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Solute uptake into *B. divergens* and *P. falciparum* infected erythrocytes: same theme but different mechanisms

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Kaushik N, **Sharma J**, Sahal D (2012) Anti-plasmodial action of *de novo*-designed, cationic, lysine-branched, amphipathic, helical peptides. Malar J. 11:256. doi: 10.1186/1475-2875-11-256.

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Jackson A, Otto T, Darby A, Ramaprasad A, Xia D, Echaide I, Farber M, Gahlot S, Gamble J, Gupta D, Gupta Y, Jackson L, Malandrin L, Malas T, Moussa E, Nair M, Reid A, Sanders M, **Sharma J,** Tracey A, Quail M, Weir W, Wastling J, Hall N, Willadsen P, Lingelbach K, Shiels B, Tait A, Berriman M, Allred D, Pain A (2014) The evolutionary dynamics of variant antigen genes in *Babesia* reveal a history of genomic innovation underlying host-parasite interaction. Nucl. Acids Res. doi: 10.1093/nar/gku322.

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Patent on Mechanism of action and specificity of novel synthetic dodecapeptides against *Candida albicans*. Maurya IK, Thota CK, **Sharma J**, Prasad R, Chauhan VS. Patent application no. is 2893/DEL/2012.

Eigenständigkeitserklärung

Ich versichere hiermit, dass ich meine dem Fachbereich Biologie der Universität Marburg vorgelegte Dissertation, mit dem Titel

"Solute uptake into *B. divergens* and *P. falciparum* infected erythrocytes: same theme but different mechanisms"

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Die Dissertation wurde in der jetztigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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(Ort/Datum) (Unterschrift mit Vor- und Zuname)

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IV. Abbreviations

µCi	Microcurie
µl	Microliter
µM	Micromolar
Cis-ACBD	1-aminocyclobutane-1, 3-dicarboxylate
CPM	Counts Per Minute
CtxB	Cholera toxin B subunit
DMEM	Dulbecco's Modified Eagle Medium
DoTMA	Dodecyltrimethylammonium
FSG	Fish Skin Gelatine
HC-3	Hemicholine
Hrs	Hours
iRBC	infected Red Blood Cell
KDa	Kilodalton
mA	Milliampere
min	Minute
ml	Milliliter
mM	Millimolar
nM	Nanomolar
NPP	New Permeability Pathways
PBS	Phosphate Buffered Saline
PPM	Parasite plasma membrane
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar membrane
RBC	Red Blood Cell
rpm	Revolutions per minute
RPS	RPMI complete medium
SDS-PAGE	SDS-polyacrylamidegel electrophoresis

V. Summary

Plasmodium falciparum and *Babesia divergens* are parasites of the same phylogeny apicomplexa and they invade and replicate in the human erythrocytes. Both the parasites differently modify the host cell membrane during and after infection, providing an excellent example of parallel but distinct adaptations of the parasites to survive in the RBC.

During invasion of the host erythrocyte, the parasite targets specific entry sites on the RBCs membrane, which is also the initial step in the biogenesis of the parasitophorous vacuole (PV). The parasitophorous vacuolar membrane (PVM) surrounds the parasite and thus *P. falciparum* remains surrounded by the vacuolar membrane for most of the parasite's development. On the other hand in *B. divergens*, vacuolar membrane fate is unclear, owing to the lack of suitable markers. The electron microscopic studies suggest the disintegration of the PVM in the later stages of parasite maturation. As the differentiated erythrocyte does not endocytose or phagocytose, events leading to the formation of the PVM, in particular the contribution of the erythrocyte membrane (RBCM) are unknown. In order to understand better the internalization of RBCM proteins by parasites, we wanted to determine whether the lipid rafts, which are concentrated patches of lipids and proteins on the erythrocyte membrane, play any such role as `specific entry site´ in the biogenesis of PV and also determine whether both parasites respectively internalize the same type of lipids/proteins or not. Recent work has shown that there is no internalization of major RBCM proteins, but the proteins which were found internalized are comparatively present in low abundance in the RBCM and are associated with the lipid rafts. In this work I used *B. divergens* and also *P. falciparum* infected erythrocytes to do a comparative investigation of the host cell lipids/proteins, whether they are included or excluded in PV. As a result (i) we found that both parasites recruit and exclude the same set of proteins. (ii) we used cholera toxin B subunit which binds to the ganglioside GM1, to follow the fate of this lipid raft associated glycophingolipid during PVM formation and disintegration. (iii) we observed that GM1 remains in the vicinity of *P. falciparum* during maturation but disappears from the vicinity of *B. divergens* as the parasite matures, consistent with a disintegration of the PVM. In conclusion, it appears that there is similarity between the early events in the PVM formation in both the parasites.

All the biochemical pathways are dependent on the balance of the transport mechanisms and counteractions like the import of extracellular metabolites, export of the intracellular metabolites and proper waste disposal of unused metabolites. Thus the second part of my work was focused on the increased permeability of the host cell membrane after infection and appearance of new permeability pathways (NPP) in the infected erythrocyte. The erythrocyte which is `save heaven´ for the parasite have limited resources and metabolism, that the parasite can use. The intracellular parasite is far more active inside the erythrocyte than outside cell. After invasion the small metabolically active merozoite stage of each genus (*Plasmodium* and *Babesia*) grows rapidly into

other developmental stages (ring form and trophozoite form) inside the erythrocyte and thereby synthesizing DNA, RNA, proteins and lipids in large amounts. In order to fulfil all the essential requirements, the growing parasite induces changes in the membrane permeability of the erythrocyte and takes up solutes from the extracellular media via NPP. One among the other important amino acids required by the parasite is L-glutamate. The main aim of my work was to observe and characterize the transport of L- glutamate into *B. divergens* infected erythrocytes, and compare it with *P. falciparum* infected erythrocytes. As it is known that *P. falciparum* infected erythrocytes have both low- and high-affinity (EAAT3) glutamate transporter activated. The transport system present in *B. divergens* infected erythrocytes is unclear, therefore it is interesting to know the details of transport mechanism and see if it is parasite specific mechanism. Like *P. falciparum*, after infection of erythrocytes, *B. divergens* also induces the uptake of several solutes which are not taken up by non-infected erythrocytes. We found that *B. divergens* infected erythrocytes show uptake of glutamate via a low-affinity transport system. The other important characteristics of the glutamate transport observed was Na^+-independency, non-saturability and non-stereo selectivity. There was also enhanced uptake of glutamate in the presence of choline. In conclusion, it appears that both parasites induce different mechanisms or transport systems for glutamate uptake and also the activation of the transport system is parasite specific.

1. Introduction

Malaria is a well known disease mainly of the tropics. It is an infectious disease caused by protozoan parasites of the phylum Apicomplexa. They are eukaryotic intracellular parasites of medical and veterinary relevance. *Plasmodium* and *Babesia* belonging to this phylum of Apicomplexa are characterized by the so called apical complex located at one pole of the cell. *P. falciparum* is the most virulent species that infect humans and are a cause of severe malaria. *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* are other *Plasmodium* species that also affect humans. In the case of *Babesia* over 100 species are known to infect different mammals (Spielman *et al*., 1985), but the two species which are found to infect humans (immunosuppressed) are *Babesia microti* and *Babesia divergens* (Fitzpatrick *et al*., 1968; Zintl *et al*., 2003).

Malaria is an endemic disease affecting humans in more than over 100 countries across the globe and territories in Africa, Asia, Latin America, the Middle East and the South Pacific. In 2010 there were reported 216 million episodes of malaria, 81% of which were in Africa. Also, 91% of the malaria deaths, out of a total of 655000, were in Africa and affected children under the age of 5 (WHO 2011).

1.1 The life cycle of *Plasmodium falciparum*

P. falciparum has a complex life cycle which involves two organisms, an insect vector and a vertebrate host (human). There are three phases in its life cycle: the pre-erythrocytic cycle, the erythrocytic cycle and the sporogonic cycle.

1.1.1 Pre-erythrocytic cycle – The Female *Anopheles* mosquito carries *Plasmodium* in the salivary glands and the gut. During the blood meal, the infected mosquito injects the parasite in its infectious form (sporozoite) into the blood stream. Once inside the human host these sporozoites invade the liver cells (hepatocytes). There, they undergo asexual reproduction and sporozoites develope into schizonts which contain ~ 40,000 merozoites (Khan *et al*., 1999).

1.1.2 Erythrocytic cycle – The Merozoites produced inside the liver cells are released into the blood stream, thereby invading the erythrocytes. Inside the erythrocytes, they undergo a trophic period with different morphological and metabolic stages: ring stage, trophozoite stage and schizont stage. During the trophozoite stage the parasite enlarges by consuming

hemoglobin. The active metabolism of hemoglobin includes indigestion and digestion of host cytoplasm and proteolysis of hemoglobin into amino acids. In the schizont stage the merozoite go through another round of asexual amplification, producing ~ 36 merozoites per schizont. When the schizont ruptures the merozoites released reinfect other red blood cells. The cycle of infection, multiplication and bursting of red blood cells continues.

1.1.3 Sporogonic cycle – Some of the merozoites inside the erythrocytes differentiate into male and female gametocytes, which are ingested by the mosquito during the blood meal. Within the mosquito midgut, the male gametocyte undergo nuclear division to produce microgametes that fertilize the female macrogamete, producing a zygote. The zygote develops into an oocyst which penetrates the mosquito´s midgut and rests on the exterior wall of the midgut. This oocyst grows and undergoes multiple round of asexual replication resulting in the production of sporozoites. These sporozoites are released into the hemocoel i.e. body cavity of the mosquito and later migrate to the salivary glands, thus completing the life cycle.

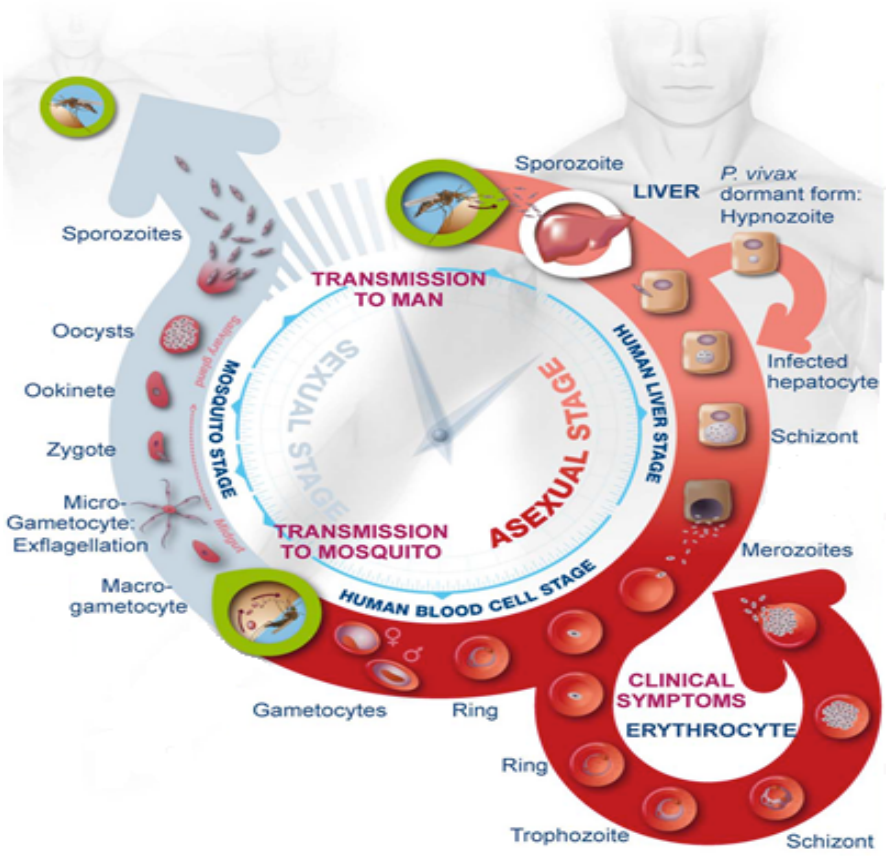


Figure 1.1: Schematic view of the life cycle of *Plasmodium falciparum*. Adapted from Delves *et al*., 2012

1.2 *Babesia divergens*, epidemiology and illness

The apicomplexan parasites of the genus *Babesia* are responsible for babesiosis an infectious disease causing malaria-like symptoms (hemolytic anemia, fever and chills) in wide variety of vertebrate hosts (Homer *et al*., 2000; Young *et al*., 1986). *Babesia* parasites are transmitted by a family of ticks (*Ixodus*), that mainly causes bovine babesiosis and human babesiosis. Bovine babesiosis is the most life threatning disease of the livestocks (eg. cattle) and is responsible for economical loss in the cattle industry, especially for tropical and sub-tropical regions of the world (Gray *et a*l., 2009). Human babesiosis occur in immunocompromised, typically splenectomized humans (Fitzpatrick *et al*., 1968; Zintl *et al*., 2003) were the parasite causes chronic disease (Herwaldt *et al*., 2011). The humans can be infected with *Babesia* by several ways, the main way is through the bite of an infected tick and the less common ways are by getting a blood transfusion from a donor or from an infected mother to her baby during pregnancy (Vial *et al*., 2006). Over the past 50 years, the research focus on babesiosis (bovine/human) has been escalated and more recognized in terms of severity from few isolated areas to global endemic areas (Vial *et al*., 2009; Lau 2009).

1.2.1 *Babesia divergens* life cycle

In contrast to *P. falciparum*, *Babesia* exclusively infects one type of cell inside the vertebrate host-the erythrocytes. The life cycle of *Babesia* can be divided into three stages: (i) Gamogony i.e. the sexual stage where the gamete differentiation and zygote formations occur inside the tick intestinal cells (ii) Sporogony i.e. the asexual division of the ookinetes (sporogonics) occurring in various tick organs, but the final differentiation of sporozoites occurs in salivary glands of the tick (iii) Merogony i.e. the asexual reproduction occurs in the erythrocytes of vertebrates resulting into merozoites and other dividing forms (Young *et al*., 1986; Vial *et al*., 2006).

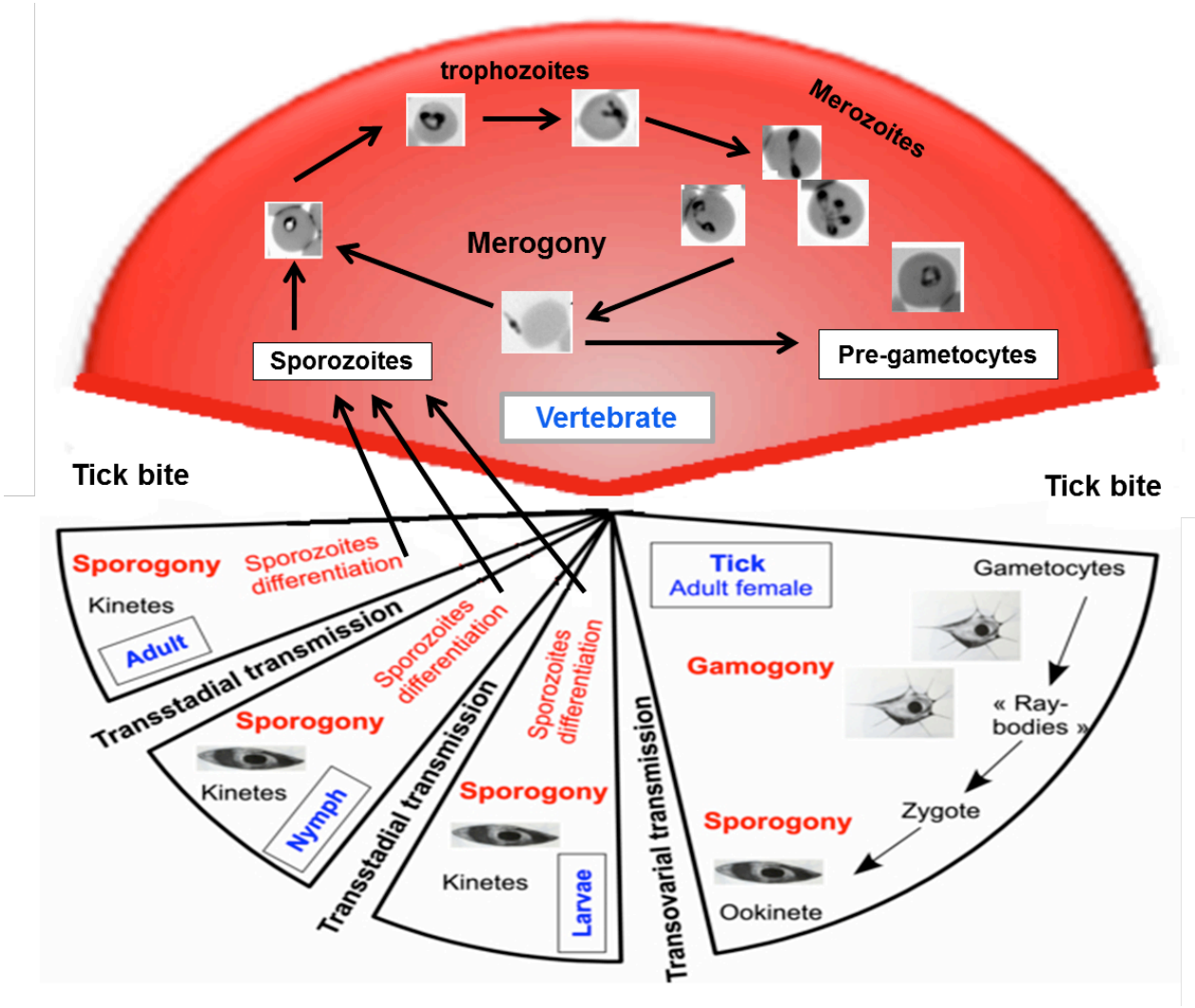


Figure 1.2: Schematic illustration of the life cycle of *Babesia* spp. Modified from Chauvin *et al*., 2009

After being bitten by an infected tick, the sporozoites directly target the erythrocytes and invade them, where the asexual division takes place. During the intraerythrocytic, asexual development cycle, trophozoite multiplies and forms two to four separate merozoites by binary fission (budding) (Mackenstedt *et al*., 1990). The rapid growth and multiplication leads to the destruction of the host cells, i.e. cell lysis, which leads to the spreading of the merozoites and reinfection of new cells. This one cycle of asynchronous, asexual parasitic growth takes about approximately 8-10 hours *in vitro* (Valentin *et al*., 1991). In the host bloodstream there is a reservoir of several developmental stages, all present at the same time point (Chauvin *et al*., 2009).

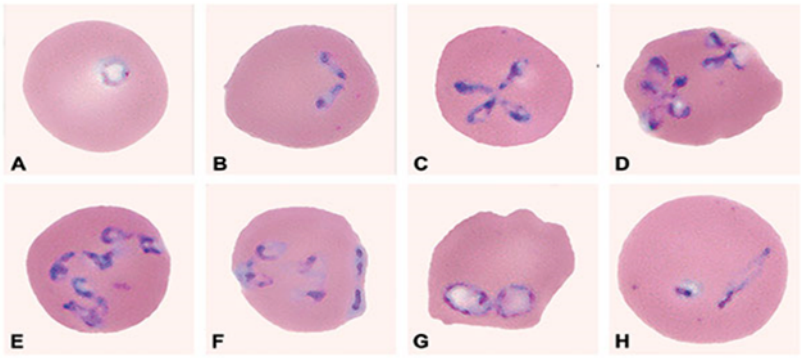


Figure 1.3: Intraerythrocytic forms of *B. divergens* inside bovine erythrocytes. Modified from Herwaldt *et al*., 2004 (A) ring-like trophozoite, (B) paired merozoites, (C) maltese-cross (tetrad), (D) and (E) multiple merozoites, (F) applique form on right border of the erythrocyte (G) and (H) degenerated forms.

A small proportion of the merozoites do not divide. Instead, they remain inside the erythrocytes to be ingested by a competent tick, inside which the sexual cycle begins again (Mackenstedt *et al*., 1990).

1.3 The Erythrocyte

The mammalian erythrocytes are biconcave, disk-shaped and are also flexible in nature. The diameter of these cells is 6-8 µm, thickness of 2-2.5 µm at the edges and thickness of 0.8-1µm at the center (Turgeon, 2004). The human RBCs are produced from the proliferation and differentiation of pluripotent cells during erythropoiesis in the bone marrow. These cells circulate for about 100-120 days in the blood before they are recycled by the macrophages in the spleen (Doohan, 2000). There are approximately 5 x 10^6 cells per microliter of blood.

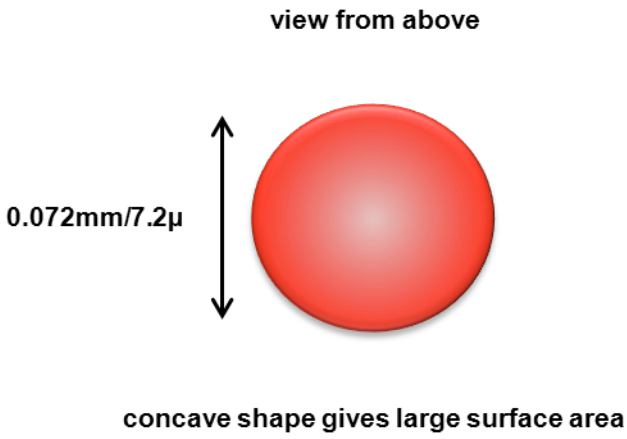


Figure 1.4: The human red blood cell.

Amongst the main function of erythrocytes is its remarkable ability to deform in order to pass through narrow capillaries in order to transport oxygen from the lungs to the tissues and organs and also supports carbon dioxide transport. The exchange of gases across the red blood cell membrane is highly dependent on the hemoglobin molecules present in the cells, cell membrane integrity and cell homeostasis.

The erythrocytes are non-nucleated cells (Yoshida *et al*., 2005) and these cells also lack other cellular organelles like endoplasmic reticulum, mitochondria and golgi bodies (Kundu *et al*., 2008; Mortensen *et al*., 2010). Thus, in mammalian erythrocytes, lack of organelles leave more room for the hemoglobin molecules and the absence of mitochondria also prevents use of oxygen for metabolic respiration, thereby increasing the efficiency of oxygen transport by erythrocytes. The differentiated erythrocytes are metabolically reduced, they lack genetic program, no synthesis of lipid and proteins and no protein transport and trafficking pathways (Schrier, 1985; Chasis *et al*., 1989). There is no phagocytosis and endocytosis in the mature erythrocytes (Burns *et al*., 1988; Zweig *et al*., 1979). The majority of vital biochemical pathways are absent in the erythrocytes but some prominent pathways which are still present are glycolysis of glucose followed by lactic acid production (Van Wijk *et al*., 2005), ATP synthesis (Miwa, 1983), glutathione synthesis (Raftos *et al*., 2006), and purine and pyrimidine metabolism (Sandberg *et al*., 1955). Thus the malaria parasite invades erythrocytes, which are terminally differentiated cells lacking subcellular organelles and many functions typical of the vast majority of cells. The erythrocytes also have limited nutrient supply requirements.

1.4 Host cell - 'Safe Heaven' for parasites

Intracellular protozoan parasites need a host cell for their survival and growth. Because of this obvious reason, the importance of a detailed understanding of the host cell environment has recently gained more attention and is being acknowledged in the field of parasitology research. The extend of exploitation and manipulation of the host cell function by protozoan parasites has been known for example *Theileria spp*. infected cells, where uncontrolled proliferation of host cells occur (Dobbelaere *et al*., 2004) and in Toxoplasma gondii infected cells, the microarray analysis highlights different regulatory and biosynthetic activities as compared to non infected cells (Blader *et al*., 2001). Therefore, host cell provides pathogens an ideal 'milieu' for the molecular interactions and development pathways which strives parasite toward successful survival. Hence host cell environment and composition should not be considered as a 'blackbox'.

From the cell biological point of view, it appears that the RBC lack all obligatory conditions such *de novo* protein and lipid synthesis (Schrier, 1985; Chasis *et al*., 1989) and many others for a parasite to survive. Despite of so many limitations, RBCs are a 'safe heaven' for the apicomplexan

parasites, *Plasmodium falciparum* and *Babesia divergens*. These parasites are protected from the host cell immune system, as there is no mechanism to represent these foreign antigens (Stanisic *et al*., 2013) and they also seclude themselves in a compartment termed as parasitophorous vacuole (PV). The parasitophorous vacuole surrounds the parasite and separates it from the host cell cytosol (Lingelbach and Joiner, 1998).

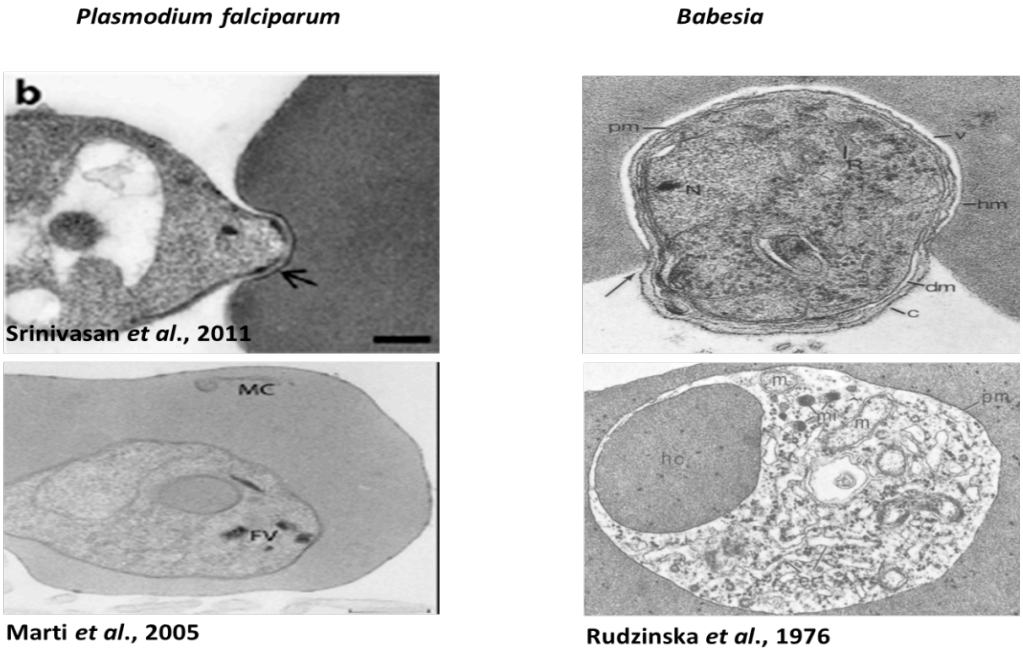


Figure 1.5: Ultramicroscopic structures of *Plasmodium falciparum* and *Babesia*. pm= plasma membrane, v= vacuole, N= nucleus, dm= double membranes, c= dense surface coat, hm= host membrane, R= rhoptry, m= mitochondria, mi= micronemes, hc= host cytoplasm, FV= food vacuole, MC= maurer's cleft.

The intracellular parasitism as a lifestyle of eukaryotic pathogens, may have co-adapted with human-race throughout our existence. Hence parasitic 'protists' display a broad range of diversity than their current hosts. And also protozoan parasites have selected intracellular lifestyle as obligatory unlike many bacterial intracellular parasites (Haas., 2009). The intracellular parasitism thereby puts a lot of challenges to the parasites to adapt to this lifestyle, and among the difficulties faced by the successful parasite are that they must find a way to enter the host cell and then escape from the host immune system. The different strategies of entry by parasites differ widely from injection through a hypodermic needle (eg. Microsporidia) (Yanji et al., 2005), active penetration of host cell (eg. *Toxoplasma*, *Plasmodium*, *Babesia*) (Morisaki *et al*., 1995; Farrow *et al*., 2011; Yokoyama *et al*., 2006), multiple endocytic pathways (eg. *Trypanosoma*) (Barrias *et al*., 2013) and host mediated phagocytosis (eg. *Leishmania*) (Shio *et al*., 2012). The resulting niches

are equally diverse, ranging from cytosolic (eg. *Theleria*, *Trypanosoma*), inside non fusigenic vacuole (eg. *Toxoplasma*) (Mordue *et al*., 1999), a phagolysosome (eg. *Leishmania*) (Shio *et al*., 2012) and residing within a vacuole called PVM (eg. *Toxoplasma, Plasmodium, Babesia*) (Lingelbach and Joiner, 1998). These adaptations of a parasite to survive influence directly its ability to access nutrients, interactions, metabolic and signaling pathways.

The group Apicomplexa (*Plasmodium* and *Babesia*), which plans to adapt a intracellular lifestyle and resides in a unique compartment termed 'PV' (Scholtyseck and Piekkarski, 1965) surrounded by 'PVM' is the topic of my research and through this I would like to answer questions like how they invade and survive in the same host cell (red blood cells), how they receive nutrition inside the nutrient deficient host cell, what are the modifications done by these parasites to the host cell membrane etc. It may be that each of these parasites which share same host cell, may serve as a partial model for each other.

1.5 Host cell invasion and compartmentation

The first logistical problem faced by merozoites is invasion of a cell which does not normally undergo phagocytosis. The *P. falciparum* merozoites during invasion undergo a series of steps including (i) recognition and attachment to the host cell, this takes place with the help of GPI-anchored membrane proteins present on the surface of the merozoites such as MSP-1 (merozoite surface protein-1) which plays an important role in the primary contact (Goel *et at*., 2003; Cowman *et al*., 2006) and AMA-1 (apical membrane protein-1) is another protein present in the apical organelles (micronemes) which is translocated to the surface before invasion (Bannister *et al*., 2003; Cowman *et al*., 2006) (ii) reorientation and junction formation, possible candidates for tight junction formation are the DBL (Duffy Binding Like) proteins family such as EBA-175 or EBA-140 (Miller *et al*., 2002) and the reticulocytes binding proteins homolog family (PfRh) (Duraisingh *et al*., 2003) and (iii) parasitophorous vacuole formation, the inward motion driven by actinomyosin motor helps parasite entry, followed by formation of PVM (Keeley *et al*., 2004) and finally resealing of the RBCs and parasitophorous vacuolar membrane (Cowmann *et al*., 2006).

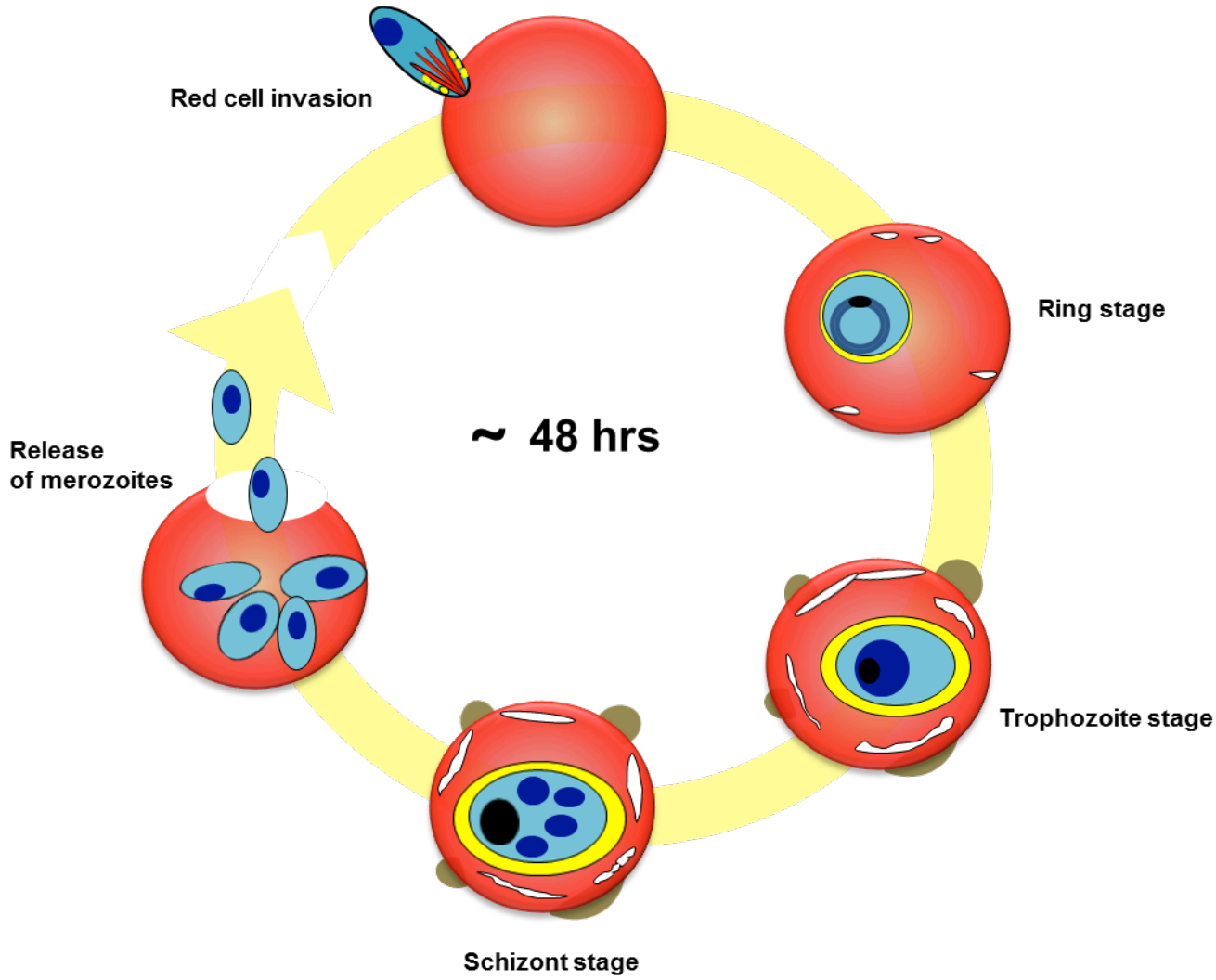


Figure 1.6: The different stages of *Plasmodium falciparum* development. Modified from Maier *et al*., 2009

In case of *Babesia* merozoites, surface proteins involved in host cell recognition and adhesion are Bd37 (Delbecq *et al*., 2008) and a protein family of variable merozoites surface antigens (VMSA) eg. MSA-1, MSA-2a1, MSA-2a2, MSA-2b in *B. bovis* (Florin-Christensen *et al*., 2002; Cowman *et al*., 1984). A homolog of AMA1 (BdAMA1) is present in the micronemes for invasion (Montero *et al*., 2009). Another important protein, which is involved in erythrocyte binding and parasitophorous vacuolar formation is RAP-1 (Rhoptry Associated Protein-1) (Sun Y *et al*., 2011).

Following the process of invasion, there is a discharge from the apical organelles (mainly rhoptries and micronemes) and the parasite is pushed by the force of an actinomyosin motor into the host cell, forming a parasitophorous vacuole (PV) around the parasites *P. falciparum* and *B. divergen*. The indentation of RBCM leads to the formation of PV surrounded by PVM, in which the parasite

reside (Soldati et al., 2004). The PVM has a large surface area of 30-33 μm^2 and its *de novo* formation takes 10-20 seconds (Suss-Toby *et al*., 1996; Lingelbach *et al*., 1998).

1.6 Origin, Composition and Function of PVM

The PV is a membrane bound vacuole that surrounds a symbiont organism inside a host cell, like any other Apicomplexan parasites (including *Toxoplasma*) *Plasmodium* and *Babesia*, are intracellular, obligatory parasites which reside, differentiate and multiply inside PV. This PV is surrounded by PVM which forms an interface between the host cell cytosol and parasite. It expands as the parasite grows in size inside the erythrocyte, it prevents acidification of proteins present in the parasitophorous vacuole (PV), prevents access of host cell proteins into the vacuolar lumen and it forms an important barrier and act as a molecular sieve for the transport of selective solutes of approximately 600 Da between the host cell and the parasite (Desai and Rosenberg., 1997; Nyalwidhe *et al*., 2002; Schwab *et al*., 1994). These unique biological properties of PVM attract interest of many research groups to look into the molecular basis of PVM biogenesis, maintenance and functions. There are different hypothesis behind the formation of PVM, but the exact mechanism is still unknown. The two main models accepted for the formation of PVM are bilayer insertion model and induced invagination. In the former model during invasion apical organelles, micronemes and rhoptry, of the parasite secrete lipids and insert into the host cell membrane, which form PVM (Bannister *et al*., 1990). Therefore, the evidence in support of the bilayer insertion model is the absence of the host cell proteins from the PVM (Atkinson *et al*., 1987; Dluzewski *et al*., 1989; Aikawa *et al*., 1981; McLaren *et al*., 1979). In the latter model invagination during invasion occurs in erythrocyte membrane followed by insertion of lipids from the erythrocyte membrane into newly formed PVM (Ward *et al*., 1993; Pouvelle *et al*., 1994).

1.6.1 Formation and maintenance of the parasitophorous vacuole membrane (PVM)

The biogenesis and maintainence of PVM is prerequisite for a parasite survival and to achieve this aim different parasites adapt different mechanisms. As in case of Trypanosoma, the complex process is dependent on many molecules from the host cell such as components of endolysosomal system and also cytoskeleton (microtubules, kinesins, actin filaments) (De Souza *et al*., 2010). In case of Plasmodium and Babesia the formation of PVM is a controversial but fascinating issue of cell biology, with little known about the actual process involved. The contribution of erythrocyte lipids and proteins in the composition of PVM is a matter of debate (Ward *et al*., 1993; Dluzewski *et al*., 1995; Susstoby *et al*., 1996), as the complexity to dissect individual pathways leading to their incorporation on the parasite surface is multiplex. In context to the lipids composition of the PVM previous studies showed that, the parasites apical organelles, micronemes and rhoptry, might

contribute some part in the formation of the PVM (Aikakawa *et al*., 1981; Bannister *et al*., 1990; Dluzewski *et al*., 1995; Joiner *et al*., 1991); there may be a bulk contribution of erythrocyte membrane lipids in the PVM formation (Ward *et al*., 1993; Pouvelle *et al*., 1994) and also contribution of lipids from both the parasite and the erythrocyte membrane (Lingelbach *et al*., 1998). But unlike lipids, earlier studies showed no contribution of erythrocyte proteins in the parasitophorous vacuolar formation in *P. falciparum* infected erythrocytes (Atkinson *et al*., 1987; Dluzewski *et al*., 1989; Aikawa *et al*., 1981; McLaren *et al*., 1977). In similar studies performed on the related apicomplexan parasite *Toxoplasma gondii*, during invasion it selectively excludes the internalization of host cell proteins into the vacuole formation (Mordue *et al*., 1999). However, according to the recent studies completed in the parasite *P. falciparum* on the internalization of the host cell proteins in PVM formation, shows the insertion of some apparent host cell membrane proteins into PVM. And also the recent identification of microdomains in the plasma membrane of eukaryotic cells, including mammalian RBC (Lingwood and Simons, 2010) has given interesting leads to re-examine the PVM composition in *P. falciparum* infected erythrocytes (Murphy *et al*., 2004). Some examples of microdomains associated proteins, found to be internalized are GPI-anchored proteins, heteromeric G protein alpha subunit (Gs) and the membrane spanning protein (Duffy) (Lauer *et al*., 2000; Murphy *et al*., 2004; Samuel *et al*., 2001). Bietz and colleagues have also found that human aquaporin 3, which is an abundant plasma membrane protein of erythrocytes, is internalized into *P. falciparum* infected erythrocytes, presumably during or soon after the invasion (Bietz *et al*., 2009).

As the parasites surface area grows in size, there is also growth in PVM to support the expanding parasite. Among the other changes, there is an increase in the dynamic network of tubulovesicular membranes (TVM) (Lauer *et al*., 1997) which extend into the host cell cytoplasm. This rapidly growing parasite is in need for large amounts of lipids and proteins. Such high demands are satisfied by *de novo* synthesis of lipids (Raph *et al*., 2004; Déchamps *et al*., 2010) and protein (Hussain *et al*., 2015) in parasites. These membranes also participate in the transfer of parasite proteins to the host cell membrane by ATP-dependent protein translocation (Ansorge *et al*., 1996) and through a translocation machine called PTEX (*P. falciparum* translocon of exported proteins) (De Koning-Ward *et al*., 2009). Among the known proteins being transferred to the erythrocyte surface are PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), a member of variant protein family (Desai *et al*., 2014). There are also a no. of secreted proteins which remain within the PV (Nyalwidhe *et al*., 2006). Thus, all these events play an important role in malaria virulence.

1.6.2 Biogenesis of PV through specific entry sites on erythrocyte membrane

Most apicomplexan parasites invade and enter their host cell in a rapid process which takes place less than a minute, without major modifications of the host cell surface. Thus the entry of a parasite

within a fraction of time, must be by some specific or preferred sites, in order to allow easy and fast entry. Also the entry of the parasite into the cell is an important milestone in the development of the infection. Thus, the infection by *Plasmodium falciparum* of the erythrocytes leads to complex mechanisms such as membrane signaling and sorting of different molecules (Murphy *et al*., 2006; Di Girolamo *et al*., 2008) in order to enter and survive. The insights into the host molecular determinants that participate in the vacuolar formation are recently studied, identified and characterized as cholesterol rich detergent resistant membranes (DRMs) or lipid rafts, which provide concentrated microenviorment for signaling pathways (Murphy *et al*., 2006; Samuel *et al*., 2001). The lipid rafts are submicron (70-200 nm) clusters or concentrated 'islands' of proteins and lipids held together with high levels of cholesterol (Simons *et al*., 1997; Brown *et al*., 1998). They are found in abundance in the plasma membrane of various cells and are also found in exocytic and endocytic compartments (Dupree *et al*., 1993; Gagescu *et al*., 2000). These are mobile, dynamic entities that move laterally along the plane of the plasma membrane and traffic continuously between the plasma membrane and internal compartments (Nichols *et al*., 2001). Many molecular players are involved in response to specific stimuli during the internalization of lipid rafts in cells, important molecules are such as GPI-anchored proteins, ligands eg. folic acid, growth hormones and also various other pathogens like cholera toxin and virses eg. SV40 and HIV (Parton *et al*., 2003).

