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Characterisation of *Plasmodium falciparum* proteins expressed on infected red blood cell surfaces as potential drug targets for severe malaria therapy

by

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Abstract

The most severe form of malaria in humans is caused by the intracellular parasite *Plasmodium falciparum*. The African continent bears the greatest burden of malaria with 90% of all malaria deaths occurring in sub-Saharan Africa where the high risk populations include pregnant woman and children under the age of five. Fatal cases of malaria are often a result of the progression of the disease to a life threatening syndrome where intravenous quinine or artesunate are administered as an emergency treatment, however a 15-20% mortality rate is still observed among treated individuals.

Pathogenesis of severe malaria is associated with the mature or late trophozoite stage of the parasite's intra-erythrocyte life cycle. At this stage the intracellular parasite expresses parasite derived proteins on the surface of the red blood cell (RBCs) that bind to host endothelial receptors. This cytoadhesion ultimately allows the parasite to multiply unhindered by the host resulting in high parasitaemia levels which is associated with the extent of the symptoms associated with severe malaria. There is currently no effective vaccine available for malaria and available antimalarial drugs are often compromised by rapidly developing drug resistance. Therefore there is an urgent need to identify novel drug targets for the treatment of severe malaria and to explore therapeutic agents that can be used as adjuncts to the currently available anti-malarial drugs. Statins, also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been identified as a potential adjunctive therapy for severe malaria due to their pleiotropic effects which include anti-cytoadhesive activity. The aim of this study was to characterise cytoadhesive related parasite proteins that are expressed on the surface of parasitised RBCs and then to assess the effects of a classic statin, lovastatin, on these parasite proteins. This was done in order to provide improved insight and extend existing knowledge on the potential role statins may have in treating severe malaria.

The *P. falciparum* 3D7 strain was efficiently synchronised and cultured according to standard culturing procedures and selective harvesting provided a 90% enrichment of narrowly synchronised cultures at the late trophozoite stage (35 -40 hours post invasion). Scanning electron microscopy (SEM) was used to successfully illustrate parasite induced

morphological changes to the surface of parasitised RBCs which included parasitic knob structures, a characteristic feature of severe malaria.

Isolation of membranes of equivalent RBCs and parasitised RBCs that were untreated and treated with 10 µM of lovastatin was optimised and surface proteins were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bands of interest that were visually apparent on Stain Free imaged gels and Coomassie brilliant blue stained gels were excised and in-gel trypsinised followed by peptide sequencing by liquid chromatography tandem mass spectrometry (LC-MS/MS). Advanced proteomic software was then used to successfully confirm the identity of key RBC structural membrane proteins as well as several parasite surface proteins, such as *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), *P. falciparum* erythrocyte membrane protein 3 (*Pf*EMP3), *P. falciparum* knob associated histidine rich protein (*Pf*KAHRP), *P. falciparum* mature infected erythrocyte surface antigen (*Pf*MESA) and *P. falciparum* cytoadhesion linked asexual gene (*Pf*CLAG) proteins. These cytoadhesive related proteins were identified on the membranes of parasitised RBCs that were treated with lovastatin thus suggesting that although lovastatin has the ability to retard cytoadhesion it is apparently not through the inhibition of expression of parasite derived adhesion proteins. The study also highlighted the need for stringent controls when using label-free proteomics to assess differential protein expression. The effects of lovastatin on protein abundance were inconclusive due to the low abundance of these proteins. This study demonstrated the potential of combining pharmacological studies with advanced proteomic techniques where the effects of physiological relevant concentrations of a drug were successfully assessed at the parasite protein expression level.

Keywords: Severe malaria, *Plasmodium falciparum*, cytoadhesion, *Plasmodium falciparum* knob associated histidine rich protein (*Pf*KAHRP), *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), SDS-PAGE, LC-MS/MS, lovastatin.

Glossary of Abbreviations

°C	Degree Celsius
µl	microliter
µM	micromolar
µm	micrometre
%	Percent
2-DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
$AgNO_3$	Silver nitrate
BCA	Bicinchoninic acid
CBB	Coomassie Brilliant Blue
CD36	Cluster Determinant 36
CID	Collision induced dissociation
cm	centimetre
cm^2	Square centimetre
CS	Cell separating
CSA	Chondroitin sulphate A
Da	Dalton
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DDT	1,1,1,trichloro2,2 bis-(4-chlorophenyl) ethane
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ESI-MS	Electrospray ionisation mass spectrometry
EtOH	Ethanol

FA	Formic acid
g	Gram
g	Gravity
g/mol	Grams per mol
G-3-PD	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1- piperazine ethanesulfonic acid
HMDS	Hexamethyldisilazane
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IAA	Iodoacetamide
ICAM-1	Intracellular adhesion molecule 1
IRS	Indoor residual spraying
ITN	Insecticide treated nets
kDa	kiloDalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoprotein
M	Molar
m/z	Mass to charge ratio
MC	Maurer's cleft
MeOH	Methanol
mg	milligram
mg/ml	milligram per millilitre
ml	millilitre
mM	millimolar
MS	Mass spectrometry
$NaHCO_3$	Sodium bicarbonate
ng/µl	nanogram per microlitre

NH_4HCO_3	Ammonium bicarbonate
nm	nanometre
OsO_4	Osmium tetroxide
PBS	Phosphate buffered saline
Pf	*Plasmodium falciparum*
*Pf*CLAG 2	*Plasmodium falciparum* cytoadherence linked asexual gene 2
*Pf*CLAG 3.1	*Plasmodium falciparum* cytoadherence linked asexual gene 3.1
*Pf*CLAG 8	*Plasmodium falciparum* cytoadherence linked asexual gene 8
*Pf*CLAG 9	*Plasmodium falciparum* cytoadherence linked asexual gene 9
*Pf*EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
*Pf*EMP2	*Plasmodium falciparum* erythrocyte membrane protein 2
*Pf*EMP3	*Plasmodium falciparum* erythrocyte membrane protein 3
*Pf*KAHRP	*Plasmodium falciparum* knob associated histidine rich protein
*Pf*MESA	*Plasmodium falciparum* mature parasite infected erythrocyte surface antigen
PMSF	Phenylmethylsulphonyl flouride
pRBC	Parasitised red blood cell
PV	Parasitophorous vacuole
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TNF-α	Tumour necrosis factor alpha
v/v	Volume per volume
VarioMacs	Vario magnetic cell sorter

VCAM-1	Vascular cell adhesion molecule 1
WHO	World Health Organisation

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Chapter 1: Introduction

1. Literature review

1.1 Malaria

Malaria is a widespread parasite related communicable disease that predominantly affects the poverty stricken population of sub-Saharan Africa (Who, 2014). Excluding human immune deficiency virus and tuberculosis, it has been reported that malaria is the leading cause of death of more people than any other communicable disease in Africa (Bates *et al.*, 2004). Victims of this devastating disease generally live in secluded areas, where access to public health services or institutes for diagnosis and appropriate treatment is limited which in turn contributes to the high mortality rate associated with this curable disease (Breman and Holloway, 2007). According to the World Malaria Report that was issued by the World Health Organisation (WHO) in 2014, approximately 198 million cases of malaria were reported in 2013 resulting in more than a half a million deaths. Africa is the region that is worst affected as 90% of all deaths caused by malaria reportedly occur on the continent. Moreover, almost 80% of all reported deaths are of children under the age of five (Who, 2014). A global distribution of reported malaria deaths worldwide is shown in Figure 1.1.

Malaria may be averted by various preventative methods such as chemoprophylaxis treatment and vector control initiatives which involve the use of insecticidal nets and indoor residual spraying (Elphinstone *et al.*, 2014). However, the lack of continuous implementation of these existing vector control strategies in conjunction with emerging drug and insecticidal resistance plays a crucial role in the failure of eradicating and eliminating malaria (Trape *et al.*, 2002). Currently there is no effective vaccine available for malaria prevention but there is a range of effective anti-malarial drug regimens that are available and used to treat infected individuals (Corradin and Engers, 2014). Although these anti-malarial drugs are effective in treating patients, late initiation of treatment or sub-standard drugs still result in an exceedingly high death toll.

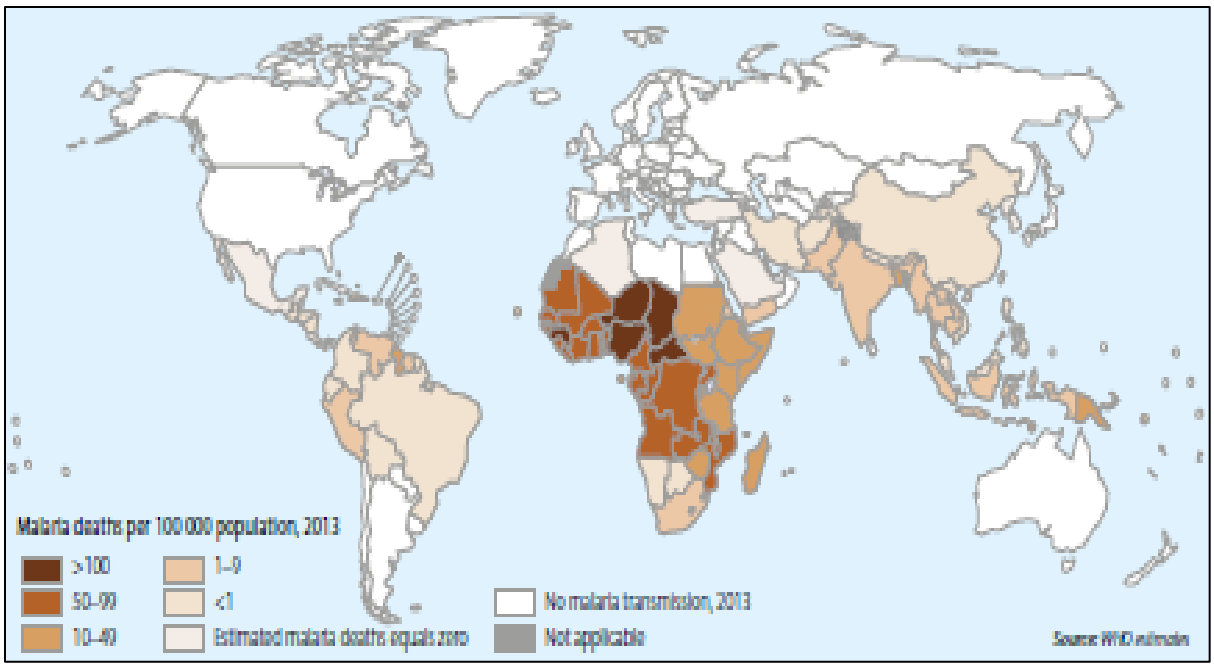


Figure 1.1: A map representing the number of malaria deaths per 100 000 population in 2013 (Who, 2014).

1.2 The life cycle of *Plasmodium falciparum*

The causative agent of malaria was discovered by a French army surgeon, Dr. Charles Louis Alphonse Laveran, who received the Nobel-prize in 1907 for his contribution to malaria research (Laveran *et al.*, 1982). Based on Dr. Laveran's findings, it was established that malaria is caused by an intracellular Apicomplexan protozoan parasite that belongs to the genus, *Plasmodium* (Escalante and Ayala, 1994). Furthermore, it was reported by Sir Ronald Ross that the *Plasmodium* parasite is transmitted via mosquitoes, more specifically the female *Anopheles* mosquito (Cox, 2010). Following these two ground-breaking discoveries regarding malaria, the five main *Plasmodium* species that are the causative agents of human malaria were identified as *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium knowlesi* and *Plasmodium malariae* (Snounou *et al.*, 1993; Cowman and Crabb, 2006). It has been found that individuals who experience the most severe and virulent form of malaria are infected with *P. falciparum* as this species is able to infect a greater percentage of red blood cells (RBCs) and prolong its survival within the host by inducing attachment of the host erythrocyte to the endothelial cells of blood vessels (Gupta *et al.*, 1994).

