malaria pigment which is a haemoglobin degradation product was easy to detect as crystals in the food vacuole and the parasitophorous vacuole membrane (PVM) was clearly distinguishable.

Figure 3.26 shows EM pictures of infected erythrocytes of the -/- genotype. The pictures show trophozoite stages. Hemozoin was also present and it was easy to identify the parasitophorous vacuole membrane.

At a first glance the EM pictures showed that the parasites looked better in erythrocytes of +/+ erythrocyte but this could be based on the quality of the sections that were imaged. I studied the EM pictures of infected erythrocytes of the two genotypes +/+ and -/- more closely and could not detect any clear differences in the over all morphology of the parasite.

I did not observe any clear aberrant development of features like the parasitophorous vacuole membrane (PVM), membranes of inner compartments or hemozoin crystals of the parasite cultured in -/- erythrocytes.

4. Discussion

The immense diseases burden of malaria on the world in particular on Sub-Saharan Africa, renders malaria the most important parasitic disease today. The control of this disease has been hampered by drug resistance in the *Plasmodium* parasite and by the lack of an effective vaccine. A better understanding of the pathogenesis of malaria, including the identification of innate or adaptive host defence mechanisms against blood stage parasite, may provide new targets for intervention of this disease.

The malaria parasite and its human host have co-evolved for a very long time and are permanently in interaction with each other. The parasite has various mechanisms to exploit its host cell thereby ensuring its survival and replication. The selective pressure of the parasite on its human host has resulted in selection of genetic polymorphisms which offer its carriers protection against malaria. The erythrocyte being the main spot for parasite infection and replication is also the main scene for genetic alterations that reduce the susceptibility to malaria infection (Min-Oo and Gros 2005; Williams 2006). This is one of the clearest evidences to suggest co-evolution of the *Plasmodium* parasite and its human host in areas endemic for the disease. The role of host genetic factors conferring resistance to malaria was discovered half a century ago. Due to the complex nature of host-parasite interaction, the mechanisms of protection are still not fully understood (Kwiatkowski 2000).
The haemoglobinopathies (α and β thalassaemia, HbAS, HbC, and HbE) are genetic polymorphisms that are well known to protect against malaria. Several studies have provided evidence for this assumption (Roberts and Williams 2003; Richer and Chudley 2005).

Genetic alterations of erythrocytic enzymes that affect susceptibility/resistance to malaria have also been observed. The most common one is the glucose 6-phosphate dehydrogenase (G6PD) deficiency which has been intensively studied (Bienzle, Ayeni et al. 1972; Cappadoro, Giribaldi et al. 1998; Guindo, Fairhurst et al. 2007). G6PD deficiency is linked to the X chromosome and over 300 variants of this trait have been reported. Recently, it has been reported that deficiency in the erythrocytic pyruvate kinase and gluthathione reductase is associated with reduced susceptibility to malaria. However, these two enzymopathies and their association with malaria

have not been intensively studied (Min-Oo, Fortin et al. 2003; Gallo, Schwarzer et al. 2009).

In general, the alteration of erythrocytic enzyme affects the coding region of the respective gene. Most variants of G6PD causing enzyme deficiency also have altered biochemical properties and this is solely caused by point mutations affecting the coding sequence (Chang and Liu 1995; Mehta, Mason et al. 2000). Pyruvate kinase (PK) deficiency is the second most common erythrocyte enzyme disorder. More than 130 different mutations, mostly missense, have so far been described in association with PK deficiency and are exclusively located in the coding region of the gene as well (Zanella and Bianchi 2000; Ayi, Min-Oo et al. 2008).

Pyridoxal kinase (PdxK) is involved in the metabolism of vitamin B_6. Thirty years ago it was reported that the activity of the erythrocytic pyridoxal kinase is lowered in American blacks as compared to American whites and that lowered activity of the erythrocytic pyridoxal kinase in Africans is probably associated with resistance to malaria infection (Chern and Beutler 1976; Martin, Miller et al. 1978).

In contrast to the above described enzyme disorders associated with malaria, genetic polymorphism at the erythrocytic pyridoxal kinase gene locus, seems to be the result of an insertion/deletion (In/Del) event in the promoter region of its gene. An 8 bp insertion in the *pdxk* promoter introduces a binding site for a putative core promoter binding protein (CPBP) that is known to enhance transcription. This insertion event has been postulated to result in an increase in PdxK activity in erythrocytes. This also suggests that the absence of the 8 bp insertion would lead to a lower activity of the enzyme. Flanagan and Beutler were able to show that individuals homozygous for the insertion indeed had a higher erythrocytic PdxK activity when compared to individuals without the insertion event (Flanagan and Beutler 2006).

In this thesis, I wanted to test the hypothesis that the lowered erythrocytic PdxK activity is another genetic trait offering protection against malaria by observing its effect on *P. falciparum* in an in vitro culture system.

I developed two PCR based methods for the screening of human genomic DNA for the presence or absence of the 8 bp insertion in the promoter region of the *pdxk* gene. One was an allele specific PCR designed exclusively for the detection of the 8 bp In /Del event which was required for this project. The second method was a PCR amplifying a small fragment of 80/88 bp around the region of the 8 bp In/Del event

and then detecting the difference in size on a 4 % metaphor gel (see material and methods 2.1). However this second PCR based method was not used for screening as it was difficult detecting the heterozygotes and this could lead to a misinterpretation of the results (see results 3.1).
The result of the allele specific PCR screen was verified by sequencing and the method proved to be reliable.

Yet, sequencing the PCR fragments showed that there are other polymorphisms, *e.g* a second In/Del (see results) event of 7 bp,in the *pdxk* promoter region 130 bp upstream of the In/del event which has been previously reported (Flanagan and Beutler 2006).. Whether these additional polymorphisms which I observed, affect the transcription of the *pdxk* gene and whether they are under a selective pressure caused by malaria needs to be established. For this purpose, other more suitable PCR based methods need to be developed for their detection than the ones which have been used in this study. However, this was not the main focus of this project.

Screening genomic DNA of Caucasians and Africans for the presence (+/+) or absence (-/-) of the 8 bp in the *pdxk* promoter, showed clearly that the 8 bp deletion event (-/-) which is associated with the lowered PdxK activity in erythrocytes is more prevalent among East Africans (malaria endemic) when compared to Caucasians (no malaria risk). The result of my PCR screen corresponded with the results of various population studies which have shown that genetic polymorphisms correlating with resistance to malaria are distributed among individuals in endemic regions (Cooke and Hill 2001; Craig, Hastings et al. 2001).

Since this project was carried out in Heidelberg, the blood of Caucasian donors was used for all investigations with *Plasmodium falciparum*. The advantage of using blood from Caucasians for the in vitro experiments is that the prevalence of other genetic factors that are known to reduce the susceptibility to malaria can almost be ruled out. Of the 42 Caucasians, which were screened for the polymorphism in the promoter of the *pdxk* gene, only 2 individuals were homozygous for the deletion event. Accordingly, all in vitro experiments were carried out with erythrocytes from these individuals. Experiments conducted with erythrocytes from only 2 individuals bearing the deletion genotype (-/-) may be considered as not statistically relevant. It is

therefore recommended to conduct this study in an endemic region with a higher frequency of this genetic trait. This would increase the sample size for all in vitro experiments.

A number of promoter polymorphisms are known to affect the expression of the respective genes resulting in an increase or decrease in protein levels. It has been reported that two allelic variants of the interleukin 6 promoter (-572 and -174 G→C) influences the IL-6 activity both in vivo and in vitro. Another example is a genetic variation (1 bp In/del event) in the promoter of the human stromelysin-1 which influences the expression of the gene (Ye, Eriksson et al. 1996; Ferrari, Ahn-Luong et al. 2003).