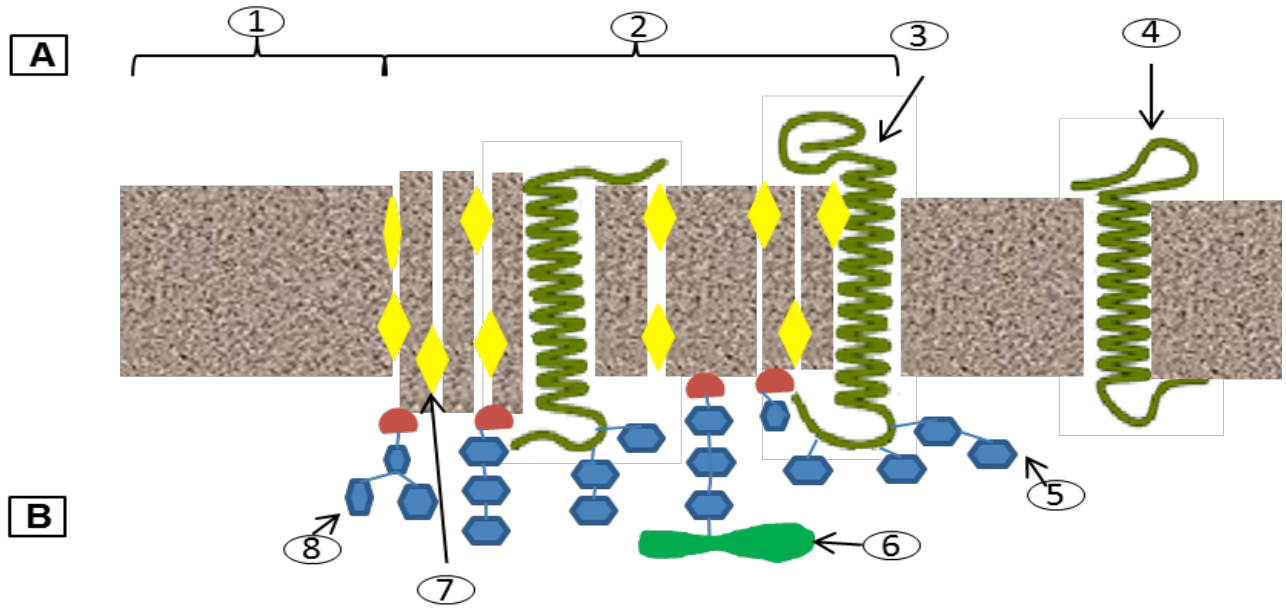


Figure 1.7: Structure of Lipid Rafts. Modified from Simons *et al*., 1997.

A = Intracellular space or cytosol; **B** = Extracellular space; **1 =** Non-raft membrane; **2 =** Lipid raft; **3** = Lipid raft associated transmembrane protein; **4** = Non-raft membrane protein; **5** = Glycosylation modifications; **6 =** GPI-anchored protein; **7** = Cholesterol; **8** = Glycolipid

In the past, studies have characterized the major proteins present in the lipid rafts and their role in parasite invasion of the erythrocytes. Murphy and Samuel have found some erythrocyte proteins associated with the lipid rafts (Murphy *et al*., 2006; Samuel *et al*., 2001), and some of which are found to be internalized during vacuole formation in *P. falciparum* infected erythrocytes like CD 59 (an inhibitor for complement lysis), scramblase (allow phospholipids movement in membranes) etc.

There are reports present showing that there is involvement of lipid raft proteins in the induction and formation of the PVM in *Plasmodium falciparum* infected erythrocytes. Harrison and colleagues have shown that lipid raft proteins, like erythrocyte adrenergic receptor (β_2-AR) and guanine nucleotide binding proteins (Gαs) mediates parasite entry into the erythrocytes (Harrison *et al*., 2003). There are also major other lipid rafts proteins such as flotillin-1 and flotillin-2 (Salzer *et al*., 2001; Murphy *et al*., 2004) and multiple spanning membrane water transport proteins namely the aquaporins 1 and 3 have been found to be recruited in the vacuolar membrane (Murphy *et al*., 2004; Bietz *et al*., 2009). In contrast, major erythrocyte proteins such as Band 3, glycophorins and spectrin are excluded from the PVM in *Plasmodium falciparum* infected erythrocytes whereas recently it has been shown that *B. divergence* infected erythrocytes internalize Band 3, spectrin and glycophorin into the PVM (Repnik *et al*., 2015). Therefore, in vacuolar formation, lipid rafts proteins play an important role. It is unclear whether the insertion of these lipid rafts proteins into the newly formed PVM is a selective or specific process.

Thus, the biological questions about entry through specific sites on the host cell have no precedence. Recent research has shed light on the specific host cell receptors which allow entry of the parasite into the host cell. The presence of two types of groups on the host cell surface (1) sialic acid-dependent (SA-dependent) entry sites, which bind to EBA (erythrocyte binding antigens) and PfRH (*P. falciparum* reticulocyte binding homology) (Camus *et al*., 1985) and (2) sialic acid-independent (SA-independent) sites which interacts with PfRH (Persson *et al*., 2008). And other increasing evidence comes from the erythrocyte G-protein and heteromeric guanine nucleotide binding regulatory proteins (Harrison *et al*., 2003) present in the microdomains of the host cell. However, as non infected erythrocytes do not undergo phagocytosis or pinocytosis, host cell pathways may not be involved in the parasite entry. Hence, how a parasite invades a tightly fixed cell in a fraction of time is unresolved mystery.

In this study I tried to explore the involvement of detergent resistance rafts (lipid rafts) as preferred entry sites for the parasite. In the past reports have shown that GM1 and cholesterol are characteristics of one type of lipid rafts (Gomez-Mouton *et al*., 2001; Janich *et al*., 2007; Fujita *et al*., 2007). A molecule of GM1, is a ganglioside which contain one sialic acid residue.

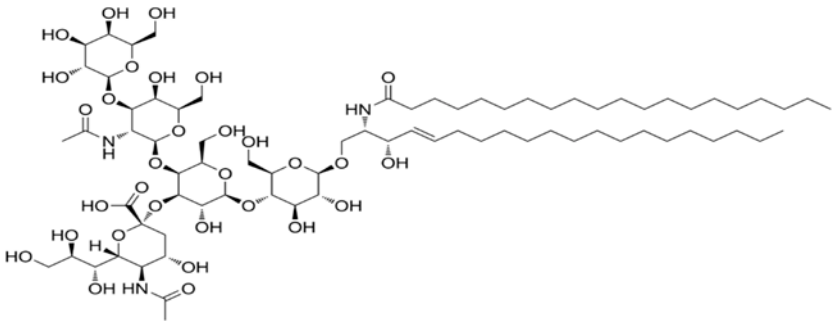


Figure 1.8: Strucure of GM1.

In mammalian cells, binding of the GM1-ganglioside as a receptor for the B subunit of cholera toxin is used as a strategy to initiate toxin action by uptake and delivery. In the first step of a cell intoxication B subunit of cholera toxin binds to GM1, which then helps the toxin (Ctx) to penetrate the cell membrane and further triggering uptake and delivery of toxin subunit A into the cells (Aman *et al*., 2001). Hagerstrand and collegues have reported accumulation of GM1 by cholera toxin subunit B plus anti-CTB on the surface of the erythrocytes, resulting in 40-60 GM1 patches (Mrowczynska *et al*., 2008). The cholera toxin association with GM1 is a 2-fingered grip: the Gal(β1-3)GalNAc “forefinger” is inserted into a deep pocket of the binding site, while the sialic acid “thumb” occupies a shallower depression on the surface of the toxin (Merritt *et al*., 1994).

1.7 Nutrient acquisition

The rapidly growing parasite exhibits a high metabolic rate and is in large demand for molecular metabolites. Thus, the parasites which choose erythrocyte with its sluggish metabolism and limited metabolic capabilities poses problems in front of the actively growing parasite. Therefore, the intracellular parasites in principle can take nutrients from two different sources: the host cell cytoplasm and the extracellular medium. *P. falciparum* ingests approximately 65% of the host cell hemoglobin into a lysosome like compartment called the food vacuole. The proteome analysis of the food vacuole has shed light on the proteins associated with PVM (Lamarque *et al*., 2008). Therefore, the growing parasite serves its needs for essential amino acids from hemoglobin and this also helps in maintaining proper osmotic balance for the expanding parasite. The infected erythrocytes also possess

increased membrane permeability to low molecular weight solutes as compared to non infected erythrocytes. The increased permeability rate can be represented either by activation or involvement of endogenous erythrocytes transporters or by formation of new permeation pathways (NPP) after infection (Kirk. 2004). The nutrients also need to cross the PVM and the parasite plasma membrane. A pore permeable to molecules upto size 1400 Da has been found on the PVM (Desai *et al*., 1997) of *P. falciparum* infected erythrocytes. The evidences from *Toxoplasma* also show similar results, a presence of a pore on the PVM which allow transport of molecules upto 1200 Da (Schwab *et al*., 1994). Therefore,the results show that PVM acts as a high capacity, low affinity molecular sieve, transporting molecules from the host cell to the PV and finally into the parasite through parasite transporters at the PPM (Schwab *et al*., 1994; Desai *et al*., 1997).

1.7.1 Erythrocyte transporters and channels

The erythrocyte plasma membrane is composed of lipids and proteins, which help the erythrocytes to achieve important functions like transport of oxygen to the tissues and also to maintain structural stability while traversing in the circulatory system.

An important property of a cell is to maintain and establish an environment different from the extracellular medium. In order to achieve cell homeostasis the erythrocytes are endowed with a variety of different membrane transporters and channels that play a key role in erythrocytes physiology. For example:

- glucose transporter (GLUT1) - which provides the erythrocytes with primary energy substrate (Tanner, 1993).
- lactate transporter - monocarboxylate transport protein (MCT1) which transports lactate and other monocaboxylate anions (Poole *et al*., 1993; Deuticke *et al*., 1982; Koho *et al*., 2006, 2008).
- aquaporins (AQP) - are water selective pores (Agre, 2006; Roudier *et al*., 1998).
- Band 3 (AE1) is a member of anion transporters family, which carry out Cl^-/HCO_3^- exchange (Cabantchik *et al*., 1972; Tanner, 1993).

According to the `pump-leak´ concept (Tosteson *et al*., 1960) the colloidosmotic swelling and bursting of the erythrocytes is prevented by very low membrane permeability to cations, the pumps maintaining this function are:

- Na^+ / K^+-ATPase which pumps Na^+ out and K^+ into the cells (Lee *et al*., 1988).

- Ca^{2+}-ATPase maintains Ca^{2+} level inside the erytrocytes cytosole by extruding Ca^{2+} (Lew *et al*., 1982; Schatzmann *et al*., 1983; Carafoli *et al*., 1987).
- Na^{+} / K^{+} / $2Cl^{-}$ pumps sodium, potassium and chloride into and out of the cells (Haas *et al*., 1989) and Na^{+} / H^{+} exchanger (Semplicini *et al*., 1989) which efflux hydrogen ion and influx sodium ion.
- Calcium-activated potassium channels- maintains ionic distribution across the red blood cells membrane (Gardos, 1958; Brugnara *et al*., 1987; Berhardt *et al*., 1991) and K(+)-specific membrane proteins (the Gárdos channels) are responsible for the phenomenon called the Gárdos effect.

The infection by *P. falciparum* with genome size of 23 megabase and encoding 5300 genes leads to interesting modifications in the host cell physiology. The genome analysis has predicted that there are limited amount of major membrane transporters like ATP-binding cassette family (ABC), major facilitator superfamily (MFS) and amio acid/ polyamine/ choline (APC) family, encoded approximately 31% in *P. falciaparum* infected erythrocytes as compared to other eukaryotes (Gardner *et al*., 2002). There are four main classes of transporters present in eukaryotes based on the transport and energy coupling mechanism, molecular phylogeny and substrate specificity and this is termed as transporter classification system (TC) which resembles the enzyme commission system (EC) (Paulsen *et al*., 2000).

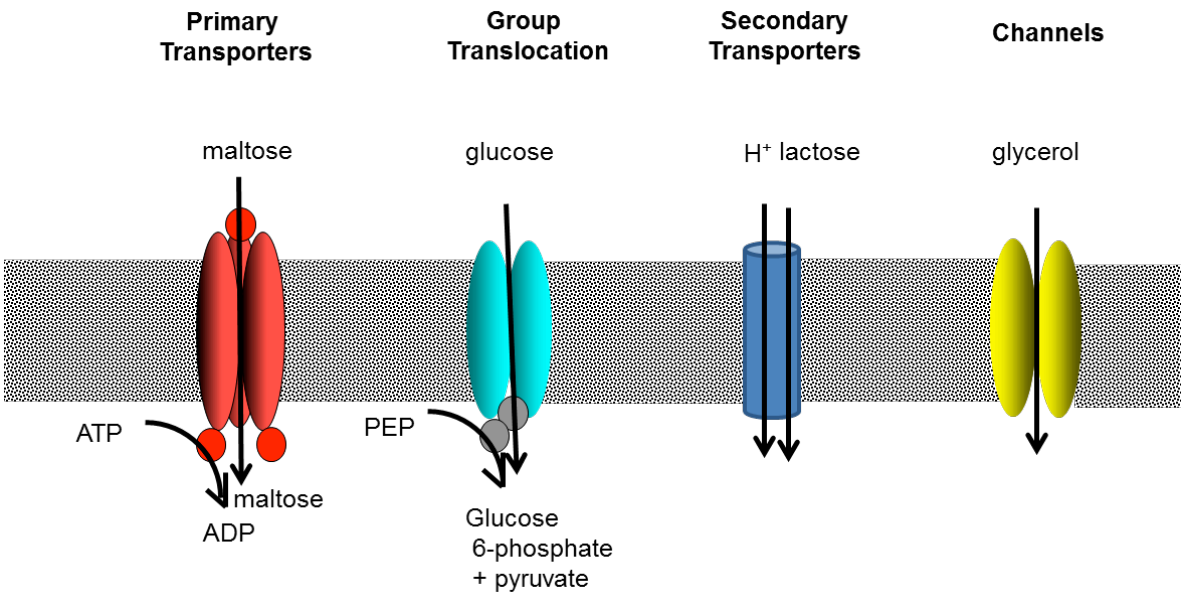


Figure 1.9: The four main classes of transporters present in eukaryotes based on the transport and energy coupling mechanism, molecular phylogeny and substrate specificity. Modified from Paulsen *et al*., 2000.

The different transporters encoded by *P. falciparum* are glucose/proton symporter (Woodrow *et al*., 2000), water/glycerol channel (Hansen *et al*., 2002), lactate and pyruvate proton symporter (Elliott *et al*., 2001) and also nucleotide/nucleobase transporter (Rager *et al*., 2001). Several other transporters for inorganic ions, drugs and hydrophobic compounds are also encoded (McIntosh *et al*., 2001). No obvious amino acid transporters has been found to be encoded, which thereby emphasize the importance of hemoglobin digestion and uptake from extracellular medium, as a source of amino acid for parasite growth. Therefore, soon after infection by the malaria parasite the erythrocytes undergo many modifications of host membrane transport both physiologically and chemically. The most important change analysed by patch clamp (Deasi *et al*., 2000; Staines *et al*., 2007) and by the uptake of labelled tracer molecules (Saliba *et al*., 1998), is the permeability of the erythrocyte membrane and therefore, appearance of New Permeability pathways (NPP) in the infected cells as compared to non infected cells.

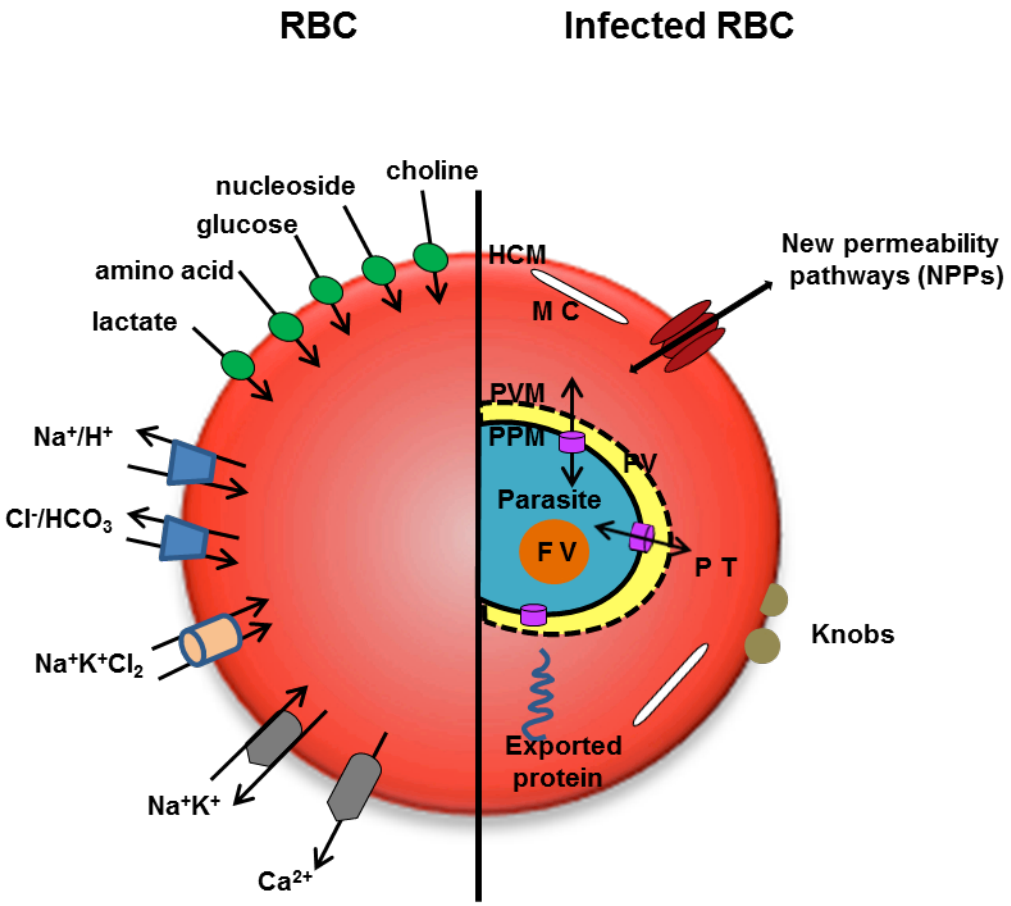


Figure 1.10: Transport systems in non-infected and infected red blood cell.

1.8 The New permeability pathways (NPP)

1.8.1 In *Plasmodium falciparum*

After infection of the human erythrocytes by the malaria parasite *Plasmodium falciparum*, the parasite, is faced to the nutrient poor environment and thus make some adaptations such as (a) it degrades hemoglobin and (b) increases the permeability of the cell for several solutes. As the parasite grows, it ingests and degrades up to 75% of the host cell hemoglobin (Loria *et al*., 1999) and less than 20% of the amino acids derived from the degradation are utilized by the parasite for protein synthesis (Krugliak *et al*., 2002). The catalysis of hemoglobin take place inside the food vacuole (Ragheb *et al*., 2011). Even though hemoglobin is the main source of amino acids for the parasite, it does not contain isoleucine (Kirk, 2001), which is an essential amino acid for the parasite and the parasite is dependent on the uptake of isoleucine from the extracellular medium. The other 'dialyzable' solutes such as glucose, hypoxanthine (purine), pantothenic acid (vitamin), glutamate, methionine, cysteine or proline (amino acids) are also obtained from the extracellular medium by the parasite (Kirk *et al*., 2007), in comparison to the other amino acids which are available in sufficient amount after digestion of hemoglobin by the parasite (Liu *et al*., 2006). There is a list of known NPP solutes mentioned in Table 1.3.

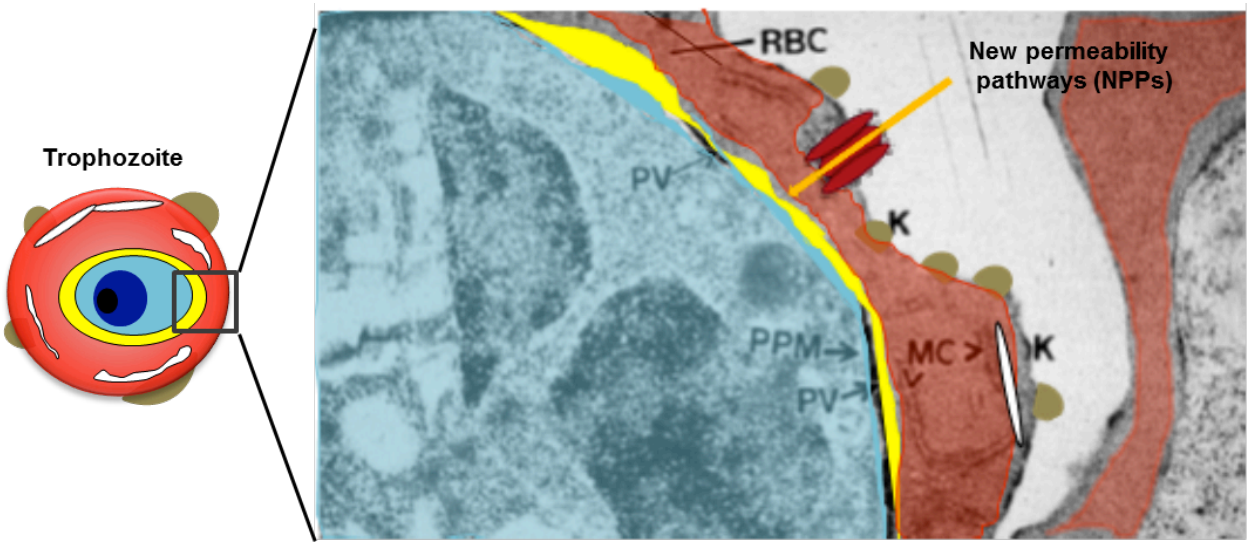


Figure 1.11: The new permeability pathways in the trophozoite stage of the parasite. Modified from Baumeister *et al*., 2010. PV = parasitophorous vacuole, PPM = parasite plasma membrane, MC = maurer's cleft, K = knobs, RBC = red blood cell.

In order to acquire several nutrients from the extracellular medium the parasite modifies the host cell membrane permeability. These modifications appear after 10-20 hours, post

infection (Staines *et al*., 1998). The profound changes found in the erythrocyte membrane leads to an increase in its permeability to a diverse range of low molecular weight solutes such as amino acids (Ginsburg *et al*., 1985; Kirk *et al*., 1994; Erfold *et al*., 1985., Martin *et al*., 2007), peptides (Ginsburg *et al*., 1985; Atamma *et al*., 1997), sugars (Ginsburg *et al*., 1983 and 1985; Kirk *et al*., 1994 and 1996), nucleosides (Upston *et al*., 1995; Gati *et al*., 1990), pantothenic acid (Saliba *et al*., 1998), choline (Kirk *et al*., 1991b; Staines *et al*., 1998) and different anions and cations (Staines *et al*., 2000; Crammer *et al*., 1995). The uptake of solutes is a result of NPP (new permeability pathways) present in the infected erythrocytes (Ginsburg *et al*., 1983; Kirk *et al*., 1999). The flux by NPP can be blocked by different reagents, the most potent reagents known are, NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) and furosemide (Kirk *et al*., 1994). These pathways have broad specificity and selective for anions over cations (Kirk *et al*., 1994) and also their rate of influx of different solutes, depends on size and hydrophobicity for different solutes (Kirk *et al*., 1994). Table 1.1 lists some of the known solutes which permeate through NPP.

In addition to its role in nutrient uptake, NPP also performs other physiological roles: (a) facilitating disposal of some metabolic wastes such as lactate (Kanaani *et al*., 1991; Cranmer *et al*., 1995) and oxidized glutathione (Atamna *et al*., 1997) from the malaria infected cells (b) helping in volume regulation because of the colloid osmotic pressure (Staines *et al*., 1999) and (c) maintaining ion balance of the host cell (Ginsburg *et al*., 1986; Kirk *et al*., 1994).

Table 1.1: Solutes known to permeate through the NPP. Adapted from Baumeister *et al*., 2010.

Carbohydrates	Amino acids	Nucleotides	Anions/Cations	Others
Glycerol	Alanine	D/L-adenosine	Cl^-	Thiourea
Erythrytol	Asparagine	D/L-thymidine	Gluconate	Phloridzin
D/L-arabitol	Cysteine	Uridine	Lactate	di- and tri-peptides
Arabinose	Cysteine	NBMPR	DNDS	Glutathione
Ribose	Glutamate		Dipicolinic acid	GSSG
Sorbitol	Glycine		Pantothenic acid	Polypeptides
Xylitol	Isoleucine			Oligonucleotides
Mannitol	Leucine		Na^+, K^+, Rb^+	Iron chelators
D/L-glucose	NBD-taurine		Mg2+, Ca2+	Sulfo-NHS-Biotin
2-deoxyglucose	etc		Fe2+/Fe3+	
etc			choline	

1.8.2 Different models for nutrient acquisition in *P. falciparum* infected RBCs

There are various models proposed to explain how the parasite takes up the nutrients from the extracellular space. The most accepted three models known for the nutrient acquisition are: (1) Sequential uptake model (Kirk, 2001; Baumeister *et al*., 2003) in which the solutes move from the extracellular medium to the parasites via the erythrocyte cytosol, thus crossing the red blood cell membrane (RBCM), the parasitophorous membrane (PVM) and the parasite plasma membrane (PPM) (2) the parasitophorous duct model (Pouvelle *et al*., 1991) shows a continuous duct connecting the external medium with the parasite surface, thus allowing free access of the solutes to the parasite and (3) the metabolic window model (Lauer *et al*., 1997) shows tubular shaped specialized junctions that extends from the parasite vacuolar membrane to the periphery of the red blood cell membrane and across which exchange of the solutes can take place.

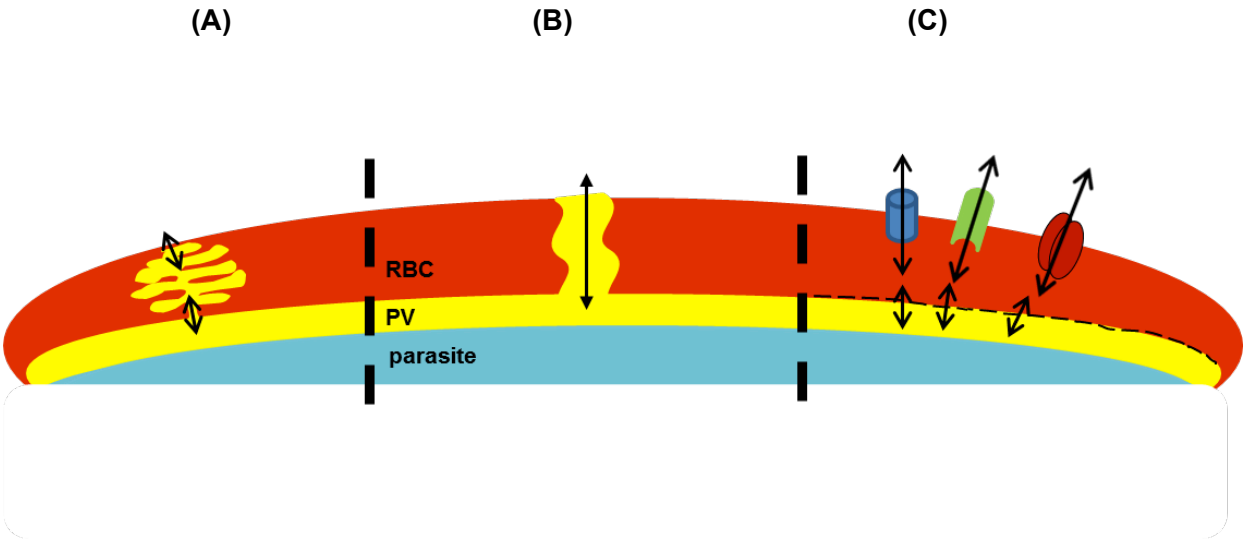


Figure 1.12: Different models for nutrient acquisition. (A) Metabolic window model (Lauer *et al*., 1997) (B) Parasitophorous duct model (Pouvelle *et al*., 1991) (C) Sequential uptake model (Baumeister *et al*., 2003; Staines *et al*., 2007)

1.8.3 In *Babesia*

There are less reports present about membrane permeability of *Babesia*-infected erythrocytes to nutrients from the extracellular medium. In some of the previous studies uptake of D- and L-glucose by *Babesia rodhaini* infected mouse erythrocytes and also by *Babesia microti* infected mouse erythrocytes has been observed (Ohmori *et al*., 2004; Homewood *et al*., 1974; Momen, 1979). Similarly, Gero and his colleagues have shown the

uptake of nucleosides in *Babesia bovis* infected bovine erythrocytes (Gero, 1989). In the past, a study conducted on *B. rodhaini* infected rat erythrocytes utilizing glucose and nucleoside, showed that uptake of glucose was inhibited by the addition of nucleoside to the basal medium, apparently by competitive inhibition (Barry, 1982). Recently, studies conducted by Alkhalil and colleagues have characteriszed the permeability alterations in *Babesia* infected erythrocytes. They compared *Babesia* and *Plasmodium* and showed increased host erythrocyte permeability through distinct mechanisms (Alkhalil *et al*., 2007). Their group performed uptake of some solutes such as sorbitol, methionine, proline, serine, D-alanine, uridine, NHS biotin derivatives into *Babesia divergens* infected erythrocytes (Alkhalil *et al*., 2007). Another interesting study was conducted by Baumeister and colleagues, they observed uptake of a drug Fosmidomycin into *B. divergens* infected erythrocytes (Baumeister *et al*., 2011) which has no metabolic relevance. Recent genome analysis of *Babesia*, describe that there is presence of variant antigen proteins on the *Babesia* infected cell surface (Jackson *et al*., 2014) and their transport mechanism are less complex as compared to *Plasmodium* infected cells. *Babesia* also modifies its host cell, but to a lesser extent as compared to *Plasmodium*.

1.9 The nature and origin of the NPP - Host or parasite derived?

While there is a general consensus with respect to the sequential transport model (i.e. transport of the solutes across the erythrocyte membrane, the PVM, and finally the parasite plasma membrane), the nature of the proteins mediating the transport across the erythrocyte membrane is a matter of debate (Ginsburg *et al*., 2004; Staines *et al*., 2007). Presently the reports from the genomics and proteomics studies of the erythrocytes also do not provide us with any useful clues for the composition of these pathways. Some groups have reported the involvement of endogenous host cell membrane transporters or channels in the formation of NPP, such as the increase in the ion conductance of the non-infected erythrocytes by the exposure to the external stimuli or oxidative stress (Huber *et al*., 2002), effect by serum components (Staines *et al*., 2003), by cytosolic protein kinase A (Egee *et al*., 2002; Merckx *et al*., 2009; Staines *et al*., 2003) and by extracellular ATP (Huber, 2012; Tanneur *et al*., 2006). Bouyer and colleagues found that after infection endogenous host cell membrane channels like VDAC (voltage dependent anion channel)/PBR (peripheral type benzodiazepine receptor) play an important role in the formation of NPP (Bouyer *et al*., 2011). Also reports are present for the activation of endogenous host cell membrane transporters (glutamate transporter) after infection by *P. falciparum* (Winterberg *et al*., 2012) and there are also evidences for the involvement of parasite encoded proteins in the formation of NPP like PSAC (plasmodial surface anion channel) (Desai, 2012).

However, the true nature of NPP remains an enigma. There are several hypotheses to explain the increased permeability of the host erythrocyte after infection. In the figure 1.1 some of the possible reasons are explained such as: activation of native proteins (endogenous host transporter) after infection by the parasite (Huber *et al*., 2002; Decherf *et al*., 2004; Duranton *et al*., 2002; Verloo *et al*., 2004); Egee *et al*., 2002), parasite causing changes in the host endogenous transporter by insertion of parasite-derived polypeptides (Ginsburg *et al*., 1987), parasite inserts a pump/channel through the host cell membrane (Marchesini *et al*., 2005) and there might be fluidization of membrane lipids which causes leak of the solutes across membrane (Taraschi *et al*., 1986; Ramya *et al*., 2002).

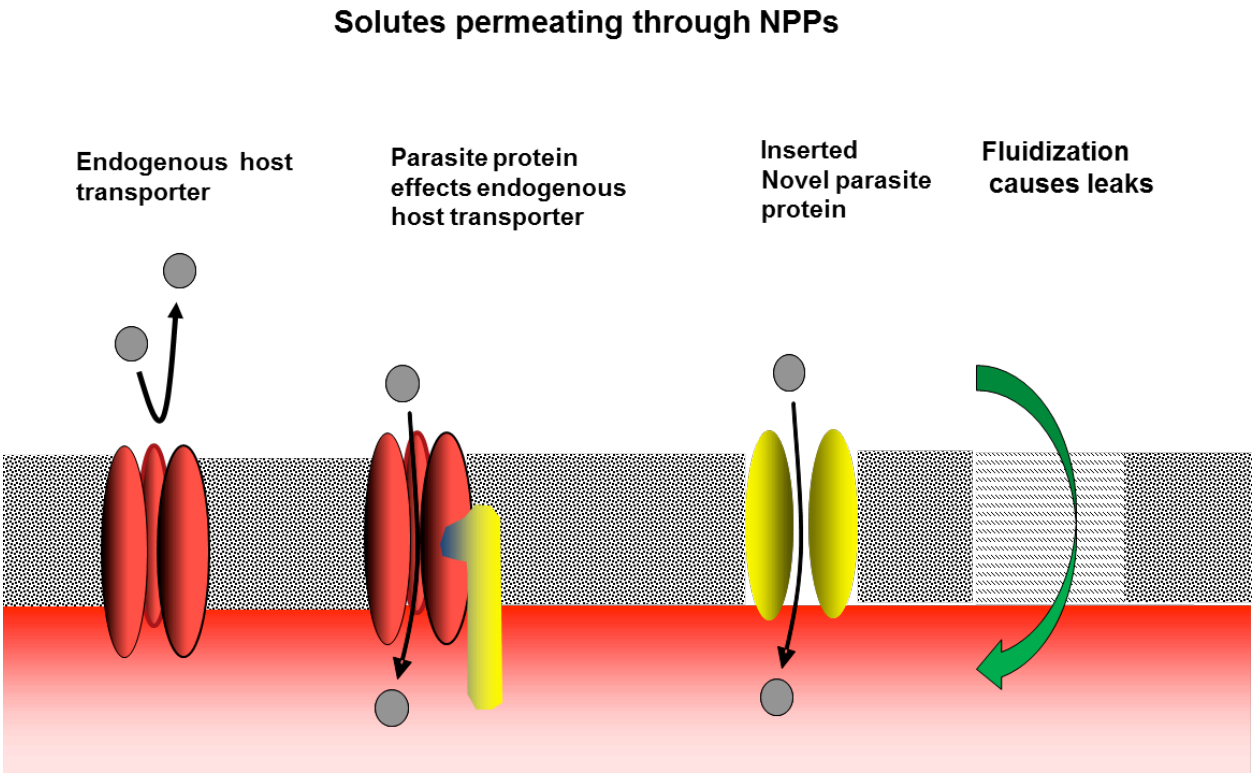


Figure 1.13: Possible reasons for increased permeability of the host cell.

1.10 Origin of NPP

The origin and activation of solute transport proteins within the erythrocyte membrane may be by: (a) single transporter present in the erythrocyte membrane, possibly encoded by the parasite, which is responsible for the uptake of all solutes for which the membrane of the non-infected erythrocyte is impermeable like plasmodial surface anion channel (PSAC) (Alkhalil *et al*., 2004), (b) The phenomenon of NPP is mediated by at least four host encoded

and the endogenous channel proteins (three anion channels and one cation channel), and the activation of these quiescent channels occurs via infection induced oxidative stress (Huber *et al*., 2002a), (c) The NPP constitute of three anion channels, one of which is parasite encoded and the other two are host encoded transport proteins whose activation occurs via phosphorylation (Egee *et al*., 2002; Merckx *et al*., 2008), (d) An infection induced CFTR (cystic fibrosis transmembrane conductance regulator) dependent anion transport is proposed which is not essential for the parasite survival. All other solutes gain access to the host cell cytosol via a separate channel/pore (Verloo *et al*., 2004) and (e) presence of two distinct pores both of which are parasite encoded might be responsible for NPP (Ginsburg *et al*., 2004).

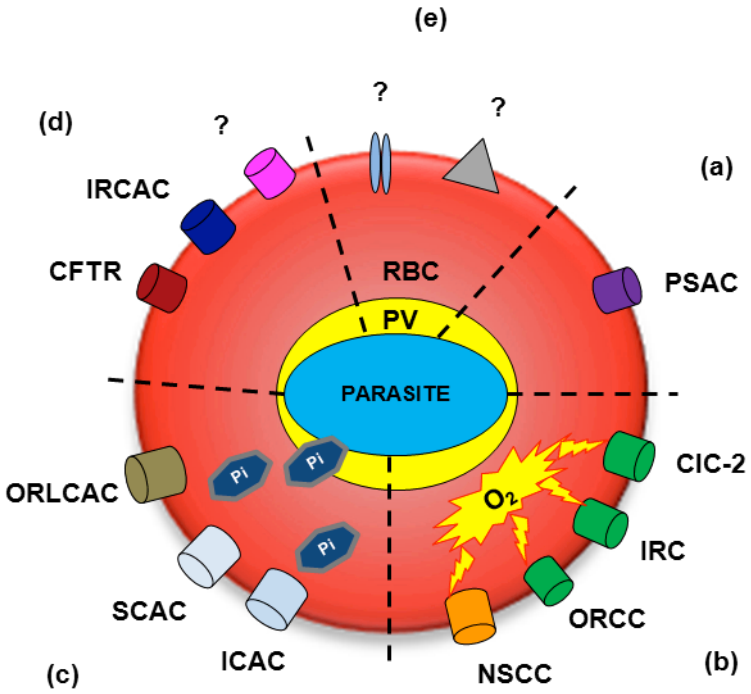


Figure 1.14: Different hypothesis of the activation and origin of protein transporters on the erythrocyte membrane. (a) unique transporter uptakes all solutes and it might be possible parasite-encoded (Alkhalil *et al*., 2004), (b) oxidative stress produced by infection activates host endogenous channel proteins (Huber *et al*., 2002), (c) phosphorylation activates host encoded transport proteins (Merckx *et al*., 2009), (d) and (e) involve parasite-encoded transport proteins (Ginsburg *et al*., 2004). Modified from (Staines *et al*., 2007). (Abbreviations: PSAC plasmodial surface anion channel, CIC-2 chloride channel family member 2, IRC inward rectifying channel, ORCC outward rectifying conductance channel, NSCC nonspecific cation channel, ICAC intermediate conductance anion channel, SCAC

small conductance anion channel, ORLCAC outwardly rectifying large conductance anion channel, CFTR cystic fibrosis transmembrane regulator, IRCAC inwardly rectifying conductance anion channel).

1.11 Amino acid transport

The amino acids are the building blocks of proteins required by the parasite for its normal growth inside the erythrocytes, and thus the parasite fulfills its need for the amino acids by three primary mechanisms (i) by digestion and ingestion of the host cell proteins, i.e. hemoglobin, (ii) uptake from the extracellular medium and (iii) biosynthesis of amino acids from other carbon sources eg. alanine, aspartate, glutamate (Sherman, 1977; Divo *et al*., 1985; Elford *et al*., 1985). The metabolic profiling of the isolated parasites has shown the presence of free amino acids in large amounts (Teng *et al*., 2009). The growing parasite degrades approximately 65% hemoglobin into amino acids and most of these amino acids are effluxed from the infected erythrocyte and approximately 16% of them are utilized by the parasite (Krugliak *et al*., 2002). Thus, requirement for most of the amino acids by the parasite is satisfied by the hemoglobin degraded inside the food vacuole, but the need for the amino acids which are absent in hemoglobin such as isoleucine is met by importing the amino acids from the extracellular medium (Martin *et al*., 2007). Therefore, the import of acidic as well as neutral amino acids as compared to cationic amino acids is done by the induction of the NPP in the infected erythrocytes by the parasite (Ginsburg *et al*., 1985). In order to maintain its normal growth for longer periods of time the parasite requires extracellular supplies of methionine (Cobbold *et al*., 2011) glutamine, cysteine, proline, tyrosine (Divo *et al*., 1985; Francis *et al*., 1994) and all of which are imported by the NPP. Many amino acids serve as fuel sources and interact with other pathways for energy production (through transamination reactions) and they also act as precursors for other metabolic pathways eg. glutamate metabolism or methionine and polyamine metabolism. Another important function is in redox metabolism, in which glutamate dehydrogenase provides the reduced NADPH needed for glutathione reductase (Krauth-Siegel *et al*., 1996).

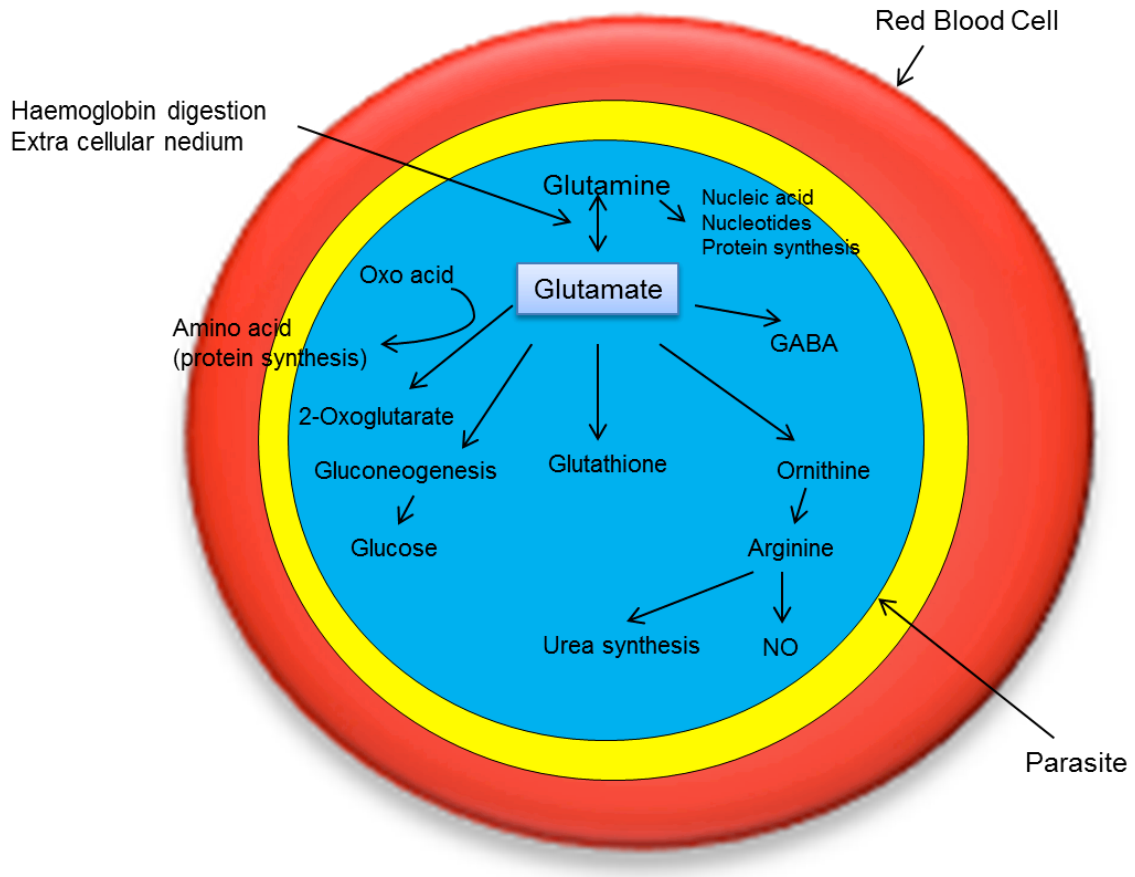


Figure 1.15: Intracellular glutamate metabolic pathways in parasite infected red blood cell.