The rather complex life cycle of the *Plasmodium* parasite consists of 3 stages, the human liver stage, the human blood stage and the infective mosquito stage. The intricate life cycle of the *Plasmodium* species is shown in Figure 1.2. Commencement of the multifaceted life cycle occurs when the *Plasmodium* infected mosquito consumes a blood meal and injects the sporozoite form of the parasite into the human host. Subsequently, the parasite then migrates via the bloodstream to the liver where it invades hepatocytes. Within the infected hepatocytes, the parasite undergoes intracellular asexual replication during an asymptomatic interval of approximately 7 days. This process makes up the human liver stage, also known as the exo-erythrocytic stage, of the life cycle of the *Plasmodium* parasite (Silvie *et al.*, 2004).

After this period, the hepatocytes rupture and merozoite forms of the parasite are released into the bloodstream. This is the start of the asexual human blood-stage of the life cycle that is linked to the typical febrile episodes that are accompanied with other clinical manifestations associated with malaria (Miller *et al.*, 2002). The free merozoites invade circulating RBCs by initially recognising and attaching to surface receptors on RBCs. This is followed by the alignment of the merozoite's apical region towards the membrane of the RBC. Once the apical region of the merozoite is positioned, a tight junction is formed between the apical region and the surface of the RBC. The merozoite is able to enter the RBC by penetrating the membrane of the RBC utilizing an actin-myosin motor (Bargieri *et al.*, 2014). The entry of the merozoite into the RBC is a crucial step of the life cycle as the RBC serves as an essential host and protective environment for the parasite's survival during its intra-erythrocytic development. Once the merozoite has invaded the RBC, it undergoes further differentiation into the ring stage followed by trophozoite stages. Trophozoites are situated in a parasitophorous vacuole which is bound by a membrane within the RBC (Cowman and Crabb, 2006).

The *Plasmodium* parasite utilizes cytosolic haemoglobin and metabolises this host protein into peptides which serve as a source of amino acids required for the nutritional needs and survival of the parasite (Francis *et al.*, 1997). A by-product of this biological reaction is the iron containing prosthetic haeme group which is toxic to the parasite. As a protective mechanism, the parasite is able to convert the toxic haeme into a non-toxic form known as

haemazoin. Haemazoin crystals are deposited into the parasite food vacuole within the RBC (Elliott *et al.*, 2008). The trophozoite undergoes schizogony, a form of asexual multiplication of the DNA, and develops into a schizont which is followed by the egress of multiple merozoites from a single infected RBC. The discharge of multiple merozoites into circulation marks the restart of the blood stage or erythrocytic cycle yet again (Cowman and Crabb, 2006). During this stage, the number of infected RBCs increases exponentially and may only be circumvented by immune responses by the host or chemotherapy (Good *et al.*, 1998; Plebanski and Hill, 2000).

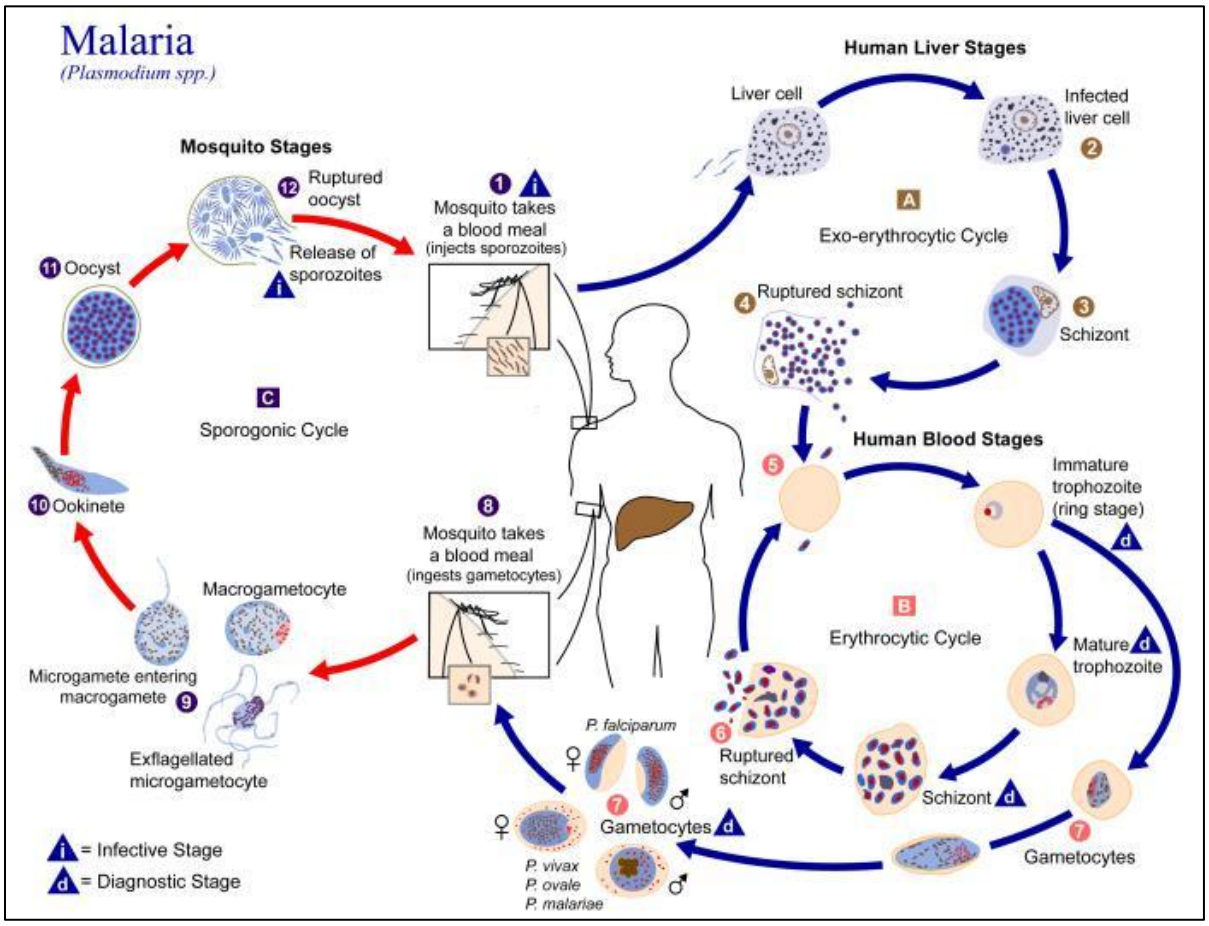


Figure 1.2: The multifaceted life cycle of the *P. falciparum* malaria parasite. (A) shows the exo-erythrocytic stage, **(B)** the erythrocytic stage and **(C)** sporogenic cycle (Garcia *et al.*, 2006) (with permission).

During the human blood stage, a small percentage of the malaria parasite additionally develops into its sexual form which is known as gametocytes. When a mosquito feeds on a host carrying the disease, it may become infected with the two sexual forms of the

gametocytes. Gametocytes mature into gametes in the gut of the mosquito and after successful fertilisation, motile ookinetes are formed. An ookinete forms into an oocyst in the mosquito midgut lining where multiple sporozoites are formed and released after which they migrate to the salivary glands of the infectious mosquito. Sporozoites may then be transferred from the malaria vector to a human host during a blood meal. This stage of the lifecycle is classified as the mosquito or sporogenic cycle (Garcia *et al.*, 2006).

1.3 Clinical features of uncomplicated malaria

Diagnosis of malaria includes rapid diagnostic tests which involves the identification of parasitic antigens, or the assessment of intra-erythrocytic parasitaemia using blood smears that are observed under a microscope (Bronzan *et al.*, 2008; Wilson, 2012). Regarding *P. falciparum* infections, the onset of clinical features of the disease often occur 8 – 15 days post infection. Patients with uncomplicated malaria generally experience flu-like symptoms and cyclic high fevers. Cyclic fever is a common feature of malaria and is a result of coordinated rupturing of RBCs. Additionally, the expression of tumour necrosis factor alpha (TNF-α) during an infection is significantly increased and consequently is associated with a rise in body temperature (Clark and Cowden, 2003). Fevers may be accompanied by headaches, fatigue, abdominal discomfort or tenderness, swelling of the spleen, vomiting as well as muscle and joint ache. Clinical symptoms occur when the parasite has advanced into the asexual blood stage of the life cycle of the *P. falciparum* parasite which makes this particular stage of the life cycle a major focal point for malaria research (Kappe *et al.*, 2010).

1.4 Severe malaria and the implications

The early clinical condition of a patient who has uncomplicated malaria may progress to complicated or severe malaria. Severe malaria is characterised by severe anaemia, respiratory distress, metabolic acidosis, impaired consciousness as well as multi-organ failure (Mackintosh *et al.*, 2004). The main pathogenic factors that are responsible for the development of severe malaria are associated with the mature or late trophozoite stage of the parasite's life cycle. At this stage the parasitised RBC has the ability to remove itself from the peripheral circulation via cytoadhesive mechanisms and sequester into organs such as the brain, lungs, kidneys and placenta. Cytoadherence is a unique feature of *P. falciparum*

infections and is not exhibited by the other less virulent *Plasmodium* species (Chen *et al.*, 2000). A serious clinical manifestation of severe malaria is cerebral malaria, a condition that occurs when parasitised RBCs adhere to the endothelium, causing micro-thrombi that can result in infarctions or inflammation of micro areas of the brain. Patients who have cerebral malaria deteriorate rapidly and their condition, if untreated, often results in convulsions, unconsciousness or even death (Idro *et al.*, 2005). In addition to cytoadhesive properties, parasitised RBCs may interact with non-parasitised RBCs, known as rosetting, and with platelets which may lead to platelet-mediated clumping. These two processes may cause blockage of the microvasculature of important organs and may lead to perfusion impairment thus resulting in further non-selective organ damage (Rowe *et al.*, 2009). The three adhesive mechanisms are illustrated in Figure 1.3.

By adhering to endothelial cells and sequestering in vennules and capillaries of vital organs, the parasite avoids circulation through the spleen, which is responsible for the elimination of impaired RBCs (Engwerda *et al.*, 2005). This pro-survival mechanism employed by the parasite thus renders the spleen incapable of successfully removing the parasitised RBCs from the peripheral circulation (Hisaeda *et al.*, 2005). Consequently, during the infection the parasite is able to thrive and grow exponentially uninhibited by the host. This is an important factor as the severity of symptoms that a patient may experience is directly proportional to the level of parasitaemia in an infected individual (Rowe *et al.*, 2009).