Given that matured erythrocytes do not have mRNA, I examined whether the presence or absence of the 8 bp insertion in the *pdxk* promoter region affects transcription of the gene by analysing the protein level of the enzyme in erythrocytes by Western blot analysis. The protein level of PdxK was lower in erythrocytes of individuals bearing the deletion (-/-) genotype when compared to erythrocytes from individuals homozygous for the insertion (+/+).

To elucidate, if the protein level of the PdxK in erythrocytes of individuals bearing the various genotypes (+/+, +/- and -/-) correlates with the activity of the protein, I conducted enzyme activity assays. The amount of PLP formed after three hours of incubation with lysate of erythrocytes from various individuals correlated poorly with the different genotypes. However, the kinetics of the enzyme reaction showed that the amount of PLP formed per minute with lysate of the +/+ genotype (4.0 nmol/L) was significantly higher than that of the -/- genotype (2.5 nmol/L). This result clearly correlated with the amount of PdxK present in the individual lysate. It furthermore confirms the findings of Flanagan and Beutler which showed that the 8 bp insertion (+/+) resulted in an increased PdxK activity in erythrocytes and that the absence of the 8 bp insertion (-/-) had the opposite effect.

Quantification of the amount of PLP in erythrocytes of the various genotypes in culture showed that erythrocytes of the +/+ genotype in pyridoxine containing medium had the highest PLP content (286 nmol/L) reflecting the higher PdxK activity as compared to erythrocytes of the -/- genotype (118 nmol/L) The low level of PLP in

erythrocytes of the -/- genotype in the presence of pyridoxine can be explained by the lowered activity of the PdxK in these cells.
The erythrocyte can only replenish its pool of vitamin B_6 by taking up the vitamins, in this case pyridoxine from the culture medium (Ink and Henderson 1984; Anderson, Perry et al. 1989). In the absence of vitamin B_6 in the medium, the exhausted pool of vitamin B_6 in the erythrocytes cannot be replenished. Determining the PLP content of erythrocytes of the various genotype in the absence of pyridoxine confirmed this as the level of PLP (90.3 nmol/L in average) was significantly less than that of +/+ erythrocytes cultured in the presence of pyridoxine containing medium.

An important part of this project was to investigate the mechanism by which lowered erythrocytic PdxK may confer protection against malaria using an in vitro culture system.
A high parasite density in the blood stream is considered to be one of the factors associated with severe malaria and the high replication rate with which parasites multiply in the bloodstream is also thought to increase the risk of progression to severe disease (Field 1949; Mockenhaupt, Ehrhardt et al. 2004). The majority of erythrocyte polymorphisms that are known to reduce susceptibility to malaria reduce parasite density. This has been illustrated by in vitro growth experiments. It has been shown that the parasite's growth is impaired when cultured in erythrocytes from individuals bearing the HbAS, HbCC, HbEE and the G6PD deficiency trait as compared to controls. This effect is most pronounced in erythrocytes with the HbAS trait. Parasitemia was 10 times higher in HbAA erythrocytes than in HbAS or HbSS erythrocytes. In the case of the G6PD deficiency trait, parasitemia was about three times higher in normal erythrocytes than in G6PD deficient cells (Friedman 1978; Roth, Raventos-Suarez et al. 1983; Olson and Nagel 1986; Chotivanich, Udomsangpetch et al. 2002).

The result of my in vitro growth experiments showed that the growth of the parasite is impaired when cultured in erythrocytes from individuals bearing the -/- genotype as compared to individuals bearing the +/+ genotype. The parasitemia in -/- erythrocytes was about half that of +/+ erythrocytes. I also observed this retarded growth phenotype when using erythrocytes from two unrelated individuals bearing the deletion (-/-) genotype. In these growth experiments, the retarded growth phenotype

was first detected after 8 days of culture. One explanation for this apparent delayed onset of effect could be that at the beginning of the experiment, infected erythrocytes (genotype unknown) were added to uninfected erythrocytes of the respective genotype in a ratio of 1:10. Thus, at the beginning of the experiment 1/10 of the erythrocytes in culture were not of the respective genotype. Since the cultures were splitted every third day, eventually only erythrocytes of the respective genotype were present in the culture. On day 8, only 1/1000 of the erythrocytes present in the culture were not of the particular genotype. An indication that this indeed may be true comes from the short term growths experiments for which the parasites were cultured in erythrocytes of the various genotypes for a period of 4 weeks before the start of the experiment. I observed an instant impairment of the parasite's growth in erythrocytes of the -/- genotype and an intermediate growth phenotype for most parasite strains in erythrocytes of the +/- genotype. This also mimics an in vivo situation where the parasites are confronted with only erythrocytes of the particular genotype. Moreover, the short term growth assays, which were performed for 3 days, showed that the impaired growth phenotype applies to *P. falciparum* strains with different drug resistance patterns, suggesting that the genetic trait affects all parasite strains regardless of their drug resistance background.
Another explanation for the delayed impairment of growth in erythrocytes of the -/- genotype observed in the in vitro growth experiments, could be that the vitamin B_6 stores of the parasite were filled at the beginning of the experiment. In light of the quite long biological half life of vitamin B_6, which is somewhat between 15 and 21 days, one could imagine that there is a certain delay until the parasite is affected by pyridoxine depletion, even more since it is in part able to counterbalance the depletion by upregulation of PLP de novo biosynthesis (see results 3.6)

The results of the in vitro growth experiments suggest that in an in vivo situation the parasite density in the blood stream of individuals bearing the deletion genotype (-/-) is likely to be less than in individuals with the insertion genotype (+/+). This is consistent with what is known of other genetic alterations of the erythrocyte that reduce parasite infection and growth thereby reducing the risk of severe malaria (Hutagalung, Wilairatana et al. 1999; Aidoo, Terlouw et al. 2002; Mockenhaupt, Ehrhardt et al. 2004). Presumably, infections are most susceptible to resistance mechanism during bottlenecks of the *Plasmodium* life cycle. One of these bottlenecks

for blood stage parasites is the first replication cycle in red blood cells after the release of liver stage merozoites into the blood stream (Baer, Klotz et al. 2007).
However, to exclude the possibility that the observed impaired growth phenotype of parasites cultured in erythrocytes of the -/- genotype is as a result of other genetic alterations, it is absolutely necessary to conduct these growth experiments with blood from more donors bearing the -/- genotype.

Comparing the parasitemia of cultures in the presence or absence of pyridoxine in the growth medium showed that the growth of the parasite is somewhat slower in the absence of pyridoxine regardless of the erythrocyte's genotype. This effect was seen more clearly when the parasites were cultured in erythrocytes of the -/- genotype in the absence of pyridoxine. This implies that the parasite is affected by the limited resources of vitamin B_6 in its erythrocytic environment. It has to be taken into consideration that although the parasites were cultured in pyridoxine depleted medium, a small amount of vitamin B_6 is still brought into the culture system by the addition of fresh erythrocytes during the splitting of the culture. The amount of vitamin B_6 brought into the culture by the addition of fresh erythrocytes is approximately 1.9 - 2.9 nmol/L. Bearing in mind that the half life of vitamin B_6 is 15-21 days, vitamin B_6 was never totally absent in the culture as the culture was splitted every 3 days by the addition of fresh erythrocytes.
Yet the presence of pyridoxine in the culture medium would lead to a considerable increase in the erythroctyic pool of vitamin B_6. Upon uptake into the erythrocyte, pyridoxine is phosphorylated by the pyridoxal kinase to PNP which is converted to PLP by the erythrocytic PNP oxidase. PLP and PNP can be dephosphorylated by the alkaline phosphotase to pyridoxal and pyridoxine which can then be taken up by the parasite (Ink and Henderson 1984).
The impaired growth of the parasite in pyridoxine depleted medium regardless of the genotype of the erythrocyte implies that the parasite needs to take up vitamin B_6 from the host erythrocyte to support maximal growth. The growth of the parasite within its host erythrocyte is dependent on the uptake of a number of essential nutrients from the extracellular medium. This has been shown by various studies demonstrating the supply of nutrients and disposal of waste products by the host cell (Huber, Uhlemann et al. 2002; Wrenger, Eschbach et al. 2006; Downie, Kirk et al.