1.12 Glutamate transport

The acidic amino acid glutamate is required for the parasite growth and is among 1 of the 5 amino acids essential for the parasite growth *in vitro* (Divo *et al*., 1985). Since glutamate is the essential component of glutathione, and it is known that glutathione is required for long-term survival of erythrocytes *in vitro* (Freeman, 1983), normal cell function and defence against oxidative stress (Ballatori *et al*., 2009), therefore it is important to understand the glutamate transport in infected erythrocytes. The glutamate transport is facilitated by Excitatory Amino Acid Transporters (EAATs) in neurons and in the CNS at the excitatory synapses. The glutamate is cleared up by these high affinity transporters (EAATs) present in the membranes of neurons and glia (Danbolt, 2001). In different cell types the glutamate uptake is regulated through protein kinase-C-mediated phosphorylation (Casado *et al*., 1993), other kinases (Flatman, 2005), arachidonic acid (Trotti *et al*., 1995) and free radicals of oxygen such as peroxynitrite and hydrogen peroxide, which target an oxidant-sensitive site shared by different isoforms of the glutamate transporter (Trotti *et al*., 1996). In previous

studies it has been shown that non-infected erythrocytes do not possess a functional glutamate transporter and thus in non-infected erythrocytes the glutamate is not transported to any significant extent across the membrane. This is in contrast to malaria parasite infected erythrocytes which possess functional high-affinity, Na^+-dependent glutamate transport, which is characteristic of the "excitatory amino acid transporter" (EAAT) family and similar results were observed in arsenite treated non-infected erythrocytes (Winterberg *et al*., 2012). Thus, after infection by *P. falciparum*, the erythrocytes showed an activated glutamate transporter with both the high-affinity and low-affinity components for glutamate transporter (Winterberg *et al*., 2012). In this work, we investigated the transport of glutamate into *B. divergens* infected erythrocytes and also compared with *P. falciparum* infected erythrocytes. Here we show that *B. divergens* infection results in the activation of Na^+-independent glutamate uptake of low affinity. The implications of these results for our understanding of increased membrane permeability via NPP by both the parasites *B. divergens* and *P. falciparum* infecting erythrocytes and inducing an increase in the transport of amino acids across the infected erythrocyte membrane are discussed.

1.13 Objective

The focus of my thesis was to investigate about: (i) to what extent these parasites utilize the properties of the host cell for biogenesis of PV and does the host cell have specific "entry sites"? (ii) what is the mechanism of nutrient acquisition by the parasite?

The formation and fate of the PVM in *Babesia* infected erythrocytes is unclear due to the absence of suitable marker proteins. The reports show that there is a disintegration of the PVM and loss of the PV in cells infected with *B. divergens* (Rudzinska *et al*., 1976). Therefore, we compared host cell membrane proteins recruitment in cells infected with either of the two parasites (*P. falciparum* and *B. divergens*), in order to see if the recruitment is the result of biochemical or biophysical characteristics of the erythrocyte plasma membrane or if it is species specific mechanisms. For this we wanted to determine whether the lipid raft, which are concentrated patches of lipids and proteins on the erythrocyte membrane, plays any role as a `specific entry site´ in the biogenesis of PV and also determine whether both parasites respectively internalize the same type of lipids/proteins or not. To investigate this hypothesis, we did morphological analysis of lipid rafts (GM1 associated lipid rafts) using cholera toxin subunit B (Alexa labelled), in order to determine inclusion and exclusion of proteins.

After infection of erythrocytes with the malaria parasite, one of the parasite induced changes occurring after 10-12 hours, post infection is the increased permeability of the erythrocyte

membrane by the formation of NPP (New Permeability Pathways). The presence of sizable no. of transporters for organic acids and nearly no transporter present for the amino acids in the erythrocytes, it reflects their availability either from the intracellular environment or from the extracellular media surrounding the cell. Thus, these amino acids are obtained from different sources such as digestion of hemoglobin in the food vacuole or from the extracellular medium via the new permeability pathways (NPP) formed in the host cell membrane. The main objective is to characterize the transport of L-glutamate into *B. divergens* infected erythrocytes and to compare it with *P. falciparum* infected erythrocytes and uninfected erythrocytes treated with arsenite. It has been shown that *P. falciparum* infected erythrocytes have high-affinity (EAAT3) glutamate transporter activated after infection. But on the other hand the transport system involved in the uptake of L-glutamate in *B. divergens* infected erythrocytes is unknown. Therefore, my focus is to dissect the transport system involved and see if it is a parasite specific mechanism.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and consumables

Balance 1205 MP	Sartorius, Göttingen
Blotting apparatus	Phase, Lübeck
Cold centrifuge Mikro 22R	Hettich Zentrifugen
Eppendorf tubes	Eppendorf, Hamburg
Falcon tubes	Sarstedt, Nümbrecht
Fluorescence confocal microscope	Zeiss, Göttingen
Gel loader tips	Eppendorf, Hamburg
Incubator	Heraeus, Hanau
Nitrocellulose membranes	Schleicher & Schuell, Dassel
Pasteur pipettes	COPAN (Italy)
pH meter	Calimatic, Mering
Pipettes	Sarstedt, Nümbrecht
Pipette tips	Sarstedt, Nümbrecht
Precision balance	Sartorius, Göttingen
Scintillation counter LS6500	Beckman Coulter (USA)
Thermomixer 5436	Eppendorf, Hamburg
Whatman paper	Schleicher & Schuell, Dassel
XCell SureLockTM	Invitrogen, Germany
Balance 1205 MP	Sartorius, Göttingen
Blotting apparatus	Phase, Lübeck

Cold centrifuge Mikro 22R	Hettich Zentrifugen
Eppendorf tubes	Eppendorf, Hamburg
Falcon tubes	Sarstedt, Nümbrecht
Fluorescence confocal microscope	Zeiss, Göttingen
Gel loader tips	Eppendorf, Hamburg
Incubator	Heraeus, Hanau
Nitrocellulose membranes	Schleicher & Schuell, Dassel
Pasteur pipettes	COPAN (Italy)
pH meter	Calimatic, Mering
Pipettes	Sarstedt, Nümbrecht
Pipette tips	Sarstedt, Nümbrecht
Precision balance	Sartorius, Göttingen
Scintillation counter LS6500	Beckman Coulter (USA)
Thermomixer 5436	Eppendorf, Hamburg
Whatman paper	Schleicher & Schuell, Dassel
XCell SureLockTM	Invitrogen, Germany

2.1.2 Chemicals and reagents

a-Chymotrypsin from bovine pancreas Grade I	AppliChem, Darmstadt
1,4-dithio-DL-threitol (DTT)	Sigma-Aldrich, Seelze
5-[Aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)amino] benzoic acid (Furosemide)	AppliChem, Darmstadt
5a-Pregnan-3a-ol-11,20-dione (Alphaxalone)	Sigma-Aldrich, Seelze

5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	Sigma-Aldrich, Seelze
5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)	Sigma-Aldrich, Seelze
Ammoniumpersulfat (APS)	Roth, Karlsruhe
Azur-eosin-methylen blue (Giemsa dye)	Merck, Darmstadt
BisTris gel 4-12%	Novex, Germany
Bovine serum albumin (BSA)	PAA, Cölbe
Bromophenol blue	Merck, Darmstadt
Choline bromide	USBiological
Choline chloride	Sigma-Aldrich, Seelze
Cis-ACBD	ToCris Bioscience
Coomassie Brilliant Blue G250	Fulka Chemie GmbH
D(+)-Saccharose	Roth, Karlsruhe
Dimethylsulfoxid (DMSO)	Roth, Karlsruhe
Dodecyltrimethylammonium bromide (DoTMA)	Sigma-Aldrich, Seelze
Dulbecco's PBS (1x) without Ca & Mg (PBS)	PAA, Cölbe
Ethylenediamine-tetraacetic acid (EDTA)	Roth, Karlsruhe
Gelafundin	Braun, Marburg an der Lahn
Glycerol	Applichem, Darmstadt
Hemicholinium-3 (Hem-3)	Sigma-Aldrich, Seelze
Hydrochloric acid (HCl)	AppliChem, Darmstadt
Hypoxanthine	C.C.Pro, Germany
Immersion oil for microscopy	Roth, Karlsruhe
L-Glutamate	Roth, Karlsruhe

Methanol (MeOH)	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
Nitro blue tetrazolium (NBT)	Sigma-Aldrich, Seelze
Neomycin solution	Sigma-Aldrich, Seelze
N-Ethylmaleimide (NEM)	AppliChem, Darmstadt
N,N,N',N'-Tetramethyethylenediamine (TEMED)	Roth, Karlsruhe
RPMI 1640	PAA, Cölbe
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt
Sodium hydroxyde (NaOH)	Merck, Darmstadt
Sodium(meta)arsenite	Sigma-Aldrich, Seelze
Ponceau stain	Applichem, Darmstadt
Protease Inhibitor Cocktail (PIC)	Calbiochem, Darmstadt
Scintillation buffer	Roth, Karlsruhe
Trishydroxymethylaminomethan (Tris)	Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Seelze

2.1.3 Radiolabeled substances

D-[3H]-Glutamate	Hartmann Analytic
L-[3-3H]-Alanine	Perkin Elmer, Rodgau
L-[3,4-3H]-Glutamate	Perkin Elmer, Rodgau
[methyl-3H]-Choline chloride	Perkin Elmer, Rodgau

2.1.4 Analysis software

AxioVision 4.3.	Fluorescence confocal microscope

ImageJ	Image editing, gel analysis
R software	Statistical analysis and graphs

2.1.5 Others

Human erythrocyte concentrate (A/Rh+)	Uni-Klinikum Marburg
Human plasma (blood group A/Rh+)	Uni-Klinikum Marburg

2.1.6 SDS-PAGE and Western blot

Ammonium peroxo-sulfate (APS)
10% APS in ddH2O

Blocking-Milk
5% Milk powder in PBS pH 7.4

Alkaline phosphatase buffer (AP buffer)
100mM Tris-HCl (pH 9), 150mM NaCl,
1mM MgCl2

Glycin buffer
100mM Glycin in PBS pH 7.6

Lysis buffer
100mM Tris-HCl (pH 8), 1mM EDTA, PIC
(1:500), 10mM NEM

Ponceau stain solution
0.2% Ponceau S, 3% Trichloroacetic

PageRuler Prestained Protein Ladder
Fermentas, St. Leon

SDS-PAGE running buffer
124mM Tris, 960mM Glycin, 0.05% SDS

Sample buffer (2x)
100mM Tris-HCl (pH 6.8), 5mM EDTA,
20% Glycerol, 4% SDS, 0.2%
Bromophenol blue, 100mM DTT

Stacking gel 4x
500mM Tris-HCl (pH 6.8), 0.4% SDS

Separating gel 4x
1.5M Tris-HCl (pH 8.8), 0.4% SDS

Western Blot transfer buffer
48mM Tris-HCl (pH 9.5), 39mM Glycin,
0.0375% SDS, 20% MeOH

2.1.7 Cell culture solutions

Culture media
RPMI 1640, 100µg/ml Neomycin, 200µM
Hypoxanthin, 1/10 in volume human
Plasma

Giemsa solution
1 volume Giemsa stain in 9 volumes
Giemsa phosphate buffer

2.1.8 Other solutions

Choline-chloride solution
150mM choline-chloride, 10mM Tris-HCl,
20mM sucrose (pH 7.2)

DoTMA solution
100µM DoTMA in PBS or chol-chloride
Solution
Hem-3 solution

100μM Hemicholinium-3 in PBS or choline chloride
Solution

NaCl solution

150mM NaCl, 10mM Tris-HCl, 20mM
Sucrose (pH 7.4)

Choline bromide solution

150mM Choline Bromide, 10mM Tris-HCl, 20mM sucrose
(pH 7.2)

2.2 Methods

2.2.1 Cell Culture

The human erythrocytes and human plasma of blood group A, Rhesus factor positive, was purchased from the Blood Bank Marburg and Geissen.

P. falciparum – The *in vitro* culturing of *P. falciparum* (isolate 3D7) was coducted under standard conditions (Trager *et al*., 1976). The parasite was cultured in the human erythrocytes, incubated at 37ºC in 250 ml petriplates and with a hematocrite of 2%. The culture media was 35ml RPMI media supplemented with 10% plasma. The plates were flushed with a gas mixture of 90% N_2, 5% O_2, 5% CO_2 and the media was changed everyday.

B. divergens – The *in vitro* culturing of *B. divergens* (isolate Rouen 1987) was also conducted under standard conditions. The blood stage parasites were cultured in the human erythrocytes, incubated at 37ºC in 250 ml petriplates and with a hematocrite of 5%. The culture media was 25ml RPMI media supplemented with 10%plasma. The plates were flushed with a gas mixture of 90% N_2, 5% O_2, 5% CO_2. The media was changed every day.

2.2.2 Enrichment of mature Trophozoites by gelafundin

From the cell culture plate extra media was removed and the remaining suspension was centrifuged in a 15 ml tube, at 3000 rpm for 2 min. Later, the sedimented erythrocytes were resuspended in a gelafundin solution (62.5% gelafundin + 37.5% RPMI) and incubated at 37ºC for 15 min.

The reason behind this separation is that the mature trophozoites have “knobs” like structures on the surface of the infected cell and thus the sedimentation of these cells is much slower as compared to the non-infected and the ring infected erythrocytes. Thus, after 15 min of incubation the supernantant was collected in a new 15 ml tube, centrifuged at 3000 rpm for 2 min. The resulting pellet was re-suspended in a volume of 10 fold RPS (complete media RPMI + serum) and the parasitemia was calculated.

2.2.3 Synchronization of rings with sorbitol

The sedimented erythrocytes are re-supended with five times its volume of sorbitol (5% sorbitol in ddH_2O). Due to the fact that the treatment causes the osmotic lysis of the mature trophozoites and also schizonts, only ring infected cells survive.

The suspension was incubated for 5 min at 37ºC and later centrifuged at 3000 rpm for 2 min. The pellet was washed twice with 10 times volume of RPMI and was observed with geimsa staining for parasitemia. After the calculation, the cells were returned to the cell culture plate.

2.2.4 Treatment of non-infected human erythrocytes with Sodium arsenite

The chemical treatment of non-infected human erythrocytes were performed by using sodium arsenite – a method which was also used by Flatman and colleagues for activating erythrocytes (Flatman *et al*., 1999). Before activation, the cells were incubated in a media at 37ºC overnight at 2% hematocrite. The cells were then sedimented in a 15 ml tube, from which 50µl of cells (5×10^8) were taken in a reaction tube, washed three times with PBS and later incubated with 1ml of 1mM sodium arsenite solution in PBS at 37ºC for 90 min. Subsequently the cells were washed twice with PBS at 3000 rpm for 2 min and then the cells were ready for the experiment.

Similarly, for the treatment of *Babesia*-infected cells. The parasites were incubated at 2% hematocrite overnight at 37ºC. The cells were sedimented in a 15 ml tube and centrifuged 3000 rpm for 2 min, from which 50µl of infected cells (5×10^8) were taken in a reaction tube and washed three times with PBS and later incubated with 1ml of 1mM sodium arsenite in PBS at 37ºC and shaken for 30 min. Later the cells were washed twice with PBS and used for the experiment.

2.2.5 Uptake assays for radioactive substrates

In these assays, the unidirectional influx of radioactive labeled substrates was measured in the non-infected RBCs, infected RBCs and sodium arsenite treated RBCs. The radioactive labelled substrates used were D-[^{3}H]-Glutamate, L-[^{3}H]-Glutamate, D-[^{3}H]-Aspartate, L-[^{3}H]-Alanin, L-[^{3}H]-Lysin. To measure the radioactive uptake the infected and non-infected erythrocytes were washed three times with PBS and then kept in PBS at 37ºC shaken for 30 min. After the incubation time was over, the cells were ready for the experiment. The above treatment, thereby establishes no significant membrane transport at the cellular state. This helps to provide a more specific measurement of the transport performance of individual

transporter classes for the added substrate (radiolabelled) without the interference by parallel other transport pathways.

For the uptake of a specific radioactive labelled substrate, the cells are incubated in a specific solution which contains the radioactive labelled substrate for which the unidirectional influx has to be measured. The influx measurement was done at defined time intervals (0, 5, 10, 20 min.).

2.2.6 Separation of cells and media

For the measurement of radioactive substrate influx in non-infected, infected and sodium arsenite treated erythrocytes, we took a definite no. of cells and the amount of radioactive substrate. The cells were incubated in the radioactive solution containing 1µCi substrate in 100µl for $2x10^7$ cells. In order to quantify the amount of radioactive substrate uptake into cells at pre-defined time points, the cells needed to be separated from the surrounding medium.

Thereby, 100µl of the cells suspension was loaded on a cushion of 500µl Dibutylphthalate in a reaction tube. The cells were sedimented at 18000x g for 2 min. After centrifugation the cells were separated from the extra surrounding medium, and thus the further influx of the radioactive substrate is hindered. Finally, the reaction tube was placed into liquid nitrogen to completely stop the uptake and lock the phase in the reaction tube. To separate the resulting cells pellet from the medium phase, the tip of the reaction tube containing the sedimented cells was cut off with a tongs and was dropped in a scintillation vial containing 2 ml scintillation buffer. The quantity of intracellular radioactive substrate was measured by means of a scintillation counter.

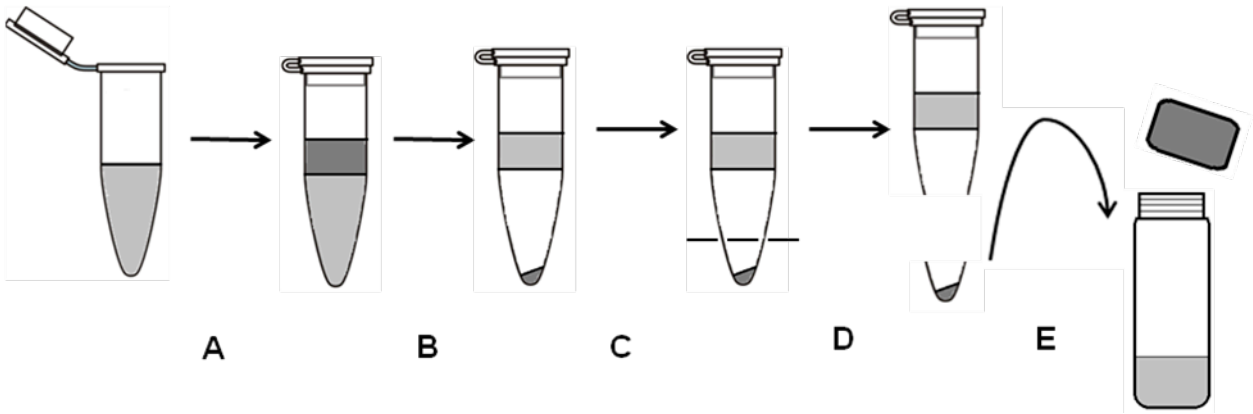


Figure 2.1: Schematic diagram for the separation of medium and cells. The suspension of the cells and media with radioactive substrate was loaded on cushion of Dibutylphthalate (A) subsequently centrifuged (B). Due to the lower density of the medium, after centrifugation the cells sediment at the bottom. Then the reaction tube is put in liquid nitrogen to stop the uptake and lock the phase (C) and the cells pellet is cut and separated from the medium (D). The cells pellet is then suspended into the scintillation buffer (E), and determine the quantity of contained radioactive substrate by scintillation counter.

2.2.7 Time course for the measurement of the absorption of radioactive labeled substrates

In this assay 3,0 x 10^8 non-infected erythrocytes, infected erythrocytes and sodium arsenite activated erythrocytes were taken in 1360 µl of 1x PBS pH 7.4, which contained 14µCi of radioactive labelled substrate. The cells suspension was kept at 37º C with shaking. At defined time points (1, 5, 10, 20 minutes) aliquotes were taken, which contained 2 x 10^7 cells (1µCi x 100µl^{-1}) and processed as described in section 2.6. Each time point had triplicates.

2.2.8 Measurement of the absorption of radioactive labeled substrate in the presence of Inhibitors

In this assay we took 1,5 x 10^8 infected erythrocytes and sodium arsenite activated erythrocytes in 1x PBS pH 7,4. The inhibitors used were NPPB, Furosemide and cis-ACBD in concentration of 100µM in 1x PBS pH 7,4. Before the radioactive substrate uptake was recorded, the cells were treated for 10 min. with the inhibitors at 37ºC. This was performed to confirm the blocking of the transport pathways with the inhibitors before the actual uptake.

Once the cells were ready, they were sedimented and the solution containing radioactive substrate and the inhibitor was added to the cells and incubated again for 10 min. Aliquots were taken at 0 min. and 10 min. in triplicates. They were processed further as mentioned in the section 2.6.

2.2.9 Biotinylation of intact infected erythrocytes

For biotinylation, 1,5 x 10^8 *Babesia* infected erythrocytes were washed three times with PBS and then incubated in biotin derivative (sulfo-NHS-LC-biotin) in PBS at a concentration of 1mg/ml for 30 min. on ice. Biotin solution was always prepared fresh just before treatment.

After 30 min. cells were washed three times with PBS to remove the unbound biotin. Subsequently cells were ready for the experiment. For the experiments investigating the recovery of the NPP, biotin treatment and subsequent cultivation was performed in RPMI1640 media.

2.2.10 Protease treatment of intact infected erythrocytes

B. divergens infected erythrocytes were washed with PBS three times and then incubated with different proteases in PBS for 30 min at 37ºC, routinely using the following concentrations; 1mg/ml for chymotrypsin and 1mg/ml for trypsin. For the experiments investigating the recovery of the NPPs, using chymotrypsin treatment and subsequent cultivation was performed in RPMI1640 media.

2.2.11 To measure the influx of radioactive amino acids in competition with non-radioactive amino acids

In the assay 1,5 x 10^8 infected erythrocytes were taken to investigate the effect of non-radioactive amino acid (L-glutamate) on the uptake of radiolabelled amino acid (L-[^{3}H]-Glutamate) in the presence and absence of extracellular Na+ (with the extracellular solutions 150mM NaCl, 10mM Tris-HCl, 20mM, sucrose (pH 7.4) and 150mM Choline Bromide, 10mM Tris-HCl, 20mM, sucrose (pH 7.2) respectively). In each case the uptake of L-[^{3}H]-Glutamate was measured at 37ºC for 10 min. with shaking, with the concentration of non-radioactive (L-glutamate) ranging from 0, 0.1, 1, 10, 100 and 1000µM in the extracellular medium.

2.2.12 Choline and Sodium chloride treatment

The uptake of different radiolabelled amino acids was measured in the presence and absence of extracellular Na+. With the extracellular solutions containing 150mM NaCl, 10mM Tris-HCl, 20mM, sucrose (pH 7.4) and 150mM choline bromide, 10mM Tris-HCl, 20mM, sucrose (pH 7.2), therefore 100mM sodium was replaced by 100mM choline. This was performed to determine the dependency of the transport pathway on ions such as sodium (Na+). The cells were washed five times before the actual uptake of the radioactive substrate, and the uptake was performed in the respective buffers containing radioactive substrate.

2.2.13 SDS-Polyacrylamide gel electrophoresis

To analyze the protein expression levels, the abundance of proteins in different compartments and protein-protein interactions, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed.

The samples were prepared in definite volume of PBS or lysis buffer and 1 volume of sample buffer. Further the samples were boiled at 100ºC for 10 min and were loaded in definite cells equivalents eg. 2 x 10^7 or 1 x 10^7 cells per plane. The markers Prestained Protein Ladder (Fermentas) or Page Ruler Unstained Protein Ladder (Fermentas) were used.

2.2.14 Hemolysis assay

The non-infected erythrocytes and *B. divergens* infected erythrocytes (1.5 x 10^8 cells) were washed and incubated in either of the two solutions PBS or Choline Chloride. At defined time points (0, 5, 10, 20 min) aliquots were removed and centrifuged for 3 min. at 3000 × g. The degree of hemolysis was determined from the absorbance of hemoglobin at 570 nm in the supernatant. Absorbance at 100% hemolysis was determined by adding 10 µl of Triton X-100 (10% (v/v) to the erythrocyte suspension having the same number of cells as above.

2.2.15 10% TCA Protein Precipitation

Cells were washed and incubated with L-(^{3}H) glutamate for 10 min. in either of the two solutions PBS and Choline Chloride. An aliquot containing approximately 1.5 x 10^8 cells and 3 x 10^8 cells were sampled and layered on top of 500µl of oil (dibutyl phthalate; Sigma-Aldrich) in a microcentrifuge tube, which was centrifuged at 18000g for 2 min, therefore separating the cells from the extracellular media. The supernatant solution was removed and the tubes were washed 3 times with either of the two solutions (choline Chloride and PBS) to remove the residual radioactivity. After the final washing, oil was removed and the cells were suspended with 100µl of 10% trichloroacetic acid (TCA). The suspension was kept on ice for 20 min. and the proteins were precipitated in the presence of TCA. The precipitated protein was sedimented by centrifugation at 18000g for 20 minutes and the supernatant was transferred to a scintillation vial, the pellet was washed 2 times with cold acetone and centrifuged at 18000g for 5 min. The pellet was then dried and transferred to the scintillation vial for counting.

2.2.16 Erythrocyte volume measurement by Multisizer 3 COULTER COUNTER®

The Multisizer 3 coulter counter is used to analyze the cell sizing, cell volume and counting within a size range of 0.4µm to 1200µm. It is unaffected by particle color, shape, composition or refractive index. This is based on Electrical sensing zone (ESZ) method. In this method the suspension of cells is made to flow through an aperture of a cylindrical glass tube, separating two electrodes between which electric current flows. The magnitude of the current is low (1mA), the current density within the aperture is created by the resistance produced by the electrode separations. As the particle passes through the aperture it produces its own volume of conducting liquid, which therefore increases the impedance of the aperture. This change in impedance is proportional to the current flow, which is converted into voltage pulse. The basic principle behind the working of this counter is that the amplitude of the voltage pulse produced is directly proportional to the volume of the particle that produces it.

The non-infected RBCs and *B. divergens* infected RBCs were washed in either of the two solutions Dulbecco's PBS (DPBS) and Choline Chloride. The cells were incubated for 30 min. in the respective solutions and analyzed in the coulter counter. Before the readings, the aperture tube was equilibrated with either of the two solutions in order to avoid any shock to the cells from one media to the other in the aperture tube. This allows for more accurate readings.

2.2.17 To measure the Efflux to radiolabelled amino acid.

In these assays, the efflux of radiolabelled amino acid was measured in the *B. divergens* infected RBCs. To measure the efflux of the radiolabelled amino acid firstly the infected cells (1.5×10^8) were washed 5 times with choline chloride and then incubated in choline chloride solution which contains the radiolabelled amino acid for wihich the efflux has to be measured for 20 min. at 37ºC. The above treatment results in cells filled with the radiolabelled amino acid. Later after incubation, the cells were washed with choline chloride 3 times for the removal of extra amino acid in the surroundings of the cell. Now for the efflux, the radiolabelled filled cells were incubated again in specific solutions (choline chloride and sodium chloride) with no radioactivity and therefore the efflux of amino acid was measured at specific time intervals (0, 10 min.). At each time interval cells (4×10^7) were removed and centrifuged at 3000rpm, 2min., after that the supernatant was removed in a scintillation vial for the reading and to separate the resulting cells pellet, they were plced in liquid nitrogen, followed by cutting of the tip of the reaction tube containing the sedimented cells with a tongs

and was dropped in a scintillation vial containing 2 ml scintillation buffer. The quantity of radioactive substrate was measured by means of a scintillation counter.

2.2.18 Cholera toxin beta subunit (CtxB) labeling and patching GM1.

The labeling and patching of GM1 on human erythrocytes with cholera toxin B subunit has been performed previously (Mrowczynska *et al*., 2008). Using the same procedure as used by Hagerstrand and colleagues, we blocked the erythrocytes with 1% fish skin gelatine in RPMI-1640 media for 30 min. at room temperature. The supernatant was discarded and cells were mixed with 10µl of 1:125 dilution of CtxB and incubated at room temperature for 30 min. The cells were washed and incubated with the anti CtxB antiserum for 30 min. (which patches the surface bound CtxB) at room temperature. We used anti CtxB antibody as primary antibody and fluorescently labeled secondary antibody as secondary antibody. The surface labeling and patching was observed using inverse epifluorescence microscopy.

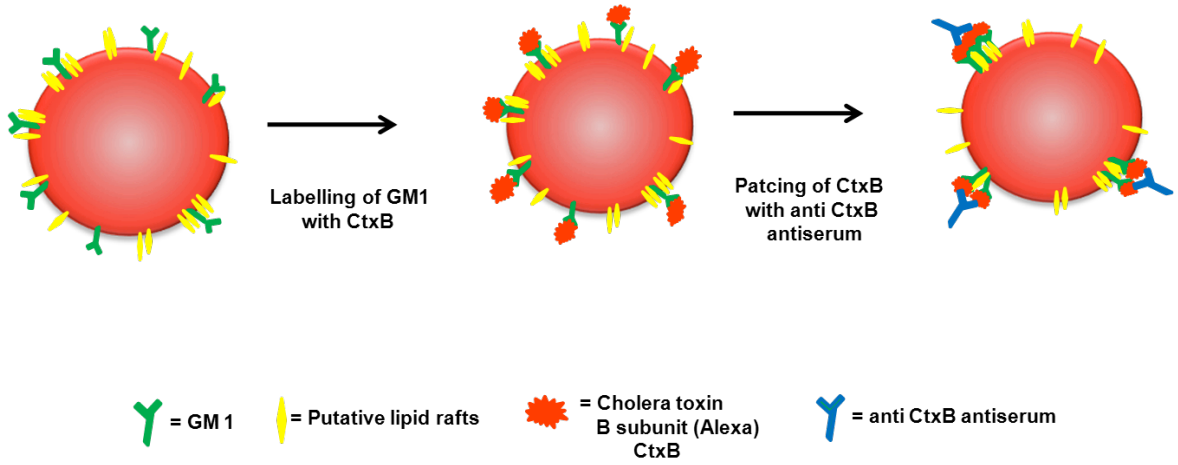


Figure 2.2: Labeling and patching of GM1 with cholera toxin. The cells were labeled with cholera toxin (CtxB) and later patching of CtxB with anti-CtxB antiserum was done. The surface labeling was observed using epifluorescence microscopy.

During the invasion study, the non-infected RBCs were labeled with CtxB and mixed with *P. falciparum* and *B. divergens* infected RBCs to a final parasetemia of 3% and cultured under standard conditions. The cells were harvested, treated as described above and then incubated with anti CtxB antiserum (as 1st antibody) and fluorescently labeled (Cy3) antibody as 2nd antibody. A batch was also analyzed by immunofluorescence.

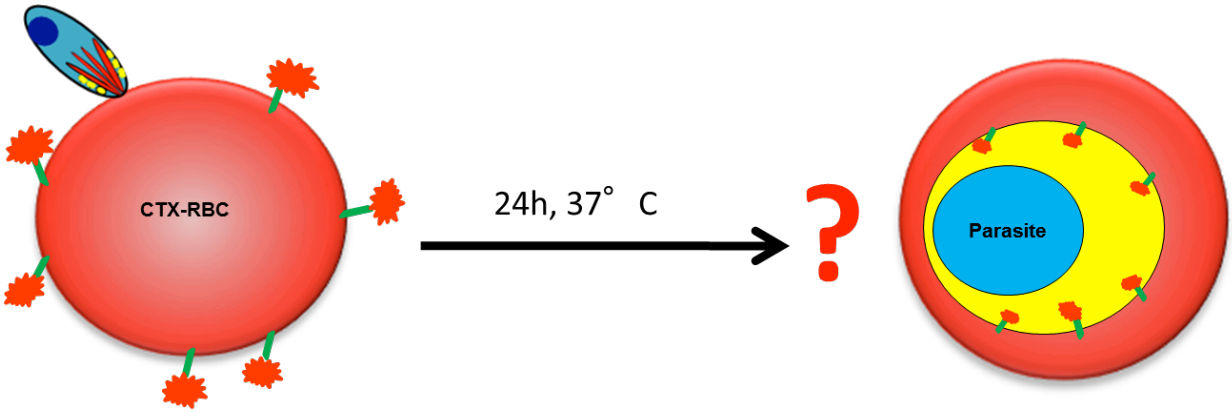


Figure 2.3: Invasion of CtxB labeled cells with parasites. Cells were CtxB labeled and then incubated with parasites (*Babesia divergens* and *Plasmodium falciparum*) in culture plates. Later, after 4 hours and 24 hours respectively observed for the fluorescence.

2.2.19 Immunoflorescence assay

The cells were resuspended and washed 3 times in 1ml PBS, then centrifuged at 3000 rpm for 2 min. The washed cells were fixed in 1ml PBS containing 4% paraformaldehyde and 0.0075% glutaraldehyde, incubated at 37ºC for 30 min. and mixed gently. Then 100µl of 1.25M glycine/PBS was added, and cells were mixed gently for 10 min. Centrifuge at 3000 rpm for 2 min. and the supernatant was discarded. The pellet was resuspended in 1ml of 0.1% TritonX-100/PBS, mixed gently for 10 min., then centrifuged at 3000 rpm for 2 min. and the supernatant was discarded. Then 500µl of 125mM glycine/PBS was added to the pellet for 10 min, later centrifuged at 3000 rpm for 2 min. The pellet was blocked in 3% BSA/PBS for 1 hour at room temperature. After blocking, the pellet was incubated with the 1st antibody overnight at 4ºC, then washed 3 times with PBS for 10min. A 2nd antibody was added for 2 hours at room temperature. Finally the pellet was washed 3 times with PBS for 10 min. and with the last washing step add Hoechst (1/100.000).

3. RESULTS

The intraerythrocytic malaria parasites *P. falciparum* and *B. divergens* induces considerable changes in the host cell membrane and the cytosol. As noted in the objective section of this thesis, the aim of my work was to observe the biogenesis of parasitophorous vacuolar membrane (PVM) through specific entry sites and parasite induced permeability changes in the erythrocyte membrane after infection.

3.1 Biogenesis of PV through specific entry sites

The erythrocyte membrane is a biological membrane, which separates intracellular components such as cytoplasm, organelles of a living cell form the extracellular enviorment. These highly specialized cells maintain their membrane stability solely by the underlying meshwork of cytoskeleton eg. spectrin, actin and associated proteins. Therefore, it is challenging for the parasite to enter these cells in less than a minute. The molecular mechanisms underlying the formation of the parasitophorous vacuolar membrane and their protein composition in *P. falciparum* and *B. divergens* infected erythrocytes are not completely understood. Recently, it was shown that the internalization of band 3, spectrin and glycophorins seems to be parasite-specific (Repnik et al., 2015). Another set of evidence for the internalization of a subset of host cell proteins comes from the detergent resistance membranes (DRM) or lipid rafts proteins in Plasmodium infected erythrocytes example heteromeric G protein or beta (2) adrenergic receptor (Murphy et al., 2006; Samuel et al., 2001). It has also been reported that Aquaporin 3, an abundant plasma membrane protein of various cells, including mammalian erythrocytes, is internalized into the infected erythrocytes, presumably during or soon after the invasion (Bietz *et al*., 2009).

3.1.1 Cholera toxin Beta subunit (CtxB) labeling of GM1 in non-infected RBCs, *B. divergens* and *P. falciparum* infected RBCs.

In our study, we wanted to investigate whether GM1 is internalized into the parasitophorous vacuole. This ganglioside is a component of a specific type of lipid rafts (Gomez-Mouton *et al*., 2001; Janich *et al*., 2007; Fujita *et al*., 2007) and may indicate rafts as a preferred entry site for the parasite. In the mammalian cells binding of the B subunit of cholera toxin to GM1 as a receptor on the cell membrane is the first step of cell intoxication, which then helps the toxin (Ctx) to penetrate the cell membrane and further triggering uptake and delivery of toxin subunit A into the cells (Aman *et al*., 2001). Hagerstrand and collegues have reported binding of CtxB to GM1 on the surface of erythrocytes and further accumulation of GM1

bound to CtxB was done by using anti-CTB, resulting in 40-60 GM1 patches represented by small bright spots on the erythrocytes surface (Mrowczynska *et al*., 2008), which indicates CtxB (Cholera toxin Beta subunit) binds to GM1 (Merritt *et al*., 1994; Harder *et al*., 1998) which is present on the outer lipid bilayer membrane of RBCs.

Therefore, in this part of my project, I investigated the internalization of fluorescent CtxB bound to GM1 during invasion of the erythrocytes by *B. divergens* and *P. falciparum*. To achieve this at first, different procedures were used to label and patch GM1 using CtxB on the surface of RBCs.

3.1.1.1 Monitoring the binding of CtxB to GM1 on non-infected RBCs.

In a previous study, the patching behavior of GM1 with CtxB on erythrocytes has been observed (Mrowczynska *et al*., 2008). The Cholera toxin is pentavalent and thus causes the formation of clusters of five GM1 molecules, into small GM1 lattices (Merritt *et al*., 1994; Harder *et al*., 1998). Therefore the first important question was, whether we can obtain the similar binding pattern (CtxB-GM1) as observed by Hägerstrand and colleagues in the non-infected RBCs. In my study, experiments were performed incubating cells directly with different concentrations of CtxB (1µg/ml, 10µg/ml and 100µg/ml). The dilutions of CtxB were made in different media in order to achieve the binding of GM1 to CtxB on intact erythrocytes. The media used were (i) RPMI complete media (RPS) (ii) Buffer (145mM NaCl, 5mM KCl, 4mM Na_2HPO4, 1mM $Na_2H_2PO_4$, 1mM $MgSO_4$, 1mM $CaCl_2$, 10mM glucose, pH 7.4) plus fish skin gelatin (FSG) (iii) RPMI plus fish skin gelatin (FSG) After incubation the cells were analyzed using an inverse epifluorescence microscopy.

(i) In RPMI complete media (RPS), we could not observe the pattern of spots on the surface of the non infected erythrocytes in Fig. 3.2. The reason was not clear. Perhaps the serum present in the RPS media was interfering with the binding of GM1 to CtxB, or there was no recognition GM1 by CtxB on the surface of RBCs.

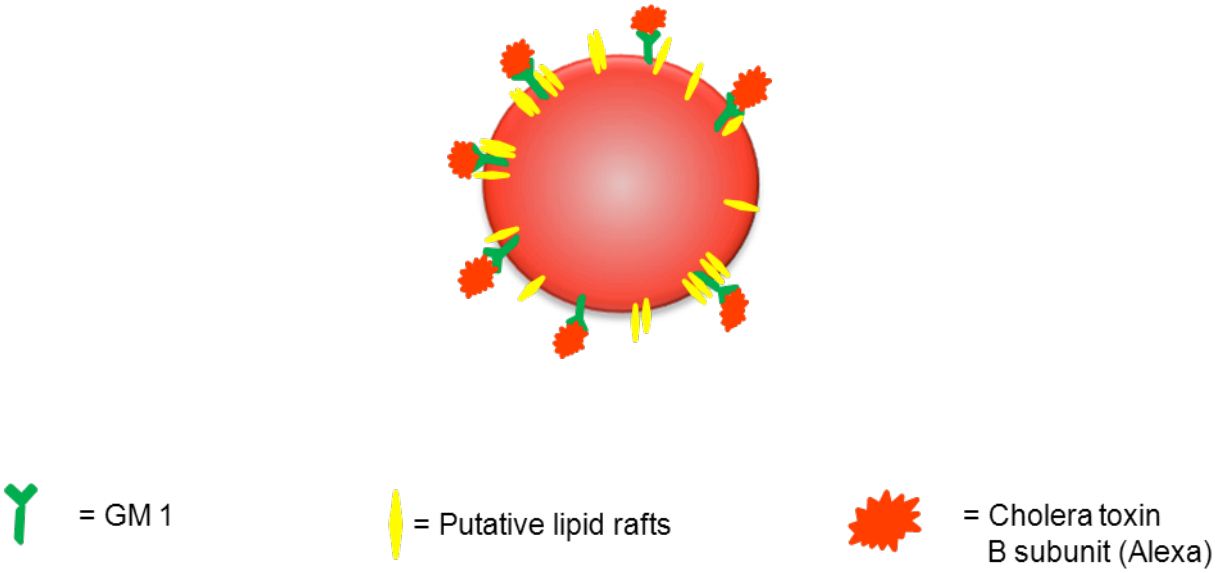


Figure 3.1: Schematic diagram of fluorescent CtxB binding to GM1 on the surface of non-infected RBCs.

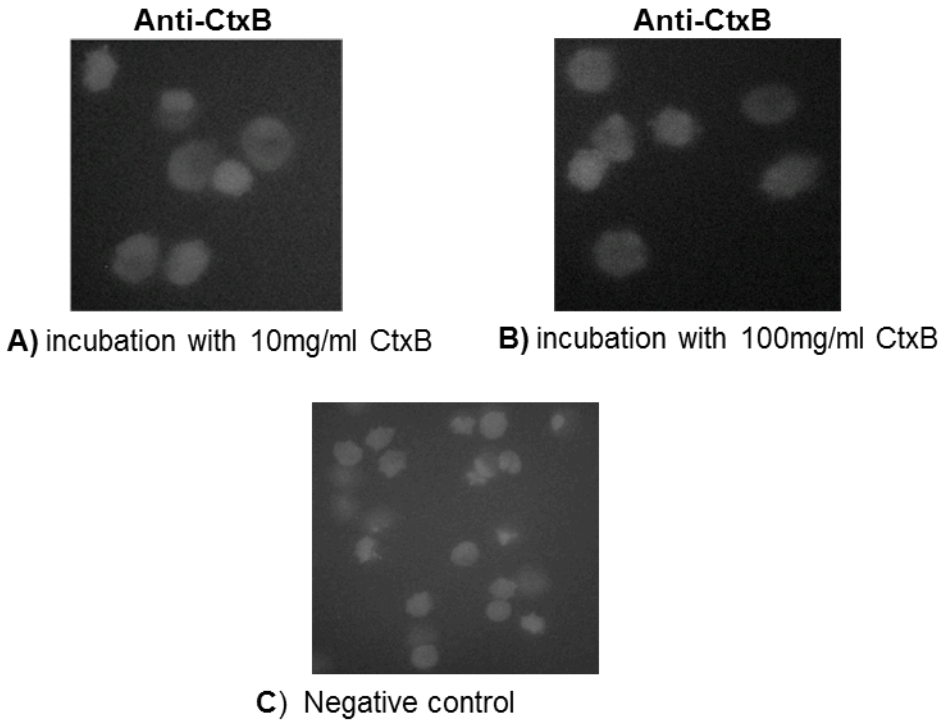


Figure 3.2: Fluorescent CtxB binding to GM1 on the surface of non-infected RBCs in RPS. Non-infected RBCs were washed with RPMI complete media and incubated with (A) 10µg/ml (B) 100µg/ml CtxB for 30 min. in darkness and (C) no CtxB. The cells were washed and observed using an inverse epifluorescence microscope.