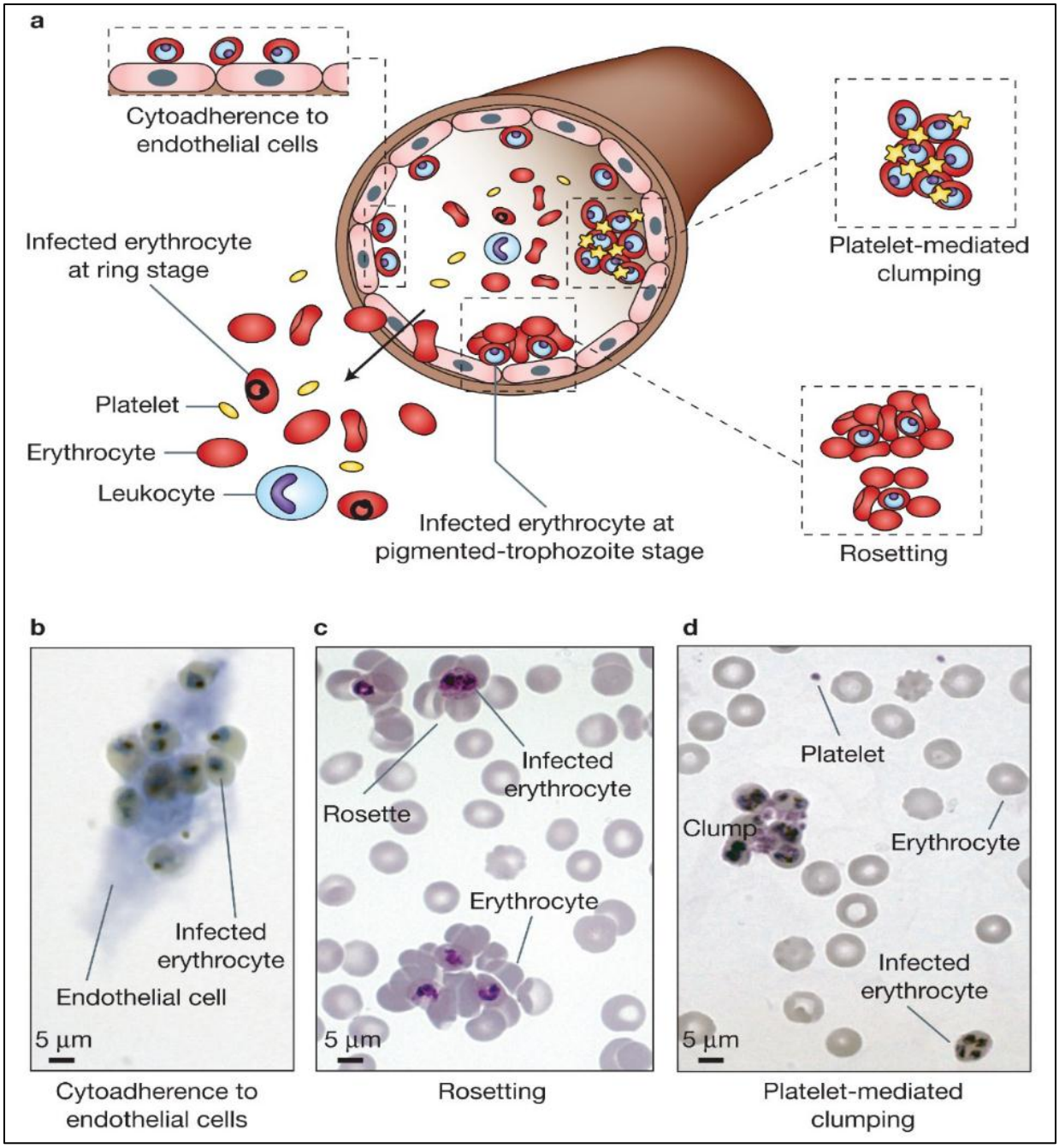


Figure 1.3: Pathological factors associated with severe malaria. (A) The three mechanism of adhesion, **(B)** shows the cytoadherence of parasitised RBCs to endothelial cells, **(C)** illustrates the interaction of non-parasitised RBCs with parasitised RBCs which is known as rosetting and **(D)** shows platelet mediated clumping (Rowe *et al.*, 2009) (with permission).

1.5 Modification to the red blood cell membrane by *P. falciparum*

The RBC is a smooth flexible biconcave disc bound by a membrane that represents a complex biological structure which comprises mostly of lipids supporting many proteins and carbohydrates. The RBC membrane has many transmembrane proteins such as Band 3,

which is a highly abundant anion transporter found in RBCs as well as kidney tubule cells, and the glycophorin protein family that are in turn heavily glycosolated. The inner cytoskeletal compartment of the cell membrane comprises mainly of spectrin (α and β subunits), ankyrin, actin and protein 4.1 (Mohandas and Gallagher, 2008). An illustration of the RBC membrane and the arrangement of key membrane components is shown in Figure 1.4.

Several of the surface expressed proteins including the glycosylated proteins of the RBC are implicated in the invasion process of the *Plasmodium* parasite. In addition, the developing intracellular parasite appears to export proteins to the surface of the parasitised RBC. After invasion of a RBC, the malaria parasite is able to alter the distribution of the proteins to its advantage by modifying the morphology and adhesive properties of the RBC membrane (Craig and Scherf, 2001). The parasite successfully alters the host membrane by trans-locating parasitic proteins to the cytoskeletal membrane and the outer surface of the RBC (Marti *et al.*, 2004). The parasite is able to synthesise and express a number of proteins throughout the life cycle (Cooke *et al.*, 2004), however this study investigated proteins that are expressed on the RBC outer surface during the mature trophozoite stage and which have a direct role in cytoadhesion

During the late trophozoite stage, the RBC membrane undergoes morphological alterations to the smooth outer surface that can be easily observed microscopically when analysing parasitised RBCs (Deitsch and Wellems, 1996). When visualising parasitised RBCs, the surface of the RBC membrane is covered with dome shaped protrusions, commonly known as knob-like structures, a morphological alteration induced by parasite proteins. Knobs are made up of electron dense particles and estimated to be 70 – 100 nm in size. The density of knobs on the surface of a RBC membrane increase from 25 hours to 40 hours post-invasion with a decline of knob densities during the remainder of the life cycle (Quadt *et al.*, 2012). The formation of the knob-like structures is dependent on the expression *P. falciparum* knob-associated histidine-rich protein (*Pf*KAHRP). This *P. falciparum* encoded protein is 70 - 100 kiloDalton (kDa) in size and is translocated to the cytoplasmic side of the RBC membrane where it interacts with host cytoskeletal proteins such as spectrin, ankyrin and actin. Additional proteins that are associated with parasitic *Pf*KAHRP and that are needed

for knob formation include *P. falciparum* erythrocyte membrane protein 3 (*Pf*EMP3) and *P. falciparum* mature parasite infected erythrocyte surface antigen (*Pf*MESA) (Cooke *et al.*, 2001). It has been observed that in the absence of knob structures, cytoadhesion does not take place efficiently as the knob structures serve as points of attachment for parasitic proteins that bind to adhesion molecules expressed on endothelial cell surfaces (Horrocks *et al.*, 2005). Therefore, it is proposed that the disruption of *Pf*KAHRP or other molecular components of the knob protrusions may lead to the inhibition of cytoadherence, thus indicating *Pf*KAHRP and associated molecules as potential drug targets for severe malaria (Ho and White, 1999).

P. falciparum erythrocyte membrane protein 1 (*Pf*EMP1) is a parasite derived protein that can be found on the surface of the host cell's membrane fixed to parasitic knobs and is considered to be the principal determinant of the parasite infected RBC's cytoadhesive ability (Waller *et al.*, 1999). *Pf*EMP1 is 250 -350 kDa in size thus characterising the parasitic antigen as one of the larger proteins inserted into the membrane of parasitised RBCs during the late trophozoite stage. *Pf*EMP1 is encoded by approximately 60 genes that belong to the diverse *var* gene family (Scherf *et al.*, 2008). The *Plasmodium* parasite is able to switch to various forms of *Pf*EMP1 during an infection thus avoiding a targeted immunological response by the host (Pasternak and Dzikowski, 2009). This unique feature is known as antigenic variation and aids to prolong the survival of the parasite within the host, thus resulting in high parasitaemia burdens in an infected individual. This antigenic phenomenon that is utilised by the parasite makes *Pf*EMP1 a significant virulent factor of malaria and a prominent drug target for potential chemotherapeutic agents for severe malaria (Marti *et al.*, 2004). However, due to the polymorphic attributes of the *Pf*EMP1 antigen, many difficulties arise when attempting to characterise the parasitic protein. Transcriptional switching of *Pf*EMP1 among different isolates makes it challenging to successfully target *Pf*EMP1 for a potential vaccine due to the high antigenic variance but chemotherapeutic agents may show more promise. Nevertheless, the distinct role of *Pf*EMP1 in the pathogenesis of severe malaria would make it a valuable target to explore with respect to inhibition of the expression of this antigen or impede the binding of this parasitic ligand to endothelial receptors (Pasternak and Dzikowski, 2009). Figure 1.4 shows the complex arrangement of host cell membrane proteins and the points of insertion of the parasite

derived proteins. Additional parasite proteins that are inserted into the membrane include rifin proteins and sequestrin however their role in cytoadhesion is yet to be elucidated (Alister *et al.*, 2012)

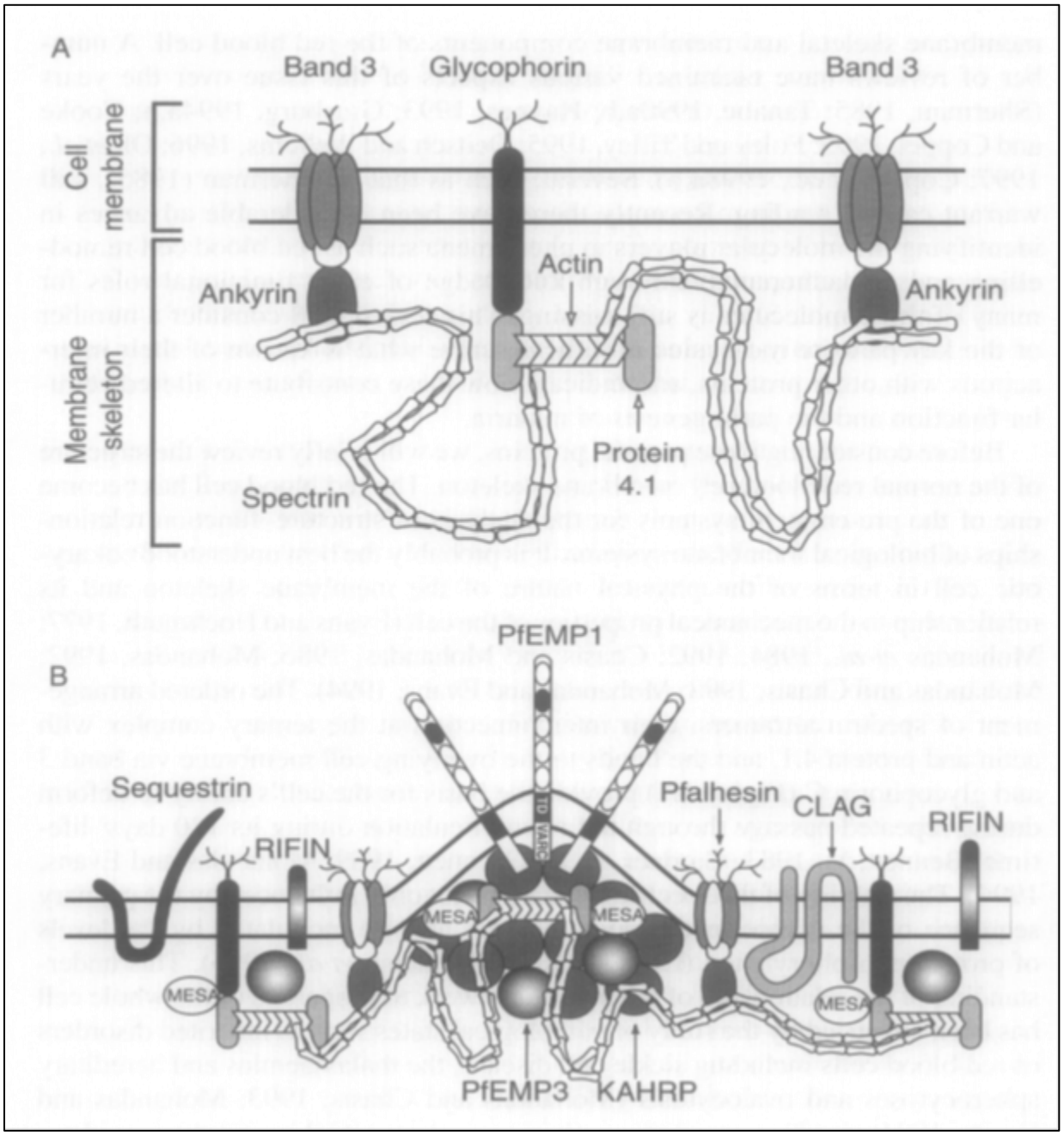


Figure 1.4: Comparison of the membranes of a normal RBC and a parasitised RBC at the late trophozoite stage. **(A)** shows the structural arrangement of proteins on the membrane of a normal RBC and **(B)** illustrates the membrane modifications and insertion of parasite derived proteins into the RBC membrane at the late trophozoite stage. *Pf*EMP1 is *Plasmodium falciparum* erythrocyte membrane protein 1; *Pf*CLAG is cytoadherence linked asexual gene; *Pf*MESA is mature parasite infected erythrocyte surface antigen; *Pf*EMP3 is *Plasmodium falciparum* erythrocyte membrane protein 3; *Pf*KAHRP is knob associated histidine rich protein (Cooke *et al.*, 2001) (with permission).