2008). An example is the rapid uptake of pantothenate (vitamin A) by the parasite from the erythrocyte´s cytosol for its survival (Saliba, Horner et al. 1998).

Knowing that the parasite possesses a de novo biosynthesis pathway for pyridoxal 5-phosphate, I investigated if lowered erythrocytic PdxK activity could have an effect on the parasitic vitamin B_6 homeostasis. Analysis of the expression profile of *pfpdx1* which is involved in the de novo synthesis of PLP and of *pfpdxk* which is involved in the salvage of vitamin B_6 by the parasite, showed that the mRNA levels of *pfpdx1* (2.5 fold) and *pfdxk* (2.0 fold) were increased when the parasite is cultured in erythrocytes bearing the deletion genotype (-/-) as compared to those cultured in erythrocytes of the insertion genotype (+/+). This data suggest that the parasite may respond to deficient B_6 supply by upregulating compensatory mechanisms. This interpretation is supported by data showing that in the absence of pyridoxine, the mRNA levels of *pfpdxk* of parasites cultured in the erythrocytes of the diverse genotypes were much higher than in the presence of pyridoxine. This could imply that the parasite is desperately trying to salvage the limited resources of vitamin B_6 in its environment.

It is known that during the blood stage development of the malaria parasite, the parasite takes up and degrades enormous amounts of haemoglobin from the host erythrocyte (Francis, Sullivan et al. 1997; Elliot 2008). Haemoglobin is digested in the acidic food vacuole of the parasite by proteinases. It also has been shown that pyridoxal 5-phosphate binds tightly to haemoglobin. PLP reacts with the N terminus of the β globin chain of haemoglobin via Schiff`s base formation (Benesch, Yung et al. 1973; Benesch, Benesch et al. 1982). Hydrolysis of haemoglobin bound PLP in the acidic food vacuole of the parasite would lead to the release of PL which can diffuse through the membrane of the food vacuole into the parasite's cytosol. In the parasite´s cytosol, PL can be converted to PLP by the parasitic PdxK.
It can be assumed that the amount of PLP bound to haemoglobin is higher in erythrocytes of the +/+ genotype than of the -/- genotype. This would also lead to the release of higher levels of PL into the parasitic cytosol upon uptake of haemoglobin bound PLP by the parasite in erythrocytes of the +/+ genotype as compared to erythrocytes of the -/- genotype. This implies that the parasite has a higher access to vitamin B_6 from host erythrocytes of the +/+ genotype as compared to the -/-

genotype. This hypothesis is supported by the observed upregulation of the parasitic genes *pfpdx1* and *pfpdxK* following culturing in erythrocytes of the -/- genotype. To test this assumption, the uptake of vitamin B_6 by the parasite from the erythrocyte, should be investigated. This can be done by conducting uptake experiments with radioactive labelled (^{3}H) pyridoxine (Ink, Mehansho et al. 1982).

In vitro growth experiments have shown that the growth of *P. falciparum* is impaired in G6PD deficient erythrocytes and this is due to oxidative stress imposed on the parasite (Roth, Raventos-Suarez et al. 1983; Guindo, Fairhurst et al. 2007). Furthermore, it has been shown that this impaired growth phenotype is abolished after two or three growth cycles of the parasite in G6PD deficient cells. There is evidence that the parasite can produce its own G6PD enzyme which may compensate for the deficient host G6PD enzyme and could account for the resumption of normal growth in G6PD deficient host cells. It has also been shown that the expression of *P. falciparum* antioxidant enzymes is enhanced when the parasite is cultured in G6PD deficient cells enabling the parasite to resist oxidative stress (Roth 1988; Akide-Ndunge, Tambini et al. 2009).These are examples of the capacity of the parasite to adapt to environmental changes.

In contrast, an upregulation of transcription of the *pfpdx1* and *pfpdxk* genes, which are involved in maintaining the vitamin B_6 homeostasis of the parasite, did not fully rescue the impaired growth phenotype when the parasites were cultured in erythrocytes bearing the -/- genotype with the reduced PdxK activity. This indicates that the parasite is not capable of totally overcoming the limited bioavailability of vitamin B_6 in its environment. Measuring the amount of PLP in the parasite cultured in erythrocytes of the various genotypes in the presence or absence of pyridoxine showed no significant differences in the parasitic PLP levels. Even the roughly 30 fold upregulation of the parasitic PfPdxK expression did not lead to an increased parasitic level of PLP when the parasites were cultured in erythrocytes of the -/- genotype. This suggests that there is a kind of optimal PLP concentration which is sensed by the parasite.

My observations in this work support the hypothesis that the lowered erythrocytic PdxK activity could be a genetic trait that reduces the susceptibility to malaria

infection. However, the mechanism of protection remains unclear. In an attempt to elucidate the mechanism behind the protective effect of this trait, I decided to evaluate the effect of reduced PdxK activity on the deformability of the erythrocyte.

The extreme deformability of erythrocytes allows the red blood cells with a diameter of 8 µM to pass trough a 4 µM capillary lumen. Aged, abnormal or damaged erythrocytes have an altered membrane cytoskeleton, which results in loss of deformability. Erythrocytes with a more rigid membrane cytoskeleton get stuck in the sinusoids of the spleen, where they are selectively removed by spleen macrophages (Bennett and Kay 1981).

During its intraerythrocytic development, the parasite causes structural, biochemical and mechanical changes in the erythrocyte, which results in a loss of erythrocyte deformability (Park, Diez-Silva et al. 2008). Furthermore, the parasite exerts oxidative stress on the host erythrocyte, which can also lead to a loss in membrane flexibility (Hunt 1990) Thus, infected erythrocytes become more rigid and are therefore removed from circulation in the spleen (Dondorp 1999). Erythrocyte polymorphisms such as the sickle cell trait and α,β thalassaemia have been linked with reduced erythrocyte deformability (Dondorp 1999). However it is not clear if this could also play a role in the mechanism of their protective effects against malaria infection. Southeast Asian ovalocytosis is also associated with reduced red blood cell deformability and this has been shown to inhibit invasion of the parasites (Jarolim 1991; Genton 1995).

Conversely, membrane active substances with antioxidative properties can positively influence the erythrocyte deformability. Pyridoxal is an antioxidative substance and it has been reported that pyridoxal positively influences the elasticity of the erythrocyte membrane (Beder, Kittova et al. 2003). Given that the lowered erythrocytic PdxK activity would lead to lower PLP levels in the erythrocyte of the -/- genotype, it is assumed that infected erythrocytes of the -/- genotype could be less deformable than those of the +/+ erythrocytes. This would also suggest that infected erythrocytes of the -/- genotype are more likely to be selectively removed from the circulation by the spleen than those of the +/+ genotype.