(ii) In Buffer plus FSG and (iii) in RPMI plus FSG, as shown previously in the work conducted by Mrowczynska and colleagues, the patching of GM1 using

CtxB in the human erythrocytes using Fish Skin Gelatine (FSG) (Mrowczynska et al., 2008). In my previous experiment in RPMI media only, I could not achieve similar patching behavior of GM1 using CtxB in non-infected RBCs, therefore I decided to use Fish Skin Gelatine (FSG) according to Mrowczynska in a buffer and in RPMI-1640 media. And as a result, I could observe patterns of lateral bright spots on the surface of the non-infected RBCs in Fig 3.3.

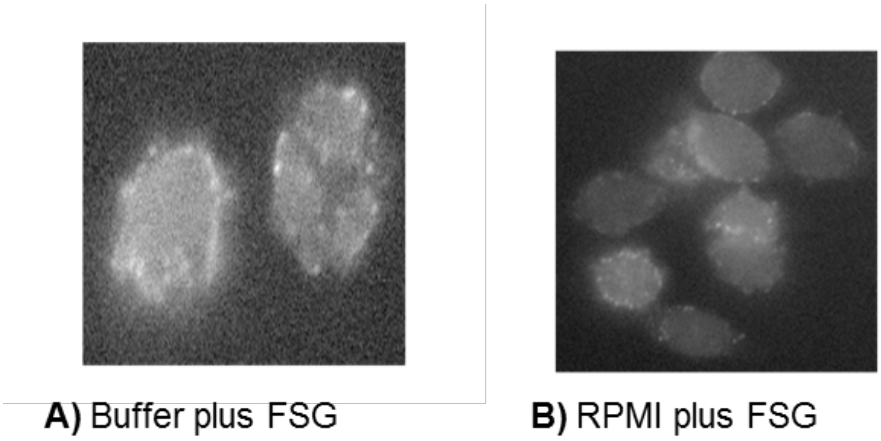


Figure 3.3: CtxB binding to GM1 on non- infected RBCs using Fish Skin Gelatine (FSG) in Buffer (A) and RPMI media (B). The non-infected RBCs were washed with the buffer/RPMI and blocked with 1% FSG for 30 min. at room temperature. The supernatant was discarded and cells were mixed with 10µl of 1:125 dilution of CtxB and incubated at room temperature for 30 min. The cells were washed and observed using inverse epifluorescence microscopy.

3.1.1.2 CtxB binding to GM1 on Fibroblasts in Dulbecco's Modified Eagle Medium (DMEM)

In order to look whether the experimental setup for binding of GM1 to CtxB is working or not, we tried to use another type of cells “fibroblast’s” for which it is published that GM1 can be patched (Pralle *et al*., 2000).

The binding of GM1 to CtxB was monitored on fibroblasts in Fig. 3.4. The observation of red spots on the surface of cells marked the binding of CtxB to GM1. It confirmed that CtxB binding to GM1 was working.

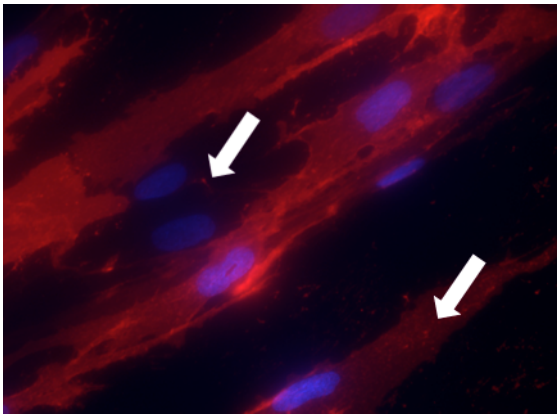


Figure 3.4: Binding of CtxB to GM1 on fibroblast cells. Fibroblasts attached to the coverslips were incubated with 10µl of 1:125 dilution of CtxB and incubated for 45 min. at room temperature in dark. The cells were washed and fixed to observe using inverse epifluorescence microscopy. The blue dots represent the nucleus, and the red dots with white arrows represent binding of CtxB to GM1.

3.1.1.3 Accumulation of patches (CtxB binding to GM1) by anti CtxB antiserum.

In all the experiments performed above for binding of CtxB with GM1 present on human erythrocytes, we observed spots having no or weak signals on the surface of the erythrocytes. In order to accumulate the patches of CtxB bound to GM1 we used anti CtxB antiserum in Fig 3.6. The erythrocytes were blocked with 1% fish skin gelatin in RPMI-1640 media, incubated with CtxB (100µg/ml) and subsequently incubated with anti-CTB. The surface labeling and patching was observed using inverse epifluorescence microscope.

The treatment of erythrocytes with CtxB plus anti-CTB formed distinct patches of GM1 on the erythrocyte surface. The appearance of bright spots like units confirmed the binding of GM1 to CtxB.

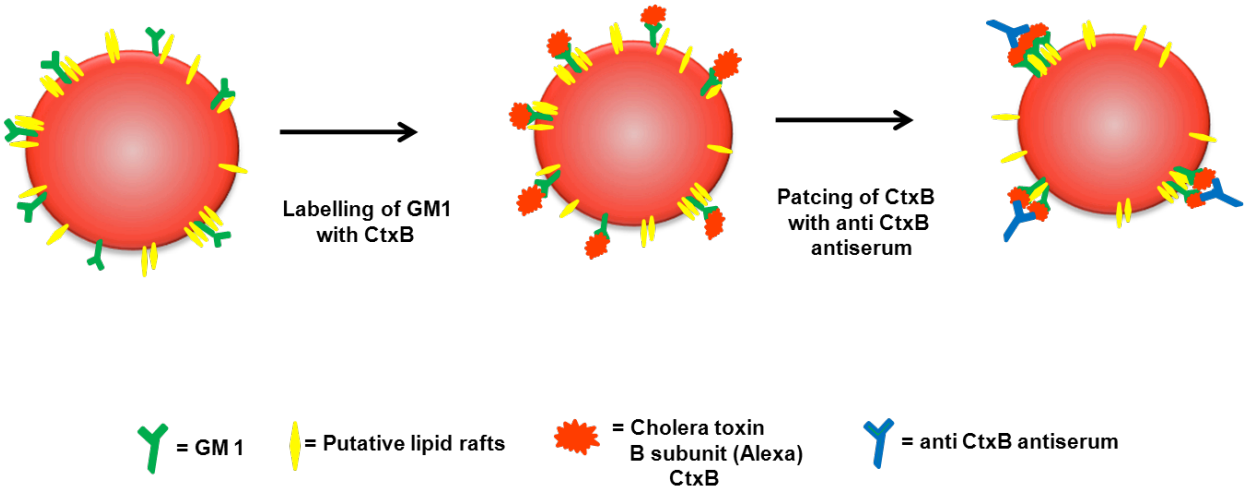


Figure 3.5: Binding and patching of GM1 with CtxB and anti-CTB.

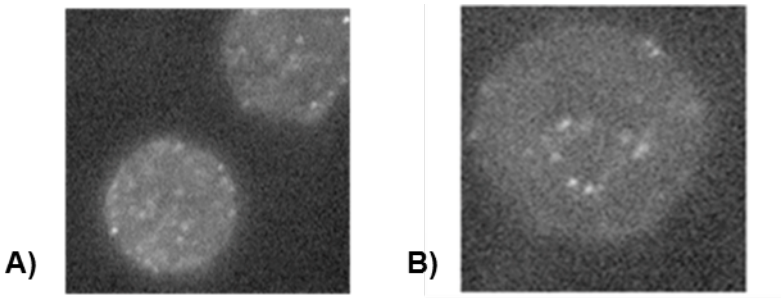


Figure 3.6: GM1 labeling using CtxB and patching with anti CtxB antiserum on non-infected RBCs. The non-infected RBCs were washed with RPMI-1640 media and blocked with 1% FSG for 30 min. at room temperature. The supernatant was discarded and the cells were mixed with 10µl of 1:125 dilution of CtxB and incubated at room temperature for 30 min. The cells were washed and incubated with the anti CtxB antiserum for 30 min. at room temperature. The cells were washed and observed using inverse epifluorescence microscope. The white dots in (A) and (B) show patching of GM1 using CtxB and anti CtxB antiserum.

3.1.2 CtxB forms stable pentamers, which partially dissociate in SDS-PAGE.

In order to see the efficiency and specificity of the anti CtxB antiserum, we performed a western blot of the membrane fraction of the CtxB treated non-infected RBC and recombinant CtxB in Fig. 3.7. The *in vitro* disassembly and reassembly of CtxB (the B subunit pentamer of cholera toxin) has been already investigated, this characteristic of CtxB

in denaturing conditions could help us in characterizing CtxB in bound and unbound to the RBC membrane (Lesieur *et al*., 2002; Zrimi *et al*., 2010). In our study we found that recombinant CtxB forms stable pentamers which partially dissociate in SDS-PAGE. The anti CtxB antiserum was able to detect different oligomeric forms of the toxin (monomer, dimer, trimer, tetramer and pentamer). The western blot analysis of the membrane fraction of non-infected RBC labelled with CtxB, showed the same pattern as for recombinant CtxB.

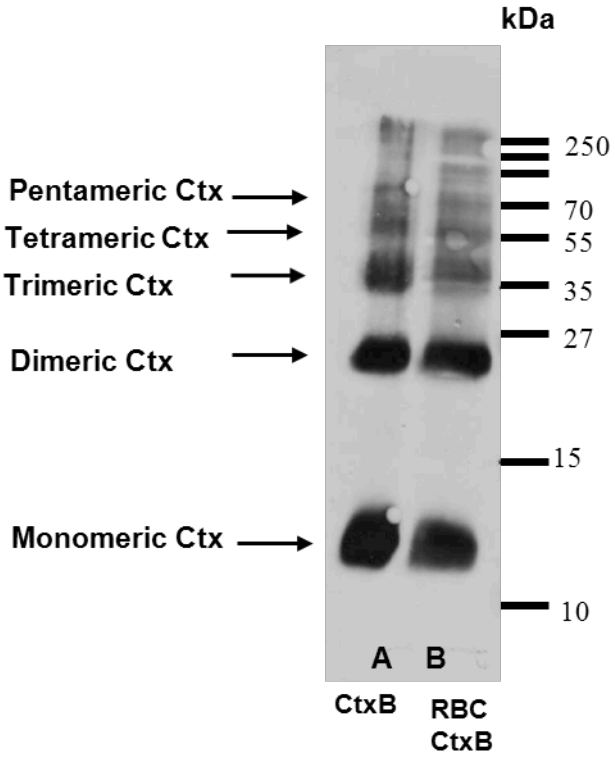


Figure 3.7: Western blot showing specificity of antiserum to CtxB. The anti CtxB antiserum specificity detects different oligomeric states of CtxB. The monomer of Ctx is 12 kDa. Lane A: recombinant CtxB (2.5µg); lane B: membrane fraction of RBC *in vitro* treated with recombinant CtxB (2.5 X 10^7 cells per lane). The anti CtxB antiserum was in a dilution of 1:1000.

3.1.3 Specificity of anti CtxB antiserum

It has been investigated, and we also found in our study, that anti CtxB antiserum recognizes different oligomeric forms of CtxB on western blot. In Fig. 3.8, the experiment was done to analyze that there was no cross reactivity of the anti CtxB antiserum with other proteins present on the membrane fractions of the non-infected RBCs or on *P. falciparum* and *B. divergens* infected RBCs, which might give us misleading results. We also observed no reactivity in the membrane fractions of CtxB labeled non-infected RBCs treated with

Proteinase K. The latter control suggests that CtxB binds to the surface of non-infected RBCs and is sensitive to protease K treatment, which indicates that CtxB is not gaining access to the inner leaflet of the RBCs membrane or to the RBCs cytoplasm.

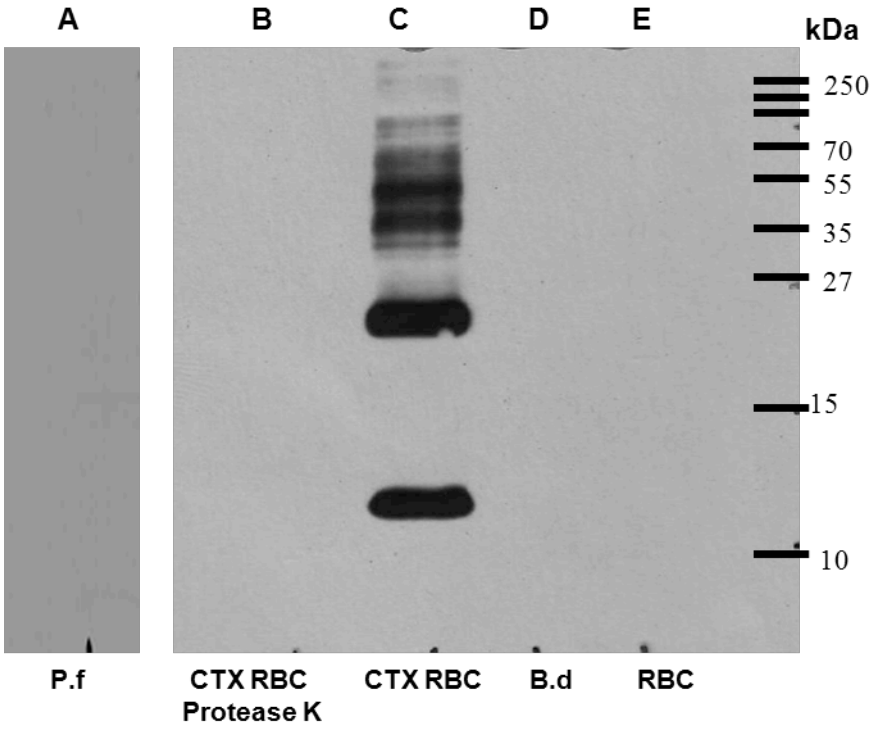


Figure 3.8: Western Blot showing specificity of anti CtxB. In this specificity of antiserum binding to CtxB on membrane fractions was analysed. Lane A (P.f), *P. falciparum* infected RBC, Lane B (CTX RBC Protease K), CtxB labeled RBC treated with Protease K, Lane C (CTX RBC), CtxB labeled RBC, Lane D (B.d), *B. divergens* infected RBC and Lane E (RBC), RBC. All lanes have 2.5 X 10^7 cells per lane.

3.1.4 Internalization of CtxB in *P. falciparum* and *B. divergens* infected RBCs.

We investigated the internalization of CtxB bound to GM1 on the RBCs membrane into *P. falciparum* and *B. divergens* infected erythrocytes as shown in Fig. 3.9. To study this, a batch of non-infected RBCs were labeled with CtxB and mixed with *P. falciparum* and *B. divergens* infected RBCs to a final parasitemia of 3% and cultured under standard conditions. After reinvasion two batch of cells were harvested (i) one was used for immunoflouresence assay and (ii) for direct observation of cells using a glass slide. Both the batches of the cells were

incubated with anti CtxB antiserum (as 1st antibody) and subsequently fluorescently labeled antibody as 2nd antibody. The cells were then observed using fluorescent microscope.

In conclusion, microscopic analysis revealed that before the parasite infection, CtxB was bound to the surface of the non-infected RBCs whereas after invasion CtxB was internalized and associated around the parasite.

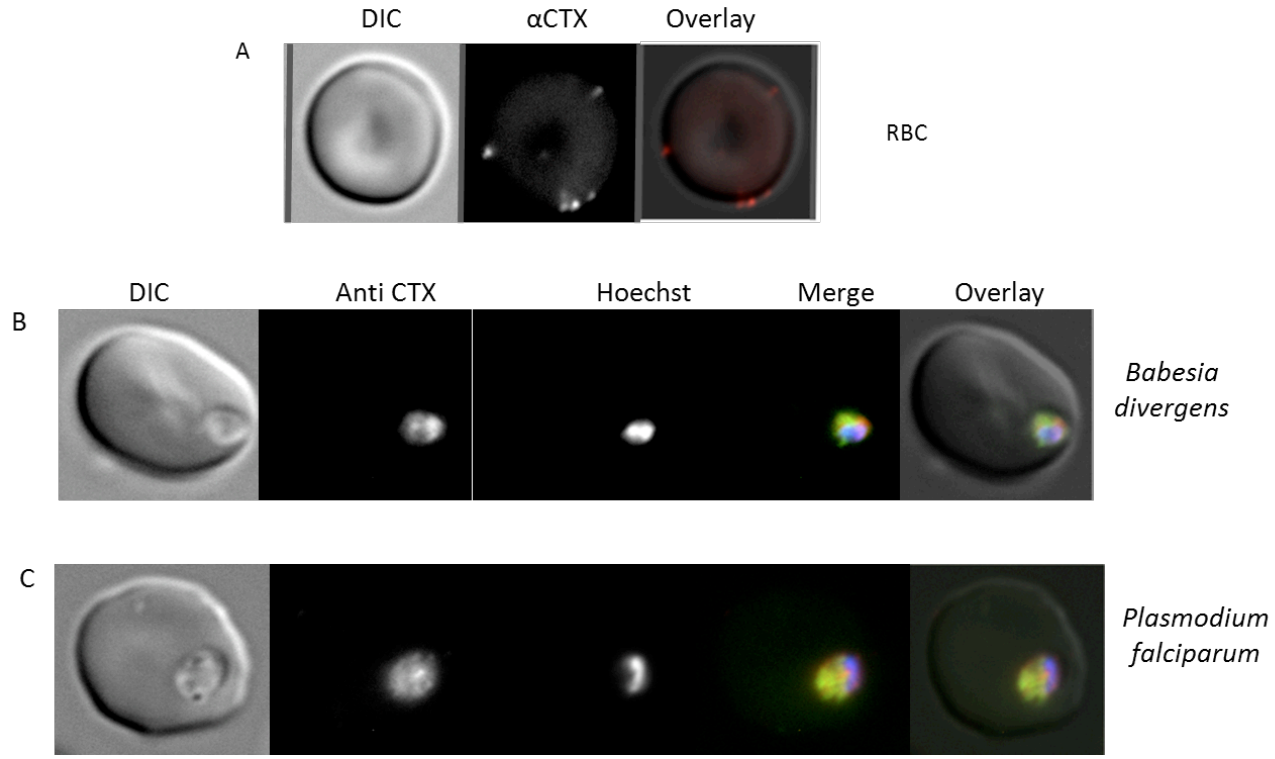


Figure 3.9: CtxB internalizes into *P. falciparum* and *B. divergens* infected RBCs. Panel A shows non-infected RBCs labeled with CtxB. Panel B shows internalization of CtxB into *B. divergens* infected RBCs. Panel C shows internalization of CtxB into *P. falciparum* infected RBCs.

3.2 Uptake study of different amino acids in *Babesia* and *Plasmodium* infected erythrocytes.

After infection of erythrocytes with the malaria parasite, the latter has limited ability to synthesize amino acids required for the parasite growth. Thus, these amino acids are obtained from different sources like the digestion of hemoglobin in the food vacuole (Lew, 2003), new permeability pathways (NPP) formed in the host cell membrane after infection and also increased influx by the endogenous host cell transporters like NaK-ATPase pump or Band 3 (Staines *et al.* 2001; Parker *et al.* 2004). The detection of NPPs in the infected erythrocyte is around 12-15 hours post invasion and then indeed the rate of transport of low-molecular-weight solutes via NPPs increases throughout the course of infection. This increased permeability of erythrocyte membrane display the activation of channels which allow influx and efflux of ions, nutrients and metabolic waste of the infected cell. In my work, I investigated the influx of amino acids L-glutamate, D-aspartate, D-glutamate and L-alanine into intact non-infected and infected erythrocytes. All experiments were carried out using radio labeled amino acids at concentrations approximately found in adult human plasma (Iwasaki *et al*., 1992): L-glutamate (200nM), D-aspartate (885nM), D-glutamate (1µM) and L-alanine (120nM). The uptake of amino acids under *in vitro* conditions at concentrations approximately in the range as found in blood plasma allows us to estimate the relative contributions of different transport systems of amino acid uptake in infected erythrocytes as found under *in vivo* conditions. Similarly, non-infected cells were incubated for 24 hrs before the experiment and were from the same donor as the erythrocytes used for the parasite culture.

The results from the time-course experiments (Fig. 3.10) showed a significant diffirence in the rate of uptake of the amino acids in cells infected by *B. divergens* and *P. falciparum*. Therefore the cells infected with *P. falciparum* take up significantly higher amounts of the amino acids than *B. divergens*-infected cells in the same time period, this relative difference in the rate of uptake might be due to involvement of different transporters involved or same transporter with different substrate affinity in the uptake of amino acids. Hence, this led us to investigate further the characteristic of the transporter involved in the uptake of glutamate in *B. divergens* infected erythrocytes. It is interesting to investigate about glutamate transport in *B. divergens* infected erythrocytes as there are no reports present until now about the characteristic of transport systems in RBCs infected with *B. divergens*. Most interesting is to know how the transport of different amino acids is done by the parsites *P. falciparum* and *B. divergens* which infect same type of host cell (RBCs).

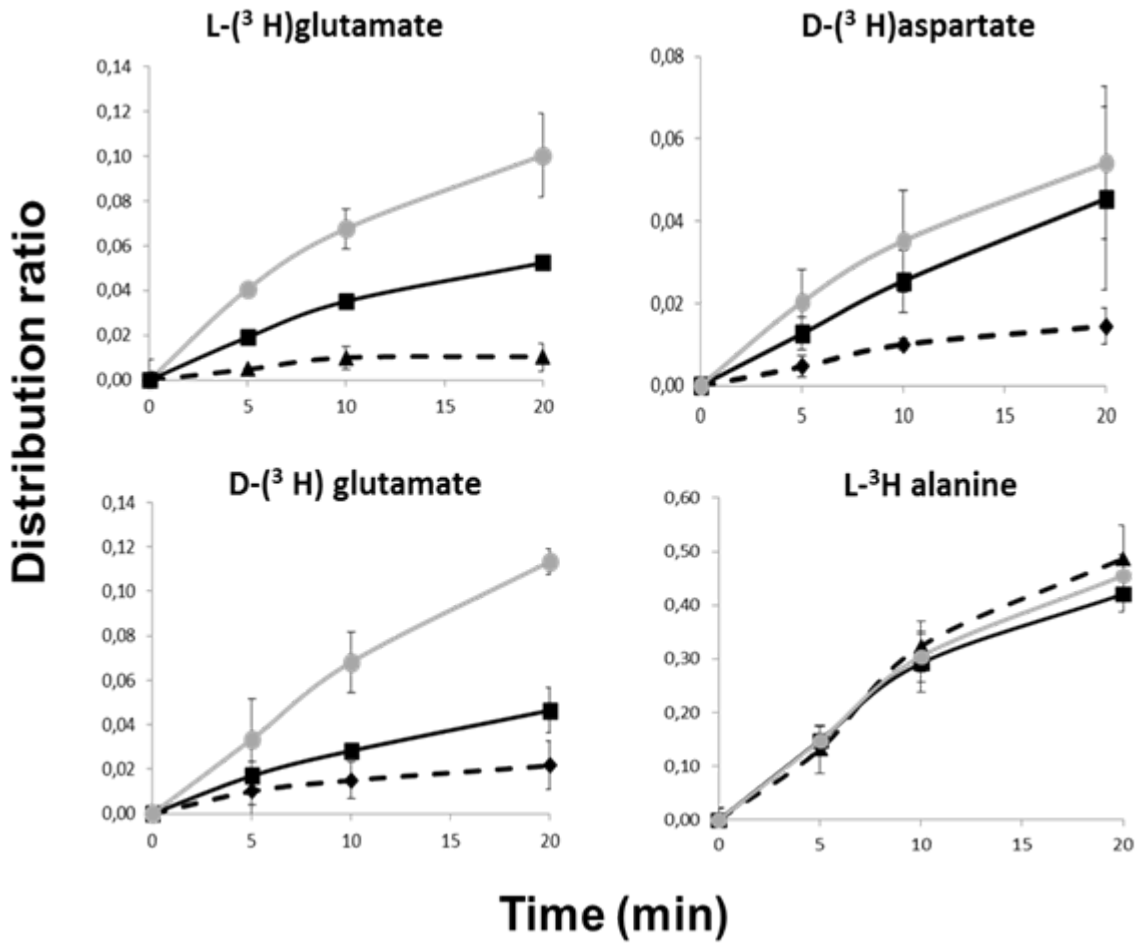


Figure 3.10: Time-course for the uptake of different amino acids. The uptake data are from non-infected red blood cells ( ) and red blood cells infected with *B. divergens* ( ) or *P. falciparum* ( ) for 100% parasitemia was measured. The influx of L-[^{3}H]-glutamate, D-[^{3}H]-aspartate, D-[^{3}H]-glutamate and L-[^{3}H]- alanin was measured at different time points (0, 5, 10 and 20 minutes) at 37ºC. The cells (2 x 10^7) were suspended in (Mg2+, Ca2+ free) PBS. The extracellular medium contained the radioactive amino acids. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data has been averaged from three independent experiments ± SEM.

3.2.1 NPP inhibitors, but not EAAT3 inhibitors, affect L-(^{3}H) glutamate uptake in *B. divergens* infected RBCs.

A transporter is a protein present in the plasma membrane of cells involved in the movement of ions, micro/macro molecules across a biological membrane. Depending upon the substrate specificities they exhibit two transport systems - low and high affinity transport. A well-known example of a transporter is EAAT3 (Excitatory amino acid transporters), present in neurons having both low- and high- affinity for glutamate (Danbolt, 2001). Recently it has been observed that chemical treatment (arsenite) of human erythrocytes activates high affinity glutamate transport (EAAT type) and also *P. falciparum* infected erythrocytes show activation of both low- and high-affinity (EAAT3) glutamate transporter (Winterberg *et al*., 2012). Winterberg and colleagues have also shown the inhibition of glutamate uptake via EAAT3 using cis-ACBD (1-aminocyclobutane-1, 3-dicarboxylate) which is an L-glutamate analog (Griffiths *et al*., 1994). Several inhibitors of anion channels, such as NPPB (5-nitro-2(-3-phenylpropylamino) benzoic acid) and furosemide, are known to block NPP activity in *P. falciparum* (Kirk *et al*., 1994).

To investigate the effects of a broad-specific NPP blocker and of an EAAT3 inhibitor (cis-ACBD) on the glutamate uptake into *B. divergens* infected erythrocytes, we pretreated non-infected RBCs, arsenite treated non-infected RBCs, *B. divergens* infected RBCs and *P. falciparum* infected RBCs with NPPB (100µM) or cis-ACBD (100µM) for 10 minutes before the glutamate uptake. Later, after 10 minutes of pretreatment glutamate uptake was measured for 10 minutes in the presence of respective inhibitors.

We found that NPPB, an inhibitor of parasite induced new permeability pathways had inhibitory effects on arsenite activated non-infected RBCs, on *B. divergens* infected RBCs and on *P. falciparum* infected RBCs (Fig. 3.11). However, the results from cis-ACBD inhibition were different. Cis-ACBD had no effect on glutamate uptake in *B. divergens* infected RBCs but on the other hand *P. falciparum* infected RBCs and arsenite activated RBCs showed inhibition as described previously (Winterberg *et al*., 2012). Therefore the results argue against the activation of EAAT3 in *B. divergens* infected RBCs.

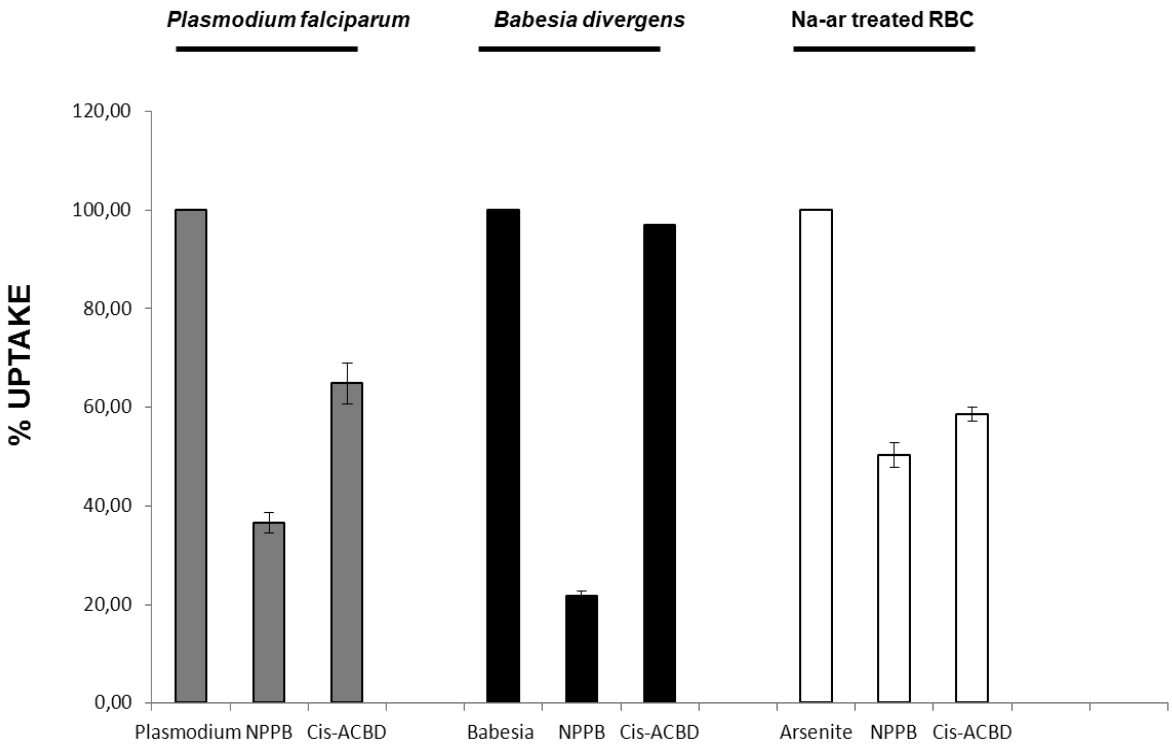


Figure 3.11: Effect of EAAT3-inhibitor cis-ACBD and NPPB on L-(^{3}H) glutamate uptake. The inhibitory effect of NPPB and cis-ACBD was obsereved on *B. divergens* infected RBCs (black bars), *P. falciparum* infected RBCs (grey bars) and arsenite activated RBCs (white bars). Where indicated, the cells were pretreated with NPPB (an inhibitor of the parasite-induced New Permeability Pathways (NPP)), at a concentration of 100µM and cis-ACBD (specific EAAT3 inhibitor) at a concentration of 100µM for 10 minutes at 37ºC. Later on the L-(^{3}H) glutamate uptake was measured, again in the presence of respective inhibitors for 10 minutes. The y-axis represents the % uptake. The data has been averaged from three independent experiments ± SEM.

3.2.2 Sulfo-NHS-LC-Biotin blocked the uptake of L-glutamate in *B. divergens* infected erythrocytes.

The novel permeability pathways appear in the *P. falciparum* infected erythrocytes after 10-15 hrs post invasion (Kirk, 2001) for the uptake of nutrients from the extracellular medium. In *P. falciparum* infected erythrocytes, the NPP allow the uptake of solutes which are not normally encountered under physiological conditions, such as certain biotin derivatives. Baumeister and colleagues showed in *P. falciparum* infected erythrocytes uptake of biotin derivative (sulfo-NHS-LC-biotin), which also block the uptake of other solutes, suggesting the

involvement of a biotinylatable transport protein (Baumeister *et al*., 2003). In my studies, Sulfo-NHS-LC-Biotin was used to label membrane proteins of intact-infected erythrocytes to test its effect on the uptake of L-glutamate in *B. divergens* infected erythrocytes. The Sulfo-NHS-LC-Biotin (Sulfosuccinimidyl-6-(biotinamido)hexanoate) used was non-cleavable, water soluble, membrane impermeable and binds to the membrane proteins. The uptake of L-glutamate into *B. divergens* infected erythrocytes was measured either in the presence of furosemide or after pretreatment with Sulfo-NHS-LC-Biotin.

We observed in Fig. 3.12, that the influx was inhibited equally into cells pretreated with Sulfo-NHS-LC-Biotin and with furosemide. The results showed that biotinylation of membrane proteins of intact-infected erythrocytes inhibited the influx of L-glutamate. Thus, Sulfo-NHS-LC-Biotin blocks novel permeability pathways induced in *B. divergens* infected erythrocytes.

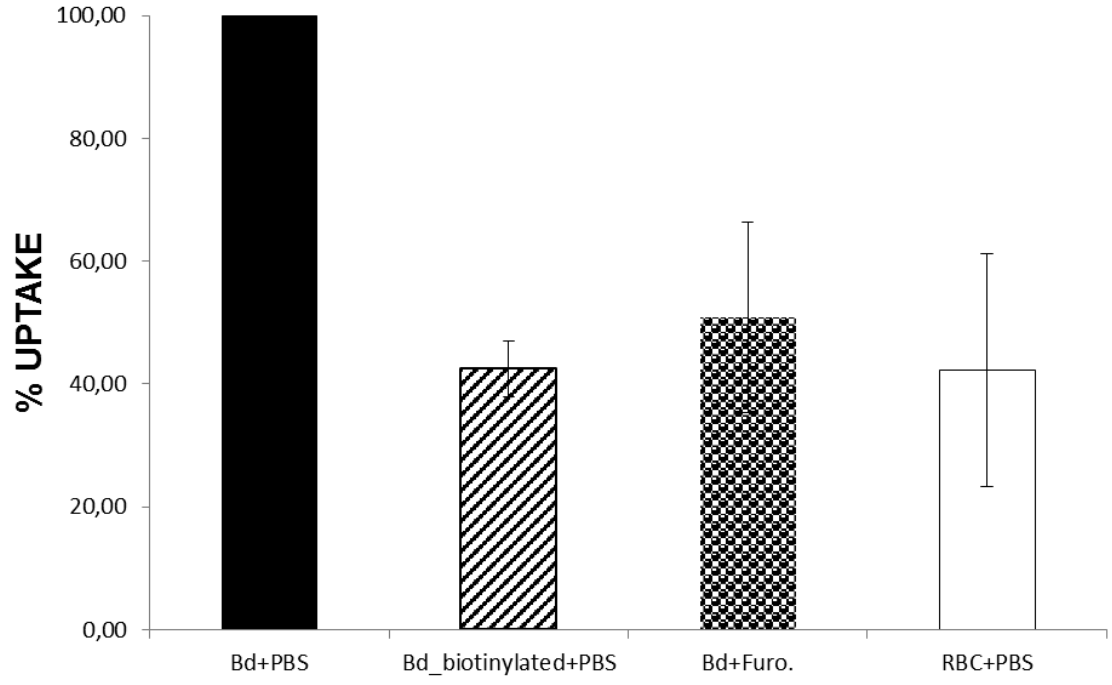


Figure 3.12: Biotinylation blocks influx of L-glutamate into *B. divergens* infected erythrocytes. The inhibitory effect of Sulfo-NHS-LC-Biotin (diagonal lines bar), furosemide (sphere bar) was observed and also compared with non-infected erythrocytes (white bar), *B. divergens* infected erythrocytes (black bar) for the uptake of L-glutamate. Where indicated, the cells were pretreated with Sulfo-NHS-LC-Biotin (1mg/ml) for 30 min. on ice and with furosemide (a known inhibitor for novel permeability pathways) at a concentration of 100µM for 10 min. at 37ºC. Subsequently the L-(^{3}H) glutamate uptake was measured, again in the presence of respective inhibitors for 10 minutes. The y-axis represents the % uptake. The data has been averaged from three independent experiments ± SEM.

3.2.3 Effect of Protease on the uptake of L-(^{3}H) glutamate in *B. divergens* infected RBCs.

Further, I wanted to observe the nature of the proteins, involved in mediating the transport of amino acids across erythrocyte membrane by using protease (chymotrypsin and trypsin). In a previous study it has been shown that the parasite induced amino acid uptake in *P. falciparum* infected RBCs is affected by the protease chymotrypsin but not by trypsin (Baumeister *et al*., 2006). In a similar way we looked for the effect of protease on intact RBCs infected by *B. divergens* in Fig. 3.13. And therefore we observed that chymotrypsin treatment of *B. divergens* infected RBCs reduced the L- glutamate uptake by 46% and trypsin reduced the uptake by 20%. Thus the proteins involved in the uptake are protease sensitive in *B. divergens* infected erythrocytes..

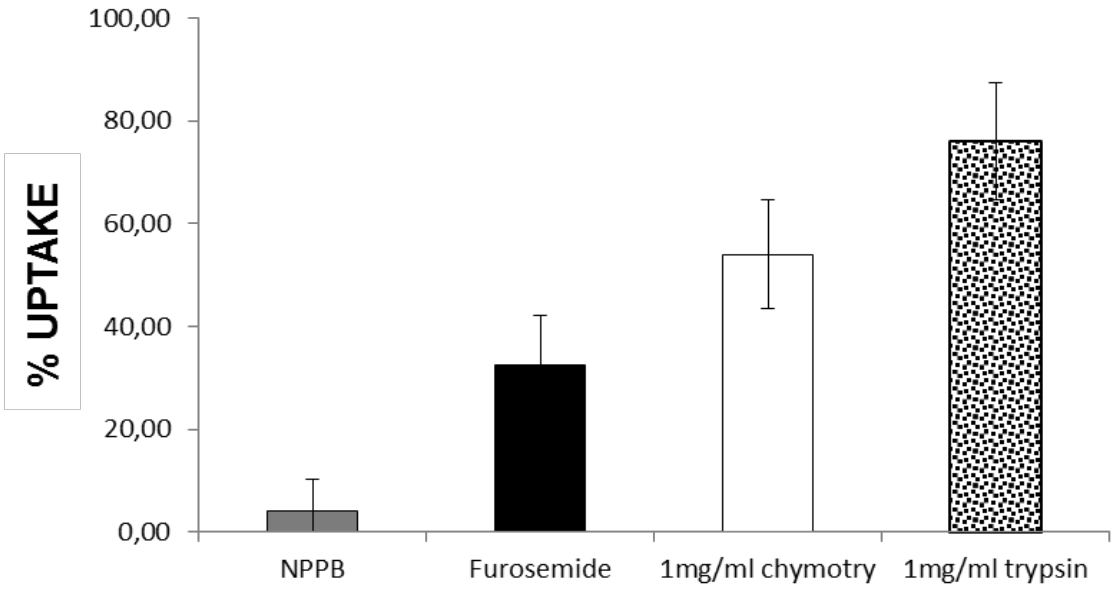


Figure 3.13: Protease sensitive uptake of glutamate in *B. divergens* infected RBCs. Effect of NPPB (100µg), furosemide (100µg) and protease (Chymotrypsin (1mg/ml) and Trypsin (1mg/ml) on *B. divergens* infected RBCs. As control were also observed. Uptake was measured for 10 min. at 37ºC. The data has been averaged from three independent experiments ± SEM.

3.2.4 Effect of Biotinylation and Chymotrypsinization on the restoration of NPP activity in *B. divergens* infected erythrocytes.

The NPP pathways are induced in the erythrocytes by the parasite after infection and can be prevented completely by inhibiting the protein synthesis of the parasite (Baumeister *et al*., 2006) and the partial inhibition of the transport activity can be done by using NPP inhibitors like NPPB, furosemide or proteases or by biotin derivatives (Baumeister *et al*., 2003). It has been shown in previous studies that in *P. falciparum* infected erythrocytes, in the absence of biotin and chymotrypsin, there is restoration of the NPP activity (Baumeister *et al*., 2003 and 2006) which means that there is involvement of parasite proteins during NPP activity.

Therefore similar experiments were also performed on *B. divergens* infected erythrocytes, the results in Fig 3.12 and 3.13 show that there is involvement of biotinylatable and protease sensitive transport proteins in *B. divergens* infected erythrocytes. Now the question was whether the NPP activity after biotin/chymotrypsin treatment of *B. divergens* infected erythrocytes is restored or not. Therefore the uptake of glutamate into cells was subjected to different rounds of biotin/chymotrypsin treatment. The infected erythrocytes were treated with biotin/chymotrypsin and an aliquot of treated cells was taken for the purpose of measuring the uptake of glutamate. The treatment was performed at time point zero and a batch of treated cells at time point zero were placed back into the culture media to observe the restoration of the NPP after time point of 4 hrs in case of Biotin treatment and at time points 2 hrs & 4 hrs in case of chymotrypsin treatment.

The results in Fig 3. 14 (A) and (B) showed that there was no restoration of NPP activity in *B. divergens* infected erythrocytes after these treatments. The possible reason might be due to non-involvement of parasite proteins during NPP activity or may be initially parasite proteins were involved in the NPP activity but later after the treatment could not be restored due to short life cycle of *B. divergens* (8-10 hours) and thus no wasting of energy and metabolites in the restoration process.