1.6 Ligand-receptor interactions involved in cytoadhesion

Multiple ligand-receptor interactions facilitate cytoadhesion, however only few have been studied in any detail and may be potential candidates for an anti-malarial drug target (Rowe *et al.*, 2009). *Pf*EMP1 has been shown to interact with a number of adhesion molecules which facilitate the adhesion of parasitised RBCs to host endothelium. These adhesion molecules include intracellular adhesion molecule 1 (ICAM-1), a transmembrane glycoprotein, that is found in high density on activated endothelial cells. ICAM-1 has been identified as a prominent adhesion molecule that is involved in severe malaria (Chakravorty and Craig, 2005). A study showed that the level of ICAM-1 adhesion to the surface of *P. falciparum* parasitised RBCs was significantly increased in isolates that were analysed from children suffering from cerebral malaria (Ochola *et al.*, 2011). A second adhesion receptor for *Pf*EMP1 is cluster determinant 36 (CD36) which has also been reported to play a role in cerebral associated adhesion mechanisms (Winograd *et al.*, 2004). Moreover, *Pf*EMP1 binds to chondroitin sulphate A (CSA), a glycosaminoglycan polysaccharide found on placental syncytiotrophoblasts. Interaction between the CSA receptor and *Pf*EMP1 results in sequestration of parasitised RBCs in the placenta of malaria infected pregnant women (Reeder *et al.*, 1999). Additional molecules that *Pf*EMP1 may interact with include thrombospondin, vascular cell adhesion molecule 1 (VCAM-1), P-selectin and E-selectin, however the exact role that these interactions play in cytoadherence is yet to be defined (Janes *et al.*, 2011). Figure 1.5 is a representation of the three main cytoadhesive receptors for the *Pf*EMP1 ligand.

A high parasitaemia level in a malaria infected individual is associated with the progression of uncomplicated malaria to severe malaria. Cytoadhesion and sequestration processes facilitate the uninhibited multiplication of parasites during an infection, thus resulting in high parasite burdens. It is therefore proposed that if the above mentioned ligand-receptor interactions can be disrupted, it would result in a significant decrease in parasitaemia during a malaria infection and this would ultimately reduce or prevent the serious symptoms associated with severe malaria. (Chotivanich *et al.*, 2000).

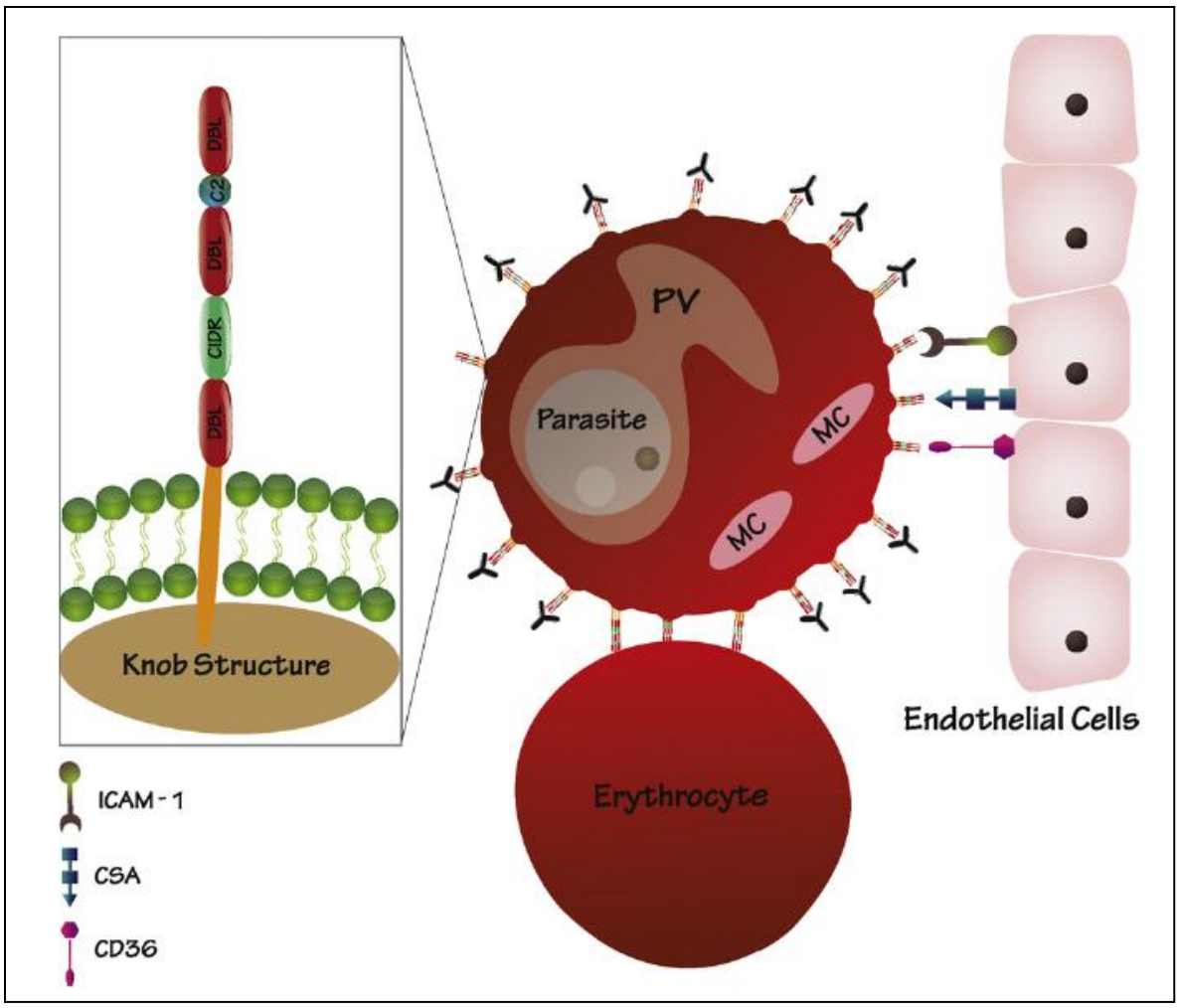


Figure 1.5: Schematic representation of the interaction between *Pf*EMP1 and host receptors. *Pf*EMP1 is expressed on knob structures that are formed on the surface of parasitised RBCs and binds to endothelial receptors. PV is parasitophorous vacuole; MC is Maurer's cleft; ICAM-1 is intracellular adhesion molecule; CSA is chondroitin sulphate A; CD36 is cluster determinant 36; DBL is duffy binding like domain and CIDR is cysteine-rich interdomain regions. (Pasternak and Dzikowski, 2009) (with permission).

1.7 Control and treatment of malaria

1.7.1 Vector control of malaria

Vector control strategies are put in place with the main aim to reduce the transmission of malaria and prevent people from being bitten by mosquitoes (Ulrich *et al.*, 2013; Hemingway, 2014). Vector control includes any measures that are utilised to prevent malaria transmission via mosquitos and includes the use of active and passive actions such as insecticide treated bed nets (ITN), indoor residual spraying (IRS), use of repellents to prevent mosquito bites, draining of water bodies that serve as breeding areas and use of

dead-end hosts for the parasite. The different insecticides that are recommended by the World Health Organisation for the prevention of malaria transmission are listed in Table 1.1.

Table 1.1: A list of recommended insecticides by class for malaria vector control. DDT is 1,1,1,trichloro2,2 bis-(4-chlorophenyl) ethane; IRS is indoor residual spreading; ITN is insecticide treated bed nets (Who, 2014).

Group/class	Insecticidal compound	Use
Organochlorines	DDT	IRS
Organophosphates	Malathion	IRS
	Fenitrothion	IRS
	Pirimiphos-methyl	IRS
Carbamates	Bendiocarb	IRS
	Propoxur	IRS
Pyrethroids	Alpha-cypermethrin	IRS and ITN
	Bifenthrin	IRS and ITN
	Cyfluthrin	IRS and ITN
	Deltamethrin	IRS and ITN
	Etofenplox	IRS and ITN
	Lambda-cyhalothrin	IRS and ITN
	Permethrin	ITN

To date, the most effective compound used for insecticidal spraying is 1,1,1,trichloro2,2 bis-(4-chlorophenyl) ethane (DDT). DDT is classified as one of the twelve persistent organic pollutants that belongs to the organochlorine class of insecticides and was introduced as an insecticide for malaria control in 1946. However, due to pressing concerns regarding the toxic and long term harmful effects on the environment, mainly through inappropriate use, DDT was prohibited for agricultural purposes in the 1970s. This led to the subsequent restriction of DDT for malaria control and in some countries the total discontinuation of DDT, even for prevention of malaria transmission. Consequently, the number of reported malaria cases increased significantly resulting in the limited reinstatement of DDT for controlled insecticidal spraying with specific restrictions (Turusov *et al.*, 2002; Sadasivaiah *et al.*, 2007). Other classes of insecticides that are used for malaria control include

organophosphates, carbamates and the widely used pyrethroids (Prato *et al.*, 2012). A major issue surrounding the control of malaria is the inaccessibility to vector control measures, the lack of funding to sustain the implementation of vector control strategies and most importantly the development of insecticidal resistance. It is for these reasons that chemoprophylaxis and antimalarial therapy is critical for the prevention and treatment of malaria despite the important role that vector control might play in the elimination of malaria. (Hemingway, 2014; Killeen *et al.*, 2014).

1.7.2 Anti-malarial treatment and prophylaxis

As mentioned, there is currently no effective vaccine for malaria and the disease is generally treated using a number of available chemotherapeutic antimalarial agents that target a particular stage of the parasite's life cycle with the ultimate goal of inhibiting the survival of the parasite within the human host. Antimalarial treatment that is currently used is based on three main categories of drugs which include quinoline derivatives, artemisinin derivatives and antifolates. The main antimalarial drug classes that are currently in use today and the site of action of the *Plasmodium* life cycle are shown in Figures 1.6 and 1.7 respectively.

1.7.2.1 Quinoline derivatives

Quinine was discovered in the 17th Century and it was the first isolated agent that was used to effectively treat malaria. Quinine is an alkaloid obtained from the bark of the Cinchona tree (Meshnick and Dobson, 2001). The well-known antimalarial compound is sometimes used to treat uncomplicated malaria and pregnant patients if the first line of treatment is ineffective. However, the main use of quinine is intravenous administration for the treatment of severe and complicated malaria (Achan *et al.*, 2011). Due to its effectiveness, quinine has been modified into a number of effective synthetic anti-malarial compounds demonstrating less adverse side effects. Chloroquine is an example of a 4-aminoquinoline derivative of quinine. Although chloroquine was developed in 1934 by Hans Andersag, a German scientist, the pharmacological use of this particular antimalarial drug was only established in 1946 (Krafts *et al.*, 2012). Chloroquine is found to be more tolerable than quinine and may be used as a prophylactic drug. However, the use of chloroquine including

the prophylactic indication of the drug has become limited due to drug resistance by the parasite (Foote *et al.*, 1989). The mechanism of action of chloroquine, similar to that of quinine and its other derivatives, involves inhibition of the haeme-polymerase enzyme which catalyses the biotransformation of haeme to haemazoin crystals. Consequently, due to enzyme inhibition, the parasite is exposed to the toxic free haeme resulting in the intra-erythrocytic blood stage death of the parasite (Slater and Cerami, 1992). Quinoline derivatives include mefloquine and primaquine. Mefloquine is used as a chemoprophylactic that targets the intra-erythrocytic development of the parasite. However, similar to chloroquine, the use of mefloquine is restricted to mefloquine sensitive areas. Primaquine is a tissue schizonticide that targets early liver stage parasites prior to the release of merozoites into the bloodstream resulting in the delay of primary attacks and preventing relapse. Primaquine also targets gametocytes thus blocking transmission to the mosquito. (Rang, 2007).