However, the investigation of the deformability of infected and uninfected erythrocytes of the various genotypes using the filtration method (Mataseje, Beder et al. 2003) revealed no differences in the filterability of the erythrocytes. This suggests that erythrocytes of the -/- genotype do not exhibit a reduced deformability as

compared to those of the +/+ genotype. In order to rule out the limitation of the method used for the assay as the cause of the obtained results, further investigations with more precise and detailed methods may be required (Nash 1989; Dondorp 1999; Dondorp 1999; Park 2008).

A number of studies have shown that some genetic traits affecting the erythrocyte impair the growth of the parasite in vitro. Yet, not much is known about the morphology of the parasite during its development in erythrocytes with these genetic variants. One study conducted by Fairhurst et al. illustrated abnormal development of the parasite in HbCC cells and linked it to the reduced multiplication rates of the parasite in HbCC cells. In this study the parasites were examined with light microscopy and electron microscopy. Electron microscopy of the parasite showed that the intracellular compartments were lost when the parasite was cultured in HbCC cells (see figure 4.1) (Fairhurst, Fujioka et al. 2003).

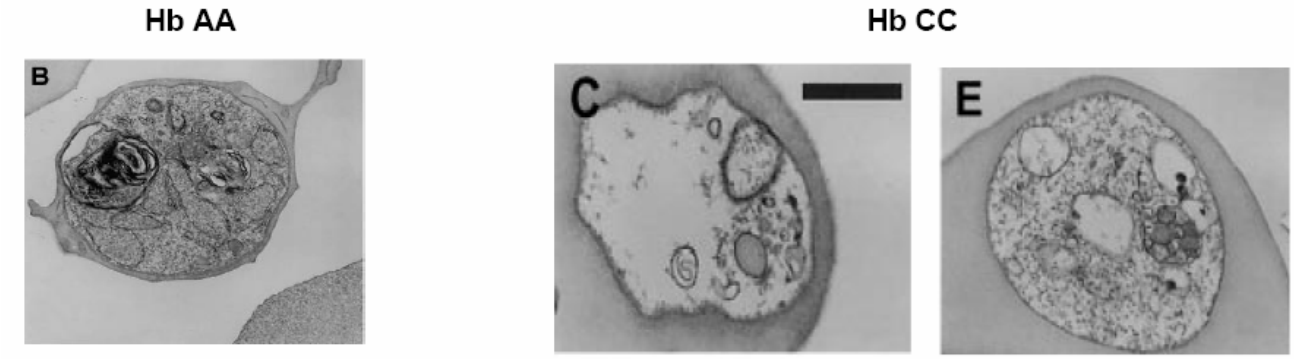

Figure 4.1: Abnormal morphology of the parasite in HbCC erythrocytes. B: Parasite in HbAA erythrocytes, C and E: Parasite in HbCC erythrocytes. The bar stands for 1µm. Figure were taken from Fairhurst et al. (Fairhurst, Fujioka et al. 2003).

It has been reported that vitamin B_6 and its derivates are sufficient singlet oxygen quenchers and potential antioxidants (Ehrenshaft, Bilski et al. 1999; Bilski, Li et al. 2000). This led to the assumption that erythrocytes of the +/+ genotype containing higher levels of PLP would provide a more conducive redox environment for the

parasite than erythrocytes of the -/- genotype. The parasite in the -/- erythrocyte is therefore likely to be exposed to increased oxidative stress when compared to the +/+ genotype. One of the main features of oxidative stress in cells is membrane damage due to phospholipid oxidation (Sies 1997; Pradines 2005) and this is escorted by abnormal morphology of the cells.

I examined the morphology of the parasites cultured in -/- erythrocytes with light microscopy and for more details with electron microscopy. For electron microscopy the method as proposed by Tokuyasu was used. This method is ideal for the preservation of membrane structures during fixation (Tokuyasu 1980; Geiger B 1981).

Although light microscopy of Giemsa stained parasites showed that some trophozoite stages cultured in -/- erythrocytes looked unusual as compared to those cultured in +/+ erythrocytes, a closer examination with electron microscopy showed no clear differences in the morphology of the parasites. The membranes were intact in both cases and intracellular compartments were present. A failure to establish the parasitophorous vacuole, intracellular compartments and membranes in erythrocytes of the -/- genotype can consequently be ruled out as the cause of impaired growth of the parasites in erythrocytes with lowered PdxK activity.

4.1 Conclusions

Early studies on the lowered erythrocytic PdxK activity in blacks and its possible role in protection against malaria allowed for the hypothesis that this could be a trait which reduces the susceptibility of the human host to malaria. For over thirty years no additional investigations were carried out to prove this hypothesis. The relatively recent discovery of the genetic basis for the reduced PdxK activity in erythrocytes enables the screening of individuals for this polymorphism followed by in vitro studies to investigate the effect of this trait on *P. falciparum*.

Screening Africans and Caucasians showed that the 8 bp deletion in the promoter region of the *pdxk* gene which is associated with the lowered activity of the enzyme in erythrocytes is more prevalent among Africans in agreement with the geographical

distribution of red blood cell polymorphisms that protect against malaria (Cooke and Hill 2001). The lower protein level of PdxK in erythrocytes of the deletion genotype -/- suggests that the reduced activity of the erythrocytic PdxK is strictly associated with low protein levels. This is also supported by the lower PLP level measured in erythrocytes of the -/- genotype. The impaired growth phenotype of the parasite in erythrocytes of the -/- genotype shows that the lowered erythrocytic PdxK activity has an in vitro effect on *P. falciparum.* Although the parasitic de novo or salvage pathways for vitamin B_6 were upregulated in the -/- genotype by roughly 2-3 fold, upregulation could not counterbalance this phenotype. Figure 4.2 illustrates a hypothetical model demonstrating the effect of this genetic trait on the blood stages of *P. falciparum.*

In summary, my data provide a plausible explanation for the previously observed association between lowered erythrocytic PdxK activity and the risk of malaria. infection. It also suggests directions for further research such as investigating the in vivo effect of this erythrocytic alteration on the clinical outcome of malaria. Population studies correlating this polymorphism in the *pdxk* promoter with the susceptibility to malaria in endemic regions are therefore highly recommended.