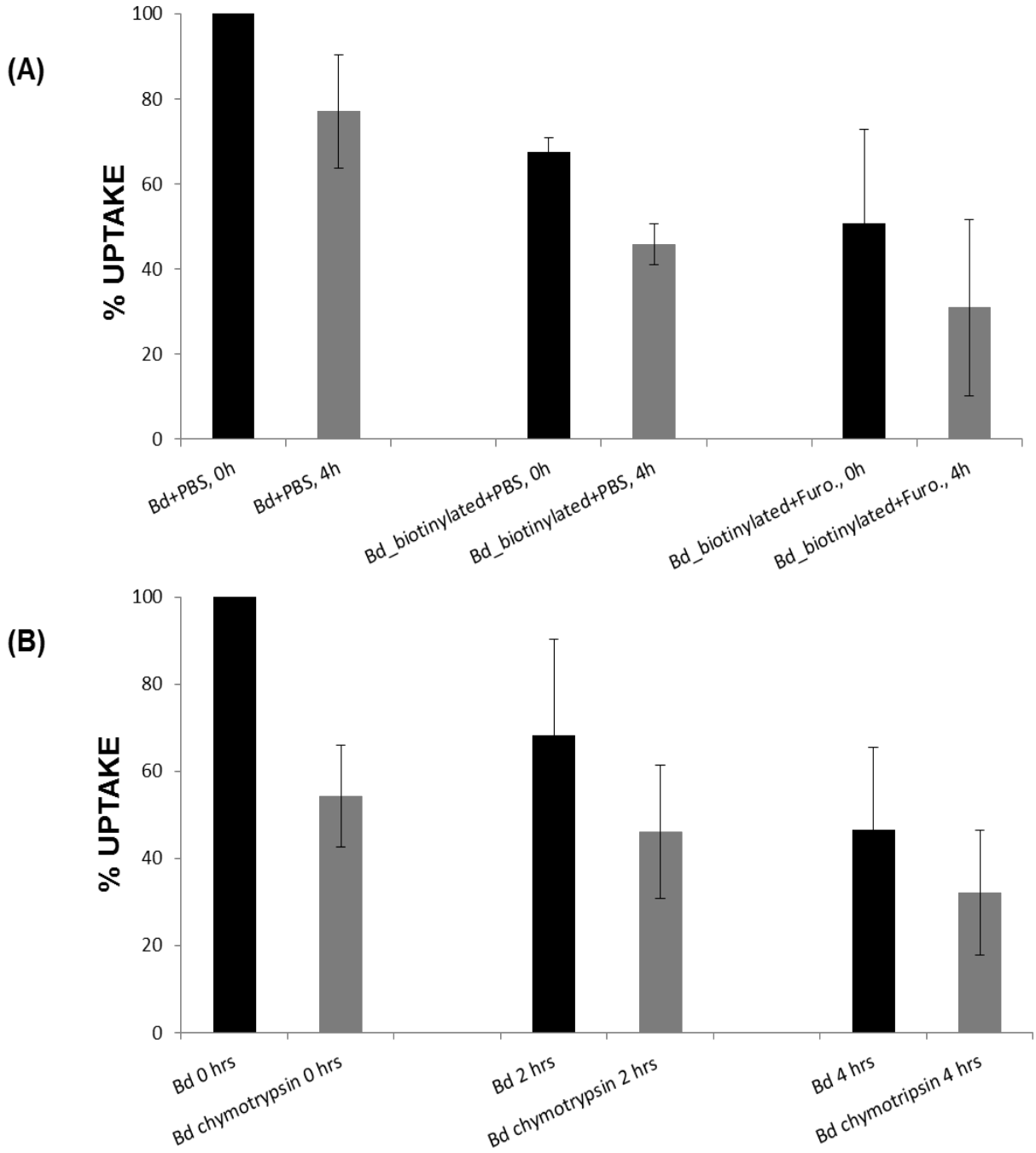


Figure 3.14: Effect of biotinylation and chymotripsinization on the uptake of glutamate in *B. divergens* infected erythrocytes. (A) Infected erythrocytes were incubated with biotin derivative (sulfo-NHS-LC-biotin) in PBS at a concentration of 1mg/ml for 30 min. on ice. (B) Similarly infected erythrocytes were incubated in chymotrypsin in PBS at a concentration of 1mg/ml for 30 min. at 37ºC. After all treatments the uptake of glutamate was measured for 10 min. at 37ºC and expressed in terms of % uptake. The data has been averaged from independent three experiments and are shown ± SEM.

3.2.5 The L-glutamate transport in *B. divergens* infected erythrocytes is Na^+ independent uptake.

The "Excitatory Amino Acid Transporter" (EAAT3) is also known as a glutamate transporter. Arsenite treatment of the non-infected RBCs has been proven to activate a high affinity, Na^+-dependent glutamate transport which is characteristic of the EAAT family (Flatman *et al*., 1999; Winterberg *et al*., 2012). Studies based on the comparison for the transport of L-glutamate in the non-infected erythrocytes treated with arsenite and in *P. falciparum*-infected cells showed the existence of both high-affinity and low-affinity components. Consequently, when glutamate uptake into *P. falciparum* is determined under conditions in which NaCl is replaced by choline chloride, the high affinity and saturable component of the glutamate uptake which is attributable to EAAT3 activation is abolished while the low affinity component (the EAAT3 independent) is hardly affected (Winterberg *et al*., 2012).

We therefore investigated the effects of Na^+ replacement by choline on the glutamate uptake into *B. divergens* infected RBCs. We pretreated the *B. divergens* infected RBCs with either of the solution containing NaCl (150mM) or Choline chloride (150mM) and L-glutamate uptake into cells was measured over time. In Fig. 3.15 we observed that unlike in *P. falciparum*, the replacement of Na^+ did not reduce glutamate uptake into *B. divergens* infected RBCs. By contrast, it appeared that choline stimulated the uptake of glutamate into *B. divergens* infected RBCs.

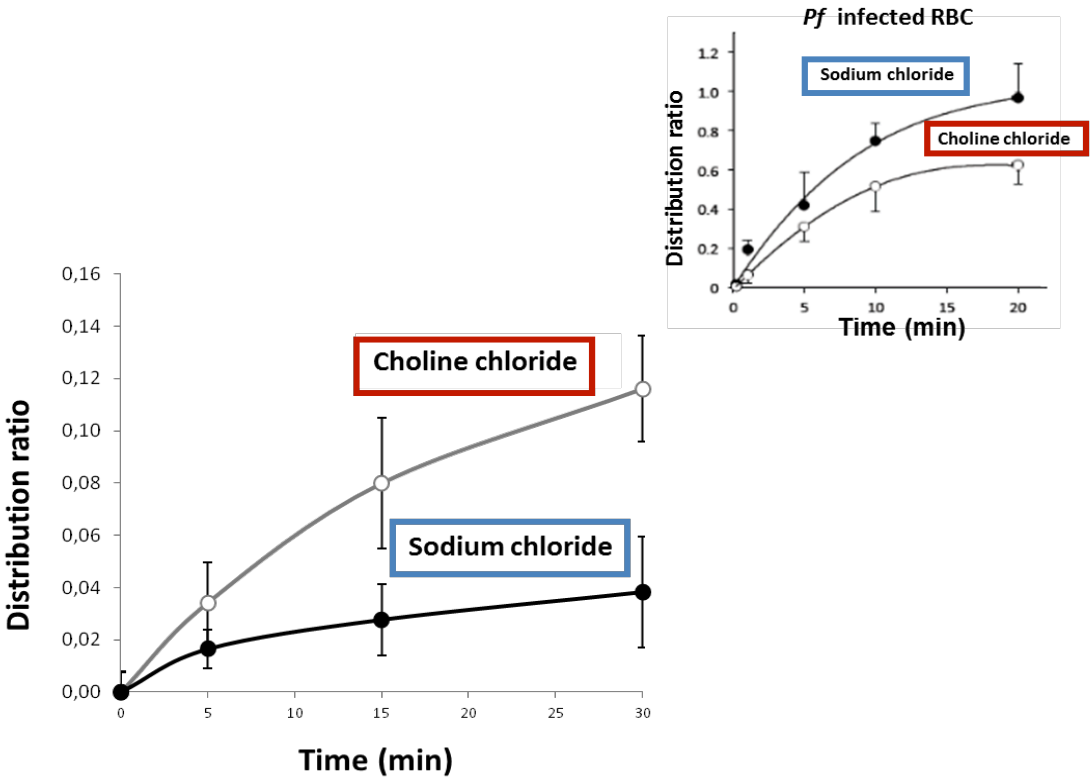


Figure 3.15: Choline stimulation of L-glutamate uptake into red blood cells infected with *B. divergens* (Bd iRBCs). The influx of L-[^{3}H]-glutamate was measured at 0, 5, 10 and 20 minutes at 37ºC. Cells were suspended in solution containing 20mM Sucrose and 10mM Tris-HCl (PH 7.4) together with either sodium chloride 150mM (Na-Cl) or choline-chloride 150mM (Chol-Cl). Radiolabeled L-glutamate was the only extracellular amino acid present at a concentration of 200nM. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data has been averaged from different independent experiments ± SEM. The inset-figure shows influx of glutamate in *P. falciparum* infected cells (modified from Winterberg et al., 2012)

We further investigated the uptake of L- glutamate in *B. divergens* infected RBCs in the presence of different cations such as sodium chloride (Na+), choline chloride (cho+) and N-Methyl-D-glucamine (NMDG) (CH3+). The cells were pretreated with the respective solutions (five times washing) and later influx of L-glutamate was observed over time. The observation from Fig. 3.16 was that the uptake of L- glutamate into *B. divergens* infected RBCs was higher in presence of choline (cho+) in comparision to other cations (Na+) and (CH3+).

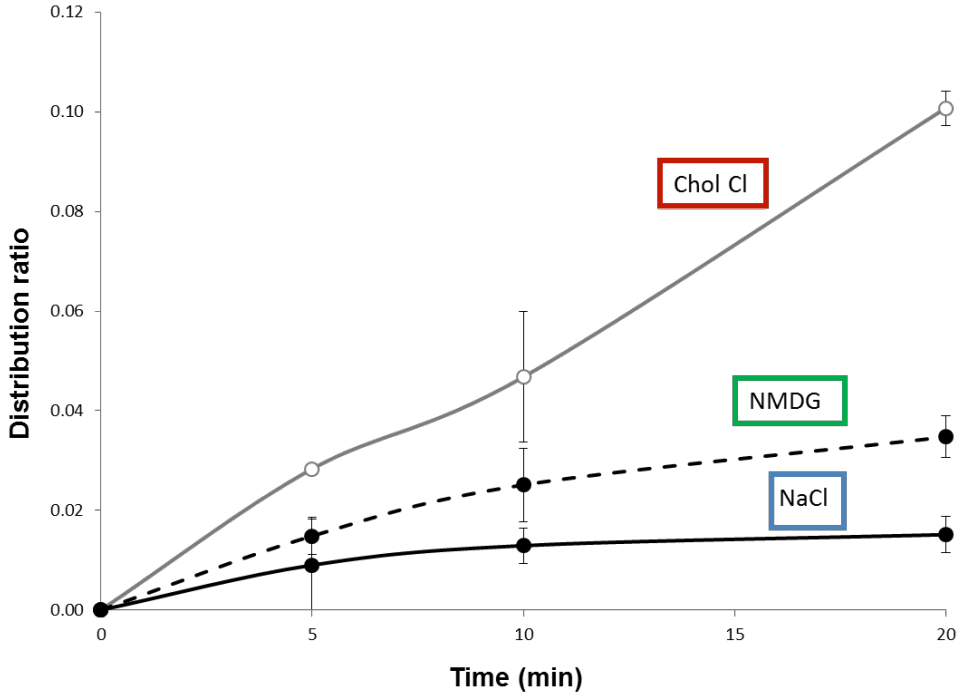
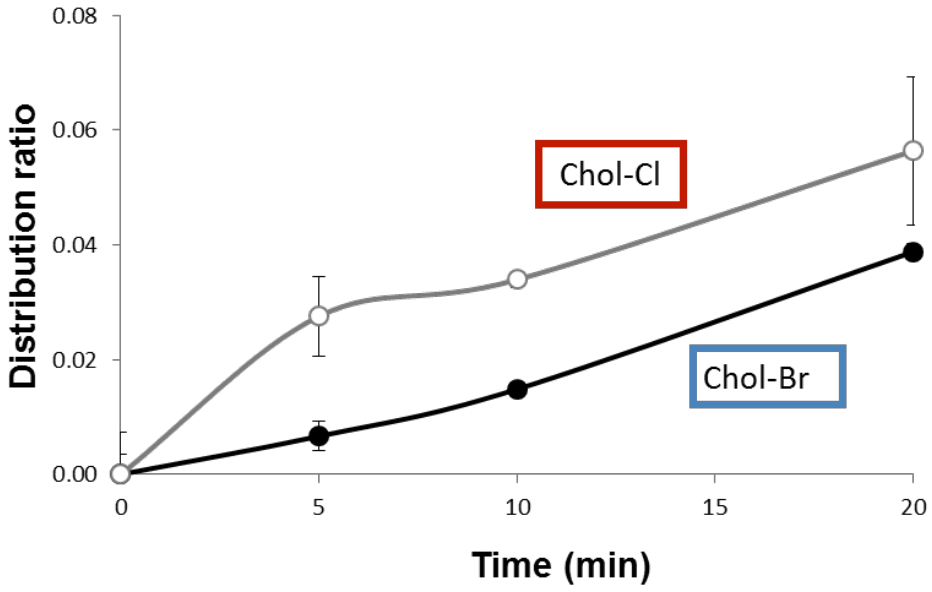


Figure 3.16: Effect of different cations on L-glutamate uptake into red blood cells infected with *B. divergens* (Bd iRBCs). The influx of L-[^{3}H]-glutamate was measured at 0, 5, 10 and 20 minutes at 37ºC. Cells were suspended in solution containing 20mM Sucrose and 10mM Tris-HCl (PH 7.4) together with either sodium chloride 150mM (Na-Cl), choline-

chloride 150mM (Chol-Cl) or NMDG 150mM. Radiolabeled L-glutamate was the only extracellular amino acid present at a concentration of 200nM. The uptake (y- axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data has been averaged from different independent experiments ± SEM.

We also investigated the uptake of glutamate in *B. divergens* infected RBCs in presence of different anions: chloride (Cl^-), bromide (Br^-) and acetate ($CH_3\ CO_2^-$). The cells were pre-treated with the respective solutions (five times washing) and later time course for the influx of glutamate was performed. The observation from Fig. 3.17 (A) was that the uptake of glutamate into *B. divergens* infected RBCs in choline chloride solution was significantly higher as compared to choline bromide solution. Similarly in Fig. 3.17 (B) the uptake of glutamate was more in choline chloride solution as compared to sodium chloride and sodium acetate solutions. The reason behind this observation might be that the glutamate uptake is choline stimulated and not by any anions.

(A)



(B)

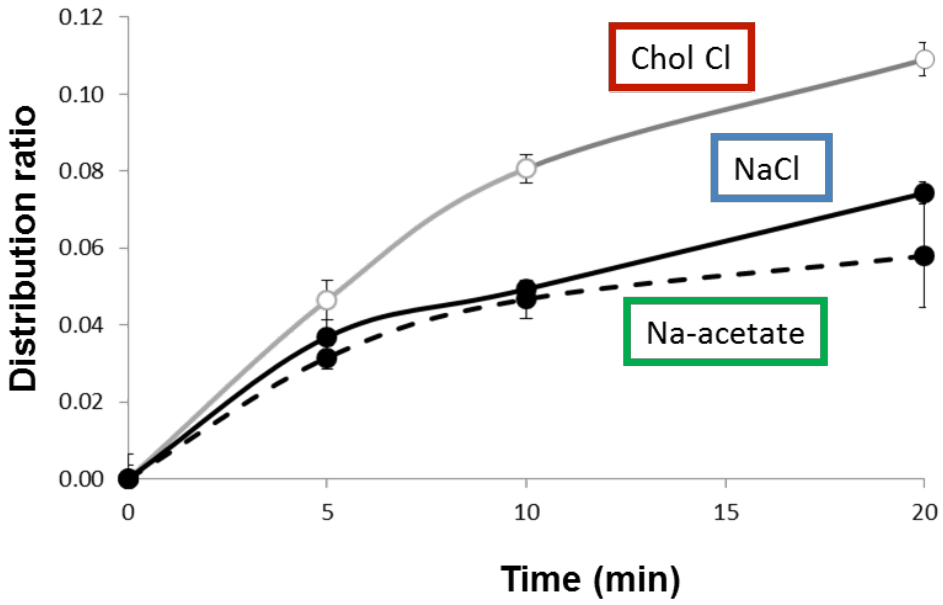
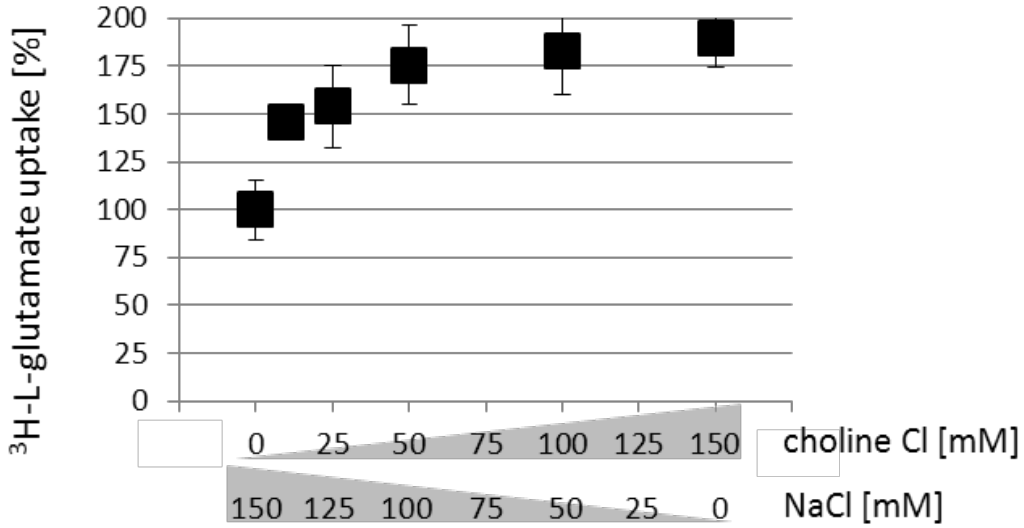


Figure 3.17: Effect of different anions on L-glutamate uptake into red blood cells infected with *B. divergens* (Bd iRBCs). The influx of L-[^{3}H]-glutamate was measured at 0, 5, 10 and 20 minutes at 37ºC. Cells were suspended in solution containing 20mM Sucrose and 10mM Tris-HCl (PH 7.4) together with either (A) choline-chloride 150mM (Chol-Cl), or choline bromide 150mM (chol-Br) and (B) choline-chloride 150mM (Chol-Cl), sodium chloride 150Mm (NaCl) and sodium acetate 150mM (Na-acetate). Radiolabeled L-glutamate was the only extracellular amino acid present at a concentration of 200nM. The uptake data (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate). On the other hand x-axis represents time (t) in minutes. The data was averaged from three independent experiments ± SEM.

Further, we looked into the effect of increasing choline concentration and decreased sodium concentration on the uptake of glutamate in Fig. 3.18. The set of experiments performed in this direction was to look up into the effect of different concentrations of Choline chloride (0, 25, 50, 75, 100, 125, 150 mM), although the final molarity (150mM) was maintained by substituting choline with sodium and potassium.

The results showed that at lower sodium concentrations (125mM and 100mM) in Fig. 3.18 (A) and (B), the rate of uptake increased compared to 150mM and thus remain stationary at and below 100mM of sodium concentration. Therefore, there might be an effect of decreasing sodium concentration on the uptake of glutamate.

(A)



(B)

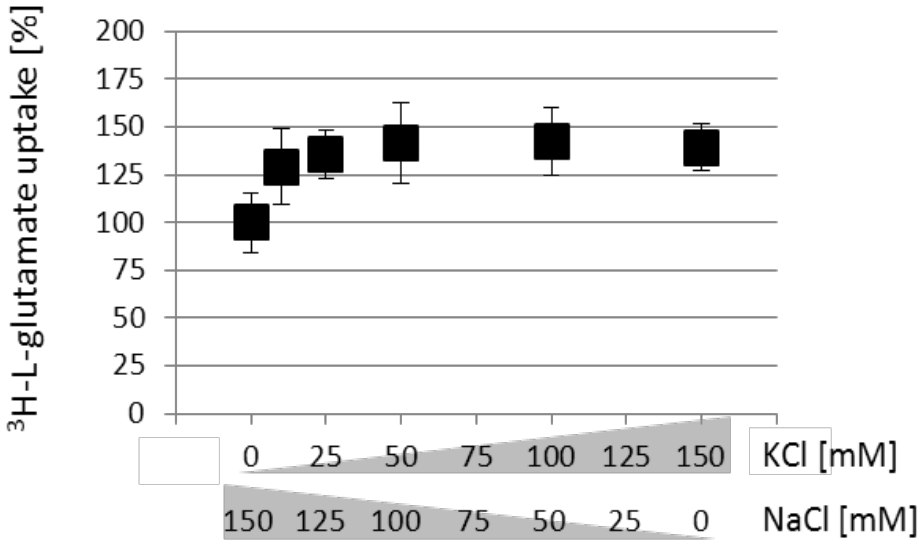
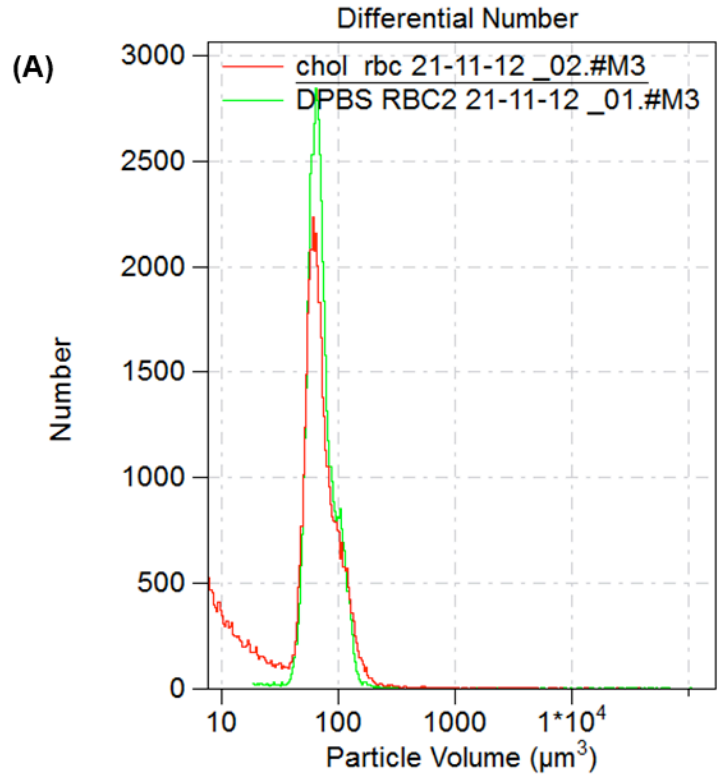


Figure 3.18: Effect of different concentrations of choline chloride on L-glutamate uptake into red blood cells infected with *B. divergens* (Bd iRBCs). Cells were washed and suspended 0, 25, 50, 75, 100, 125, 150 mM of choline chloride substituted with either (A) sodium or (B) potassium to achieve final molarity of 150mM of the solution. The influx of L-[^{3}H]-glutamate was measured after 10 minutes at 37ºC. Radiolabeled L-glutamate was the only extracellular amino acid present at a concentration of 200nM. The uptake data (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate). On the other hand x-axis represents time (t) in minutes. The data was averaged from three independent experiments ± SEM.

3.2.6 Neither Erythrocyte size nor hemoglobin release changed in two solutions PBS and Choline chloride

An important reason for the increased uptake of glutamate into cells can be due to osmosis or lysis in both the solutions. In order to exclude the effects of increased volume or lysis of erythrocytes in presence of choline chlorideor PBS, we compared these parameters by incubating *B. divergens* infected RBCs in either PBS (150mM) or in choline chloride (150 mM).

The erythrocyte size measurement was done using a coulter counter. The Coulter Principle, also known as ESZ (Electrical Sensing Zone Method), the Multisizer 3 COULTER COUNTER® provided mass, number, volume and surface area size distributions in one measurement, with an overall sizing range of 0.4 µm to 1,200µm. Its response is unaffected by particle color, shape, composition or refractive index. The cells (infected RBCs and non infected RBCs) were incubated in either of the two solutions (PBS and choline chloride) for 30 min. and measured in the coulter counter as shown in Fig. 3.19. The results showed that there was no size variation in the two solutions over a period of 30 min. Thus the solutions provide isotonic environment to the non-infected erythrocytes and infected erythrocytes.



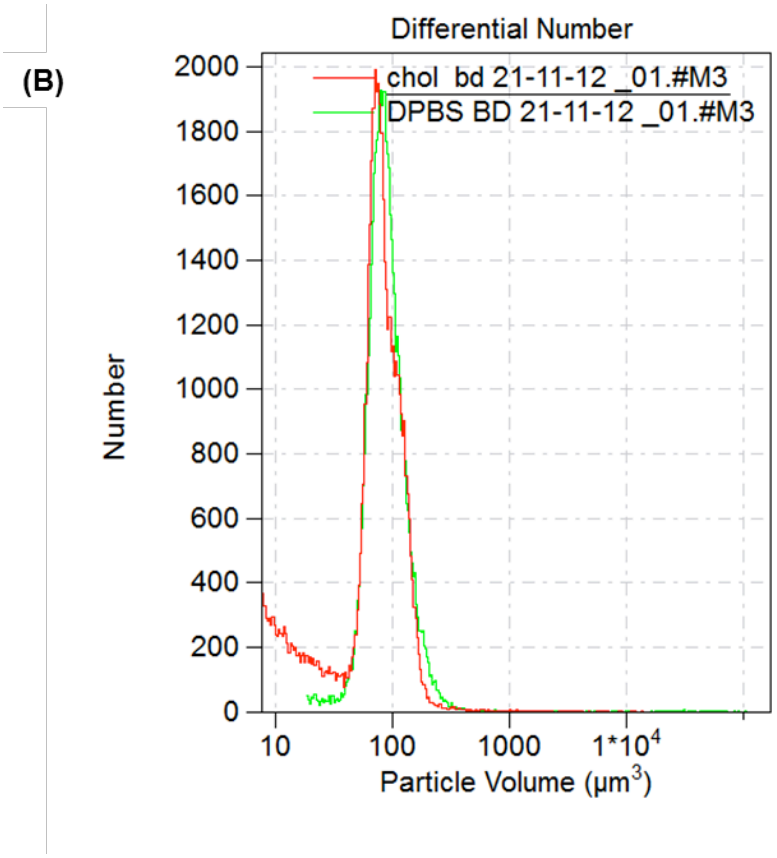


Figure 3.19: Effect of choline chloride and PBS on non-infected and *B. divergens* infected RBCs in terms of volume. Cell volume analysis of (A) non-infected RBCs and (B) *B. divergens* infected RBCs in presence of Choline Chloride (red peak) and PBS (green peak) was done. The cells were washed and incubated for 30 min. in either of the two solutions (Choline Chloride and PBS). And later the effect on volume was measured in the coulter counter. The y-axis represents the number of cells analyzed by the coulter counter. The x-axis represents the volume of the cells analyzed. The data was averaged from three independent experiments ± SEM.

Hemolysis

In order to observe the cell lysis or perhaps the release of hemoglobin from the non-infected RBCs in PBS and Choline choline, the cells were incubated for 30 min. in either of the two solutions and hemoglobin was observed photospectroscopically. In Fig. 3.20, the results showed no release of hemoglobin from the non infected RBCs in either of the two solutions over 30 min. period.

Therefore, both the solutions PBS and Choline chloride do not physically disrupt the cell membrane, thereby providing a stable pH at room temperature.

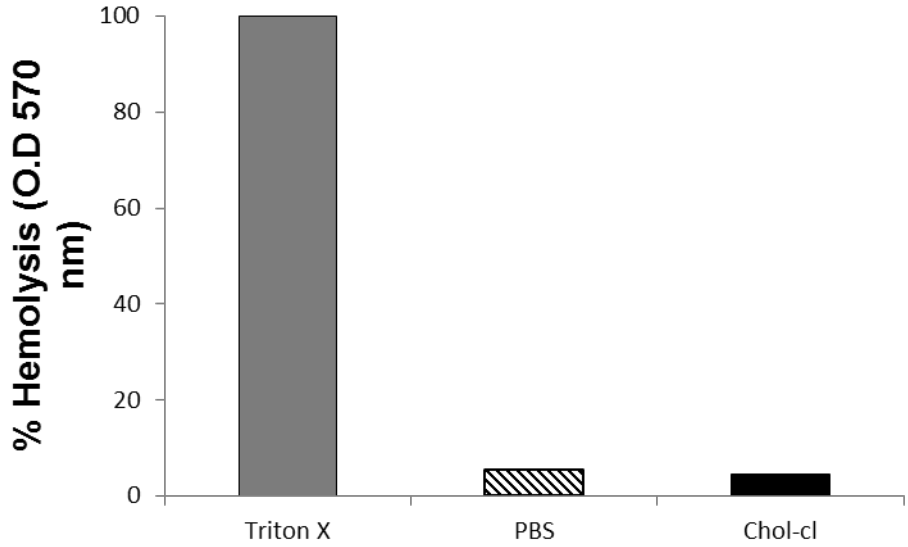


Figure 3.20: Hemolysis analysis of non-infected RBCs in solutions Choline Chloride and PBS. Analysis of cell lysis in non-infected RBCs. The cells were incubated for 30 min. in either of the two solutions Choline Chloride and PBS. Later measured photospectroscopically at O.D 570 nm. Cells lysed with Triton X 100 was taken as positive control. The y-axis represents % hemolysis. The data was averaged from three independent experiments ± SEM.

3.2.7 No sink formation in infected RBCs for the uptake of glutamate in the presence of choline chloride.

We further investigated (Fig. 3.21) for the phenomenon of sink formation, that might be increasing the uptake of glutamate in presence of choline chloride in *B. divergens* infected RBCs which might be facilitating the incorporation of glutamate into newly synthesized proteins, thereby providing a sink for the radiolabelled glutamate. In order to observe the result of this phenomenon, glutamate uptake was performed into *B. divergens* infected RBCs, either in the presence of PBS or choline chloride. Cells were lysed in 10% TCA and the supernatant was separated from the pellet containing the precipitated proteins and thereby both the fractions (pellet and the supernatant) were transferred to a vial for counting radioactivity. Hence the ratio of the precipitable to non-precipitable radioactivity did not vary significantly between different samples with different cell numbers (1.5×10^8 and 3×10^8). Thus glutamate is not influxed into infected cells by the formation of a sink, there is activation of a specific transporter for glutamate uptake in *B. divergens* infected erythrocytes.

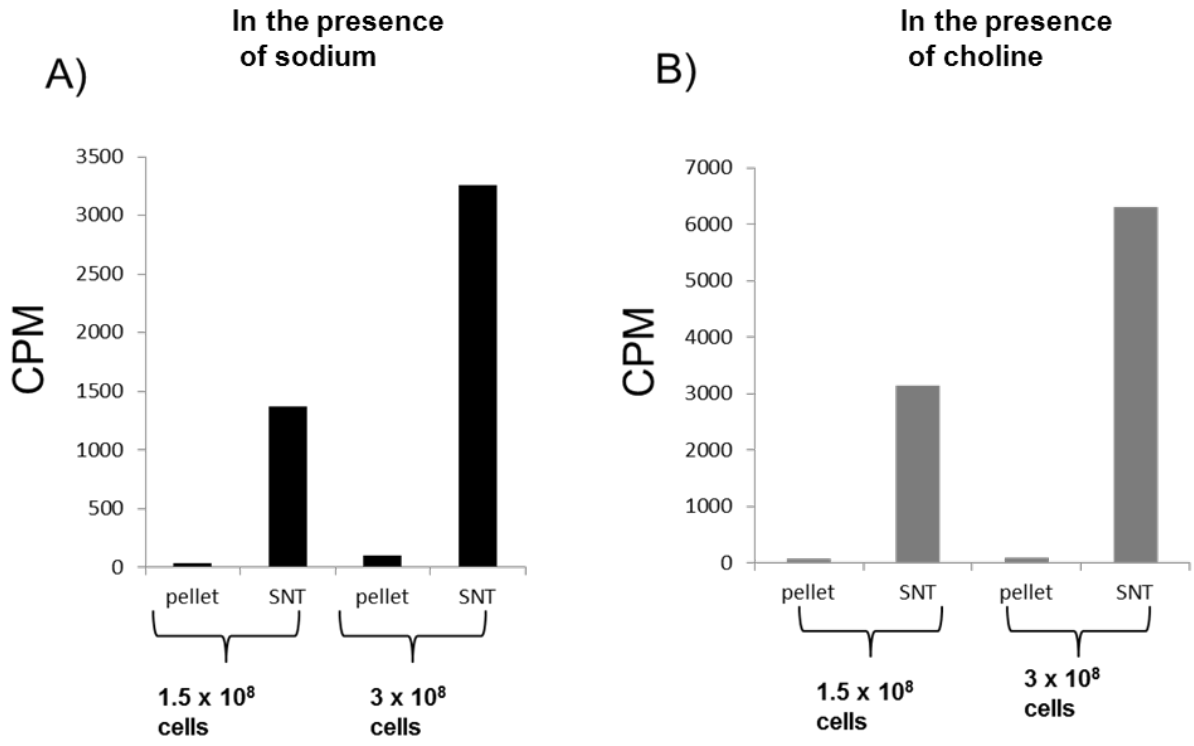


Figure 3.21: TCA analysis of glutamate incorporated protein in *B. divergens* infected RBCs. Cells were washed and incubated with L-(^{3}H) glutamate for 10 min. in either of the two solutions (A) PBS and (B) Choline Chloride. An aliquot containing approximately 1.5 x 10^8 cells and 3 x 10^8 cells was sampled and layered on top of 500µl of oil in a microcentrifuge tube, which was centrifuged at 18000g 2 min, therefore separating the cells from the extracellular media. The supernatant solution was removed and the tubes were washed 3 times with either of the two solutions (choline Chloride and PBS) to remove the residual radioactivity. After the final washing, oil was removed and the cell pellet was resuspended in 100µl of 10% trichloroacetic acid (TCA). The suspension was kept on ice for 20 min. and subsequently the precipitated protein was sedimented by centrifugation at 18000g for 20 minutes. The supernatant was transferred to a scintillation vial, and the remaining pellet was washed 2 times with cold acetone, was dried and also transferred to the scintillation vial for counting. The y-axis represents CPM (counts per minute). The data was averaged from three independent experiments ± SEM.

3.2.8 No activation of EAAT3 in arsenite treated *B. divergens* infected erythrocytesIt has been known that arsenite induces oxidative stress and also activates phosphorylation/dephosporylation pathways in cells (Ventura-Lima *et al*., 2011). In a previous study conducted on the ferret erythrocytes by Flatman and colleagues, they

observed that arsenite activates the ion transporter $Na^+K^+2Cl^-$ by phosphorylation (Flatman *et al*., 1999). Likewise treatment of human erythrocytes with arsenite activates EAAT3 transporter by phosphorylation, which takes up glutamate via a high and, at least one low affinity transporter as determined by Km and Vmax values (Winterberg *et al*., 2012). In my study, all the results above indicate that the EAAT3 is not activated in *B. divergens* infected erythrocytes. We therefore investigated whether this transporter can be chemically activated in *B. divergens* infected RBCs after treatment with arsenite in Fig. 3.22. The non-infected RBCs and *B. divergens* infected RBCs were treated with sodium arsenite and observed for glutamate uptake into cells. In the experiments we compared cultures with different parasitemia (15% and 30%) in order to look for the effect of arsenite treatment on the non-infected and the infected RBCs in a same batch of cells. In the culture with higher parasitemia like 30%, we were unable to show activation of the rest 70% non-infected RBCs. But in the culture with lower parasitemia between 15%-17% we were able to show the activation of the rest 85%-83% non-infected RBCs.

Thus, sodium arsenite stimulated glutamate uptake into non-infected RBCs in a mixed culture with lower parasitemia by 3-fold over the basal levels, whereas it had slight (≈ 40 %) stimulatory effects on *B. divergens* infected RBCs, this effect might be due to the activation of non-infected RBCs present in the mixed culture of cells. Together, infection with *B. divergens* not only fails to activate EAAT3 but it also appears to block chemical EAAT3 activation by arsenite, since we could not observe the activation of non infected cells in a mixed culture with higher parasitemia.

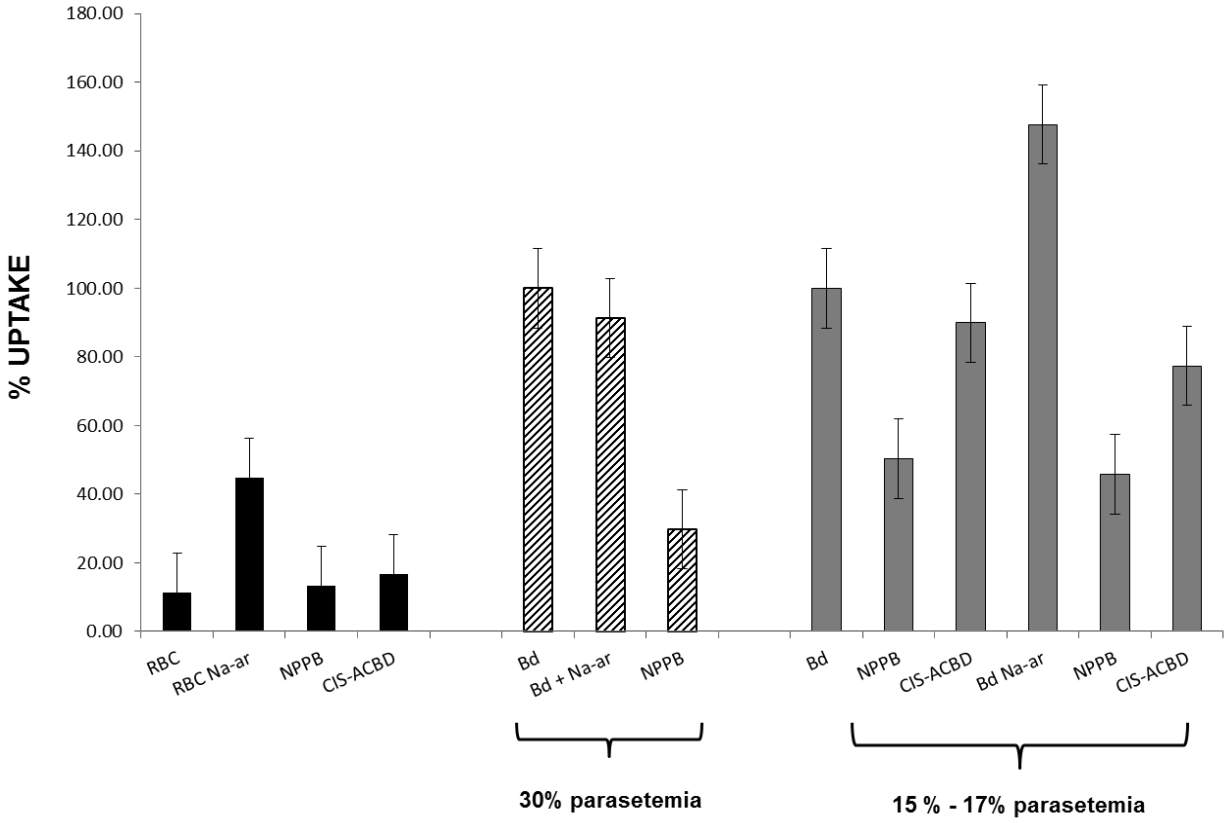


Figure 3.22: Effect of arsenite treatment on *B. divergens* infected RBCs cultures with different parasitemia and also on non-infected RBCs. The influx of L-[^{3}H]-glutamate was measured in triplicates at 0 and 10 minutes at 37ºC. The average at 0 minutes was subtracted from the average at 10 minutes. Cells used were – non-infected RBCs (RBC), arsenite activated RBCs (RBC+Na-ar), *B. divergens* infected RBCs (Bd), arsenite activated *B. divergens* infected RBCs (Bd+Na-ar). Where indicated infected and non-infected cells were treated with Na-ar (1mg/ml) for 30 min. at 37ºC and also as indicated cells were suspended in (Mg2+, Ca2+ free) PBS, NPPB (an inhibitor of the parasite-induced New Permeability Pathways (NPPs) at a concentration of 100µM and cis-ACBD (specific EAAT3 inhibitor) 100µM with radiolabelled L-glutamate. The data has been averaged from three independent experiments ± SEM.

3.2.9 Glutamate transport into *B. divergens* infected RBCs is non-saturable.

A transporter is a membrane protein which catalysis the translocation of solutes by possessing important characteristics of specificity and saturability. In nature, there is common duality of the transport system. There are mixed systems for metabolic transport into infected cell, a high affinity component capable of uptake at low extracellular substrate concentrations and a low affinity component capable to transport large amount of substrate at high extracellular concentrations. In terms of biochemistry, Michaelis-Menten kinetics (v=

Vmax (S)/ Km+(S)) determines the maximum rate (Vmax) and substrate concentration (Km) for a transporter. Winterberg and colleagues observed that *P. falciparum* infection activates the glutamate transporter, and thereby glutamate is influxed into the infected erythrocytes by two types of transport mechanisms, a saturable (high affinity, EAAT3) and a non saturable (low affinity) (Winterberg *et al*., 2012). On the other hand, detailed characteristics of the transporter involved in glutamate uptake in *B. divergens* infected RBCs in not known, this prompted us to investigate the saturability of glutamate transport into *B. divergens* infected RBCs.

Therefore, in Fig. 3.23 we looked for the competing effect of unlabeled L-glutamate (cold) at increasing concentrations (0 - 1000 µM) on the uptake of labeled L-[^{3}H]-glutamate in Na^+ containing media and choline containing media. We observed that the uptake of the radiolabel was largely unaffected by unlabeled L-glutamate, up to a concentration of 1000µM and thus the uptake is non saturable. There was no observed saturability of the transporter and also as shown before in Fig. 3.15 the uptake of glutamate into *B. divergens* infected RBCs in choline containing media was more as compared to Na^+ containing medium.

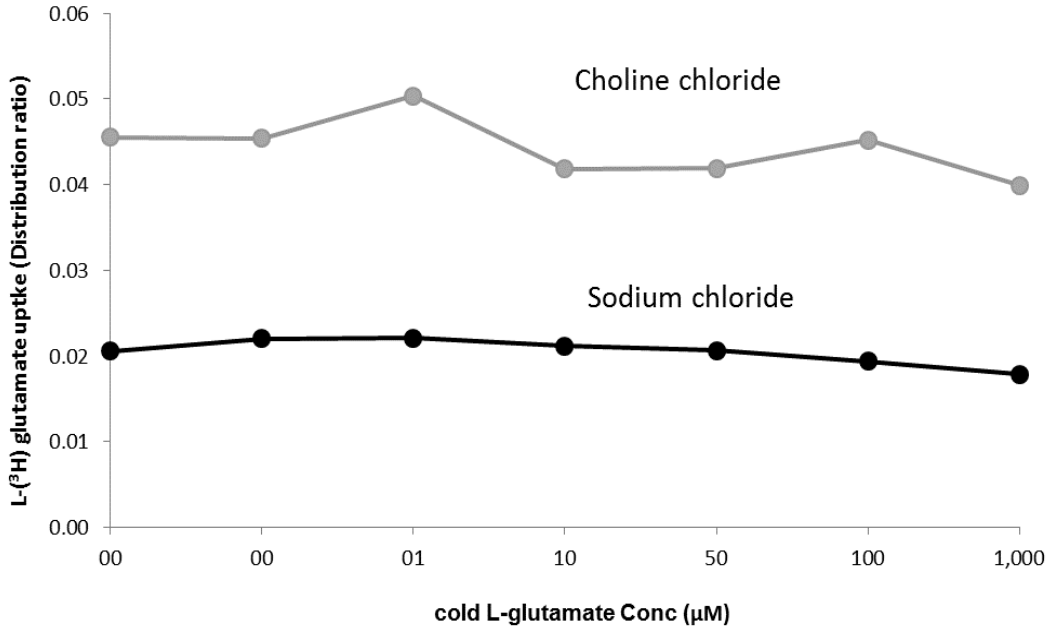


Figure 3.23: Uptake of glutamate in *B. divergens* infected RBCs is non saturable. Effect of unlabeled L-glutamate at concentrations 0 - 1000 µM on the uptake of labeled L-[^{3}H]-glutamate in Na^+ containing media and choline containing media. The influx of L-[^{3}H]-glutamate was measured in triplicates at 0 and 10 minutes at 37ºC. The average at 0 minutes was subtracted from the average at 10 minute. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis

represents concentration in micromolar (μM). The data averaged from three independent experiments ± SEM.