1.7.2.2 Artemisinin derivatives

The active compound Ginghaosu, commonly known today as artemisinin, is an extract from the herb *Artemisia annua* and was successfully isolated and characterised in 1972 (Butler and Wu, 1992). The need to improve the pharmacological profile of artemisinin lead to the derivatisation of the compound into several semi-synthetic anti-malarial agents such as artemether, artesunate, arteether and dihydroartemisinin (Balint, 2001). The proposed mode of action of artemisinin and its derivatives is the generation of free radicals that alkylate haeme thus preventing haeme polymerisation. Additionally, the compound also induces alkylation of vital parasitic proteins (Meshnick, 2002). Artemisinin is known for its potent and rapid anti-malarial activity and these drug derivatives are the cornerstone of current combination drug malaria treatment. Artemisinin based drugs are partnered with other longer acting antimalarial compounds for the treatment of malaria. However, emerging drug resistance to this efficacious agent has already been reported in South East Asia (Dondorp *et al.*, 2011; Ashley *et al.*, 2014).

1.7.2.3 Anti-folates

Similar to the host, the parasite requires folic acid for DNA replication that is essential for its survival, however contrary to its host, the parasite is capable of carrying out folic acid synthesis *de novo*. Therefore the parasite requires the functioning of enzymes, not common to the host, such as dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Antifolates are able to inhibit these enzymes ultimately resulting in the depletion of pyrimidines needed for DNA synthesis (Nzila, 2006). Anti-folate agents include DHFR inhibitors such as proguanil and pyrimethamine as well as DHPS inhibitors that consist of sulfadoxine (sulphonamide) and dapsone (sulfone).

1.7.2.4 Antimalarial drug resistance and the use of drug combinations

Successful treatment of malaria patients and the sustainability of antimalarial drugs are severely compromised by parasite drug resistance. Factors that contribute to increasing drug resistance include the mutation rate of the malaria parasite in conjunction with the use of poor quality drugs. Furthermore, misuse of available antimalarial drugs or self-treatment often result in sub-therapeutic levels which not only contributes to the progression of a patient's condition from uncomplicated to severe malaria but is also a major factor in the development of drug resistance. (Who, 2010a). An alarming fact is that signs of resistance to artemisinin, a fundamental drug in malaria treatment, has already been reported in regions such as Myanmar, Cambodia, Thailand and Vietnam with resistance to the majority of other available antimalarial drugs also prevalent in malaria endemic areas (Who, 2012). As a result of this, the WHO has strictly advised that monotherapy should not be used for the treatment of malaria and so a large part of malaria treatment involves combinational therapy (Who, 2014). The rationale behind combinational therapy is the prevention of partial or complete drug resistance against a particular anti-malarial agent and to exploit the beneficial synergistic effects that the drug components in the combination may have (Kremsner and Krishna, 2004). Artemisinin-based combination therapy (ACT) is recommended by the WHO for the current treatment of malaria. ACT is the standard regimen for uncomplicated malaria whereby fast acting artemisinin, which is responsible for the initial elimination of parasites, is combined with slow-clearing drugs that target remaining parasites in the host (Who, 2010a).

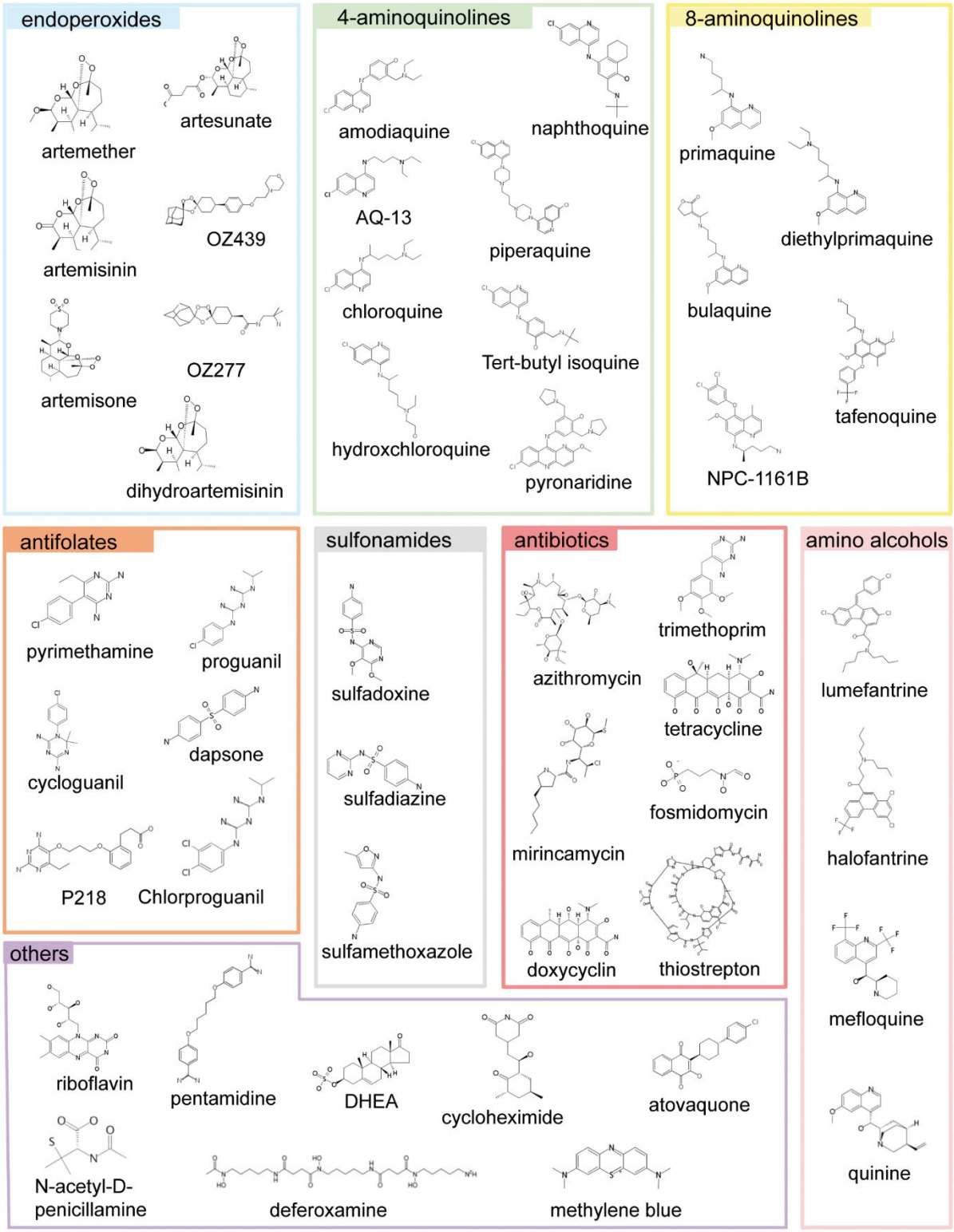


Figure 1.6: Structures of the main classes of antimalarial drugs. Colours of boxes around the different drug classes correlate to the site of drug action on the *P. falciparum* life cycle in Figure 1.7 (Delves *et al.*, 2012) (with permission).

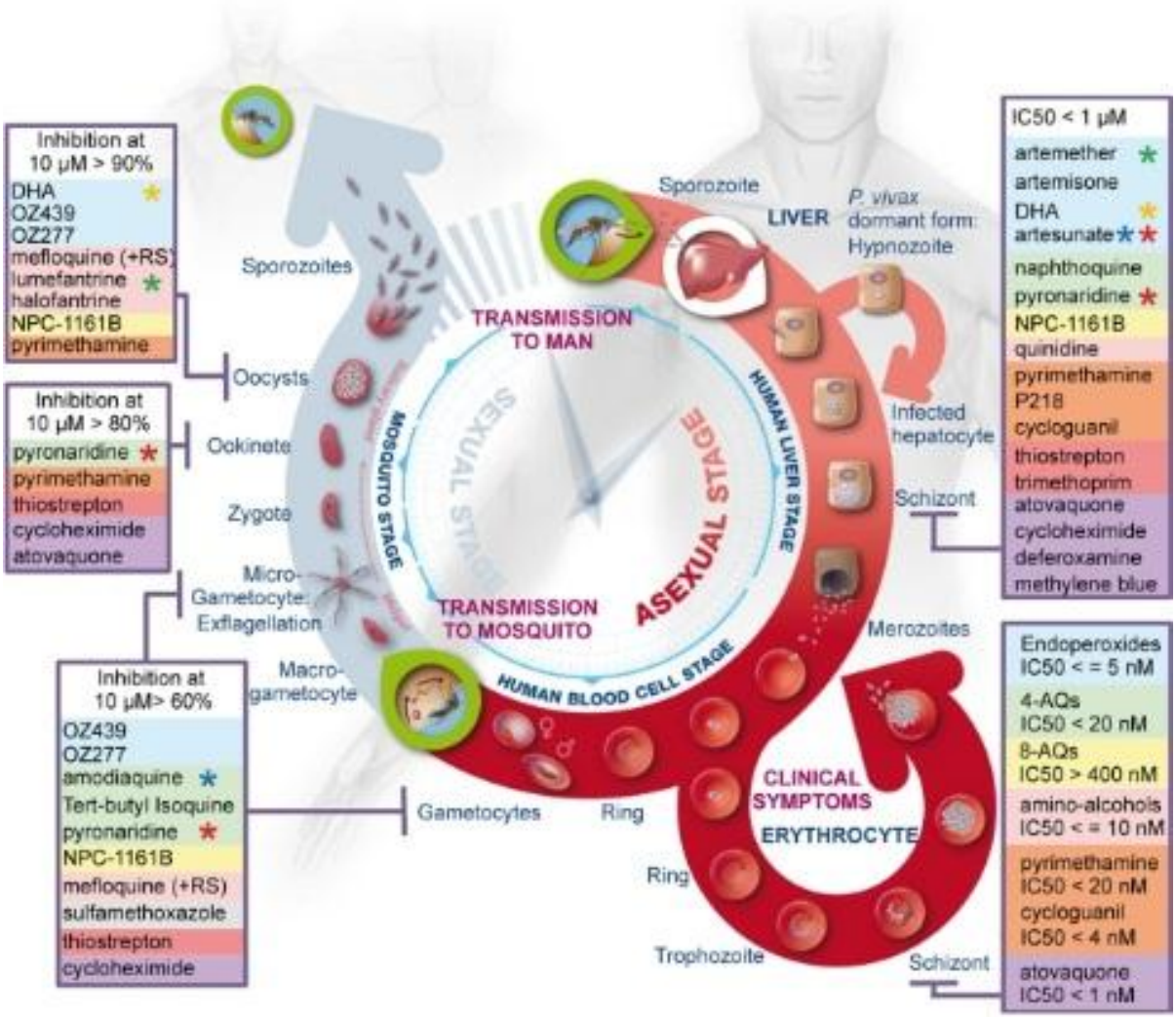


Figure 1.7: Summary of the activity of currently used antimalarial drugs on the *P. falciparum* life cycle that consists of the mosquito, human liver and human blood stage. Drugs that are inhibitors of developing parasite forms are listed in the boxes. DHA is dihydroartemisinin, 4-AQ is 4-aminoquinoline, 8-AQ is 8-aminoquinoline (Delves *et al.*, 2012) (with permission).

Artemisinin based combinations that are recommended by the WHO and that are widely used iclude dihydroartemisinin-piperaquine, artesunate-sulfadoxine-pyrimethamine, artemether-lumefantrine, artesunate-mefloquine and artesunate-amiodiaquine (Who, 2014). Other combinations that are non-artemisinin based include atovaquone-proguanil and pyrimethamine-sulfadoxine (Biamonte *et al.*, 2013). Continuous efforts and research involved in developing effective vaccines and anti-malarial agents are increasing as a result of the persistent threat of drug resistance however despite these efforts, there is currently no licensed vaccine for malaria. This is due to the parasite's ability to induce differential protein expression throughout its life cycle which makes identifying specific antigens as

possible targets for vaccines an extremely difficult task (Corradin and Engers, 2014). Hence, the continuous threat of drug resistance along with the lack of a malaria vaccine highlights the need for the identification of novel malaria drug targets as well as the implementation of new and effective treatment approaches.