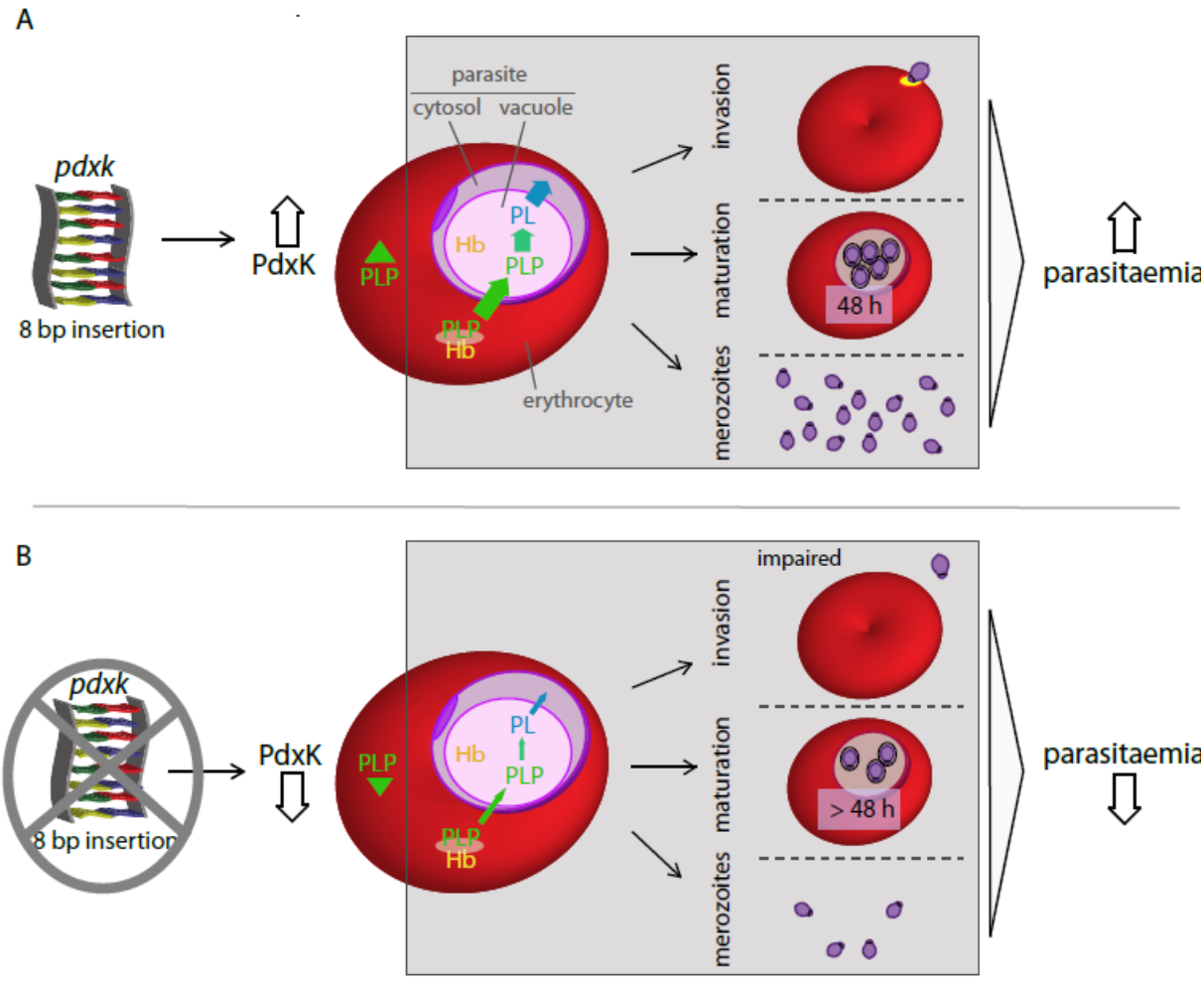

Figure 4.2: Influence of the 8 bp deletion on the *P. falciparum* blood stages
This hypothetical model demonstrates the effect of the 8 bp deletion in the promoter of the *pdxk* gene on *P. falciparum.* Data supporting the events in the grey box could not be generated in this project. This part of the model is based on facts derived from published data **A:** The presence of the 8 bp insertion leads to a higher PdxK activity in erythrocytes which is accompanied by higher levels of PLP. PLP binds to haemoglobin. Haemoglobin with a higher percentage of bound PLP is taken up by the parasite. In the parasite´s acidic food vacuole, PLP is released from haemoglobin and is hydrolysed to PL which can exit the food vacuole. High levels of PL are therefore released into the parasite´s cytosol and phosphorylated by the parasitic PdxK. The parasite has a sufficient supply of PLP and matures and replicates normally.
B: The absence of the 8 bp insertion in the promoter of the *pdxk* gene leads to a lower PdxK activity in erythrocyte resulting in lower levels of PLP. Haemoglobin with a lower percentage of bound PLP is taken up by the parasite and the level of PL available for the parasite is thus reduced. The parasite is not capable of fully compensating for the decreased resources of vitamin B_6 in the -/- genotype environment and this could affect its development in the

erythrocyte resulting in a reduced parasitemia. A reduction in parasitemia could be as a result of reduced invasion, reduced merozoite formation or a prolonged intraerythrocytic development.

References

Ahn, S. Y., M. Y. Shin, et al. (2008). Magnetic separation: a highly effective method for synchronization of cultured erythrocytic Plasmodium falciparum. Parasitol Res **102**(6): 1195-200.

Aidoo, M., D. J. Terlouw, et al. (2002). Protective effects of the sickle cell gene against malaria morbidity and mortality. Lancet **359**(9314): 1311-2.

Akide-Ndunge, O. B., E. Tambini, et al. (2009). Co-ordinated stage-dependent enhancement of Plasmodium falciparum antioxidant enzymes and heat shock protein expression in parasites growing in oxidatively stressed or G6PD-deficient red blood cells. Malar J **8**: 113.

Akompong, T., N. Ghori, et al. (2000). In vitro activity of riboflavin against the human malaria parasite Plasmodium falciparum. Antimicrob Agents Chemother **44**(1): 88-96.

Alexander, F. W., E. Sandmeier, et al. (1994). Evolutionary relationships among pyridoxal-5'-phosphate-dependent enzymes. Regio-specific alpha, beta and gamma families. Eur J Biochem **219**(3): 953-60.

Allen, S. J., A. O'Donnell, et al. (1999). Prevention of cerebral malaria in children in Papua New Guinea by southeast Asian ovalocytosis band 3. Am J Trop Med Hyg **60**(6): 1056-60.

Amino, R., S. Thiberge, et al. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med **12**(2): 220-4.

Anderson, B. B., G. M. Perry, et al. (1989). Rapid uptake and clearance of pyridoxine by red blood cells in vivo. Am J Clin Nutr **50**(5): 1059-63.

Ayi, K., G. Min-Oo, et al. (2008). Pyruvate kinase deficiency and malaria. N Engl J Med **358**(17): 1805-10.

Baer, K., C. Klotz, et al. (2007). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS Pathog **3**(11): e171.

Baird, J. K., S. Masbar, et al. (1998). Age-dependent susceptibility to severe disease with primary exposure to Plasmodium falciparum.J Infect Dis **178**(2): 592-5.

Balmer, P., H. M. Phillips, et al. (2000). The effect of nitric oxide on the growth of Plasmodium falciparum, P. chabaudi and P. berghei in vitro. Parasite Immunol **22**(2): 97-106.

Barraviera, B., P. E. Machado, et al. (1988). Glutathione reductase activity and its relation with riboflavin levels measured by methemoglobin reduction by cystamine in patients with malaria (preliminary report). Rev Inst Med Trop Sao Paulo **30**(2): 107-8.

Beder, I., M. Kittova, et al. (2003). The effect of selected membrane active substances on erythrocyte deformability. Bratisl Lek Listy **104**(2): 69-72.

Benesch, R., R. E. Benesch, et al. (1982). Labeling of hemoglobin with pyridoxal phosphate. J Biol Chem **257**(3): 1320-4.

Benesch, R. E., S. Yung, et al. (1973). Pyridoxal compounds as specific reagents for the alpha and beta N-termini of hemoglobin. Proc Natl Acad Sci U S A **70**(9): 2595-9.

Bennett, G. D. and M. M. Kay (1981). Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol **9**(3): 297-307.

Bienzle, U., O. Ayeni, et al. (1972). Glucose-6-phosphate dehydrogenase and malaria. Greater resistance of females heterozygous for enzyme deficiency and of males with non-deficient variant. Lancet **1**(7742): 107-10.

Bilski, P., M. Y. Li, et al. (2000). Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. Photochem Photobiol **71**(2): 129-34.

Bongfen, S. E., A. Laroque, et al. (2009). Genetic and genomic analyses of host-pathogen interactions in malaria. Trends Parasitol **25**(9): 417-22.

Bozdech, Z., J. Zhu, et al. (2003). Expression profiling of the schizont and trophozoite stages of Plasmodium falciparum with a long-oligonucleotide microarray. Genome Biol **4**(2): R9.

Breman, J. G. (2001). The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg **64**(1-2 Suppl): 1-11.