3.2.10 Effect of choline chloride on a non-EAAT3 mediated pathway in *P. falciparum* infected RBCs.

In a previous study it has been shown that *P. falciparum* infected erythrocytes have both low- and high-affinity glutamate transporters activated and also there is inhibition of the EAAT3 activity when Na^+ is depleted. Therefore EAAT3 activated in *P. falciparum* infected RBCs is high affinity, Na^+ dependent, saturable and stereoselective (Winterberg et al., 2012). On the other hand, in case of *B. divergens* infected RBCs, we observed that uptake of glutamate is by a low affinity transporter which is a non saturable transporter and Na^+ independent. Now the next step was to reexamine the previous data, to know whether there is any similarity or difference between the low affinity transport in *B. divergens* and *P. falciparum* infected erythrocytes. The approach was to see any effect of choline on the low affinity transporter in *P. falciparum* infected RBCs, since in *B. divergens* infected RBCs the uptake of glutamate by low afiinity transporter is enhanced by choline and now in *P. falciparum* infected RBCs do we observe same or different effect. Therefore, experiments were designed to discover the effect of choline on glutamate uptake in *P. falciparum* infected RBCs and also how to exclude the interference of EAAT3 mediated uptake in presence of choline.

Since our results showed that the uptake of glutamate into *B. divergens* infected RBCs is by a low affinity transport which is stimulated by choline chloride, we then looked for the possible effects of choline chloride on *P. falciparum* infected RBCs in Fig. 3.24. *P. falciparum* infected RBCs were treated with cis-ACBD (100μM), which blocks the high affinity pathway and also with 1mM glutamate (this concentration, saturates the EAAT3 pathway) (Winterberg *et al*., 2012) thus preventing the uptake of radiolabelled glutamate by the high affinity pathway. Further, the uptake of L-^{3}H-glutamate was determined either in the solution containing choline chloride and sodium chloride.

We observed that the presence of cis-ACBD and cold glutamate reduced the uptake of L-^{3}H-glutamate into *P. falciparum* infected RBCs by 40% as described previously by winterberg and collegues. The residual uptake (60%) of L-^{3}H-glutamate was similar in both solutions containing choline chloride and sodium chloride, suggesting that there is no enhancement in the glutamate uptake in presence of choline in *P. falciparum* infected RBCs.

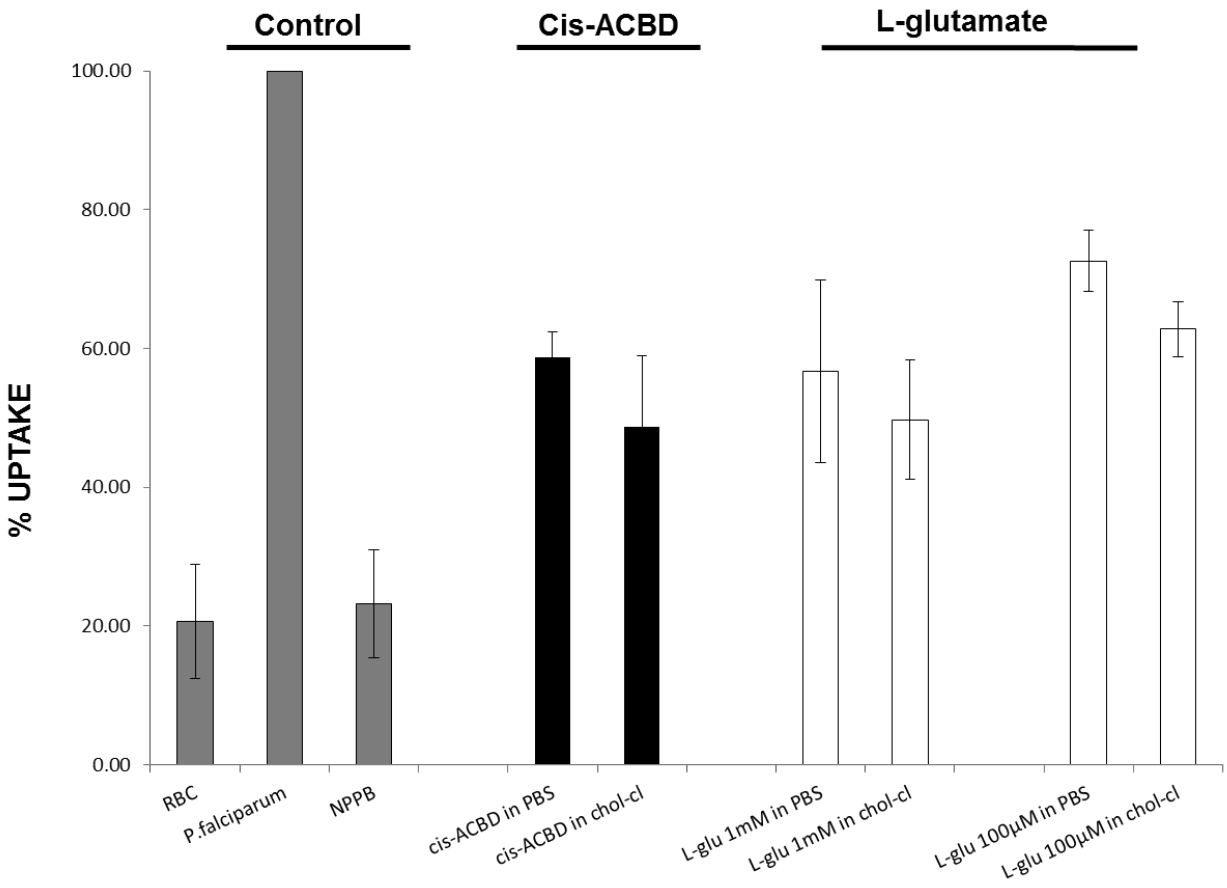


Figure 3.24: Effect of cis-ACBD (PBS/ Chol-Cl) and 1mM Glutamate (PBS/ Chol-Cl) on *P. falciparum* infected RBCs. Quantification of uptake of L-(^{3}H) glutamate into *P. falciparum* infected RBCs. The cells were treated either with cis-ACBD (100µM) (red bars) or non-radioactive 1mM L-glutamate (blue bars) in either of the two (the PBS or choline chloride) for 10 min at 37ºC prior to the uptake and later again for 10 min. at 37ºC with L-(^{3}H) glutamate into the two solutions also having cis-ACBD or non-radioactive L-glutamate. Thereby uptake was measured after the latter step. The y-axis represents the % uptake. The data has been averaged from three independent experiments ± SEM.

3.2.11 (^{3}H)-choline chloride uptake and its inhibition in *B. divergens*-infected red blood cells

Choline is a quaternary ammonium ion (trimethyl-n-propylammonium ion) and a water soluble essential nutrient for the cell. In the non-infected erythrocytes, choline is taken up by a facilitated-diffusion system involving a endogenous transporter having high specificity for choline, saturability and temperature dependence (Martin, 1968; Edward, 1973; Deves *et al*., 1979). To further investigate the role of choline in the enhanced uptake of glutamate in *B. divergens* infected RBCs, we firstly observed the uptake of radiolabelled choline into the non-

infected and into the *B. divergens* infected erythrocytes in Fig. 3.25. We observed that there is a basal level of choline transport in the RBCs, which does increases significantly in *B. divergens* infected erythrocytes when compared.

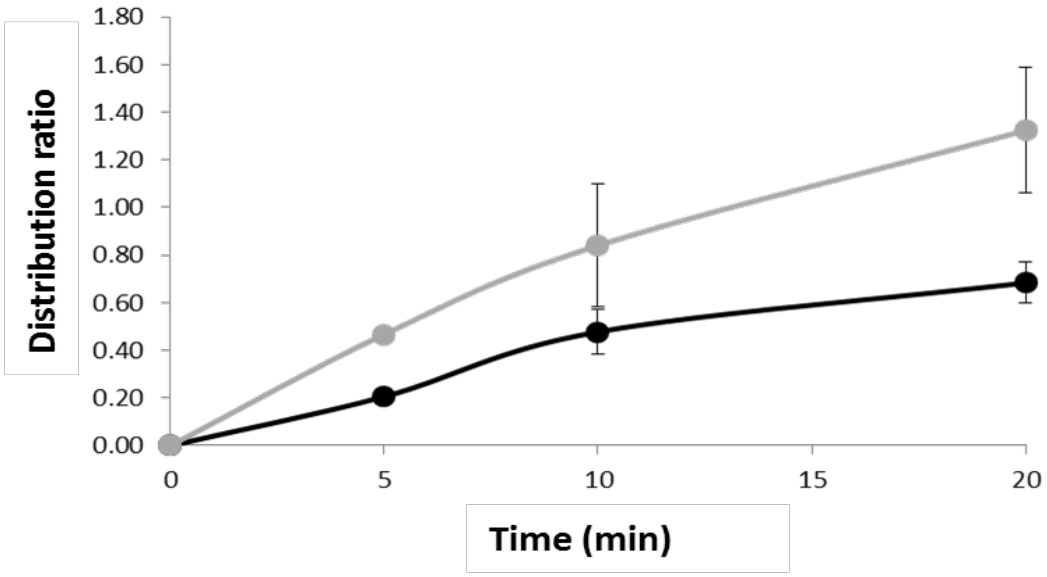


Figure 3.25: Time-course of choline chloride uptake into non-infected red blood cells (RBCs) and red blood cells infected with *B. divergens* (Bd iRBCs). Non-infected red blood cells (—●—) and red blood cells infected with *B. divergens* (—●—). The influx of [^{3}H]-choline chloride was measured at different time points (0, 5, 10 and 20 minutes) at 37ºC. The cells were suspended in PBS. The extracellular concentration of radioactive choline chloride was 116nM. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data has been averaged from three independent experiments ± SEM.

The existence of two choline transport pathways (high and low) has been described in the non-infected RBCs (Martin, 1968; Edward, 1973; Deves *et al*., 1979) and also for the *P. falciparum* infected RBCs (Kirk *et al*., 1991). One of these pathways involves a high affinity choline transporter while the other pathway is less defined and of low affinity. Choline transport can be inhibited by the competitive, high affinity inhibitor hemicholine (HC-3) (Okuda *et al*., 2011) and by the low affinity inhibitor dodecyltrimethylammonium (DoTMA) (Ancelin *et al.,* 1985 and 1991). In my study in Fig. 3. 26, I observed that the uptake of ^{3}H-choline in the non-infected RBCs was lower as compared to *B. divergens* infected RBCs by

40%. Both DoTMA and HC-3 decreased the uptake of ^{3}H choline chloride into *B. divergens* infected RBCs by 80% and 60%, respectively, whereas NPPB had no inhibitory effect. The results show that there is a transport of choline in *B. divergens* infected erythrocytes and the transport is inhibited by choline transport inhibitors, but on the other hand the broad specific NPP inhibitor (NPPB) could not inhibit the uptake which reflects that the uptake of choline chloride is not facilitated by NPP pathways, there might be a activation of a novel transport protein or the transport occurs via the host endogenous pathway.

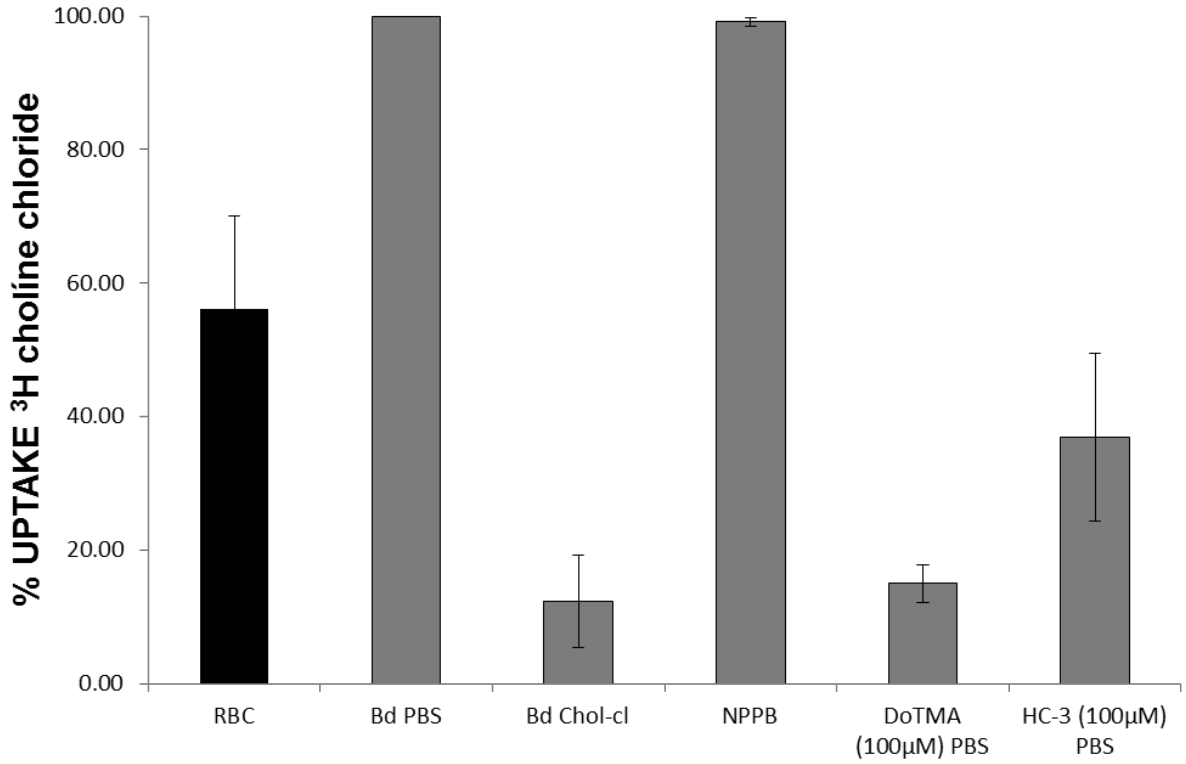


Figure 3.26: Effect of inhibitors of the choline-chloride transporter upon (^{3}H) choline chloride uptake by *B. divergens*-infected cells. The inhibitory effect of dodecyltrimethylammonium (DoTMA) and Hemicholinium-3 (HC-3) was observed on *B. divergens* infected RBCs. Where indicated the cells were pretreated with DoTMA (100µM), HC-3 (100µM) and NPPB (100µM) for 10 minutes at 37ºC. Later on the (^{3}H) choline chloride uptake was measured in the presence of the respective inhibitors for 10 minutes. The y-axis represents the % uptake. The data were averaged from three independent experiments ± SEM.

3.2.12 Choline chloride transporter inhibitors reduce L-(^{3}H) glutamate uptake in *B. divergens* infected erythrocytes

Previous studies show that in the erythrocytes a choline exchanger is present, which in addition to choline also transports other cations like Cs, Rb, K, Li, Na and Mg^2 (Martin, 1972) and also it is an unspecific transporter present mediating uptake of catecholamines (Azoui *et al*., 1996). In my work the observation that choline chloride stimulates glutamate uptake into *B. divergens* infected RBCs led us to investigate for the possible reasons of such a effect, the open questions for this effect are (i) is glutamate coupling with choline and transported via a choline transporter (symporter/antiporter) or (ii) is there any other novel pathway independently influxing glutamate and choline into the cell. We therefore decided to determine L-glutamate uptake in the presence of the choline transport inhibitors in Fig. 3.27. When transport was measured in the presence of NaCl, the high affinity inhibitor HC-3 had no significant effect on L-glutamate uptake, and the low affinity inhibitor DoTMA inhibited uptake by approximately 50%. When L-glutamate uptake was determined in the presence of choline chloride, the high affinity inhibitor again had no effect on L-glutamate transport whereas in the presence of DoTMA, uptake into *B. divergens* infected RBCs was reduced by approximately 50%. These results suggest that, whilst glutamate is transported into *B. divergens* infected RBCs via a pathway that is inhibitable by a high affinity inhibitor (HC-3) and thus this pathway is not involved in the uptake of glutamate. There might be some other transporters involved for the glutamate uptake influenced by DoTMA and its inhibitory effect on the uptake seems to be ~ 50%.

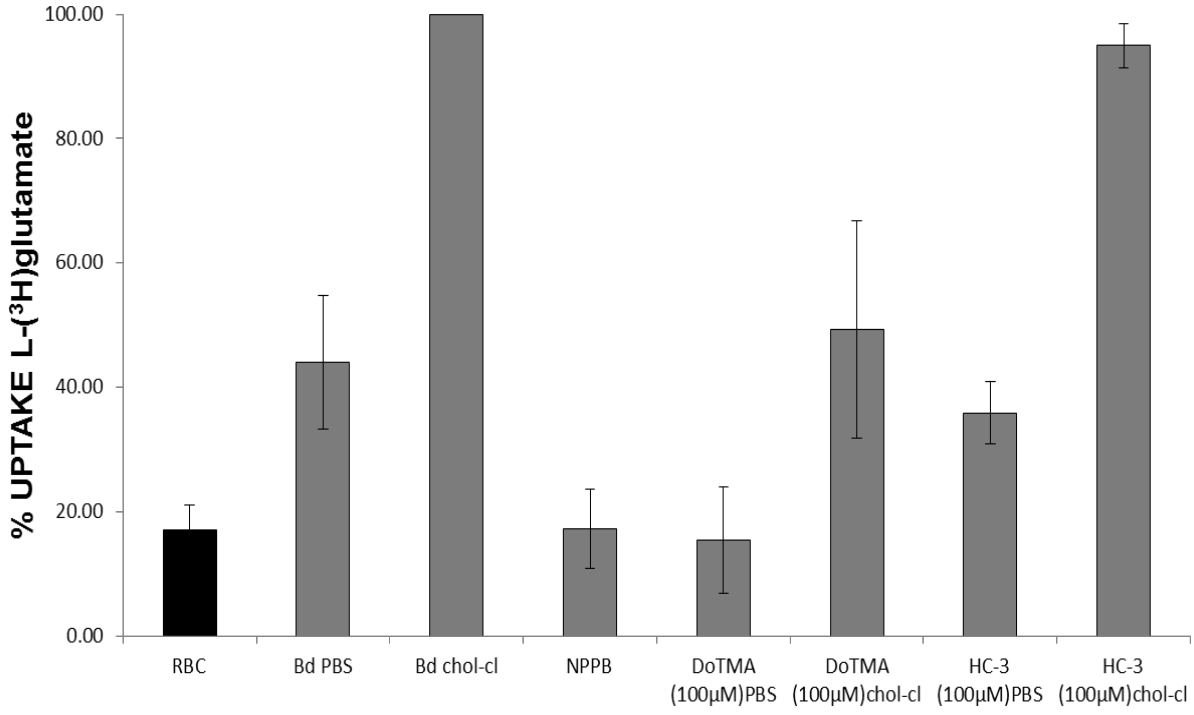


Figure 3.27: Effect of inhibitors DoTMA and HC-3 on L-(^{3}H) glutamate uptake into *B. divergens* infected RBCs. The inhibitory effect of dodecyltrimethylammonium (DoTMA) and Hemicholinium-3 (HC-3) was observed on *B. divergens* infected RBCs. The cells were pretreated with DoTMA (100µM), HC-3 (100µM) and NPPB (100µM) for 10 minutes at 37ºC. Later on the L-(^{3}H) glutamate uptake was measured in the presence of respective inhibitors for 10 minutes. The y-axis represents the % Uptake. The data averaged from three independent experiments ± SEM.

3.2.13 No involvement of cystine/glutamate antiporter for L-glutamate uptake

The bidirectional transfer of amino acids across plasma membrane by antiporters allow regulated transport of amino acids. In human fibroblast there is an Xc^- transporter, which is an Na^+ independent transport system for L-cystine and L-glutamate (Patel *et al*., 2004). *In vitro* this antiporter also functions as a glutamate/glutamate exchanger in cells. Therefore the two systems known till now to transport glutamate in mammalian cells (i) Excitatory amino acid transporters (EAATs) and (ii) cystine/glutamate antiporter. These systems can be distinguished on the basis of their ionic requirements for the transport, the EAATs are sodium dependent transporters, whereas cystine/glutamate are sodium independent and chloride

dependent transporters. Now, whether the Xc^- antiporter plays an important role in glutamate uptake in *B. divergens* infected erythrocyte was analysed by using an inhibitor L-homocysteic acid (LHC), which is a potent competitive inhibitor of this transporter (Angelo et al., 2010). Therefore, we found in Fig. 3.28 that there is no inhibitory effect of LHC on the uptake of glutamate and also as previously shown in Fig. 3.15, the transporter is choline independent. Hence, both the observations support the non involvement of a Xc^- antiporter in the uptake of glutamate.

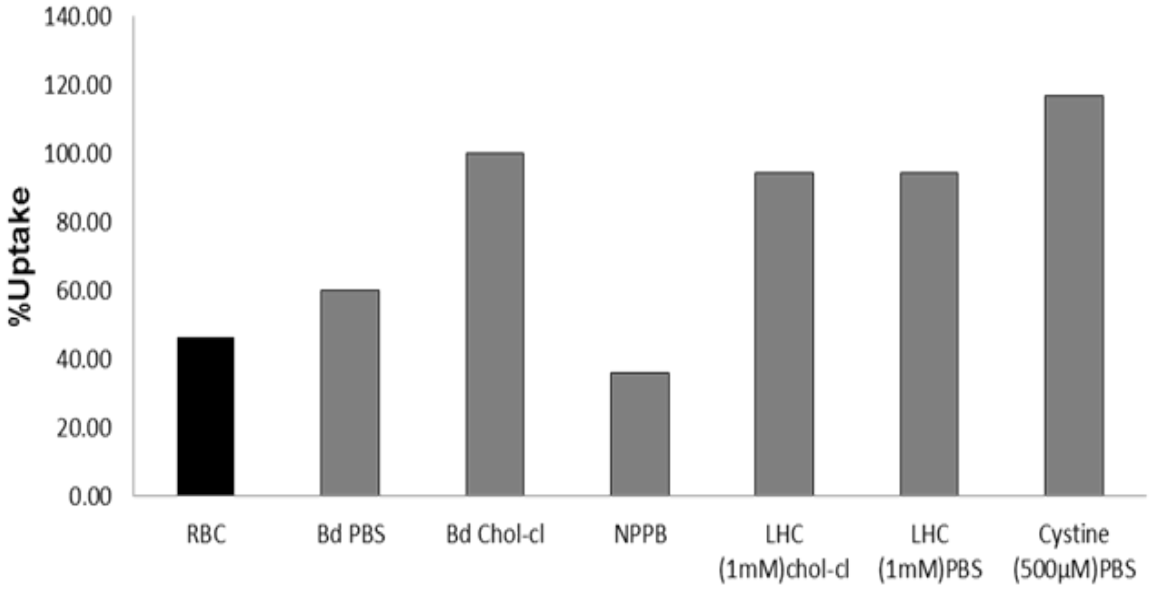


Figure 3.28: Effect of Xc- antiporter inhibitor L-Homocystine (LHC) and L- cystine on L-(^{3}H) glutamate uptake into *B. divergens* infected RBCs. The inhibitory effect of competitive inhibitor L-Homocystine (LHC) and L-cystine was observed on *B. divergens* infected RBCs. The cells were pretreated with LHC (1mM), L-cystine (500µM) and NPPB (100µM) for 10 minutes at 37ºC. Later on the L-(^{3}H) glutamate uptake was measured in the presence of respective inhibitors for 10 minutes. The y-axis represents the % Uptake. The data averaged from three independent experiments ± SEM.

3.2.14 Uptake of charged amino acids by *B. divergens* infected RBCs in presence and absence of choline

There are reports present in the past, focusing on the effect of charge, structure and affinity of different substrates on the choline transport system (Deves *et al*., 1979). As choline is a quaternary ammonium ion having a positive charge, in order to inform us about the effect of charge on the binding site of the transporter in our system, which allow increased uptake of

glutamate (negatively charged) into the *B. divergens* infected RBCs, it was important to look for any effect of charge of L-alanine (neutral), D-glutamate (negative), D-aspartate (negative) and L-lysine (positive) which are taken up in the presence and absence of choline.

The results in Fig. 3.29. showed that the uptake of negatively charged amino acids- D-aspartate and L-glutamate increases in the presence of choline, whereas D-glutamate uptake is an exception whose uptake was same in both the conditions. Also the uptake of positively charged amino acid- L-lysine and neutral amino acid- L-alanine did not increase. Therefore, we observed the effect of charge on the transport of amino acids in the presence of choline, hence this might be via a cotransport.

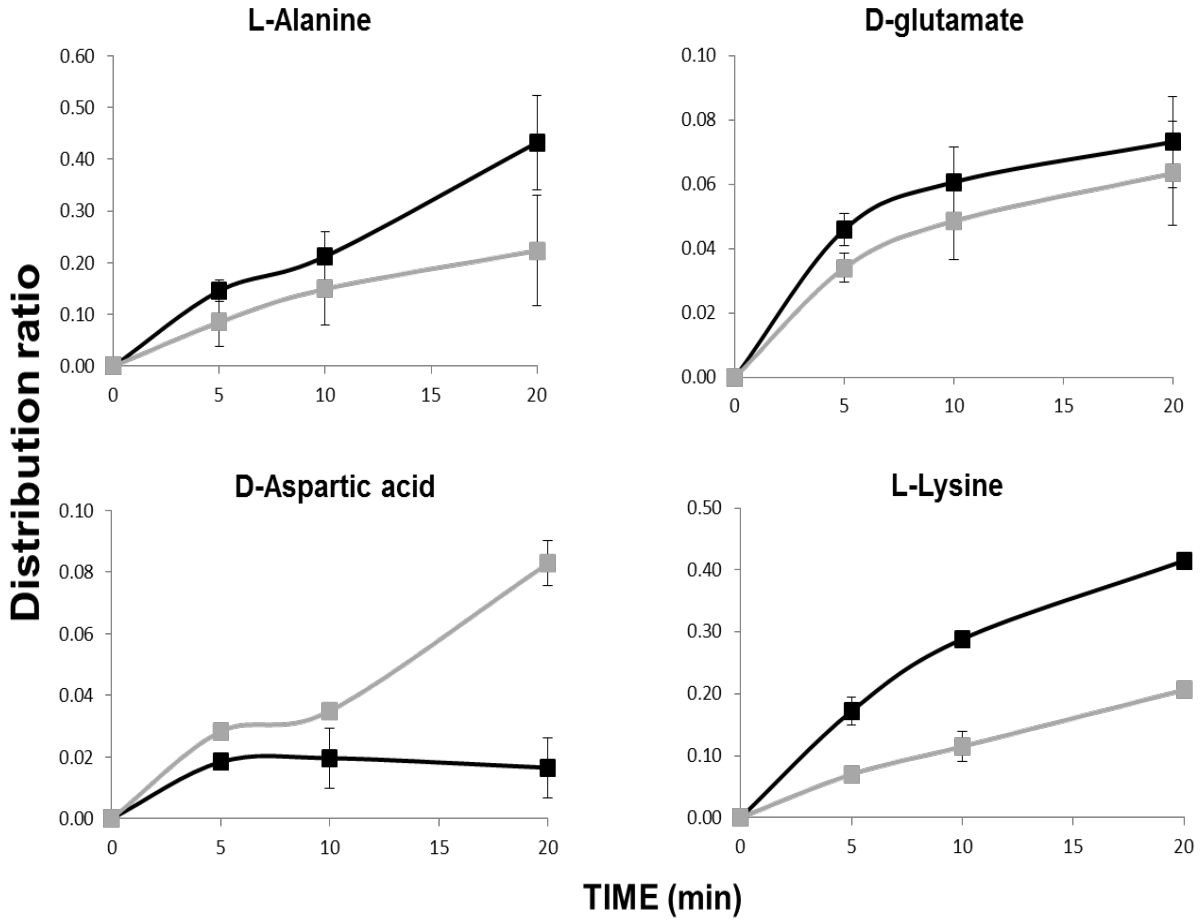


Figure 3.29: Time-course of L-Alanine, D-Glutamate, D-Aspartate and L-Lysine uptake into *B. divergens* infected RBCs (Bd iRBCs) in presence ( ) and absence ( ) of choline chloride. The influx of amino acids L-alanine, D-glutamate, D-aspartate and L-lysine was measured at different time points (0, 5, 10 and 20 minutes) at 37ºC. Cells were suspended in solution containing 20mM Sucrose and 10mM Tris-HCl (PH 7.4) together with

either sodium chloride 150mM (Na-Cl) or choline-chloride 150mM (Chol-Cl). The extracellular concentration of radioactive amino acids was 120nM, 1µM, 885nM and 106nM respectively. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data averaged from three independent experiments ± SEM.

3.2.15 Stereoselectivity observed between L-glutamate and D-glutamate uptake into *B. divergens* infected RBCs.

The data above show that choline chloride has some stimulatory effect on the uptake of L-glutamate into *B. divergens* infected RBCs. Therefore, in order to look for any role of stereoselectivity in the uptake of (L- or D-) glutamate into *B. divergens* infected RBCs we compared the transport of these stereoisomers in the presence and absence of choline chloride. We also know from past reports that *P. falciparum* infected RBCs have a glutamate transporter which is non-stereoselective, hence it was also important as a comparison, to look for the type of stereoselective present in *B. divergens* infected RBCs.

The uptake results in Fig. 3.30, show that the uptake of D-glutamate into *B. divergens* infected RBCs in the presence or absence of choline chloride was similar to uptake of D-glutamte in sodium, therefore no effect on transport of D-glutamate in presence of choline is observed which is in contrast to the uptake of L-glutamate in choline. This leads us to conclude that the transporter is stereoselective for L-glutamate in the presence of choline chloride.

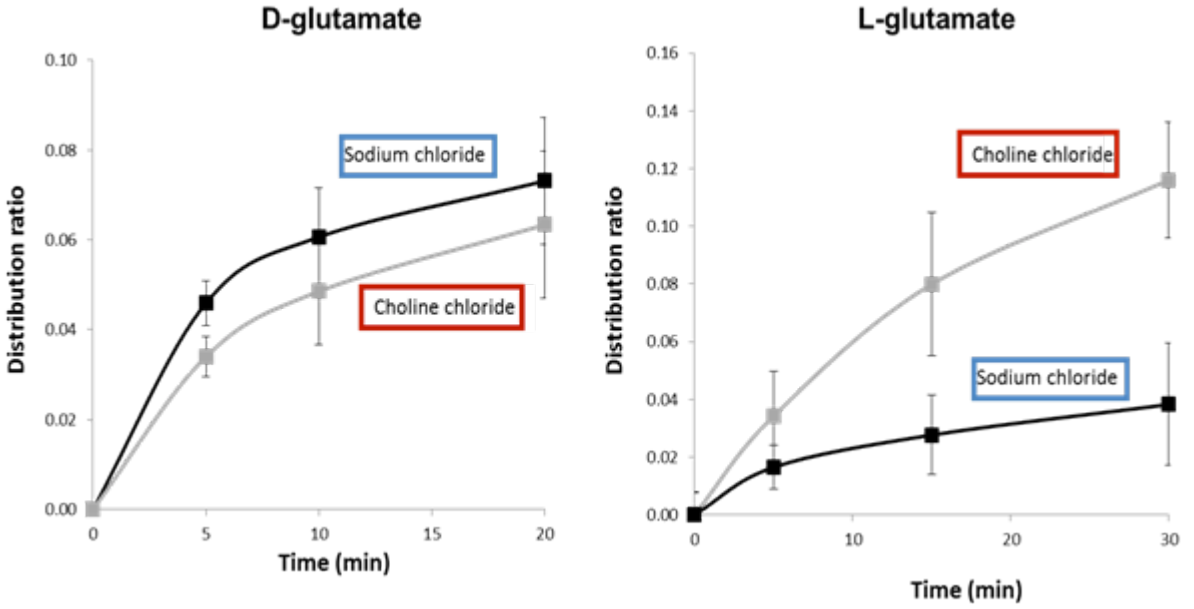


Figure 3.30: Effect of stereoselectivity in *B. divergens* infected RBCs for uptake of D- or L- (^{3}H) glutamate in presence and absence of choline chloride. The influx of D-glutamate and L-glutamate was measured at different time points (0, 5, 10 and 20 minutes) at 37ºC. Cells were suspended in a solution containing 20mM Sucrose and 10mM Tris-HCl (PH 7.4) together with either sodium chloride 150mM (Na-Cl) or choline-chloride 150mM (Chol-Cl). The extracellular concentration of radioactive amino acids was 1µM and 200nM respectively. The uptake (y-axis) is represented in distribution ratio (i.e. ratio of intracellular to extracellular radioactive substrate) and x-axis represents time (t) in minutes. The data were averaged from three independent experiments ± SEM.

Hence, I would summarize the results of my investigations on the influence of choline chloride on the L-glutamate uptake in *B. divergens* infected RBCs (i) firstly the uptake of L-glutamate was increased in choline as compared to sodium (ii) neither cell volume increase nor cell lysis was present in choline (iii) there is no sink formation for the uptake, thus gives us a hint for the activation of a specific transporter (iv) the transport is non saturable (v) the inhibitors (HC-3 and DoTMA) of choline transport, inhibited the L-glutamate transport also.
In order to investigate for the reasons of increased uptake and influence of choline chloride on the glutamate uptake, we also looked for the efflux rate of glutamate in presence and in absence of choline in *B. divergens* infected RBCs.
Therefore, in Fig. 3.31 we observed that there is a high efflux rate of glutamate in the absence of choline chloride as compared to in the presence of choline chloride, which could tell us why we see high uptake in the presence of choline chloride. This difference in efflux might explain this phenomenon of increased uptake in presence of choline than in sodium.

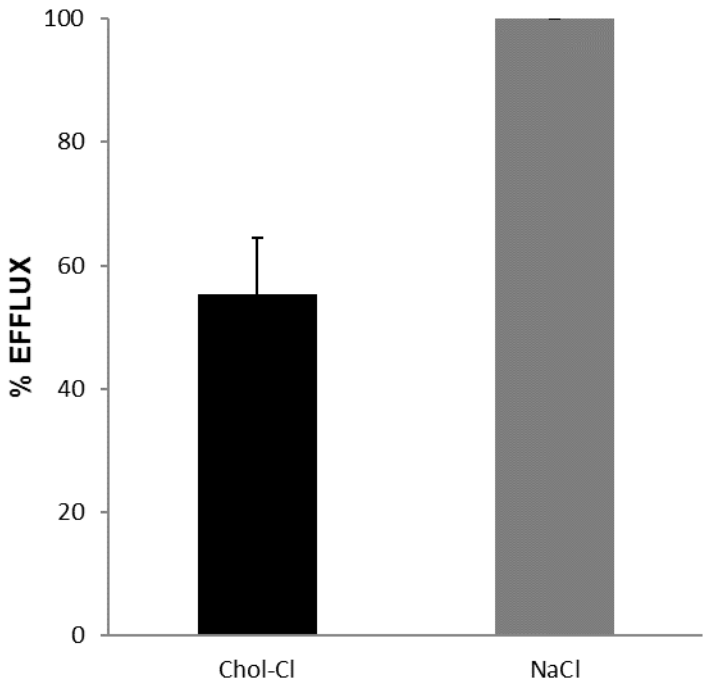


Figure 3. 31: Efflux of L-glutamate in presence and absence of choline chloride in *B. divergens* infected RBCs. The % efflux of L-glutamate in the presence (black bar) and absence (grey bar) of choline chloride in *B. divergens* infected RBCs. The efflux was measured for 10 min. at 37ºC. The data has been averaged from three independent experiments ± SEM.

4. DISCUSSION

Plasmodium and *Babesia* are unicellular eukaryotes of the phylum Apicomplexa which invade the same type of mammalian cells erythrocytes. These parasites live intracellularly with short lived extracellular stages. Intracellular parasitism is like a 'life in a safe haven' where the parasite protects itself from the host immune response and benefits from the nutrient rich environment (Sinai *et al*., 1997). These parasites penetrate their host cell by a multistepped complex process, involving special organelles (micronemes, rhoptry). The complete process of invasion of erythrocyte by parasite takes less than a minute. The differentiated mammalian erythrocytes lack mitochondria, ribosomes, endoplasmic reticulum and golgi apparatus (Baldini *et al*., 1960; Greminard *et al*., 2002; Gronowicz *et al*., 1984) and are considered metabolically inactive (Kirk, 2001; Baumeister *et al*., 2010). Despite the lack of nutrients inside the host cell, the parasite grow into the small, metabolically active merozoite form of each genus (*Plasmodium* and *Babesia*), which are the infectious stages of the complex life cycle. The intra-erythrocytic developments take place inside a compartment called the parasitophorous vacuole, which surrounds the parasite and forms an interface between the parasite and the host cell cytoplasm.

During the course of the parasite development, the host cell undergoes various alterations, which are required on one hand for parasites survival and on the other hand, directly contributes to the symptoms of severe malaria. There are various alteration/events in the host cell triggered by intracellular parasites, which require involvement of proteins, lipids and a variety of solutes. The important events induced are (i) erythrocyte membrane indentation by the parasite during the invasion (ii) formation and maintenance of the PVM and PV (iii) development of new permeability pathways (NPP) (iv) machinery to sustain import and export of parasite proteins to the vacuole and erythrocytes membrane (v) antigenic and structural changes in the membrane and cytoplasm of the erythrocyte.

Although both the parasites *Plasmodium* and *Babesia* are phylogenetically distinct apicomplexan parasites, they share a number of cell biological features. The most important amongst them is the potential for rapid and exponential growth in the erythrocytes. Also, for both the parasites intracellular survival results in host cell modifications mediated by the parasite proteins. Therefore, it is interesting to observe similarities between these distantly related parasites.

The aim of my study was to investigate (i) the internalization of GM1, a component of lipid rafts, after infection by *Plasmodium falciparum* and *Babesia divergens* into the parasitophorous vacuolar membrane (PVM). In order to look for specific entry sites (lipid rafts) for both the parasites (*Plasmodium* and *Babesia*) on the RBCs membrane for invasion,

also which is the initial step in the biogenesis of the parasitophorous vacuole (PV) and (ii) comparing the uptake of glutamate into both the parasites i.e. observing permeability changes in the erythrocyte membrane after infection by the malaria parasite (*Plasmodium* and *Babesia*).

4.1 Biogenesis of PV: through specific entry sites

After infection by the parasites, there is compartmentation and appearance of novel membrane systems in the infected erythrocytes. The most conspicuous amongst them are firstly the parasitophorous vacuole (PV), which is a unique compartment as compared to the other host cell compartments (Lingelbach *et al*., 1998) and secondly the parasite cytoplasm, also containing a unique set of proteins (Nyalwidhe *et al*., 2006). The PV is surrounded by Parasitophorous vacuolar Membrane (PVM), which is an important barrier between the parasite and the host cell. The PV provides ideal shelter for the parasite as it has a neutral pH, no hydrolases and no excess of parasiticidal cytosolic proteins. Since it is an important interface between the parasite and the host cell cytoplasm its biogenesis and cell biological functions are of considerable interest.

The erythrocytes possess no mechanism of endo- nor phagocytosis because of its unique mesh of cytoskeleton present and anchoring with various erythrocyte membrane proteins (Nans *et al*., 2011). In case of *P. falciparum* infected erythrocytes the parasitophorous vacuole (PV) remains throughout the intracellular development whereas in *B. divergens* infected erythrocytes it disintegrates soon after the invasion. The molecular mechanisms underlying the formation and disintegration of parasitophorous vacuole are not known in both the parasites *P. falciparum* and *B. divergens*. The contribution of erythrocyte membrane lipids and proteins in the composition of Parasitophorous Vacuole Membrane (PVM) is a matter of debate (Ward *et al*., 1993; Dluzewski *et al*., 1995; Susstoby *et al*., 1996). The past studies show that lipids required for the PVM are derived from the host cell (Lingelbach *et al*., 1998; Ward *et al*., 1993; Pouvelle *et al*., 1994) and the host cell proteins such as band 3 and glycophorin show no contribution in the vacuolar formation (Atkinson *et al*., 1987; Dluzewski *et al*., 1989; Aikawa *et al*., 1981; McLaren *et al*., 1977). In general, the involvement of proteins in the formation of PVM is not clear. However, recently it has been found that several erythrocyte membrane proteins are internalized into infected erythrocytes, presumably during or soon after invasion rather than retrograde trafficking from the erythrocyte membrane to the parasite (Hiller *et al*., 2003; Bietz *et al*., 2009; Repnik *et al*., 2015). The proteins like aquaporin 3, flotillin 1 and 2 are recruited to the vacuole, and are lipid raft associated proteins (Murphy *et al*., 2004, 2007). Therefore, it is important to

investigate whether the host cell membrane proteins are internalized into PVM of *B. divergens* and can be the bonafide markers of the PV formation and disintegration. It is also important to investigate the role of recuited proteins found in the PVM for parasite survival and whether these lipid raft associated proteins, although speculative, might be specific sites for the parasite entry.