1.8 Statins

Statins, also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are used as the first line of treatment for elevated plasma levels of low-density lipoprotein (LDL) cholesterol, which is commonly associated with coronary heart disease (Keaney Jr *et al.*, 2014). Statins are analogues of HMG-CoA which is a precursor of cholesterol as well as the substrate for HMG-CoA reductase. The agents from this specific drug class have a strong affinity for HMG-CoA reductase, thus competitively inhibiting the enzyme which is responsible for the conversion of HMG-CoA to mevalonic acid in the cholesterol synthesis pathway that is illustrated in Figure 1.8 (Stancu and Sima, 2001).

Lovastatin is a closed-ring lactone pro-drug and was the first successful drug used to treat dislipidaemia. Lovastatin was introduced onto the market in 1987 and its use resulted in significant developments in the treatment of cholesterol related diseases (Sirtori, 2014). Additionally, alteration to the structural form of lovastatin to produce open ring structures led to the development of improved synthetic compounds such as atorvastatin, pravastatin, rosuvastatin etc that are curently used to treat patients with elevated serum cholesterol levels (Sirtori, 2014). A summary of the old and newer generation of statins is shown in Figure 1.9.

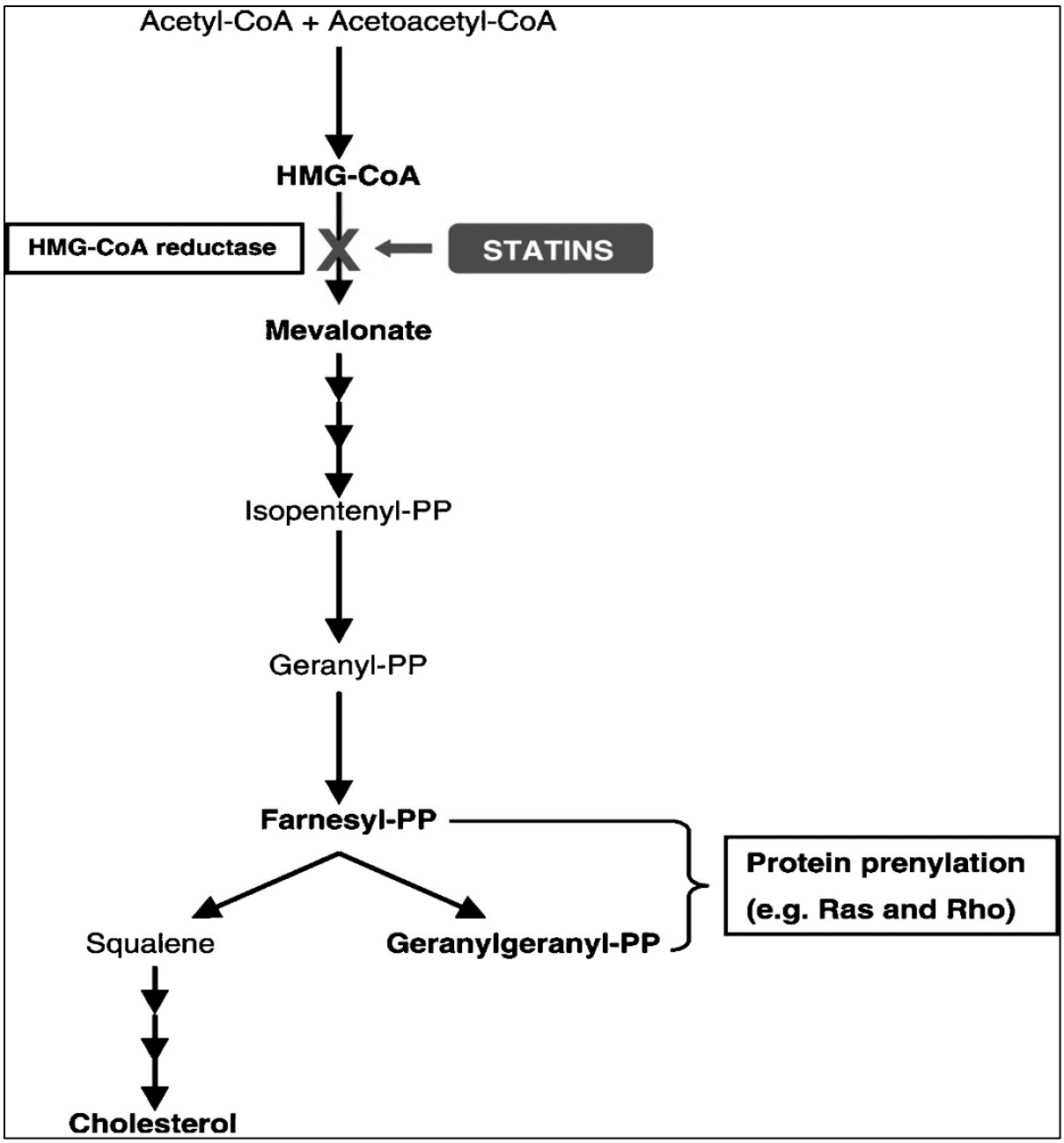


Figure 1.8: The cholesterol biosynthesis pathway. HMG-CoA is 3-hydroxy-3-methylglutaryl coenzyme A (Martin *et al.*, 1985) (with permission).

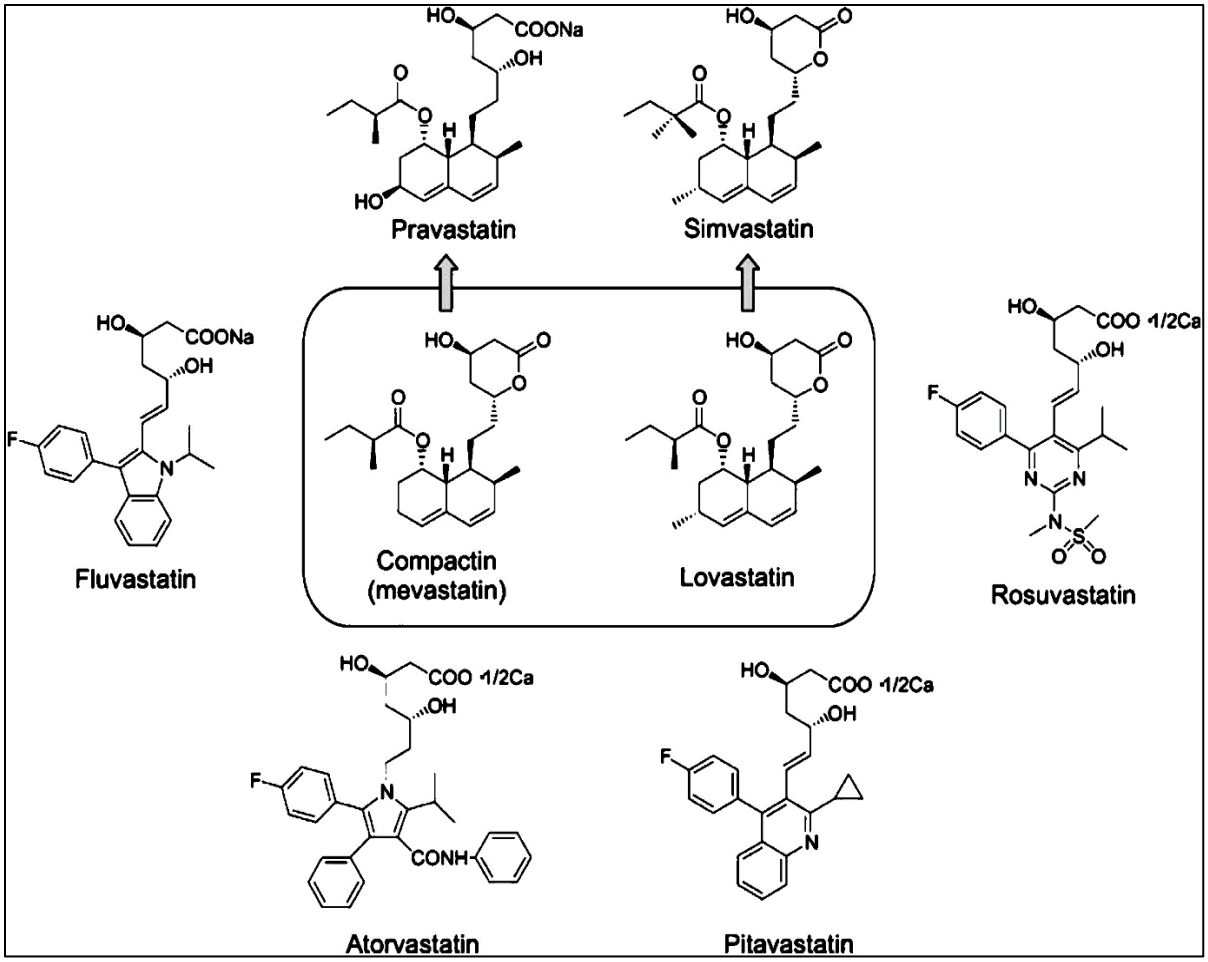


Figure 1.9: The chemical structures of compounds from the statin drug class. Compactin (mevastatin) is not currently used for the treatment of hypercholesterolemia (Sirtori, 2014) (with permission).

1.9 The potential role of statins as an adjunctive therapy for severe malaria

Due to its reliability, quinine has been the mainstay drug for the treatment of severe malaria for many years. However, injectable artesunate has been identified as an alternative drug for severe malaria followed by oral ACTs once a patient is able to take oral medication (Who, 2010b). Artesunate has been found to be the superior drug with regards to efficacy and safety profile when compared to quinine. Additionally the mortality rate among patients, who suffered from severe malaria and who were treated with intravenous artesunate instead of quinine, was seen to be reduced by approximately a quarter during two multi-national clinical trials, South-East Asia Quinine Artesunate Malaria Trial (SEAQUAMAT) and Africa Quinine Artesunate Malaria Trial (AQUAMAT) (Dondorp *et al.*, 2005; Dondorp *et al.*, 2010). Although these drugs are administered immediately to patients

who have been diagnosed with severe malaria, a mortality rate of 15-20% is still observed in treated populations, often due to being diagnosed at an advanced stage of malaria. Furthermore, severe malaria predominantly affects children between infancy and 5 years, as older individuals are able to acquire some degree of specific immunity, with an approximate 80% fatality rate in children. To date there has been no available antimalarial drug or alternative treatment to decrease this mortality rate and therefore there is an urgent need to develop novel therapeutics or to explore alternative treatment options for severe malaria (Black *et al.*, 2010; Who, 2014).

A review written by Rowe *et al* looked at the specific molecular mechanisms involved in cytoadhesion and more importantly highlighted the imperative need for adjunctive anti-adhesive therapy for severe malaria. This type of adjunctive therapy can be defined as an intervention between parasite ligands and host receptors that are involved in the cytoadhesion of parasitised RBCs to endothelial cells of the host. Characteristic features of this therapy would be drug based and moreover, a drug that is stable with very few side effects. Drugs that are already on the market would be preferable as this would be advantageous with regards to cost efficiency factors as well as the time needed for development and to establish safety parameters of the drug (Rowe *et al.*, 2009). Statins are ideal candidates for such an anti-adhesive drug therapy not only because it qualifies for most of these criteria, but agents from this drug class have been shown to exert activity against the *P. falciparum* parasite. A study carried out by Grellier *et al* reported that lovastatin and simvastatin inhibited the *in vitro* development of *P. falciparum* in human RBCs with the inhibitory effects of the two HMG-CoA reductase inhibitors corresponding to the late trophozoite stage (Grellier *et al.*, 1994). Additionally, two other studies showed that atorvastatin has the potential to work synergistically with anti-malarial drugs such as quinine and mefloquine (Parquet *et al.*, 2009; Parquet *et al.*, 2010). Furthermore, research has shown that statins have pleiotropic vascular actions including anti-inflammatory properties, anti-oxidative effects and the ability to improve endothelial function. A study done by Taoufiq *et al* proved that pre-treatment of endothelial cells with atorvastatin was shown to reduce the expression of adhesion molecules such as ICAM-1 and subsequently *P. falciparum* cytoadherence. Moreover, the use of atorvastatin was found to protect cells against apoptosis induced by adhering parasitised RBCs (Taoufiq *et al.*, 2011).