Cake, M. H., D. M. DiSorbo, et al. (1978). Effect of pyridoxal phosphate on the DNA binding site of activated hepatic glucocorticoid receptor. J Biol Chem **253**(14): 4886-91.

Cappadoro, M., G. Giribaldi, et al. (1998). Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by Plasmodium falciparum may explain malaria protection in G6PD deficiency. Blood **92**(7): 2527-34.

Carter, R. and K. N. Mendis (2002). Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev **15**(4): 564-94.

Caulfield, L. E., S. A. Richard, et al. (2004). Undernutrition as an underlying cause of malaria morbidity and mortality in children less than five years old.Am J Trop Med Hyg **71**(2 Suppl): 55-63.

Chang, J. G. and T. C. Liu (1995). Glucose-6-phosphate dehydrogenase deficiency. Crit Rev Oncol Hematol **20**(1-2): 1-7.

Chang, Y. C., R. D. Scott, et al. (1987). Function of pyridoxal 5'-phosphate in glycogen phosphorylase: a model study using 6-fluoro-5'-deoxypyridoxal- and 5'-deoxypyridoxal-reconstituted enzymes. Biochemistry **26**(2): 360-7.

Chern, C. J. and E. Beutler (1975). Pyridoxal kinase: decreased activity in red blood cells of Afro-Americans. Science **187**(4181): 1084-6.

Chern, C. J. and E. Beutler (1976). Biochemical and electrophoretic studies of erythrocyte pyridoxine kinase in white and black Americans. Am J Hum Genet **28**(1): 9-17.

Chotivanich, K., R. Udomsangpetch, et al. (2002). Hemoglobin E: a balanced polymorphism protective against high parasitemias and thus severe P falciparum malaria. Blood **100**(4): 1172-6.

Clayton, P. T. (2006). B6-responsive disorders: a model of vitamin dependency. J Inherit Metab Dis **29**(2-3): 317-26.

Cooke, G. S. and A. V. Hill (2001). Genetics of susceptibility to human infectious disease. Nat Rev Genet **2**(12): 967-77.

Cox, F. E. (2010). History of the discovery of the malaria parasites and their vectors. Parasites & vectors **3**.

Craig, A., I. Hastings, et al. (2001). Genetics and malaria--more questions than answers. Trends Parasitol **17**(2): 55-6.

Das, B. S., D. B. Das, et al. (1988). Riboflavin deficiency and severity of malaria. Eur J Clin Nutr **42**(4): 277-83.

Das, B. S., D. I. Thurnham, et al. (1990). Increased plasma lipid peroxidation in riboflavin-deficient, malaria-infected children. Am J Clin Nutr **51**(5): 859-63.

Davis, T. M., T. Q. Binh, et al. (1994). Serum vitamin A and E concentrations in acute falciparum malaria: modulators or markers of severity? Clin Sci (Lond) **87**(5): 505-11.

Deng, W. and S. G. Roberts (2006). Core promoter elements recognized by transcription factor IIB. Biochem Soc Trans **34**(Pt 6): 1051-3.

Dondorp, A. M., Angus, B.J, Chotivanich, K , White N.I (1999). Red blood cell deformability as a predictor of Anemia un severe falciparum malaria. Am.J.Trop.Med. Hyg **60**(5): 733-737.

Dondorp, A. M., Chotivanich, K.T, Fucharoen, S., Kager, P.A (1999). Red cell deformability ,splenic function and anaemia in thalassaemia. British journal of haemotology **105**: 505-508.

Doolan, D. L., C. Dobano, et al. (2009). Acquired immunity to malaria. Clin Microbiol Rev **22**(1): 13-36, Table of Contents.

Downie, M. J., K. Kirk, et al. (2008). Purine salvage pathways in the intraerythrocytic malaria parasite Plasmodium falciparum. Eukaryot Cell **7**(8): 1231-7.

Dutta, P., J. Pinto, et al. (1985). Antimalarial effects of riboflavin deficiency. Lancet **2**(8463): 1040-3.

Eaton, J. W., J. R. Eckman, et al. (1976). Suppression of malaria infection by oxidant-sensitive host erythrocytes. Nature **264**(5588): 758-60.

Eckman, J. R., J. W. Eaton, et al. (1976). Role of vitamin E in regulating malaria expression. Trans Assoc Am Physicians **89**: 105-15.

Ehrenshaft, M., P. Bilski, et al. (1999). A highly conserved sequence is a novel gene involved in de novo vitamin B6 biosynthesis. Proc Natl Acad Sci U S A **96**(16): 9374-8.

Ehrhardt, S., G. D. Burchard, et al. (2006). Malaria, anemia, and malnutrition in african children--defining intervention priorities. J Infect Dis **194**(1): 108-14.

Elliot, D., McIntosch, M.T, Joiner K (2008). Four distinct pathways of hemoglobin uptake in the malaria parasite Plasmodium falciparum. PNAS **105**(7): 2463-2468.

Fairhurst, R. M., H. Fujioka, et al. (2003). Aberrant development of Plasmodium falciparum in hemoglobin CC red cells: implications for the malaria protective effect of the homozygous state. Blood **101**(8): 3309-15.

Ferrari, S. L., L. Ahn-Luong, et al. (2003). Two promoter polymorphisms regulating interleukin-6 gene expression are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women. J Clin Endocrinol Metab **88**(1): 255-9.

Field, J. W. (1949). Blood examination and prognosis in acute falciparum malaria. Trans R Soc Trop Med Hyg **43**(1): 33-48.

Fischer, P. R. and P. Boone (1998). Short report: severe malaria associated with blood group. Am J Trop Med Hyg **58**(1): 122-3.

Fitzpatrick, T. B., N. Amrhein, et al. (2007). Two independent routes of de novo vitamin B6 biosynthesis: not that different after all. Biochem J **407**(1): 1-13.

Flanagan, J. M. and E. Beutler (2006). The genetic basis of human erythrocyte pyridoxal kinase activity variation. Haematologica **91**(6): 801-4.

Flint, J., R. M. Harding, et al. (1993). The population genetics of the haemoglobinopathies. Baillieres Clin Haematol **6**(1): 215-62.

Francis, S. E., D. J. Sullivan, Jr., et al. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu Rev Microbiol **51**: 97-123.

Frevert, U. (2004). Sneaking in through the back entrance: the biology of malaria liver stages. Trends Parasitol **20**(9): 417-24.

Friedman, M. J. (1978). Erythrocytic mechanism of sickle cell resistance to malaria. Proc Natl Acad Sci U S A **75**(4): 1994-7.

Gallo, V., E. Schwarzer, et al. (2009). Inherited glutathione reductase deficiency and Plasmodium falciparum malaria--a case study. PLoS One **4**(10): e7303.

Gallup, J. L. and J. D. Sachs (2001). The economic burden of malaria. Am J Trop Med Hyg **64**(1-2 Suppl): 85-96.

Geiger B, D. H., Tokuysau K (1981). Immunoelectron Microscope Studies of Membrane-Microfi lament Interactions :Distributions of a-Actinin, Tropomyosin, and Vinculinin Intestinal Epithelial Brush Border and Chicken Gizzard Smooth Muscle Cells. Journal of cell biology **91**: 614-628.

Gengenbacher, M., T. B. Fitzpatrick, et al. (2006). Vitamin B6 biosynthesis by the malaria parasite Plasmodium falciparum: biochemical and structural insights. J Biol Chem **281**(6): 3633-41.

Genton, B., F. al-Yaman, et al. (1995). Ovalocytosis and cerebral malaria. Nature **378**(6557): 564-5.

Genton , B., Al-Yaman, F, Mgone, C and alpers, M (1995). Ovalocytosis and cerebral malaria. Nature **378**.