In the previous studies it has been hypothesized that PVM formation in *P. falciparum* infected cells, is a parasite driven and selective process, and certain internalized host cell proteins may have signalling functions in the generation of PVM (Harrison *et al*., 2003). Thus, it is interesting to know that whether *B. divergens*, a parasite with different cell biological properties recruits, or excludes the same type of proteins as for *P. falciparum* into the newly formed PVM. In this present study, we have examined internalization of GM1, a glycosphingolipid and a lipid raft component (Harder *et al*., 1998) into PVM of *B. divergens* and *P. falciparum*. Lipid rafts are submicron (70-200 nm) clusters or 'islands' of proteins and lipids held together by high levels of cholesterol (Simons *et al*., 1997; Brown *et al*., 1998). These specific sites (Lipid rafts) are thought to play an important role in the invasion of the parasite into the erythrocytes. Therefore, we observed internalization of GM1 labelled with CtxB into PVM after infection by *P. falciparum* and *B. divergens* in the erythrocytes. The internalization study was done by labelling GM1 with fluorescent CtxB (cholera toxin B subunit) and further the treatment of the fluorescent complex GM1-CtxB by anti-CtxB was done. Initially different methods were done to standardize the binding of fluorescent CtxB with GM1. The data in Fig 3.3 and 3.6 illustrate the binding of GM1 with fluorescently labelled cholera toxin Beta subunit on non-infected cells and infected cells. GM1 is present on the outer lipid bilayer membrane of the erythrocytes and initial experiments to label GM1 with CtxB did not give us meaningful results as we did not observe any labeling (bright spots) on the erythrocytes, the reasons could be the serum present in the RPS media was interfering with the binding, or there might be no recognition of GM1 by CtxB on the surface of RBCs. But later as seen in Fig. 3.6, by using Fish skin gelatin to block the cells, labeling worked and we could observe a pattern of bright red spots on the surface of erythrocytes. The labelling and treatment of GM1 with CtxB plus anti CtxB formed distinct patches of GM1 on the erythrocyte surface. Also in order to enhance the fluorescence signal, we used anti CtxB as a primary antibody and another anti anti CtxB as a secondary antibody, which was fluorescently labelled.

Thus, the observation of bright patches of GM1 labelled with fluorescent CtxB on the erythrocytes in our results was similar to the labelling performed previously by Mrowczynska and colleagues on RBCs (Mrowczynska *et al*., 2008). In Fig 3.7 and 3.8 specificity and efficiency of the antiserum CtxB was observed by immunoblotting, which was found not

reacting with non-infected erythrocytes or with *P. falciparum* and *B. divergens* infected erythrocytes. Also, the proteinase K treatment indicated that CtxB was not gaining access to the inner leaflet of RBCs membrane or to the RBCs cytoplasm rather was present on the surface of the erythrocytes. Finally, the invasion studies were performed (Fig 3.9) for both *P. falciparum* and *B. divergens* parasites to locate the fate (internalised or not) of GM1 labelled with CtxB. The data showed that prior to invasion, CtxB was present on the surface of the non-infected erythrocytes and soon after the invasion, CtxB was found to be internalised during invasion and associated around the periphery of both the parasite *P. falciparum* and *B. divergens*. However, the methods used cannot distinguish whether the CtxB was present in the PVM or PPM of each of the two parasites. The labelling in the case of *B. divergens* was present in the young stages referring to the fact that the PVM is present during the early stages of development and disappears in the later stages. On the other hand, labeling in the case of the *P. falciparum* was present in all the stages of the life cycle. There are several unanswered questions about this observation of internalisation of CtxB such as exact location around the parasite, erythrocyte membrane remodelling after exclusion of sphingolipids (GM1), which other proteins or lipids are fortuitously involved in these signalling events and PVM expansion. However, from this research it is clear that internalisation of GM1 is a highly selective process.

In conclusion, it appears that there is some similarity between the early events in the PVM formation in both the parasites *P. falciparum* and *B. divergens*. However, there are several questions still remaining unanswered like other biophysical (cell shape, tonicity) and biochemical (oxidation stress), constrains of the erythrocyte membrane involved in the PVM formation, the disintegration of PV in *B. divergens* and most importantly, why the two parasites adopt different strategies to survive in the same type of host cell environment.

This research substantiates our hypothesis on the conservation of the molecules and processes involved in the invasion and the fate of PVM in these two parasites, inside the same type of host cell. Further, it paves the way for the comparative analysis to be investigated, involving different other molecular constrains present in the host cell and taking part in the development of parasites.

This work was discontinued later by me and was taken over by one of my colleague, focusing on extraction of DRM´s containing CTX and electron microscopic studies to revealing more about specific entry sites.

4.2 Parasite increases permeability of human erythrocyte membrane: physiological changes

One of the earliest and important changes after infection is the increase in permeability of the erythrocyte membrane and the formation of NPP (New Permeation Pathways). Since, the intracellular parasite has limited ability to synthesize amino acids required for the parasite growth. Thus, amino acids are obtained from different sources such as the digestion of hemoglobin within the food vacuole and increased influx from the extracellular medium by new permeability pathways (NPP) formed in the host cell membrane (Kirk, 2001). In case of non-infected erythrocytes, which has a variety of different amino acid transport systems, the uptake of amino acids and solutes are limited. The nature of the proteins mediating the transport of solutes across the erythrocyte membrane after infection is a matter of debate (Ginsburg *et al*., 2004, Staines *et al*., 2007). From the previous research conducted, the formation of NPP might involve activation of endogenous host cell transporters or channels such as the glutamate transporter and voltage dependent anion channel respectively (Winterberg *et al*., 2012; Bouyer *et al*., 2011) or parasite encoded proteins like PSAC (plasmodial surface anion channel) (Desai, 2012).

4.2.1 Characterisation of NPP in *B. divergens*-infected erythrocytes

As seen for *P. falciparum*, upon invasion of the host erythrocytes, *B. divergens* also induces the uptake of solutes across the erythrocyte membrane, which are not taken up by the non-infected erythrocytes. The uptake kinetics for some solutes have been compared between *P. falciparum* and *B. divergens* infected cells, and have shown distinct differences between the two parasites (Alkhalil *et al*., 2007). In *P. falciparum* infected erythrocytes NPP appear approximately 10-15 hours post invasion and coincides with the transformation time of ring stage parasites to the metabolically more active trophozoites. In *Babesia* infected cells the appearance of NPP is difficult to determine (i) because of the shorter replication cycle which is approximately around 8-10 hours (Valentin *et al*., 1991) and, more importantly, (ii) because in contrast to *P. falciparum* the developmental stages of *Babesia* can currently not be synchronized. As a consequence, *in vitro* cultures of *Babesia* parasites always contain a mixture of different developmental stages. Therefore, characterization of NPP in terms of its transport kinetics for different solutes and other biochemical properties was conducted as an aim of my research.

4.2.2 Physiological and biochemical comparisons of NPP in *B. divergens* and *P. falciparum* infected erythrocytes

Recently, in the work conducted by Alkhalil and colleagues on *B. divergens* and *P. Falciparum* infected erythrocytes, they observed increased permeability of the infected host erythrocytes to different solutes such as sorbitol, methionine, proline and glycine etc. by different transport mechanisms in both the parasites (Alkhalil *et al*., 2007). Our group extensively characterized the transport system of the amino acids glutamate and alanine, and of different biotin derivatives into *P. falciparum* infected erythrocytes (Baumeister *et al*., 2003 and 2006; Winterberg *et al*., 2012). The characteristics of the transport system involved in the uptake of L-glutamate into *B. divergens* infected erythrocytes has not been investigated earlier.

We started our investigation by comparing the influx of the amino acids L-glutamate, D-aspartate, D-glutamate and L-alanine into the non-infected and intact infected erythrocytes (*P. falciparum* and *B. divergens*) as shown in Fig 3.10. The radiolabeled amino acids used were at concentrations approximating those found in adult human plasma (Iwasaki et. al., 1992): L-glutamate (200nM), D-aspartate (885nM), D-glutamate (1µM) and L-alanine (120nM). We observed the uptake kinetics of the amino acids under *in vitro* conditions, which allows us to estimate the relative contribution of different transport systems active in the infected erythrocytes. For all the solutes, *B. divergens* showed lower uptake kinetics as compared to *P. falciparum* infected erythrocytes, reflecting that both genus might have (i) different transport mechanisms or (ii) may be the transporter has different substrate affinity in the two parasites or (iii) there are differences in the copy number of the transporters present, in context to the origin of protein (parasite or host) .

4.2.3 No activation of EAAT3 type glutamate transporter in *B. divergens* infected erythrocytes

Winterberg and colleagues have recently shown that the treatment of the non-infected human erythrocytes with arsenite activates high affinity glutamate transport (EAAT type) and also *P. falciparum* infected erythrocytes show activation of the high-affinity glutamate transporter (EAAT3) after infection (Winterberg *et al*., 2012). They have shown that the high-affinity glutamate transport can be abolished using cis-ACBD (1-aminocyclobutane-1, 3-dicarboxylate) which is an L-glutamate analog (Griffiths *et al*., 1994). There are also several inhibitors of anion channels, such as NPPB and furosemide, known to block NPP activity in *P. falciparum* infected erythrocytes (Kirk *et al*., 1994) and which also inhibit glutamate uptake in *P. falciparum* infected erythrocytes (Winterberg *et al*., 2012).

In the experiment conducted to observe the presence of (EAAT3) glutamate transporter in *B. divergens* infected RBCs, in Fig. 3.11 we found that cis-ACBD does not inhibit glutamate influx into the *B. divergens* infected erythrocytes whereas in the *P. falciparum* infected erythrocytes and the arenite treated non-infected erythrocytes (taken as controls) showed inhibition by cis-ACBD. Thus providing a clear line of evidence for no activation of EAAT3 in *B. divergens* infected erythrocytes for the uptake of glutamate. Next in order to activate chemically EAAT3 in *B. divergens* infected RBCs, an experiment was performed as shown in Fig. 3.22, we found that sodium arsenite stimulated glutamate uptake into the non-infected RBCs (mediated by EAAT3) by 3-fold over the basal levels, whereas it had no stimulatory effects on the *B. divergens* infected RBCs. Hence infection of erythrocytes with *B. divergens* not only fails to activate EAAT3 but it also appears to block the chemical activation of EAAT3. Thus, in the *B. divergens* infected erythrocytes EAAT3 obviously is not activated. The reasons behind non activation of EAAT3 in Babesia might be that the oxidative stress required to activate the transporter is not enough or there might be different kinases involved in the activation and deactivation of the transporter in both the parasites, as an evidence a recent report from phosphoproteome analysis in *Plasmodium* infected cells has concluded presence of several kinases in the host cell and parasite cytosol (Pease *et al*., 2013; Abdi *et al*., 2013).

We further investigated the characteristics of the transporter involved in the uptake of glutamate into *B. divergens* infected erythrocytes. As above, we have discussed the non activation of EAAT3 glutamate transporter in *B. divergens* infected erythrocytes. Other interesting characteristics of the glutamate transport in *B. divergens* infected erythrocytes observed were (i) the broad specific NPP blockers like NPPB (blocks ~80%) as shown in Fig. 3.11 and also furosemide (blocks ~ 60%) had an inhibitory effect on the glutamate uptake, (ii) an important feature of a transporter is its dependency on Na^+, in case of *B. divergens* infected RBCs as shown in Fig. 3.15, we observed that the transporter is Na^+ independent for the uptake of glutamate, which is in contrast to the glutamate transport (EAAT3) present in *P. falciparum* infected RBCs, (iii) the transport in *B. divergens* infected erythrocytes occur only by a non saturable pathway as shown in Fig. 3.23, whereas in case of *P. falciparum* infected erythrocytes it has been shown that the glutamate transport occur via a saturable and a non saturable pathways. In *B. divergens* infected RBC the influx of radiolabelled glutamate was found unaffected in the presence of unlabelled glutamate at concentration ranging from 0.1-1000 µM, thus the transport of glutamate into *B. divergens* infected erythrocytes seems to be via a non saturable transporter. Therefore, from the results obtained, the hypothesis about the glutamate transport activated in *B. divergens* infected erythrocytes might be (i) activation of a low affinity glutamate transporter, or (ii) the pathway induced in the erythrocyte

membrane after infection by *B. divergens* may be similar with broad-specificity anion–selective channels (Ruiz-Ramos *et al*., 2009).

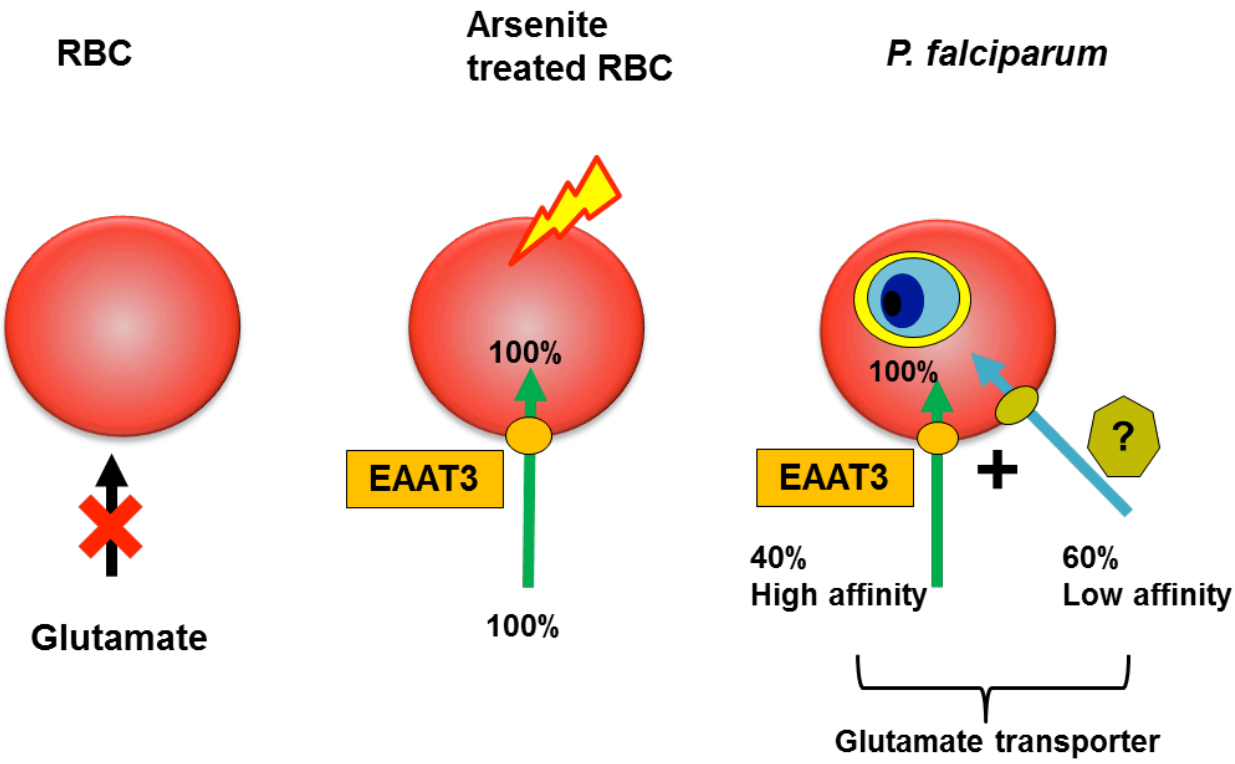


Figure 4.1: The EAAT 3 transporter is activated by *Plasmodium falciparum*.

4.2.4 Protease and Sulfo-NHC-LC-biotin blocks novel permeation pathways in *B. divergens*

Previous studies in *P. falciparum* infected erythrocytes showed that biotin derivatives (sulfo-NHS-LC-biotin) and proteases affect NPP induced by the parasite in the host cell membrane (Baumeister *et al*., 2003; Cohn *et al*., 2003). The NPP are blocked by sulfo-NHS-LC-biotin, presumably by covalent binding of the derivative to the lysine residues of the channel proteins present on the surface of the erythrocytes (Nyalwidhe *et al*., 2002; Baumeister *et al*., 2003; Cohn *et al*., 2003). In my studies performed on *B. divergens* infected erythrocytes, the data in Fig 3.12, showed that pretreatment of *B. divergens* infected RBCs with sulfo-NHS-LC-biotin inhibited the influx of glutamate to the same extent as in furosemide treated *B. divergens* infected RBCs. Another experiment done by treating *B. divergens* infected RBCs with proteases (chymotrypsin and trypsin) in Fig. 3.13 showed that the NPP involves

protease-susceptible proteins which are exposed on the surface of the infected erythrocytes. This suggests that the essential protein components which are involved in NPP are exposed on the extracellular face of the erythrocyte membrane and are susceptible to biotinylation and proteases.

Previous reports have also shown that NPP induced in *P. falciparum* infected RBCs reappear and are restored after treatment of *P. falciparum* infected erythrocytes with biotin and chymotrypsin (Baumeister *et al*., 2006). Our investigation for the regeneration of the NPP in *B. divergens* infected RBCs after biotinylation and chymotrypsinization in Fig. 3.14 shows that there is no reappearance of the NPP in *B. divergens* infected RBCs. However, different explanations for the phenomenon might be one or more distinct biotinylated/chymotrypsin-sensitive pathways involved, whether the channels are directly biotinylated/chymotrypsin-sensitive or whether the sensitivity (biotinylated/chymotrypsin) is present in one or more auxiliary proteins required to maintain channel function and which are exposed on the surface of the infected erythrocytes is not clear.

4.2.5 Effect of choline on glutamate transport

We observed in Fig. 3.15 that the uptake of glutamate in *B. divergens* infected erythrocytes increased significantly rather than a decreased or unchanged uptake in presence of choline in comparison to sodium, which was an unexpected result as we were looking for sodium dependency. To investigate the phenomenon that choline might have some stimulatory effect on the uptake, we did certain experiments. Past study demonstrates that choline is transported into erythrocytes via an endogenous choline transporter (Deves *et al*., 1979) and also the entry of choline into *P. falciaprum* infected erythrocyte is essentially through the erythrocyte choline transporter and a small amount via furosemide-sensitive NPP (Kirk *et al*., 1994). Thereby, then transported into the parasite (*P. falciparum*) by the parasites choline transporter and used to synthesize PC via the Kennedy pathway (Wein *et al*., 2012).

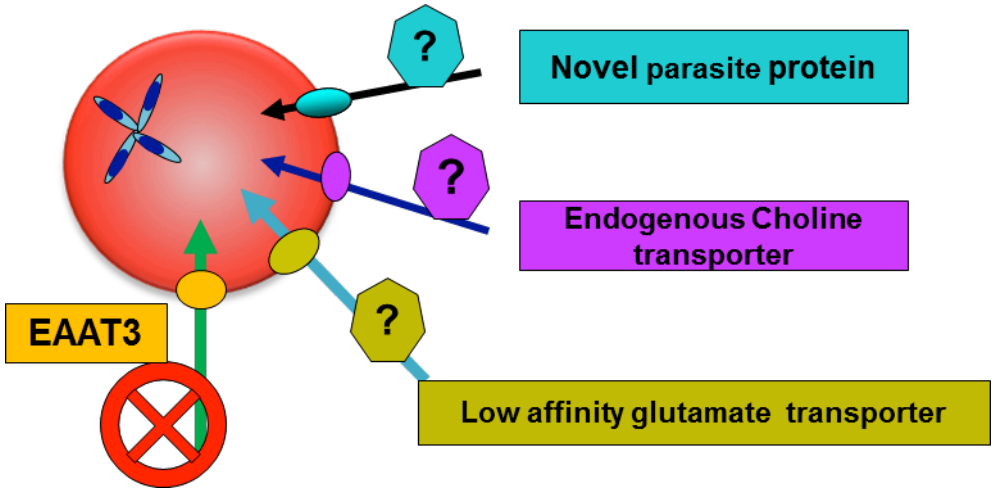


Figure 4.2: Hyopthetical model for the glutamate transport into *B. divergens* infected erythrocytes.

Previous reports show that, in the erythrocytes there is a choline exchanger present, which in addition to choline transports other cations such as Cs, Rb, K, Li, Na and Mg^2 (Martin, 1972) and also, that it is an unspecific transporter mediating uptake of catecholamines (Azoui *et al*., 1996). In our study performed on *B. divergens* infected erythrocytes there might also be involvement of this choline exchanger mediating glutamate transport.

Further, we also looked for the effect of cations/anions on the glutamate uptake into *B. divergens* infected RBCs, to know whether the uptake is affected by different ions which activate some ion specific pathways. The effect of anions was observed in Fig. 3.17 (A and B) where chloride ion (Cl^-) was compared with other anions like bromide (Br^-) and acetate (CH_3CO^-), concluding that an anion does not stimulate the higher uptake in *B. divergens* infected RBCs. On the other hand an effect of cations was observed on the increased uptake in Fig. 3.16 concluding that there was an increased uptake in choline chloride (cho+) compared to sodium chloride (Na^+), and N-Methyl-D-glucamine (NMDG) ($CH3^+$). From the results we see, it is possible that, there might be an activation of a system (transporter) which might be (i) sodium independent that needs no sodium for its activity or (ii) a system in which sodium depletion leads to higher influx via this transporter, thus it is regulated by sodium and therefore not independent. Ebel and colleagues have demonstrated the presence of a transporter which is Na^+ independent in rat erythrocytes for the efflux of Mg^+ in two conditions (i) sucrose medium (ii) in Cl^- high medium such as KCl, LiCl or Choline chloride (Ebel *et al*., 2002). Another group has shown the presence of TRP (transient receptor channels) cation channels in erythrocytes, a subfamily of this TRP channels (TRPML) can be activated in sodium-free medium (Grimm et al., 2012), which participate in cation leak (Ca^{2+}, Na^+, K^+) and

Ca^{2+} induced suicidal death (Foller *et al*., 2008). Further data collected in this direction in Fig. 3.18, shows that there might be involvement of Na^{+} independent transporter for the uptake of glutamate. As observed with the decreasing concentration of sodium (150-100 mM), the glutamate uptake increases and later as the concentration of sodium increases (beyond 100mM) the uptake remains stationary, the conclusion from the above observation might be that the transport is inhibited in the presence of sodium.

Since choline is a positively charged quaternary ammonium ion (Deves *et al*., 1979), we further investigated the effect of charge (Fig. 3.29) on the uptake of amino acids such as glutamate (negatively charged), aspartate (negatively charged), alanine (neutral) and lysine (positively charged). The results showed that the uptake of negatively charged amino acids (aspartate, glutamate) increased in the presence of choline, whereas the uptake of positively charged amino acid (lysine) and neutral amino acid (alanine) did not show any increase. This led to the conclusion that the charge might be influencing the uptake by inducing conformational changes of the choline transporter or of the low affinity glutamate transporter or of both, and there might also be the possibility of any other novel parasite protein active after infection which facilitates this mechanism. Past reports show that the charge of choline has a role in the uptake of choline analogues through choline transporter present via a symport or a antiport transport system, the known antimalarial drugs (choline analogues) are such as T4, inhibiting *de novo* phosphotidylcholine biosynthesis (Vial *et al*., 2004; Le Roch *et al*., 2008) or T16 which interact with heme (Biagini *et al*., 2003). These analogues inhibit *in vitro* parasites growth, affecting especially the asexual blood stages (Calas *et al*., 1997; Ancelin *et al*., 1998; 2003). Therefore choline charge might also have some influence on the uptake of glutamate. We also compared L- and D- glutamate uptake into *B. divergens* infected erythrocytes in the presence of choline (Fig. 3.31), and therefore the transport kinetics of both the stereoisomers seems to be stereoselective in the presence of choline.

Further, it was important to verify that the increased uptake of glutamate in *B. divergens* infected RBCs, when suspended in choline chloride solution was not an artifact due to (i) increase of volume by swelling or (ii) haemolysis. The approach used was to analyze the non-infected and *B. divergens* infected erythrocytes in a choline chloride solution using Multisizer 3 coulter counter, which is a instrument used to analyze the cell sizing, cell volume and counting. The data in Fig. 3.19 a and b showed no swelling, as there was no difference in the peaks of non infected and infected erythrocytes in different solutions (choline chloride and PBS) and Fig. 3.20 showed no haemolysis in cells (both non-infected and *B. divergens* infected RBCs) when suspended in choline chloride media as there was no hemoglobin observed. The data in the Fig. 3.21 illustrate the fact that in the presence of choline,

glutamate is not influxed into infected cells by the formation of a sink, there is activation of a specific transporter for glutamate uptake in *B. divergens* infected erythrocytes.

In this study, since we have observed a higher influx of glutamate in the presence of choline, we wanted to look for the involvement of choline transporter in glutamate uptake in *B. divergens* infected cells, by using specific choline transporter inhibitors like HC-3 (an inhibitor for high affinity uptake) and DoTMA (Fig. 3.27). The inhibitory effect of these compounds on *B. divergens* infected cells was observed in the presence and absence of choline. The inhibitor DoTMA showed ~ 50% inhibition of glutamate in the presence of choline and up to ~ 80% in the absence of choline. Whereas, HC-3 showed no inhibition in the presence of choline chloride and thus no involvement of high affinity pathway in glutamate uptake. There might be activation of transporters which are inhibited by low affinity inhibitor (DoTMA), or there might be some other novel parasite protein activated. The inhibitory action of these compounds (HC-3 and DoTMA) might be via binding to a hydrophobic region present adjacent to the active site or in the active site of the transporter which can accommodate these alkyltrimethylammonium ions, thereby affecting the glutamate uptake in the *B. divergens* infected RBCs. We also looked for the involvement of either a symporter or an antiporter for both choline and glutamate influx into *B. divergens* infected erythrocytes. For this we observed for the involvement of cystine/glutamate or glutamate/glutamate (Xc-) antiporter, by using L-homocysteic acid (LHC) in Fig. 3.28, which is a potent competitive inhibitor for Xc- antiporter and found that there is no involvement of this antiporter system in the transport of glutamate in *B. divergens* infected RBCs. Another probable reason for the phenomenon observed in Fig. 3.15 might be based on the differences in the efflux of glutamate from the erythrocytes as shown in Fig. 3.32. During the efflux experiments we found that the efflux of glutamate was less in choline rather than in sodium, which leads to increased counts of glutamate in choline containing media as compared to sodium.

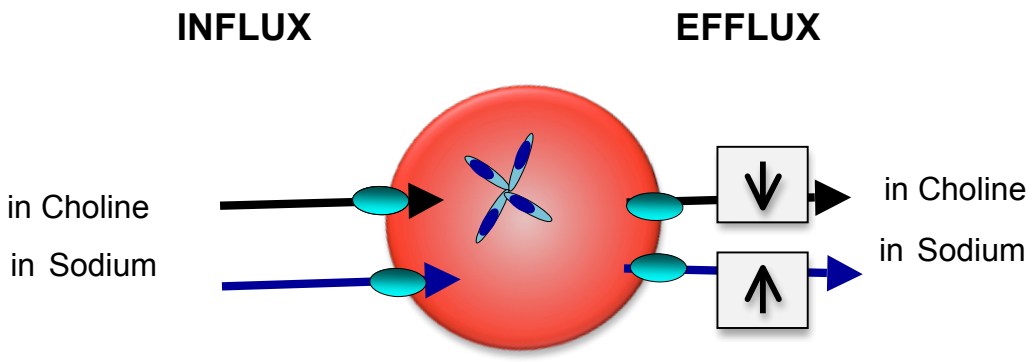


Figure 4.3: Hyopthetical model for the influx and efflux of glutamate in *B. divergens* infected erythrocytes.

In a previous study conducted on glutamate uptake into *P. falciparum* infected RBCs, it has been demonstrated that there is inhibition of the EAAT3 activity when sodium chloride (NaCl) is replaced by choline chloride (ChoCl) (Winterberg et al., 2012). However, those experiments were not designed to discover any stimulatory effects of choline chloride on the non-saturable, a low affinity pathway which is also Na^+ dependent. This prompted us to reexamine the previous data, with a focus on whether (i) the low affinity uptake is modulated by choline (ii) whether there is any similarity or difference between the low affinity uptake in *P. falciparum* and *B. divergens* infected erythrocytes. In our studies performed on *P. falciparum* infected RBCs (Fig. 3.24) we observed that the presence of cis-ACBD and cold glutamate reduced in a similar way the uptake of glutamate by 40% as described previously by Winterberg and colleagues (Winterberg *et al*. 2012). The remaining 60% uptake of glutamate was similar in the presence of Chol Cl and NaCl, suggesting that there was no choline activatable component in the EAAT3 independent pathways present in *P. falciparum* infected RBCs. This is in contrast to the low affinity transport of glutamate in *B. divergens* infected RBCs, which is increased in the presence of Chol Cl. The possible explanation for the difference in the low affinity uptake might be that the activation of a respective transporter in the erythrocyte membrane requires one or more protein kinases (Egee *et al*., 2002), infection might have induced changes in the signalling mechanisms or remodelling of the erythrocyte membrane proteins (channels and transporters) after infection by the parasites.

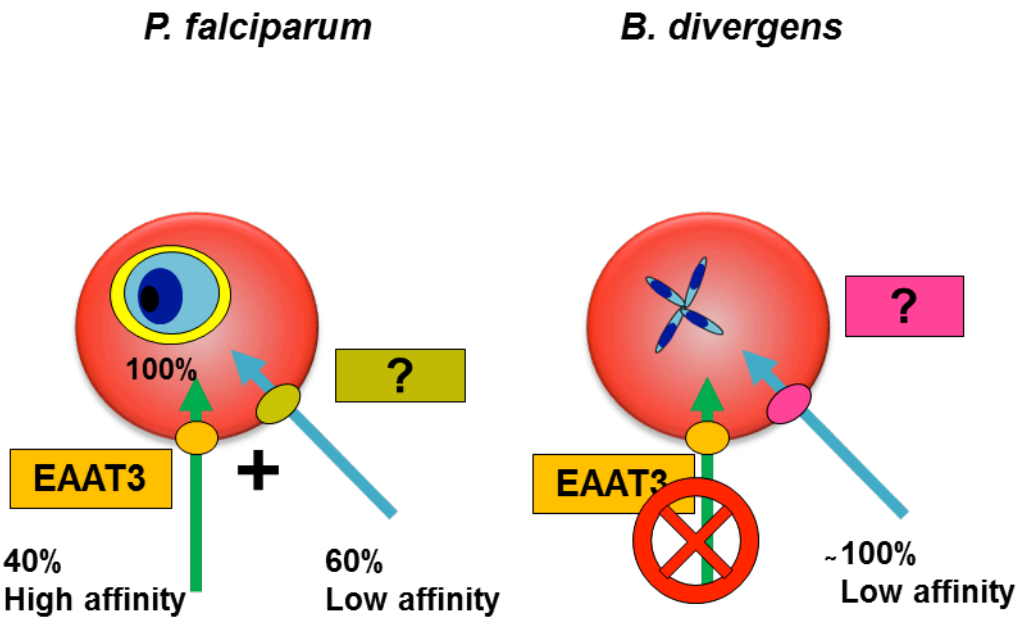


Figure 4.4: Model comparing glutamate uptake into *P. falciparum* and *B. divergens* infected RBCs.

4.2.6 Choline transport into non-infected and *B. divergens* infected human erythrocytes.

Choline is a positively charged, quaternary ammonium ion (trimethyl-n-propylammonium ion) and a water soluble essential nutrient for the cells. The choline transport system in the erythrocytes has been studied in detail in context to its structure, affinity and transport activity (Deves *et al*., 1979). Choline is transported into non-infected human erythrocytes via a endogenous choline transporter, which is a saturable carrier system and also follows Michaelis-Menten kinetics (Martin, 1977). The growing parasites *P. falciparum* and *B. divergens*, inside the erythrocyte entails the synthesis of new membranes for its survival. The essential component of the eukaryotic membranes are phospholipids (phosphatidylcholine or phosphatidyathanolamine) whose synthesis increases during the course of development (Holz, 1977). Therefore, *de novo* synthesis of phospholipids inside the intracellular parasite requires choline, which is taken up from the extracellular medium. It has been demonstrated that the transport of choline occurs through a endogenous RBC transporter in *P. knowlesi* infected erythrocytes, which is the rate limiting step for the phosphotidylcholine biosynthesis (Ancelin *et al*., 1989). There is also an active transport of choline in *P. falciparum* infected erythrocytes via a non-saturable component different from the endogenous RBC choline transporter (Kirk *et al*., 1991), and through both saturable and non-saturable processes in *P. vinckei* infected erythrocytes (Staines *et al*., 1998).

There is nothing known about the uptake of choline into *B. divergens* infected erythrocytes. In the present study performed on *B. divergens* infected erythrocytes, the data in Fig. 3.26 show that the uptake of choline in the non-infected erythrocytes as compared to *B. divergens* infected erythrocytes is different. The transport of choline in *B. divergens* infected RBC is more (~40%) than non infected RBC. There are two choline transport pathways (high and low) described for non-infected RBCs and for *P. falciparum* infected RBCs. The choline transport in the erythrocytes can be inhibited by the competitive, high affinity inhibitor Hemicholine (HC-3) (Okuda *et al*., 2011) and by the low affinity inhibitor dodecyltrimethylammonium (DoTMA) (Ancelin *et al*., 1985 and 1991). The data in the Fig. 3.26 comparing the uptake of choline in the non-infected RBCs and the *B. divergens* infected RBCs, treated and non treated with choline transporter inhibitors (HC-3 and DoTMA), show the characteristics of the choline transport after infection. The inhibitory action of compounds such as HC-3 and DoTMA on the choline transport in *B. divergens* infected erythrocytes suggests that there might be is presence of both high and low affinity choline transport via a host endogenous choline transporter or via a novel transport protein.

4.3 Summary: Different parasites induce different pathways for glutamate uptake

Interestingly, permeability increase in the host cell membrane after infection by both the parasites *P. falciparum* and *B. divergens*, this is an excellent example of parallel but distinct adaptations of parasites to survive in the RBCs. The parallel adaptations by both the parasites are the formation of NPP, which facilitate the uptake of solutes from the extracellular medium. *P. falciparum* infected erythrocytes have both low- and high-affinity (EAAT3) glutamate transporter activated (Winterberg *et al*., 2012). Like *P. falciparum*, upon infection of erythrocytes with *B. divergens*, there is increased influx of L-glutamate into infected erythrocytes. Our studies show that (i) *B. divergens* infected erythrocytes have no activation of high affinity glutamate transporter (EAAT3) as compared to *P. falciparum* infected erythrocytes. (ii) The glutamate transport into *B. divergens* infected erythrocytes is by a low affinity transport system, which is Na+-independent, non-saturable and non-stereo selective. (iii) The transport is also enhanced by choline, but the actual contribution of choline transporter to the glutamate uptake is unclear (iv) No reappearance of NPP activity (glutamate uptake) in the *B. divergens* infected erythrocytes could be observed.

In *P. falciparum* infected erythrocytes the characteristics of the glutamate transporter are:

- High affinity (EAAT3) : Na^+ dependent , saturable, stereo selective
- Low affinity (one or more) : Na+ independent, non saturable, non stereo selective

In *B. divergens* infected erythrocytes the characteristics of the glutamate transporter are:

- High affinity (EAAT3) : completely absent
- Low affinity : Na+ independent, non saturable, choline stimulatable, stereo selective
- Low affinity : Na+ independent, non saturable, non stereo selective

4.4 Outlook

These results highlight that there might be specific entry sites for the parasite as shown by the internalization of GM1 (lipid raft) in the parasitophorous vacuole. But the important challenges for the future studies is to look for the biophysical and biochemical features of the erythrocyte membrane involved in the PVM formation, and why there is a disintegration of the PV in *B. divergens* and what are the characteristics of NPP in two parasites, which adopt different mechanisms for nutrient acquisition from the extracellular enviornment.

Interestingly, in my work, it also appears that both parasites induce different pathways for glutamate uptake. In *P. falciparum* infected erythrocytes glutamate is taken up by high and

low affinity pathway where as in *B. divergens* infected erythrocytes the uptake is via a low affinity pathway. The molecular identity and genetic origin of the proteins comprising the transporter(s) and facilitating the transport across *B. divergens* infected erythrocyte membrane is yet to be established. The biological significance of glutamate import into infected cells has to be understood. The quantitative analysis of the oxidative stress in both the parasites. And also metabolomis of the infected erythrocytes and chemically stressed erythrocytes will be advantageous.

Another important question is to understand the enhanced uptake of glutamate in the presence of choline.

For all the above experiments, it would be very beneficial to develop approaches which allow synchronization and enrichment of viable *B. divergens* infected erythrocytes, which would also help with more detailed molecular and biochemical studies for this parasite.

5. References

Abdi AI, Carvalho TG, Wilkes JM, Doerig C (2013) A secreted *Plasmodium falciparum* kinase reveals a signature motif for classification of tyrosine kinase-like kinases. Microbiology, 159: 2533-47.

Agre P (2006) The aquaporin water channels. Proc Am Thorac Soc., 3: 5–13.

Aikawa M, Miller LH, Rabbege JR, Epstein N (1981) Freeze fracture study on erythrocyte membrane during malarial parasite invasion. J. Cell Biol., 91: 55-62.

Alkhalil A, Cohn JV, Wagner MA, Cabrera JS, Rajapandi T, Desai SA (2004) *Plasmodium* falciparum likely encodes the principal anion channel on infected human erythrocytes. Blood, 104: 4279-86.

Alkhalil A, Hill DA, Desai SA (2007) *Babesia* and plasmodia increase host erythrocyte permeability through distinct mechanisms. Cell Microbiol., 9: 851-60.

Aman AT, Fraser S, Merritt EA, Rodigherio C, Kenny M, Ahn M, Hol WG, Williams NA, Lencer WI, Hirst TR (2001) A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity. Proc Natl Acad Sci USA, 98: 8536-41.

Ancelin ML, Calas M, Bompart J, Cordina G, Martin D, Ben Bari M, Jei T, Druilhe P, Vial HJ (1998) Antimalarial activity of 77 phospholipid polar head analogs: close correlation between inhibition of phospholipid metabolism and *in vitro Plasmodium falciparum* growth. Blood, 91: 1426-37.

Ancelin ML, Calas M, Vidal-Sailhan V, Herbuté S, Ringwald P, Vial HJ (2003) Potent inhibitors of Plasmodium phospholipid metabolism with a broad spectrum of *in vitro* antimalarial activities. Antimicrob Agents Chemother., 47: 2590-7.

Ancelin ML, Parant M, Thuet MJ, Philippot JR, Vial HJ (1991) Increased permeability to choline in simian erythrocytes after *Plasmodium knowlesi* infection. Biochem J., 273: 701-9.

Ancelin ML, Vial HJ (1989) Regulation of phosphatidylcholine biosynthesis in *Plasmodium*-infected erythrocytes. Biochim Biophys Acta, 1001: 82-9.

Ancelin ML, Vial HJ, Philippot JR (1985) Inhibitors of choline transport into *Plasmodium*-infected erythrocytes are effective antiplasmodial compounds in vitro. Biochem Pharmacol., 34: 4068-71.

D'Angelo JA, Dehlink E, Platzer B, Dwyer P, Circu ML, Garay J, Aw TY, Fiebiger E, Dickinson BL (2010) The cystine/glutamate antiporter regulates dendritic cell differentiation and antigen presentation. J Immunol., 185: 3217-2.

Atamma H, Ginsburg H (1997) The malaria parasite supplies glutathione to its host cell-

investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*. Eur J Biochem., 250: 670-9.

Atkinson CT, Aikawa M, Perry G, Fujino T, Bennett V, Davidson E A, Howard R J (1987) Ultrastructural localization of erythrocyte cytoskeleton and integral membrane proteins in *Plasmodium falciparum* infected erythrocytes. Eur. J. Cell Biol., 45: 192-199.

Ansorge I, Benting J, Bhakdi S, Lingelbach K (1996) Protein sorting in *Plasmodium falciparum*-infected red blood cells permeabilized with the pore-forming protein streptolysin O. Biochem J., 315: 307-14.

Azoui R, Cuche JL, Renaud JF, Safar M, Dagher G (1996) A dopamine transporter in human erythrocytes: modulation by insulin. Exp Physiol., 81: 421-34.

Baldini M, Pannacciulli I (1960 The maturation rate of reticulocytes. Blood, 15: 614-29.

Ballatori N, Krance SM, Notenboom S, Shi S, Tieu K, Hammond CL (2009) Glutathione dysregulation and the etiology and progression of human diseases. Biol Chem., 390: 191-214.

Bannister LH, Dluzewski AR (1990) The ultrastructure of red cell invasion in malaria infections: a review. Blood Cells, 16: 257-92.

Bannister LH, Hopkins JM, Dluzewski AR, Margos G, Williams IT, Blackman MJ, Kocken CH, Thomas AW, Mitchell GH (2003) *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-1) is translocated within micronemes along subpellicular microtubules during merozoite development . Journal of Cell Science, 116: 3825-3834.

Barrias ES, de Carvalho TM, De Souza W (2013) Trypanosoma cruzi: Entry into Mammalian Host Cells and Parasitophorous Vacuole Formation. Front Immunol., 4: 186.

Barry DN (1982) Metabolism of *Babesia* parasites *in vitro* Glucose and energy-metabolism and survival of *Babesia rodhaini* in a basal medium with and without adenosine. Aust J Exp Biol Med Sci., 60: 159-65.

Baumeister S, Endermann T, Charpian S, Nyalwidhe J, Duranton C, Huber S, Kirk K, Lang F, Lingelbach K (2003) A biotin derivative blocks parasite induced novel permeation pathways in *Plasmodium falciparum*-infected erythrocytes. Mol Biochem Parasitol., 132: 35-45.

Baumeister S, Wiesner J, Reichenberg A, Hintz M, Bietz S, Harb OS, Roos DS, Kordes M, Friesen J, Matuschewski K, Lingelbach K, Jomaa H, Seeber F (2011) Fosmidomycin uptake into *Plasmodium* and *Babesia*-infected erythrocytes is facilitated by parasite-induced new permeability pathways. PloS One, 6:e19334.

Baumeister S, Winterberg M, Duranton C, Huber SM, Lang F, Kirk K, Lingelbach K (2006) Evidence for the involvement of *Plasmodium falciparum* proteins in the formation of new permeability pathways in the erythrocyte membrane. Mol Microbiol., 60: 493-504.

Baumeister S, Winterberg M, Przyborski JM, Lingelbach K (2010) The malaria parasite *Plasmodium falciparum*: cell biological peculiarities and nutritional consequences. Protoplasma, 240: 3-12.

Berhardt I, Hall AC, Ellory JC (1991) Effects of low ionic strength media on passive human red cell monovalent cation transport. J Physiol., 434: 489-506.

Biagini GA, Richier E, Bray PG, Calas M, Vial H, Ward SA (2003) Heme binding contributes to antimalarial activity of bis-quaternary ammoniums. Antimicrob Agents Chemother., 47: 2584-9.

Bietz S, Montilla I, Külzer S, Przyborski JM, Lingelbach K (2009) Recruitment of human aquaporin 3 to internal membranes in the *Plasmodium falciparum* infected erythrocyte. Mol Biochem Parasitol., 167: 48-53.

Blader IJ, Manger ID, Boothroyd JC (2001) Microarray analysis reveals previously unknown changes in *Toxoplasma gondii*-infected human cells. J Biol Chem., 276: 24223–24231.
Bouyer G, Cueff A, Egée S, Kmiecik J, Maksimova Y, Glogowska E, Gallagher PG, Thomas SL(2011) Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are upregulated by *Plasmodium falciparum*. Blood, 118: 2305-12.