Additionally, a study done by Reis *et al* looked at the effects of lovastatin on neuro-inflammation in cerebral malaria and reported that treatment of lovastatin in combination with chloroquine in a *Plasmodium berghei* murine model decreased neuro-inflammation and prevented cognitive impairment after cerebral malaria. Although it has been highlighted that there are various differences between malaria murine models, such as *P. berghei*, and human malaria there are also notable similarities regarding the inflammatory cascade commonly associated with cerebral malaria (Reis *et al.*, 2012). Hence, these findings along with the other cited studies further substantiate the claim that statins could be potential candidates for adjunctive anti-cytoadhesive therapy for severe malaria. The potential therapeutic role of statins in reducing the severity and complications in severe malaria treatment should therefore be explored further and this avenue of research should be one of the focal points of current malaria research to develop an improved anti-malarial treatment.

1.10 Scope of the study

1.10.1 Summary and motivation for study

Malaria is a life-threatening disease that is responsible for millions of deaths globally each year. It is known as a disease of children as it predominantly targets children under the age of 5 who are vulnerable to the *Plasmodium* parasite with death often the final outcome if not treated promptly (Who, 2014). Malaria is a curable disease that has been studied and researched for more than a century, however the disease is yet to be completely controlled or eradicated by a vaccine, drug therapy or adequate vector control measures. Thus identification of new key drug targets, the development of novel therapeutic approaches or even the exploration of existing drugs as possible tools to control malaria is vital.

Clinical manifestations of malaria can progress to a severe form of the disease. High parasite levels in the host, due to the evasion of splenic clearance by sequestration of parasitised RBCs, and cytoadhesion of parasitised RBCs to microvasculature are the underlying pathological factors responsible for the fatal symptoms associated with severe malaria (Rowe *et al.*, 2009). Therefore, the research in this present study was directed towards the

late trophozoite stage (35 – 40 hours post invasion) of the *P.falciparum* life cycle and more specifically focused on the molecules that play a key role in cytoadhesion. It is hypothesized that if the cytoadhesion process is hindered in any way, it may lead to a decrease in the parasitaemia burden during an infection thus alleviating the severe symptoms associated with malaria (Alister *et al.*, 2012).

Statins may potentially be used as an adjunctive therapy for severe malaria as agents from this cholesterol-lowering drug class have been shown to exert some activity against the malaria parasite, show synergism with antimalarial compounds and more importantly impede the interaction between parasite ligands and host receptors involved in cytoadhesion (Grellier *et al.*, 1994; Taoufiq *et al.*, 2011; Souraud *et al.*, 2012). Proteomic based methods were used to explore this possibility by identifying possible drug targets for severe malaria and determining the effect of lovastatin on cytoadhesion related parasite proteins with the ultimate aim of providing research based evidence to motivate the use of statins as a potential adjunctive therapy for severe malaria.

1.10.2 General outline of study

1.10.2.1 Culturing of *P. falciparum*

The chloroquine sensitive *P. falciparum* 3D7 laboratory strain was used for the purpose of this study as literature identifies the *P. falciparum* species as the prominent causative agent for the most fatal form of human malaria and is the main *Plasmodium* species that is associated with cytoadhesion mechanisms (Jensen *et al.*, 2004). In 1976, Trager and Jensen developed the continuous *in vitro* culturing system of *P. falciparum* that is so widely used in laboratories today (Trager and Jensen, 1976). Figure 1.10 is a representation with typical images of the *P. falciparum* parasite during the 48 hour asexual life cycle.

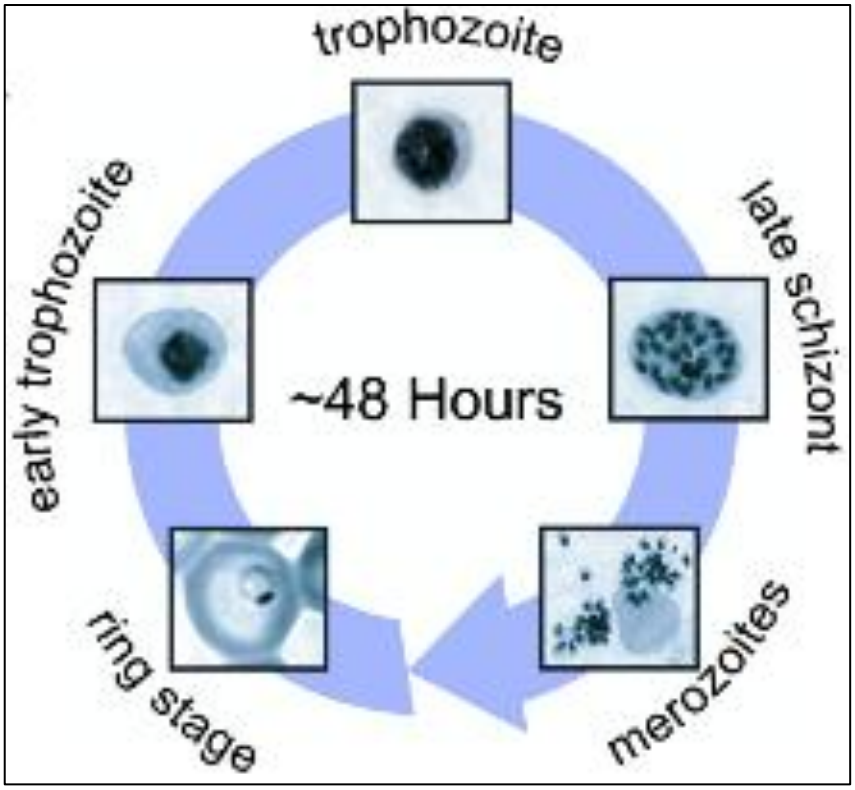


Figure 1.10: The 48-hour asexual development of the *Plasmodium* parasite (Bozdech *et al.*, 2003) (with permission).

In this study, parasitic cultures were prepared under conditions that mimic the environment of the human host, in addition to utilising modified culturing methods according to (Trager and Jensen, 1976). After culturing procedures were optimised, parasites were successfully cultivated with an effective synchronisation step to provide late trophozoite stage enriched parasitised RBCs and the morphology of these parasitised RBCs was assessed using scanning electron microscopy (SEM). Normal control RBCs were also visualised using SEM and the images were compared in order to highlight the morphological changes on the surface of the RBC that are induced by the parasite during the late trophozoite stage.

1.10.2.2 Proteomic analysis

The field of proteomics can be broadly defined as the use of biotechnology and various scientific disciplines to study the protein load expressed by a cell, tissue or organism also known as the proteome. The term 'proteomics' was initially formalised in 1995 and since then has become a distinctive and resourceful area of research (Graves and Haystead, 2002; Wilkins *et al.*, 2006). Proteomics is made up of various components and applications which

include protein-protein interaction studies, functional and structural proteomics, profiling of protein expression as well as post-translational modification studies. A particular avenue of research where the role of proteomics has been significantly established is that of drug discovery and drug target identification and validation (Graves and Haystead, 2002). In this study, proteomic based techniques were utilised to identify possible protein drug targets for severe malaria and to further elucidate the potential role that statins may have as an adjunctive therapy for severe malaria.

Exploitation of the structural, chemical and physical properties of proteins can be utilised in experimental techniques that are used for proteomic studies. An example of these techniques include sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which is an essential technique of proteomics and it is widely used for successfully resolving complex protein mixtures prior to protein identification and characterisation (Graves and Haystead, 2002). Proteins are solubilised in an anionic detergent, sodium dodecyl sulphate (SDS), which denatures the proteins while binding to most proteins at an almost fixed ratio thus resulting in single linear polypeptide chains. SDS is also used to impart a negative charge to proteins thus facilitating the migration of proteins towards the anode in an electrical field. Polyacrylamide gels are non-ionic polymers that may consist of different pore sizes which facilitate the separation of protein according to their size (Dunn, 1993). Furthermore, the movement of proteins during electrophoresis is dependent on a fixed ratio of charge to the mass of a protein. A higher charge density ratio will result in migration of proteins at a faster rate and the smaller the protein the faster it will move through the gel and vice versa for larger proteins (Hames, 1981). In this study, SDS-PAGE was used for the separation and initial characterisation of proteins located on the membrane of normal RBCs and parasite derived proteins that are transferred by the malaria parasite onto the membrane of parasitised RBCs at the late trophozoite stage. Prior to SDS-PAGE, RBC ghosts were prepared from non-parasitised and highly synchronous parasitised RBCs. RBC ghosts represent intact RBC membrane structures without the abundant intracellular proteins such as haemoglobin. Ghosts can be prepared and isolated by lysing RBCs via hypotonic wash steps. Ghost preparation followed by gel electrophoresis is a useful technique that can be utilised to study the structure and properties of the RBC membrane with minimal interference by intracellular elements (Fairbanks *et al.*, 1971; Schwoch and Passow, 1973).

Gel electrophoresis was a critical step in the progression of protein studies, however major limitations include the lack of sensitivity, poor dynamic range of concentrations that can be visualised, poor resolution of proteins of similar molecular mass and the inability to directly identify proteins represented as bands on gels. This analytical restraint resulted in the development of one of the oldest methods used for protein identification by N-terminal sequencing of proteins known as the Edman degradation method (Edman, 1950). However, over the years there has been an increased demand for more sensitive and selective analytical tools for protein identification and the Edman's sequencing method was replaced by mass spectrometry (MS) based methodology. MS has become the leading analytical instrumentation for targeted protein identification and characterisation as MS provides the required sensitivity, reproducibility and mass accuracy in addition to the high through-put required for current proteomic applications (Angel *et al.*, 2012).

Proteomic workflows that include gel electrophoresis followed by mass spectrometry has become a very dominant and useful tool in the field of proteomics (Dutt and Lee, 2000; Gygi and Aebersold, 2000). In this study, relevant protein bands that were identified on gels were excised, trypsinised and analysed by electrospray ionisation mass spectrometry (ESI-MS/MS) to sequence the peptides that were then used to identify and characterise the target proteins. ESI-MS/MS was used to generate sequence data of selected proteins isolated from membranes of normal RBCs and parasitised RBCs at the late trophozoite stage in this study. The sequence data obtained was compared to a protein sequence database of both *Plasmodium* and human proteins resulting in the matching of peptides and ultimately the identification of the expressed proteins that are implicated in the adhesion process during the late trophozoite stage. A general workflow of gel electrophoresis coupled to mass spectrometry based proteomics is illustrated in Figure 1.11.