Gilles, H. M., K. A. Fletcher, et al. (1967). Glucose-6-phosphate-dehydrogenase deficiency, sickling, and malaria in African children in South Western Nigeria. Lancet **1**(7482): 138-40.

Guindo, A., R. M. Fairhurst, et al. (2007). X-linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. PLoS Med **4**(3): e66.

Hamm, M. W., H. Mehansho, et al. (1979). Transport and metabolism of pyridoxamine and pyridoxamine phosphate in the small intestine of the rat. J Nutr **109**(9): 1552-9.

Han, Y. S., J. Thompson, et al. (2000). Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. EMBO J **19**(22): 6030-40.

Hanna, M. C., A. J. Turner, et al. (1997). Human pyridoxal kinase. cDNA cloning, expression, and modulation by ligands of the benzodiazepine receptor. J Biol Chem **272**(16): 10756-60.

Hay, S. I., C. A. Guerra, et al. (2009). A world malaria map: Plasmodium falciparum endemicity in 2007. PLoS Med **6**(3): e1000048.

Huber, S. M., A. C. Uhlemann, et al. (2002). Plasmodium falciparum activates endogenous Cl(-) channels of human erythrocytes by membrane oxidation. EMBO J **21**(1-2): 22-30.

Hunt, N. H., Stocker, R (1990). Oxidative stress and the redox status of malaria infected erythrocytes.Blood cells **16**: 499-526.

Hunter, G. A. and G. C. Ferreira (2009). 5-aminolevulinate synthase: catalysis of the first step of heme biosynthesis. Cell Mol Biol (Noisy-le-grand) **55**(1): 102-10.

Hutagalung, R., P. Wilairatana, et al. (1999). Influence of hemoglobin E trait on the severity of Falciparum malaria. J Infect Dis **179**(1): 283-6.

Ink, S. L. and L. M. Henderson (1984). Effect of binding to hemoglobin and albumin on pyridoxal transport and metabolism. J Biol Chem **259**(9): 5833-7.

Ink, S. L. and L. M. Henderson (1984). Vitamin B6 metabolism. Annu Rev Nutr **4**: 455-70.

Ink, S. L., H. Mehansho, et al. (1982). The binding of pyridoxal to hemoglobin. J Biol Chem **257**(9): 4753-7.

Jarolim, P., Palek,J,Amato, D, Hassan , K and Liu, S (1991). Deletion in erythrocyte band 3 gene in malaria-resistant Southeast-Asian ovalocytosis. PNAS **88**: 11022-11026.

Kark, J. A., M. J. Haut, et al. (1982). A rapid fluorometric assay for erythrocyte pyridoxal kinase. Biochem Med **27**(1): 109-20.

Kerry J.A, K., F (1986). Purification and Characterization of Pyridoxal Kinase from Human Erythrocytes. Preparative Biochemistry and Biotechnology **16**: 199-216.

Kirchgatter, K. and H. A. Del Portillo (2005). Clinical and molecular aspects of severe malaria. An Acad Bras Cienc **77**(3): 455-75.

Krishna, S., A. M. Taylor, et al. (1999). Thiamine deficiency and malaria in adults from southeast Asia. Lancet **353**(9152): 546-9.

Kun, J. F., B. Mordmuller, et al. (1998). Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria. Lancet **351**(9098): 265-6.

Kwiatkowski, D. (2000). Genetic susceptibility to malaria getting complex. Curr Opin Genet Dev **10**(3): 320-4.

Langhi, D. M., Jr. and J. O. Bordin (2006). Duffy blood group and malaria. Hematology **11**(5): 389-98.

Leklem, J. E. and T. D. Shultz (1983). Increased plasma pyridoxal 5'-phosphate and vitamin B6 in male adolescents after 4500-meter run. Am J Clin Nutr **38**(4): 541-8.

Levander, O. A., A. L. Ager, Jr., et al. (1989). Menhaden-fish oil in a vitamin E-deficient diet: protection against chloroquine-resistant malaria in mice. Am J Clin Nutr **50**(6): 1237-9.

Levesque, M. C., M. R. Hobbs, et al. (1999). Nitric oxide synthase type 2 promoter polymorphisms, nitric oxide production, and disease severity in Tanzanian children with malaria. J Infect Dis **180**(6): 1994-2009

Lumeng, L., A. Lui, et al. (1980). Plasma content of B6 vitamers and its relationship to hepatic vitamin B6 metabolism. J Clin Invest **66**(4): 688-95.

Mackintosh, C. L., J. G. Beeson, et al. (2004). Clinical features and pathogenesis of severe malaria. Trends Parasitol **20**(12): 597-603.

Maier, A. G., M. T. Duraisingh, et al. (2003). Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med **9**(1): 87-92.

Martin, S. K., L. H. Miller, et al. (1978). Low erythrocyte pyridoxal-kinase activity in Blacks: Its possible relation to falciparum malaria. Lancet **1**(8062): 466-8.

Mataseje, A., I. Beder, et al. (2003). The assessment of erythrocyte deformability by filtration rate. Bratisl Lek Listy **104**(4-5): 158-60.

Mehansho, H., M. W. Hamm, et al. (1979). Transport and metabolism of pyridoxal and pyridoxal phosphate in the small intestine of the rat. J Nutr **109**(9): 1542-51.

Mehansho, H. and L. M. Henderson (1980). Transport and accumulation of pyridoxine and pyridoxal by erythrocytes. J Biol Chem **255**(24): 11901-7.

Mehta, A., P. J. Mason, et al. (2000). Glucose-6-phosphate dehydrogenase deficiency. Baillieres Best Pract Res Clin Haematol **13**(1): 21-38.

Merrill, A. H., Jr., J. M. Henderson, et al. (1984). Metabolism of vitamin B-6 by human liver. J Nutr **114**(9): 1664-74.

Meydani, S. N., J. D. Ribaya-Mercado, et al. (1991). Vitamin B-6 deficiency impairs interleukin 2 production and lymphocyte proliferation in elderly adults. Am J Clin Nutr **53**(5): 1275-80.

Miller, L. H., M. F. Good, et al. (1994). Malaria pathogenesis. Science **264**(5167): 1878-83.

Miller, L. H., S. J. Mason, et al. (1976). The resistance factor to Plasmodium vivax in blacks. The Duffy-blood-group genotype, FyFy. N Engl J Med **295**(6): 302-4.

Miller, L. H., S. J. Mason, et al. (1975). Erythrocyte receptors for (Plasmodium knowlesi) malaria: Duffy blood group determinants. Science **189**(4202): 561-3.

Mills, P. B., R. A. Surtees, et al. (2005). Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox(am)ine 5'-phosphate oxidase. Hum Mol Genet **14**(8): 1077-86.

Min-Oo, G., A. Fortin, et al. (2003). Pyruvate kinase deficiency in mice protects against malaria. Nat Genet **35**(4): 357-62.

Min-Oo, G. and P. Gros (2005). Erythrocyte variants and the nature of their malaria protective effect. Cell Microbiol **7**(6): 753-63.

Mockenhaupt, F. P., S. Ehrhardt, et al. (2004). Manifestation and outcome of severe malaria in children in northern Ghana. Am J Trop Med Hyg **71**(2): 167-72.

Mockenhaupt, F. P., S. Ehrhardt, et al. (2004). Hemoglobin C and resistance to severe malaria in Ghanaian children. J Infect Dis **190**(5): 1006-9.

Mockenhaupt, F. P., S. Ehrhardt, et al. (2004). Alpha(+)-thalassemia protects African children from severe malaria. Blood **104**(7): 2003-6.