Brayton KA, Lau AO, Herndon DR, Hannick L, Kappmeyer LS, Berens SJ, Bidwell SL, Brown WC, Crabtree J, Fadrosh D, Feldblum T, Forberger HA, Haas BJ, Howell JM, Khouri H, Koo H, Mann DJ, Norimine J, Paulsen IT, Radune D, Ren Q, Smith RK Jr, Suarez CE, White O, Wortman JR, Knowles DP Jr, McElwain TF, Nene VM (2007) Genome sequence of *Babesia* bovis and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathog., 3: 1401-13.

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol., 14: 111-36.

Brugnara C, Tosteson DC (1987) Cell volume, K transport, and cell density in human erythrocytes. Am J Physiol., 252: C269–C276.

Burns ER, Pollack S (1988) *P. falciparum* infected erythrocytes are capable of endocytosis. In Vitro Cell Dev Biol., 24: 481-6.

Cabantchik ZI, Rothstein A (1972) The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. J Membr Biol., 10: 311-30.

Calas M, Cordina G, Bompart J, Ben Bari M, Jei T, Ancelin ML, Vial H (1997) Antimalarial activity of molecules interfering with *Plasmodium falciparum* phospholipid metabolism. Structure-activity relationship analysis. J Med Chem., 40: 3557-66.

Camus D, Hadley TJ (1985) A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science, 230: 553-6.

Carafoli E, Longoni S (1987) The plasma membrane in the control of the signaling function of calcium. Soc Gen Physiol Ser., 42: 21-9.

Casado M, Bendahan A, Zafra F, Danbolt NC, Aragón C, Giménez C, Kanner BI (1993) Phosphorylation and modulation of brain glutamate transporters by protein kinase C. J Biol Chem., 268: 27313-7.
Chasis JA, Prenant M, Leung A, Mohandas N (1989) Membrane assembly and remodelling during reticulocyte maturation. Blood, 74: 1112–1120.

Chauvin A, Moreau E, Bonnet S, Plantard O, Malandrin L (2009) *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet Res., 40: 1–18.

Cobbold SA, Martin RE, Kirk K (2011) Methionine transport in the malaria parasite *Plasmodium falciparum*. Int J Parasitol., 41: 125-35.

Cohn JV, Alkhalil A, Wagner MA, Rajapandi T, Desai SA (2003) Extracellular lysines on the plasmodial surface anion channel involved in Na^+ exclusion. Mol Biochem Parasitol., 132: 27–34.

Cowman AF, Bernard O, Stewart N, Kemp DJ (1984) Genes of the protozoan parasite *Babesia bovis* that rearrange to produce RNA species with different sequences. Cell, 37: 653–660.

Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. Cell, 124: 755-66.

Crammer SL, Conant AR, Gutteridge WE and Halestrap AP (1995) Characterization of the enhanced transport of L- and D-lactate into human red blood cells infected with *Plasmodium falciparum* suggests the presence of a novel saturable lactate proton cotransporter. Biol Chem., 270: 15045-52.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol., 65: 1-105.

Déchamps S, Shastri S, Wengelnik K, Vial HJ (2010) Glycerophospholipid acquisition in Plasmodium - a puzzling assembly of biosynthetic pathways. Int. J. Parasitol., 40: 1347–1365.

Decherf G, Egee S, Staines HM, Ellory JC, Thomas SL (2004) Anionic channels in malaria-infected human red blood cells. Blood Cells Mol Dis., 32: 366-71.

De Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, Papenfuss AT, Sanders PR, Lundie RJ, Maier AG, Cowman AF, Crabb BS (2010) A newly discovered protein export machine in malaria parasites. Nature, 459: 945-9.

Delbecq S, Auguin D, Yang YS, Löhr F, Arold S, Schetters T, Précigout E, Gorenflot A,

Roumestand C (2008) The solution structure of the adhesion protein Bd37 from *Babesia divergens* reveals structural homology with eukaryotic proteins involved in membrane trafficking. J Mol Biol., 375: 409-24.

Delves M, Plouffe D, Scheurer C, Meister S, Wittlin S, Winzeler EA, Sinden RE, Leroy D (2012) The activities of current antimalarial drugs on the life cycle stages of *Plasmodium*: a comparative study with human and rodent parasites. PLoS Med, 9: e1001169.

Desai SA (2012) Ion and nutrient uptake by malaria parasite-infected erythrocytes. Cell Microbiol., 14: 1003-9.

Desai SA, Miller LH (2014) Malaria: Protein-export pathway illuminated. Nature, 511: 541-2.

Desai SA, Bezrukov SM, Zimmerberg J (2000) A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. Nature, 406: 1001-5.

Desai SA, Rosenberg RL (1997) Pore size of the malaria parasite's nutrient channel. Proc Natl Acad Sci U S A, 94: 2045-9.

De Souza W, De Carvalho TM, Barrias ES (2010) Review on Trypanosoma cruzi: Host Cell Interaction. Int J Cell Biol., pii: 295394.

Deuticke B (1982) Monocarboxylate transport in erythrocytes. J Membr Biol., 70: 89-103.

Deves R, Kurpka RM (1979) The binding and translocation steps in transport as related to substrate structure. A study of the choline carrier of erythrocytes. Biochim Biophys Acta, 557: 469-85.

Di Girolamo F, Raggi C, Birago C, Pizzi E, Lalle M, Picci L, Pace T, Bachi A, de Jong J, Janse CJ, Waters AP, Sargiacomo M, Ponzi M (2008) *Plasmodium* lipid rafts contain proteins implicated in vesicular trafficking and signalling as well as members of the PIR superfamily, potentially implicated in host immune system interactions. Proteomics, 8: 2500-1.

Divo AA, Geary TG, Davis NL, Jensen JB (1985) Nutritional requirements of *Plasmodium falciparum* in culture. I. Exogenously supplied dialyzable components necessary for continuous growth. J Protozool., 32: 59-64.

Dluzewski AR, Fryer PR, Griffiths S, Wilson RJM, Gratzer WB (1989) Red cell protein distribution during malarial invasion. J. Cell Sci., 92: 691-699.

Dluzewski AR, Zicha D, Dunn GA and Gratzer WB (1995) Origins of the parasitophorous vacuole membrane of the malaria parasite: surface area of the parasitized red cell. Eur. J. Cell Biol., 68: 446-449.

Dobbelaere DA, Kuenzi P (2004) The strategies of the Theileria parasite: a new twist in host–pathogen interactions. Curr Opin Immunol., 16: 524–530.

Doohan J (2000) Circulatory System. http://www.biosbcc.net/doohan/sample/htm/Blood

cells.htm

Dupree P, Parton RG, Raposo G, Kurzchalia TV, Simons K (1993) Caveolae and sorting in the trans-Golgi network of epithelial cells. EMBO J., 12: 1597-1605.

Duraisingh MT, Triglia T, Ralph SA, Rayner JC, Barnwell JW, McFadden GI, Cowman AF (2003) Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. EMBO J., 22: 1047-57.

Duranton C, Huber SM, Lang F (2002) Oxidation induces a Cl(-)-dependent cation conductance in human red blood cells. J Physiol., 539: 847-55.

Ebel H, Hollstein M, Gunther T (2002) Role of the choline exchanger in Na(+)-independent Mg(2+) efflux from rat erythrocytes. Biochim Biophys Acta, 1559: 135-44.

Edwards PAW (1973) The inactivation by fluorodinitrobenzene of glucose transport across the human erythrocyte membrane The effect of glucose inside or outside the cell. Biochimica et Biophysica Acta (BBA) – Biomembranes, 307: 415–418.

Egée S, Lapaix F, Decherf G, Staines HM, Ellory JC, Doerig C, Thomas SL (2002) A stretch-activated anion channel is up-regulated by the malaria parasite *Plasmodium falciparum*. J Physiol., 542: 795-801.

Elford BC, Haynes JD, Chulay JD and Wilson RJ (1985) Selective stage-specific changes in the permeability to small hydrophilic solutes of human erythrocytes infected with *Plasmodium falciparum*. Mol Biochem Parasitol., 16: 43-60.

Elliott JL, Saliba KJ, Kirk K (2001) Transport of lactate and pyruvate in the intraerythrocytic malaria parasite, *Plasmodium falciparum*. Biochem. J., 355: 733-739.

Farrow RE, Green J, Katsimitsoulia Z, Taylor WR, Holder AA, Molloy JE (2011) The mechanism of erythrocyte invasion by the malarial parasite, *Plasmodium falciparum*. Semin Cell Dev Biol., 22: 953-60.

Fitzpatrick JEP, Kennedy CC, Mcgeown MG, Oreopoulos DG, Robertson JH, Soyannwo MAO (1968) Human case of piroplasmosis (babesiosis). Nature, 217: 861-862.

Flatman PW, Creanor J (1999) Regulation of Na^+-K^+-$2Cl^-$ cotransport by protein phosphorylation in ferret erythrocytes. J Physiol., 517: 699-708.

Flatman PW, Creanor J (1999) Stimulation of Na^+-K^+-$2Cl^-$ cotransport by arsenite in ferret erythrocytes. J Physiol., 519: 143-52.

Flatman PW (2005) Activation of ferret erythrocyte Na^+-K^+-$2Cl^-$ cotransport by deoxygenation. J Physiol., 563: 421-31.

Florin-Christensen M, Suarez CE, Hines SA, Palmer GH, Brown WC, McElwain TF (2002)

The *Babesia bovis* merozoite surface antigen 2 locus contains four tandemly arranged and expressed genes encoding immunologically distinct proteins. Infect. Immun., 70: 3566–3575.

Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM (2008) TRPC6 contributes to the Ca(2+) leak of human erythrocytes. Cell Physiol Biochem., 21: 183-92.

Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG, Goldberg DE (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. EMBO J., 13: 306-17.

Freeman JC (1983) Partial requirements for in vitro survival of human red blood cells. J Membr Biol., 75: 225-231.

Fujita A, Cheng J, Hirakawa M, Furukawa K, Kusunoki S, Fujimoto T (2007) Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. Mol Biol Cell, 18: 2112–2122.

Gagescu R, Demaurex N, Parton RG, Hunziker W, Huber LA, Gruenberg J (2000) The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. Mol Biol Cell., 11: 2775-2791.

Gardner MJ, Hall N, Fung E, White O, Berriman O, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DMA, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungal C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B (2002) plasmodium genomics. Nature, 419: 498-511.

Gardos G (1958) The function of calcium in the potassium permeability of human erythrocytes. Biochim Biophys Acta, 30: 653–654.

Gati WP, Lin AN, Wang TI, Young JD, Paterson AR (1990) Parasite-induced processes for adenosine permeation in mouse erythrocytes infected with the malarial parasite *Plasmodium yoelii*. Biochem J., 272: 277-80.

Geminard C, de Gassart A, Vidal M (2002) Reticulocyte maturation: mitoptosis and exosome release. Bio cell, 26: 205-15.

Gero AM, Scott HV, O'Sullivan WJ, Christopherson RI (1989) Antimalarial action of nitrobenzylthioinosine in combination with purine nucleoside antimetabolites. Mol Biochem Parasitol., 34: 87-97.

Ginsburg H, Krugliak M, Eidelman O, Cabantchik ZI (1983) New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. Mol Biochem Parasitol., 8: 177-90.

Ginsburg H, Kutner S, Krugliak M, Cabantchik ZI (1985) Characterization of permeation pathways appearing in the host membrane of *Plasmodium falciparum* infected red blood cells. Mol Biochem Parasitol., 14: 313-22.

Ginsburg H, Kutner S, Zangwil M, Cabantchik ZI (1986) Selectivity properties of pores induced in host erythrocyte membrane by *Plasmodium falciparum*. Effect of parasite maturation. Biochim Biophys Acta, 861: 194-6.

Ginsburg H, Stein WD (1987) New permeability pathways induced by the malarial parasite in the membrane of its host erythrocyte: potential routes for targeting of drugs into infected cells. Biosci Rep., 7: 455-63.

Ginsburg H, Stein WD (1988) Biophysical analysis of novel transport pathways induced in red blood cell membranes. Prog Clin Biol Res., 252: 317-22.

Ginsburg H, Stein WD (2004) The new permeability pathways induced by the malaria parasite in the membrane of the infected erythrocyte: comparison of results using different experimental techniques. J Membr Bio., 197: 113-34.

Goel VK, Li X, Chen H, Liu SC, Chishti AH, Oh SS (2003) Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. Proc Natl Acad Sci U S A, 100: 5164-9.

Gomez-Móuton C, Abad JL, Mira E, Lacalle RA, Gallardo E, Jiménez-Baranda S, Illa I, Bernad A, Mañes S, Martínez-A C (2001) Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci USA, 98: 9642-7.

Gray J, Dautel H, Estrada-Peña A, Kahl O, Lindgren E (2009) Effects of climate change on ticks and tick-borne diseases in Europe. Interdisc Perspec Infect Dis., 2009: 593232.

Griffiths R, Dunlop J, Gorman A, Senior J, Grieve A (1994) L-trans-pyrrolidine-2,4-dicarboxylate and cis-1-aminocyclobutane-1,3-dicarboxylate behave as transportable, competitive inhibitors of the high-affinity glutamate transporters. Biochem Pharmacol., 47: 267-74.

Grimm C, Jörs S, Guo Z, Obukhov AG, Heller S (2012) Constitutive activity of TRPML2 and TRPML3 channels versus activation by low extracellular sodium and small molecules. J Biol Chem., 287: 22701-8.

Gronowicz G, Swift H, Steck TL (1984) Maturation of the reticulocyte in vitro. J Cell Sci., 71: 177-97.

Haas A (2009) Everybody has a home of their own - “The phagosome zoo” In: Schaible UE, Haas A, editors. Intracellular Niches of Microbes. Weinheim: Wiley VCH, 159–190.

Haas M, McManus TJ (1989) Cation-anion cotransport. Methods Enzymol., 173: 280-91.

Hansen M, Kun JF, Schultz JE, Beitz E (2002) A single, bi-functional aquaglyceroporin in blood-stage *Plasmodium falciparum* malaria parasites. J. Biol. Chem., 277: 4874-4882

Harder T, Scheiffele P, Verkade P, Simons K (1998) Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol., 141: 929-42.

Harrison T, Samuel BU, Akompong T, Hamm H, Mohandas N, Lomasney JW, Haldar K (2003) Erythrocyte G protein coupled receptor signaling in malaria infection. Science, 301: 1734-6.

Herwaldt BL, Bruyn G, Pieniazek NJ, Homer M, Lofy KH, Slemenda SB, Fritsche TR, Persing D H, Limaye AP (2004) *Babesia divergens*-like infection, Washington State. Emerging infectious diseases, 10: 622-629.

Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, Wilson M (2011) Transfusion-associated babesiosis in the United States: a description of cases. Annals of internal medicine, 155: 509-519.

Hiller NL, Akompong T, Morrow JS, Holder AA, Haldar K (2003) Identification of a stomatin orthologue in vacuoles induced in human erythrocytes by malaria parasites. A role for microbial raft proteins in apicomplexan vacuole biogenesis. J Biol Chem., 278: 48413-21.

Holz GG Jr (1977) Lipids and the malarial parasite. Bull World Health Organ, 55: 237-48.

Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH (2000) Babesiosis. Clin Micro Rev., 13: 451–469.

Homewood CA, Neame KD (1974) Malaria and the permeability of the host erythrocyte. Nature (Lond), 252: 718–719.

Huber SM, Uhlemann AC, Gamper NL, Duranton C, Kremsner PG, Lang F (2002) *Plasmodium falciparum* activates endogenous Cl(-) channels of human erythrocytes by membrane oxidation. EMBO J., 21: 22-30.

Huber SM (2012) Purinoceptor signaling in malaria-infected erythrocytes. Microbes Infect., 14: 779-86.

Hussain T, Yogavel M, Sharma A (2015) Inhibition of protein synthesis and malaria parasite development by drug targeting of methionyl-tRNA synthetases. Antimicrob Agents Chemother., 59: 1856-67.

Iwasaki Y1, Ikeda K, Shiojima T, Kinoshita M (1992) Increased plasma concentrations of aspartate, glutamate and glycine in Parkinson's disease. Neurosci Lett., 145: 175-7.

Jackson AP, Otto TD, Darby A, Ramaprasad A, Xia D, Echaide IE, Farber M, Gahlot S,

Gamble J, Gupta D, Gupta Y, Jackson L, Malandrin L, Malas TB, Moussa E, Nair M, Reid AJ, Sanders M, Sharma J, Tracey A, Quail MA, Weir W, Wastling JM, Hall N, Willadsen P, Lingelbach K, Shiels B, Tait A, Berriman M, Allred DR, Pain A. (2014) The evolutionary dynamics of variant antigen genes in *Babesia* reveal a history of genomic innovation underlying host-parasite interaction. Nucleic Acids Res., 42: 7113-31.

Janich P, Corbeil D (2007) GM1 and GM3 gangliosides highlight distinct lipid microdomains within the apical domain of epithelial cells. FEBS Lett., 581: 1783–1787.

Joiner KA (1991) Rhoptry lipids and parasitophorous vacuole formation: a slippery issue. Parasitol. Today, 7: 226-227.

Kabanova S, Kleinbongard P, Volkmer J, Andre B, Kelm M, Jax TW (2009) Gene expression analysis of human red blood cells. Int J Med Sci., 6: 156-9.

Kanaani J, Ginsburg H (1989) Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase. J Biol Chem., 264: 3194-9.

Kanaani J, Ginsburg H (1991) Transport of lactate in *Plasmodium falciparum*-infected human erythrocytes. J Cell Physiol., 149: 469-76.

Keeley A, Soldati D (2004) The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. Trends Cell Biol., 14: 528-32.

Khan, Nasser and Andrew Lai. The malaria website: pathology/immune response. Biology Department, Brown University. (1999) <http://www.brown.edu/Courses/Bio_160/Projects1999/malaria/ldpg.html> Accessed 2007 Feb 25

Kirk K, Horner HA (1995) Novel anion dependence of induced cation transport in malaria-infected erythrocytes. J Biol Chem., 270: 24270-5.

Kirk K, Horner HA (1995) In search of a selective inhibitor of the induced transport of small solutes in *Plasmodium falciparum*-infected erythrocytes: effects of arylaminobenzoates. Biochem J., 311: 761-8.

Kirk K, Saliba KJ (2007) Targeting Nutrient Uptake Mechanisms in *Plasmodium*. Current Drug Targets, 8: 75-88.

Kirk K, Ashworth KJ, Elford BC, Pinches RA, Ellory JC (1991) Characteristics of 86Rb+ transport in human erythrocytes infected with *Plasmodium falciparum*. Biochim Biophys Acta, 1061: 305-8.

Kirk K, Horner HA, Kirk J (1996) Glucose uptake in *Plasmodium falciparum*-infected erythrocytes is an equilibrative not an active process. Mol Biochem Parasitol., 82: 195-205.

Kirk K, Horner HA, Elford BC, Ellory JC, Newbold CI (1994) Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. J Biol Chem., 269: 3339-47.

Kirk K, Horner HA, Spillet DJ, Elford BC (1993) Glibenclamide and meglitinide block the transport of low molecular weight solutes into malaria-infected erythrocytes. FEBS Lett., 323: 123-8.

Kirk K, Tilley L, Ginsburg H (1999) Transport and trafficking in the malaria-infected erythrocyte. Parasitol Today, 15: 355-7.

Kirk K, Wong HY, Elford BC, Newbold CI, Ellory JC (1991) Enhanced choline and Rb^+ transport in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. Biochem J., 278: 521-5.

Kirk K (2001) Membrane transport in the malaria-infected erythrocyte. Physiol Rev., 81: 495-537.

Koho NM, Hyyppä S, Pösö AR (2006) Monocarboxylate transporters (MCT) as lactate carriers in equine muscle and red blood cells. Equine Vet J Suppl., 36: 354-8.

Koho NM, Raekallio M, Kuusela E, Vuolle J, Pösö AR (2008) Lactate transport in canine red blood cells. Am J Vet Res., 69: 1091-6.

Krauth-Siegel RL, Muller JG, Lottspeich F, Schirmer RH (1996) Glutathione reductase and glutamate dehydrogenase of *Plasmodium falciparum*, the causative agent of tropical malaria. Eur. J. Biochem., 235: 345-350.

Krugliak M, Zhang J, Ginsburg H (2002) Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. Mol. Biochem. Parasitol., 119: 249-256.

Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, Zhang J, Selak MA, Ney PA, Thompson CB (2008) Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood, 112: 1493-502.

Lamarque M, Tastet C, Poncet J, Demettre E, Jouin P, Vial H, Dubremetz JF (2008) Food vacuole proteome of the malarial parasite *Plasmodium falciparum*. Proteomics Clin Appl., 2: 1361-74.

Lau AO (2009) An overview of the *Babesia*, *Plasmodium* and *Theileria* genomes: A comparative perspective. Mol Biochem Parasitol., 164: 1–8.

Lauer S, VanWye J, Harrison T, McManus H, Samuel BU, Hiller NL, Mohandas N, Haldar K (2000) Vacuolar uptake of host components, and a role for cholesterol and sphingomyelin in malarial infection. EMBO J., 19: 3556-64.

Lauer SA, Rathod PK, Ghori N, Haldar K (1997) A membrane network for nutrient import in red cells infected with the malaria parasite. Science, 276: 1122-5.

Lee P, Ye Z, Van Dyke K, Kirk K (1988) X-ray microanalysis of *Plasmodium falciparum* and

infected red blood cells: effects of qinghaosu and chloroquine on potassium, sodium, and phosphorus composition. Am J Trop Med Hyg., 39: 157-65.

Le Roch KG, Johnson JR, Ahiboh H, Chung DW, Prudhomme J, Plouffe D, Henson K, Zhou Y, Witola W, Yates JR, Mamoun CB, Winzeler EA, Vial H (2008) A systematic approach to understand the mechanism of action of the bisthiazolium compound T4 on the human malaria parasite, *Plasmodium falciparum*. BMC Genomics, 9: 513.

Lesieur C, Cliff MJ, Carter R, James RF, Clarke AR, Hirst TR (2002) A kinetic model of intermediate formation during assembly of cholera toxin B-subunit pentamers. J Biol Chem., 277: 16697-704.

Lew VL *et al* (2003) Excess hemoglobin digestion and the osmotic stability of *Plasmodium falciparum*-infected red blood cells. Blood, 101: 4189-4194.

Lew VL, Tsien RY, Miner C, Bookchin RM (1982) Physiological [Ca^{2+}]i level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. Nature, 298: 478-81.

Lingelbach K, Joiner KA (1998) The parasitophorous vacuole membrane surrounding *Plasmodium* and *Toxoplasma*: an unusual compartment in infected cells. J Cell Sci., 111: 1467-75.

Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. Science, 327: 46-50.

Liu J, Istvan ES, Gluzman IY, Gross J, Goldberg DE (2006) *Plasmodium falciparum* ensures its amino acid supply with multiple acquisition pathways and redundant proteolytic enzyme systems. Proc Natl Acad Sci USA, 103: 8840-5.

Lobo CA (2005) *Babesia divergens* and *Plasmodium falciparum* use common receptors, glycophorins A and B, to invade the human red blood cell. Infect Immun., 73: 649-51.

Loria P, Miller S, Foley M, Tilley L (1999) Inhibition of the peroxidativedegradation of haem as the basis of action of chloroquine and other quinoline antimalarials. Biochem. J., 339: 363-370.

Mackenstedt U, Gauer M, Mehlhorn H, Schein E, Hauschild S (1990) Sexual cycle of *Babesia divergens* confirmed by DNA measurements. Parasitology research, 76: 199-206.
Maier AG, Cooke BM, Cowman AF, Tilley L (2009) Malaria parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol., 7: 341-54.

Marchesini N, Vieira M, Luo S, Moreno SN, Docampo R (2005) A malaria parasite-encoded vacuolar H(+)-ATPase is targeted to the host erythrocyte. J Biol Chem., 280: 36841-7.

Martin K (1968) Concentrative Accumulation of Choline by Human Erythrocytes . J Gen Physiol., 51: 497–516.

Martin K (1972) Extracellular cations and the movement of choline across the erythrocyte

membrane. J Physiol., 224: 207-30.

Martin RE, Kirk K (2007) Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. Blood, 109: 2217-24.

McLaren DJ, Bannister LH, Trigg PI, Butcher GA (1977) A freeze-fracture study on the parasite-erythrocyte interrelationship in *Plasmodium knowlesi* infections. Bull. WHO, 55: 199-203.

McLaren DJ, Bannister LH, Trigg PI, Butcher GA (1979) Freeze fracture studies on the interaction between the malaria parasite and the host erythrocyte in *Plasmodium knowlesi* infections. Parasitology, 79: 125-39.

McIntosh MT, Drozdowicz YM, Laroiya K, Rea PA, Vaidya AB (2001) Two classes of plant-like vacuolar-type H+-pyrophosphatases in malaria parasites. Mol. Biochem. Parasitol., 114: 183-195.

Merckx A, Bouyer G, Thomas SL, Langsley G, Egée S (2009) Anion channels in *Plasmodium-falciparum*-infected erythrocytes and protein kinase A. Trends Parasitol., 25: 139-44.

Merckx A, Nivez MP, Bouyer G, Alano P, Langsley G, Deitsch K, Thomas S, Doerig C, Egée S (2008) *Plasmodium falciparum* regulatory subunit of cAMP-dependent PKA and anion channel conductance. PLoS Pathog, 4: e19.

Merritt EA, Sarfaty S, van den Akker F, L'Hoir C, Martial JA, Hol WG (1994) Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. Protein Sci., 3: 166-75.

Miller LH, Baruch DI, Marsh K, Doumbo OK (2002) The pathogenic basis of malaria. Nature, 415: 673-9.

Miwa S (1983) Hereditary disorders of red cell enzymes in the Embden-Meyerhof pathway. Am J Hematol., 14: 381-391.

Momen H, Chance ML, Peters W (1979) Biochemistry of intraeythrocytic parasites. Ann. Trop. Med. Parasitol., 73: 109–112.

Montero E, Rodriguez M, Oksov Y, Lobo CA (2009) *Babesia divergens* apical membrane antigen 1 and its interaction with the human red blood cell. Infect Immun., 77: 4783-93.

Mordue DG, Desai N, Dustin M, Sibley LD (1999) Invasion by *Toxoplasma gondii* establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. J Exp Med., 190: 1783-92.

Morisaki JH, Heuser JE, Sibley LD (1995) Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. J Cell Sci., 108: 2457-64.

Mortensen M, Ferguson DJ, Edelmann M, Kessler B, Morten KJ, Komatsu M, Simon AK (2010) Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia *in vivo*. Proc Natl Acad Sci USA, 107: 832-7.

Mrówczyńska L, Hägerstrand H (2008) Patching of ganglioside M1 in human erythrocytes - distribution of CD47 and CD59 in patched and curved membrane. Molecular Membrane Biology, 25: 358-365.

Murphy SC, Harrison T, Hamm HE, Lomasney JW, Mohandas N, Haldar K (2006) Erythrocyte G protein as a novel target for malarial chemotherapy. PLoS Med., 3: e528.

Murphy SC, Samuel BU, Harrison T, Speicher KD, Speicher DW, Reid ME, Prohaska R, Low PS, Tanner MJ, Mohandas N, Haldar K (2004) Erythrocyte detergent-resistant membrane proteins: their characterization and selective uptake during malarial infection. Blood, 103: 1920.

Nans A, Mohandas N, Stokes DL (2011) Native ultrastructure of the red cell cytoskeleton by cryo-electron tomography. Biophys J., 101: 2341-50.

Nichols BJ, Kenworthy AK, Polishchuk RS, Lodge R, Roberts TH, Hirschberg K, Phair RD, Lippincott-Schwartz J (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J. Cell Biol., 153: 529-541.

Nunes MC, Okada M, Scheidig-Benatar C, Cooke BM, Scherf A (2010) *Plasmodium falciparum* FIKK kinase members target distinct components of the erythrocyte membrane. PLoS One., 5: e11747.

Nyalwidhe J, Baumeister S, Hibbs AR, Tawill S, Papakrivos J, Volker U, Lingelbach K (2002) A nonpermeant biotin derivative gains access to the parasitophorous vacuole in *Plasmodium falciparum*-infected erythrocytes permeabilized with streptolysin O. J Biol Chem., 277: 40005-11.

Nyalwidhe J, Lingelbach K (2006) Proteases and chaperones are the most abundant proteins in the parasitophorous vacuole of *Plasmodium falciparum*-infected erythrocytes. Proteomics, 6: 1563-73.

Ohmori T, Adachi K, Fukuda Y, Tamahara S, Matsuki N, Ono K (2004) Glucose uptake activity in murine red blood cells infected with *Babesia microti* and *Babesia rodhaini*. J Vet Med Sci., 66: 945-9.

Okuda T, Konishi A, Misawa H, Haga T (2011) Substrate-induced internalization of the high-affinity choline transporter. J Neurosci., 31: 14989-97.

Patel SA, Warren BA, Rhoderick JF, Bridges RJ (2004) Differentiation of substrate and non-substrate inhibitors of transport system xc(-): an obligate exchanger of L-glutamate and L-cystine. Neuropharmacology, 46: 273-84.

Parker PD, Tilley L, Klonis N (2004) *Plasmodium falciparum* induces reorganization of host

membrane proteins during intraerythrocytic growth. Blood, 103: 2404-6.

Parton RG, Richards AA (2002) Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic, 4: 724-38.

Pease BN, Huttlin EL, Jedrychowski MP, Talevich E, Harmon J, Dillman T, Kannan N, Doerig C, Chakrabarti R, Gygi SP, Chakrabarti D (2013) Global analysis of protein expression and phosphorylation of three stages of *Plasmodium falciparum* intraerythrocytic development. J Proteome Res., 12:4028-45.

Persson KE, McCallum FJ, Reiling L, Lister NA, Stubbs J, Cowman AF, Marsh K, Beeson JG (2008) Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. J Clin Invest., 118: 342-51.

Poole RC, Halestrap AP (1993) Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am J Physiol., 264: C761-82.

Pouvelle B, Gormley J, Taraschi T (1994) Characterization of trafficking pathways and membrane genesis in malaria-infected erythrocytes. Mol. Biochem. Parasitol., 66: 83-96.

Pouvelle B, Spiegel R, Hsiao L, Howard RJ, Morris RL, Thomas AP, Taraschi TF (1991) Direct access to serum macromolecules by intraerythrocytic malaria parasites. Nature, 353: 73-5.

Pralle A, Keller P, Florin EL, Simons K, Hörber JK (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol., 148: 997-1008.

Paulsen IT, Nguyen L, Sliwinski MK, Rabus R, Saier MH Jr (2000) Microbial genome analyses: comparative transport capabilities in eighteen prokaryotes. J Mol Biol., 301: 75-100.

Raftos JE, Dwarte TM, Luty A, Willcock CJ (2006) Direct measurement of the rate of glutathione synthesis in 1-chloro-2, 4-dinitrobenzene treated human erythrocytes. Redox Rep., 11: 9-14.

Rager N, Mamoun CB, Carter NS, Goldberg D, Ullman B (2001) Localization of the *Plasmodium falciparum* PfNT1 nucleoside transporter to the parasite plasma membrane. J. Biol. Chem., 276: 41095-41099.

Ragheb D, Dalal S, Bompiani KM, Ray WK, Klemba M (2011) Distribution and biochemical properties of an M1-family aminopeptidase in *Plasmodium falciparum* indicate a role in vacuolar hemoglobin catabolism. J Biol Chem., 286: 27255-65.

Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, Foth BJ, Tonkin CJ, Roos DS, McFadden GI (2004) Tropical infectious diseases: metabolic maps and functions of the *Plasmodium falciparum* apicoplast. Nat Rev Microbiol., 2: 203-16.

Ramya TNC, Surolia N, Surolia A (2002) Survival strategies of the malarial parasite

Plasmodium falciparum. Current Science, 83: 818-825.

Repnik U, Gangopadhyay P, Bietz S, Przyborski JM, Griffiths G, Lingelbach K (2015) The apicomplexan parasite *Babesia divergens* internalizes band 3, glycophorin A and spectrin during invasion of human red blood cells. Cell Microbiol., 17: 1052-68.

Roudier N, Verbavatz JM, Maurel C, Ripoche P, Tacnet F (1998) Evidence for the presence of aquaporin-3 in human red blood cells. J Biol Chem., 273: 8407-12.

Rudzinska MA, Trager W, Lewengrub SJ, Gubert E (1976) An electron microscopic study of *Babesia microti* invading erythrocytes. Cell Tissue Res., 169: 323-34.

Ruiz-Ramos R, Lopez-Carrillo L, Rios-Perez AD, De Vizcaya-Ruiz A, Cebrian ME (2009) Sodium arsenite induces ROS generation, DNA oxidative damage, HO-1 and c-Myc proteins, NF-kappaB activation and cell proliferation in human breast cancer MCF-7 cells. Mutat Res., 674: 109-115.

Saliba KJ, Kirk K (1998) Uptake of an antiplasmodial protease inhibitor into *Plasmodium falciparum*-infected human erythrocytes via a parasite-induced pathway. Mol Biochem Parasitol., 94: 297-301.

Salzer U, Prohaska R (2001) Stomatin, flotillin-1, and flotillin-2 are major integral proteins of erythrocyte lipid rafts. Blood, 97: 1141-3.

Samuel BU, Mohandas N, Harrison T, McManus H, Rosse W, Reid M, Haldar K (2001) The role of cholesterol and glycosylphosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection. J Biol Chem., 276: 29319-29.

Sandberg AA, Lee GR, Cartwright GE, Wintrobe MM (1955) Purine nucleoside phosphorylase activity of blood. I. Erythrocytes. J Clin Invest., 34: 1823-9.

Schatzmann HJ (1983) The red cell calcium pump. Annu Rev Physiol., 45: 303-12.

Scholtyseck E, Piekarski G (1965) [Electron microscopic studies on merozoites of *Eimeria* (*Eimeria perforans* and *E. stidae*) and *Toxoplasma gondii*. On the systematic position of *T. gondii*]. Z Parasitenkd, 26: 91-115.

Schrier SL (1985) Red cell membrane biology – introduction. Clin Haematol., 14: 1–12.

Schwab JC, Beckers CJ, Joiner KA (1994) The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. Proc Natl Acad Sci USA, 91: 509-13.

Semplicini A, Spalvins A, Canessa M (1989) Kinetics and stoichiometry of the human red cell Na^+/H^+ exchanger. J Membr Biol., 107: 219-28.

Sherman IW (1977) Amino acid metabolism and protein synthesis in malarial parasites. Bull

World Health Organ, 55: 265-76.

Shio MT, Hassani K, Isnard A, Ralph B, Contreras I, Gomez MA, Abu-Dayyeh I, Olivier M (2012) Host cell signalling and *Leishmania* mechanisms of evasion. J Trop Med., 2012: 819512.

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature, 387: 569-72.

Sinai AP, Webster P, Joiner KA (1997) Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: a high affinity interaction. J Cell Sci., 110: 2117-28.

Soldati D, Foth BJ, Cowman AF (2004) Molecular and functional aspects of parasite invasion. Trends Parasitol., 20: 567-74.

Spielman A, Wilson ML, Levine JF, Piesman J (1985) Ecology of *Ixodes dammini*-borne human babesiosis and Lyme disease. Annu Rev Entomol., 30: 439-60.

Staines HM, Ellory JC, Kirk K (2001) Perturbation of the pump-leak balance for Na(+) and K(+) in malaria-infected erythrocytes. Am J Physiol Cell Physiol., 280: C1576-87.

Staines HM, Kirk K (1998) Increased choline transport in erythrocytes from mice infected with the malaria parasite *Plasmodium vinckei vinckei*. Biochem J., 334: 525-3.
Staines HM, Alkhalil A, Allen RJ, De Jonge HR, Derbyshire E, Egée S, Ginsburg H, Hill DA, Huber SM, Kirk K, Lang F, Lisk G, Oteng E, Pillai AD, Rayavara K, Rouhani S, Saliba KJ, Shen C, Solomon T, Thomas SL, Verloo P, Desai SA (2007) Electrophysiological studies of malaria parasite-infected erythrocytes: current status. Int J Parasitol., 37: 475-82.

Staines HM, Chang W, Ellory JC, Tiffert T, Kirk K, Lew VL (1999) Passive Ca(2+) transport and Ca(2+)-dependent K(+) transport in *Plasmodium falciparum*-infected red cells. J Membr Biol., 172: 13-24.

Staines HM, Powell T, Ellory JC, Egée S, Lapaix F, Decherf G, Thomas SL, Duranton C, Lang F, Huber SM (2003) Modulation of whole-cell currents in *Plasmodium falciparum*-infected human red blood cells by holding potential and serum. J Physiol., 552: 177-83.

Staines HM, Rae C, Kirk K (2000) Increased permeability of the malaria-infected erythrocyte to organic cations. Biochim Biophys Acta, 1463: 88-98.

Sun Y, Moreau E, Chauvin A, Malandrin L (2011) The invasion process of bovine erythrocyte by *Babesia divergens*: knowledge from an *in vitro* assay. Veterinary Research, 42: 62.

Suss-Toby E, Zimmerberg J, Ward GE (1996) *Toxoplasma* invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. Proc. Nat. Acad. Sci. USA, 93: 8413-8418.

Tanner MJ (1993) The major integral proteins of the human red cells. Baillieres Clin Haematol., 6: 333-56.

Tanneur V, Duranton C, Brand VB, Sandu CD, Akkaya C, Kasinathan RS, Gachet C, Sluyter R, Barden JA, Wiley JS, Lang F, Huber SM (2006) Purinoceptors are involved in the induction of an osmolyte permeability in malaria-infected and oxidized human erythrocytes. FASEB J., 20: 133-5.

Taraschi TF, Ellingson JS, Wu A, Zimmerman R, Rubin E (1986) Membrane tolerance to ethanol is rapidly lost after withdrawal: A model for studies of membrane adaptation. Proceedings of the National Academy of Sciences of the USA, 83: 3669–3673.

Teng R, Junankar PR, Bubb WA, Rae C, Mercier P, Kirk K (2009) Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by (1)H NMR spectroscopy. NMR Biomed., 22: 292-302.

Tosteson DC, Hoffman JH (1960) Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J Gen Physiol., 44: 169-94.

Trager W, Jensen J B (1976) Human malaria parasites in continuous culture. Science, 193: 673-5.

Trotti D, Rossi D, Gjesdal O, Levy LM, Racagni G, Danbolt NC, Volterra A (1996) Peroxynitrite inhibits glutamate transporter subtypes. J Biol Chem., 271: 5976-9.

Trotti D, Volterra A, Lehre KP, Rossi D, Gjesdal O, Racagni G, Danbolt NC (1995) Arachidonic acid inhibits a purified and reconstituted glutamate transporter directly from the water phase and not via the phospholipid membrane. J Biol Chem., 270: 9890-5.

Turgeon ML (2004) Theory and Procedures in Clinical Hematology. Lippincott Williams & Wilkins, New York pp.100.

Upston JM, Gero AM (1995) Parasite-induced permeation of nucleosides in *Plasmodium falciparum* malaria. Biochim Biophys Acta, 1236: 249-58.

Van Wijk R, Van Solinge WW (2005) The energy-less red blood cell is lost: erythrocyte enzyme abnormalities of glycolysis. Blood, 106: 4034-4042.

Valentin A, Rigomier D, Precigout E, Carcy B, Gorenflot A, Schrevel J (1991) Lipid trafficking between high density lipoproteins and *Babesia divergens*-infected human erythrocytes. Biology of the cell / under the auspices of the European Cell Biology Organization, 73: 63-70.

Ventura-Lima J, Bogo MR, Monserrat JM (2011) Arsenic toxicity in mammals and aquatic animals: a comparative biochemical approach. Ecotoxicol Environ Saf., 74: 211-8.

Verloo P, Kocken CH, Van der Wel A, Tilly BC, Hogema BM, Sinaasappel M, Thomas AW, De Jonge HR (2004) *Plasmodium falciparum*-activated chloride channels are defective in erythrocytes from cystic fibrosis patients. J Biol Chem., 279: 10316-22.

Vial HJ, Gorenflot A (2006) Chemotherapy against babesiosis. Veterinary parasitology, 138: 147-160.

Vial HJ, Wein S, Farenc C, Kocken C, Nicolas O, Ancelin ML, Bressolle F, Thomas A, Calas

M (2004) Prodrugs of bisthiazolium salts are orally potent antimalarials. Proc Natl Acad Sci USA, 101: 15458-63.

Waniewski RA, Martin DL (1984) Characterization of L-glutamic acid transport by glioma cells in culture: evidence for sodium-independent, chloride-dependent high affinity influx. J Neurosci., 4: 2237-46.

Ward GE, Miller L H, Dvorak J A (1993) The origin of parasitophorous vacuole membrane lipids in malaria-infected erythrocytes. J. Cell Sci., 106: 237-248.

Wein S, Maynadier M, Bordat Y, Perez J, Maheshwari S, Bette-Bobillo P, Tran Van Ba C, Penarete-Vargas D, Fraisse L, Cerdan R, Vial H. (2012) Transport and pharmacodynamics of albitiazolium, an antimalarial drug candidate. Br J Pharmacol., 166: 2263-76.

Winterberg M, Rajendran E, Baumeister S, Bietz S, Kirk K, Lingelbach K (2012) Chemical activation of a high-affinity glutamate transporter in human erythrocytes and its implications for malaria-parasite-induced glutamate uptake. Blood, 119: 3604-12.

Woodrow CJ, Burchmore RJ, Krishna S (2000) Hexose permeation pathways in *Plasmodium falciparum*-infected erythrocytes. Proc. Natl Acad. Sci. USA, 97: 9931-9936.

Xua Y, Weiss LM (2005) The microsporidian polar tube: A highly specialised invasion organelle. Int J Parasitol., 35: 941–953.

Yokoyama N, Okamura M, Igarashi I (2006) Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. Vet Parasitol., 138: 22-32.

Yoshida H, Kawane K, Koike M, Mori Y, Uchiyama Y, Nagata S (2005) Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. Nature, 437: 754–758.

Young AS, Morzaria SP (1986) Biology of babesia. Parasitol Today, 2: 211-9.

Zintl A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS (2003) *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. Clinical microbiology reviews, 16: 622-636.

Zrimi J, Ng Ling A, Giri-Rachman Arifin E, Feverati G, Lesieur C (2010) Cholera toxin B subunits assemble into pentamers-proposition of a fly-casting mechanism. PLoS One, 5: e15347.

Zweig S, Singer SJ (1979) Concanavalin A-induced endocytosis in rabbit reticulocytes, and its decrease with reticulocyte maturation. J Cell Biol., 80: 487-91

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