Modiano, D., G. Luoni, et al. (2001). Haemoglobin C protects against clinical Plasmodium falciparum malaria. Nature **414**(6861): 305-8.

Muldoon, T. G. and J. A. Cidlowski (1980). Specific modification of rat uterine estrogen receptor by pyridoxal 5'-phosphate. J Biol Chem **255**(7): 3100-7.

Muller, I. B., J. E. Hyde, et al. Vitamin B metabolism in Plasmodium falciparum as a source of drug targets. Trends Parasitol **26**(1): 35-43.

Muller, I. B., J. Knockel, et al. Secretion of an acid phosphatase provides a possible mechanism to acquire host nutrients by Plasmodium falciparum. Cell Microbiol **12**(5): 677-91.

Muller, S. and B. Kappes (2007). Vitamin and cofactor biosynthesis pathways in Plasmodium and other apicomplexan parasites. Trends Parasitol **23**(3): 112-21.

Nash, G. B., O´Brian, E, Dormandy J.A (1989). Abnormalities in the mechanical properties of red blood cells caused by *Plasmodium falciparum*. Blood **74**(2): 855-861.

Natta, C. L. and R. D. Reynolds (1984). Apparent vitamin B6 deficiency in sickle cell anemia. Am J Clin Nutr **40**(2): 235-9.

Olson, J. A. and R. L. Nagel (1986). Synchronized cultures of P falciparum in abnormal red cells: the mechanism of the inhibition of growth in HbCC cells. Blood **67**(4): 997-1001.

Park, Y., M. Diez-Silva, et al. (2008). Refractive index maps and membrane dynamics of human red blood cells parasitized by Plasmodium falciparum. Proc Natl Acad Sci U S A **105**(37): 13730-5.

Park, Y. D.-S., M, Popescus, G, Suresh,S (2008). Refractive index maps and membrane dynamics of human red blood cells parasitized by Plasmodium falciparum. PNAS **105**: 13730 -13735.

Pradines, B., Baret, E, Rogier,C (2005). Generation of free radicals and enhancement of hemin-induced membrane damage by a catechol iron chelator in *P.falciparum*. journal of biological sciences **5**: 463-471

Richer, J. and A. E. Chudley (2005). The hemoglobinopathies and malaria. Clin Genet **68**(4): 332-6.

Roberts, D. J. and T. N. Williams (2003). Haemoglobinopathies and resistance to malaria. Redox Rep **8**(5): 304-10.

Roth, E., Schulman,S (1988). The adaptation of Plasmodium falciparum to oxidative stress in G6PD deficient human erythrocytes.

Roth, E. F., Jr., C. Raventos-Suarez, et al. (1983). Glucose-6-phosphate dehydrogenase deficiency inhibits in vitro growth of Plasmodium falciparum. Proc Natl Acad Sci U S A **80**(1): 298-9.

Ruwende, C., S. C. Khoo, et al. (1995). Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. Nature **376**(6537): 246-9.

Salanti, A., M. Dahlback, et al. (2004). Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med **200**(9): 1197-203.

Saliba, K. J., H. A. Horner, et al. (1998). Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite Plasmodium falciparum. J Biol Chem **273**(17): 10190-5.

Samba, E. (2001). The malaria burden and Africa. Am J Trop Med Hyg **64**(1-2 Suppl): ii.

Semba, R. D. (1998). The role of vitamin A and related retinoids in immune function. Nutr Rev **56**(1 Pt 2): S38-48.

Serghides, L. and K. C. Kain (2002). Mechanism of protection induced by vitamin A in falciparum malaria. Lancet **359**(9315): 1404-6.

Shankar, A. H. (2000). Nutritional modulation of malaria morbidity and mortality. J Infect Dis **182 Suppl 1**: S37-53.

Shankar, A. H., B. Genton, et al. (1999). Effect of vitamin A supplementation on morbidity due to Plasmodium falciparum in young children in Papua New Guinea: a randomised trial. Lancet **354**(9174): 203-9.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. Exp Physiol **82**(2): 291-5.

Snow, R. W., C. A. Guerra, et al. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature **434**(7030): 214-7.

Sturm, A., R. Amino, et al. (2006). Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science **313**(5791): 1287-90.

Talbott, M. C., L. T. Miller, et al. (1987). Pyridoxine supplementation: effect on lymphocyte responses in elderly persons. Am J Clin Nutr **46**(4): 659-64.

Tambasco-Studart, M., O. Titiz, et al. (2005). Vitamin B6 biosynthesis in higher plants. Proc Natl Acad Sci U S A **102**(38): 13687-92.

Thurnham, D. I., S. J. Oppenheimer, et al. (1983). Riboflavin status and malaria in infants in Papua New Guinea. Trans R Soc Trop Med Hyg **77**(3): 423-4.

Tokuyasu, K. T. (1980). Immunochemistry on ultrathin frozen sections. Histochem J **12**(4): 381-403.

Tully, D. B., V. E. Allgood, et al. (1994). Modulation of steroid receptor-mediated gene expression by vitamin B6. FASEB J **8**(3): 343-9.

VanBuskirk, K. M., M. T. O'Neill, et al. (2009). Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design. Proc Natl Acad Sci U S A **106**(31): 13004-9.

Vaughan, A. M., A. S. Aly, et al. (2008). Malaria parasite pre-erythrocytic stage infection: gliding and hiding. Cell Host Microbe **4**(3): 209-18.

Vul'fson, P. L. (1986). [Glycogen phosphorylase of skeletal muscles]. Biokhimiia **51**(12): 1974-92.

Waymire, K. G., J. D. Mahuren, et al. (1995). Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet **11**(1): 45-51.

Wickham, M. E., J. G. Culvenor, et al. (2003). Selective inhibition of a two-step egress of malaria parasites from the host erythrocyte. J Biol Chem **278**(39): 37658-63.

Williams, T. N. (2006). Human red blood cell polymorphisms and malaria. Curr Opin Microbiol **9**(4): 388-94.

Williams, T. N., K. Maitland, et al. (1996). High incidence of malaria in alpha-thalassaemic children. Nature **383**(6600): 522-5.

Williams, T. N., T. W. Mwangi, et al. (2005). An immune basis for malaria protection by the sickle cell trait. PLoS Med **2**(5): e128.

Williams, T. N., T. W. Mwangi, et al. (2005). Negative epistasis between the malaria-protective effects of alpha+-thalassemia and the sickle cell trait. Nat Genet **37**(11): 1253-7.

Williams, T. N., S. Wambua, et al. (2005). Both heterozygous and homozygous alpha+ thalassemias protect against severe and fatal Plasmodium falciparum malaria on the coast of Kenya. Blood **106**(1): 368-71.

Wrenger, C., M. L. Eschbach, et al. (2006). Vitamin B1 de novo synthesis in the human malaria parasite Plasmodium falciparum depends on external provision of 4-amino-5-hydroxymethyl-2-methylpyrimidine. Biol Chem **387**(1): 41-51.

Wrenger, C., M. L. Eschbach, et al. (2005). Analysis of the vitamin B6 biosynthesis pathway in the human malaria parasite Plasmodium falciparum. J Biol Chem **280**(7): 5242-8.

Yang, M. Q. and L. L. Elnitski (2008). Diversity of core promoter elements comprising human bidirectional promoters. BMC Genomics **9 Suppl 2**: S3.

Ye, S., P. Eriksson, et al. (1996). Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. J Biol Chem **271**(22): 13055-60.

Zanella, A. and P. Bianchi (2000). Red cell pyruvate kinase deficiency: from genetics to clinical manifestations. Baillieres Best Pract Res Clin Haematol **13**(1): 57-81.

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