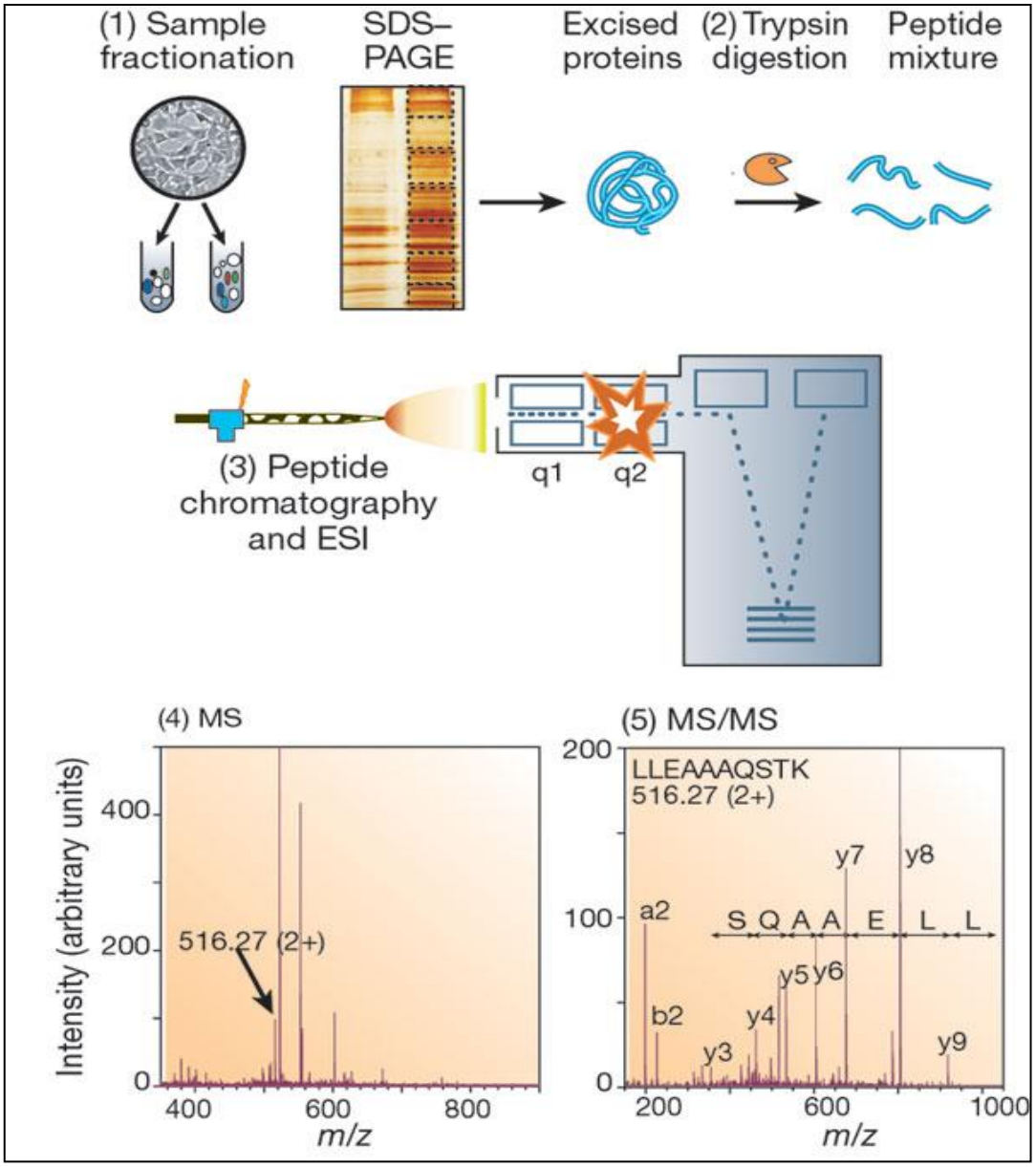


Figure 1.11: A general workflow of a mass spectrometry based proteomics experiment. SDS-PAGE is sodium dodecyl sulphate polyacrylamide gel electrophoresis; ESI is electrospray ionisation and MS is mass spectrometry (Aebersold and Mann, 2003) (with permission).

1.10.2.3 Treatment of parasitised RBCs with Statins

After successfully identifying target proteins or receptors on the parasitised RBC membrane, the secondary aim of the study was to assess whether the *in vitro* drug treatment of statins would have an effect on the expression of relevant proteins. Knowledge regarding the ability of statins to counteract parasite cytoadhesion is currently insufficient. There is also little literature pertaining to the direct interactions and effects of statins on *P. falciparum* proteins. In this study, lovastatin was utilised to study the effects of statins on *P. falciparum*

parasite protein expression on the surface of parasitised RBCs during the late trophozoite stage thus aiming to assess the potential role that statins may play in severe malaria.

1.11 Aims

The aims of the study were:

I. To identify proteomic changes that occur on membranes of parasitised RBCs at the late trophozoite stage of the *P. falciparum* life cycle

II. To assess the *in vitro* effects of therapeutic concentrations of statins on the expression of identified proteins on the membranes of parasitised RBCs at the late trophozoite stage.

1.12 Study objectives

- To optimise culturing and accurate synchronisation of *P. falciparum* parasites (3D7 strain).
- To isolate and obtain a high percentage enrichment of parasitised RBCs at the late trophozoite stage.
- To confirm the presence of knob-like protrusions on the surface of parasitised RBCs at the late trophozoite stage.
- To identify proteomic differences between normal RBCs and parasitised RBCs by using SDS-PAGE to extract target proteins and sequence peptides using LC-MS/MS.
- To assess the effects of therapeutic concentrations of selected statins on *P. falciparum* protein expression on the surface of parasitised RBCs.

Chapter 2: Materials and Methods

2.1 Culturing and maintenance of *P. falciparum* 3D7 strain

P. falciparum 3D7 cultures were originally obtained from the Department of Biochemistry, University of Pretoria after which cultures were maintained at the Council of Scientific and Industrial Research (CSIR), Pretoria. Ethical approval for the *in vitro* culturing of *P. falciparum* and collection of human blood for the culturing was obtained from the Faculty of Health Sciences Student Research Ethics Committee of the University of Pretoria (Ethical approval number: 304/2013, see appendix).

2.1.1 Materials

2.1.1.1 Reagents

I. D-glucose

D-glucose was obtained from Merck (Darmstadt, Germany). A 20% stock solution was made by dissolving 20 g of D-glucose powder in 100 ml of deionised water. The solution was filter-sterilised using a 0.22 µm pore size filter and dispensed into 10 ml aliquots which were stored at 4°C.

II. 4- (2-hydroxyethyl)-1- piperazine ethanesulfonic acid (HEPES)

HEPES was obtained from Sigma-Aldrich (St Louis, USA). A 1.25 M stock solution of HEPES was prepared by adding 119 g of HEPES powder to 400 ml of deionised water. The solution was filter-sterilised using a 0.22 µm pore size filter and the stock solution was dispensed into 50 ml aliquots that were stored at 4°C.

III. Hypoxanthine

Hypoxanthine was obtained from Sigma-Aldrich (St Louis, USA) and an 80.8 mM solution was made by dissolving 550 mg in a 500 mM solution of NaOH. The stock solution was distributed into 12.5 ml aliquots and stored at 4°C.

IV. Roswell Park Memorial Institute Medium (RPMI) 1640 Incomplete culture medium

Incomplete medium was prepared by dissolving 52 g of RPMI 1640 powdered medium, which was procured from Sigma-Aldrich (St Louis, USA), in 4835 ml of sterile deionised water. To this solution, a mass of 9 g of Sodium bicarbonate ($NaHCO_3$), supplied by Sigma-Aldrich (St Louis, USA), was added to adjust the pH. The following reagents were then added to the RPMI mixture:

- 100 ml of HEPES (12.5 mM)
- 50 ml of Glucose (20%)
- 0.48 ml of Gentamycin (undiluted)
- 12.5 ml of Hypoxanthine (80 mM)

The final volume was adjusted to 5 litres with sterile deionised water and the final solution was sterilised twice using 0.45 µm and 0.22 µm pore size cellulose acetate filters. Prepared medium was dispensed into sterile 500 ml bottles and stored at 4°C.

V. RPMI 1640 complete medium

Complete medium was prepared one litre at a time or as required by dissolving 5 g of Albumax, that was supplied by Life technologies (Johannesburg, South Africa), in 40 ml of incomplete medium. The solution was filter-sterilised into 960 ml of sterile incomplete medium with a 0.22 µm pore size filter. Complete medium was stored at 4°C and used within 2 weeks.

VI. Giemsa Stain

Pre-made Giemsa stain was purchased from Merck (Darmstadt, Germany) and a working solution was prepared by making a 1:6 v/v dilution with distilled water. The solution was stored at room temperature and made up freshly on a weekly basis.

VII. Methanol (MeOH)

MeOH was obtained from Sigma-Aldrich (St Louis, USA) and used undiluted.

VIII. Phosphate buffer saline (PBS)

FTA Hemagglutination buffer was procured from BD Biosciences (San Jose, USA). A sterile solution was prepared by dissolving 9.23 g in a volume of 1 litre of deionised water. The solution was stored at 4°C and filter sterilisation using 0.22 µm was done when required.

IX. D-sorbitol

D-sorbitol was supplied by Sigma-Aldrich (St Louis, USA). A 5% working solution was prepared by dissolving 25 g of D-sorbitol powder in 500 ml of deionised water. The solution was autoclaved immediately and stored at 4°C.

2.1.2 Methods

2.1.2.1 Collection of blood for the culturing of *P. falciparum*

The donation of human blood from healthy volunteers for the culturing of *P. falciparum* parasites was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria. Blood was obtained from donors on a monthly basis due to a one month shelf life of stored blood for culturing purposes. Blood was collected into a blood bag obtained from Adcock Ingram (Johannesburg, South Africa). Blood bags were stored at 4°C and each blood bag contained a 70 ml citrate phosphate glucose adenine anticoagulant solution. The anticoagulant solution contained 229 mg of citric acid monohydrate, 1.8 g of sodium citrate dihydrate, 175.7 mg of sodium acid phosphate dihydrate, 2.2 g of glucose monohydrate and 19.2 mg of adenine. The donated blood was transferred into sterile 50 ml tubes and allowed to sediment overnight at 4°C or centrifuged at 2000 *g* to facilitate the separation of RBCs. The plasma, platelets and white blood cells were removed using a sterile plastic Pasteur pipette. PBS was added to the remaining RBCs followed by centrifugation at 2000 *g* for 10 minutes. The supernatant and buffy-coat were removed and the purified RBCs were then placed in incomplete culture medium at a 1:1 v/v ratio to achieve a final haematocrit of approximately 50%.

2.1.2.2 Maintenance of *P. falciparum* 3D7 strain

The *P. falciparum* 3D7 strain was maintained in purified human RBCs and RPMI 1640 complete medium that was further supplemented with, D-glucose, gentamycin, HEPES,

Hypoxanthine and $NaHCO_3$. *P. falciparum* parasites were cultured in a 5% haematocrit RBC suspension at 8 - 10% parasitaemia in a 75 cm^2 tissue cell culture flask. Cultures were retained in a gaseous environment of 90% nitrogen, 5% carbon dioxide and 5% oxygen. Culture flasks that contained a final culture volume of 30 mL were then placed in an airtight rotary incubator at 37°C. The cultures were maintained by changing the culture medium daily and diluted with RBCs when parasitaemia exceeded 8 - 10%. When larger volumes of parasites were required for downstream experimental procedures, cultures were grown to a higher parasitaemia and the volume of medium added to the cultures was adjusted according to recommendations provided by the methods in malaria research guide (Kirsten *et al.*, 2008). This was done to ensure successful growth of cultures at high percentages of parasitaemia for harvesting parasite proteins at levels required for proteomic analysis.

2.1.2.3 Calculation of percentage parasitaemia

A volume of 500 µl was removed from a culture flask and added to an Eppendorf tube. The contents of the Eppendorf tube were centrifuged at 1000 *g* for 1 minute to obtain a pellet. Half of the supernatant was discarded and the pellet was re-suspended in the remainder of the supernatant. A volume of 50 µl of the sample was placed onto a microscope slide. Another slide was used to prepare a thin monolayer of cells by dragging a drop of the sample across the slide. The slide was then carefully dried using a hairdryer and then fixed in methanol for approximately 30 seconds. After fixation, the slide was placed in Giemsa stain for a minimum of 2 minutes for the ring stage and a maximum of 4 minutes for the late trophozoite stage. After staining, the slides were rinsed, dried again and then observed under the microscope using immersion oil and an objective of a 100 x magnification. A Carl Zeiss microscope with a special ocular grid (reticulocyte ocular) as shown below in Figure 2.1 was used to systematically count the parasitised cells and the percentage parasitaemia calculated. The percentage parasitaemia was determined by counting all parasitised and non-parasitised RBCs located in the small square including those situated on the top and left borders. Without moving the slide, parasitised RBCs were then counted in the whole area of the larger square (including the small square) as well as on the top and left border. This step was repeated on adjacent areas of the slide until a total sum of 100 RBCs (non-parasitised and parasitised) was counted in the small square. By extrapolation, it could be deduced that 1000 RBCs were examined. This was repeated twice and the mean number of

parasitised RBCs per 1000 RBCs was divided by 10 to get a percentage parasitaemia (Kirsten *et al.*, 2008).

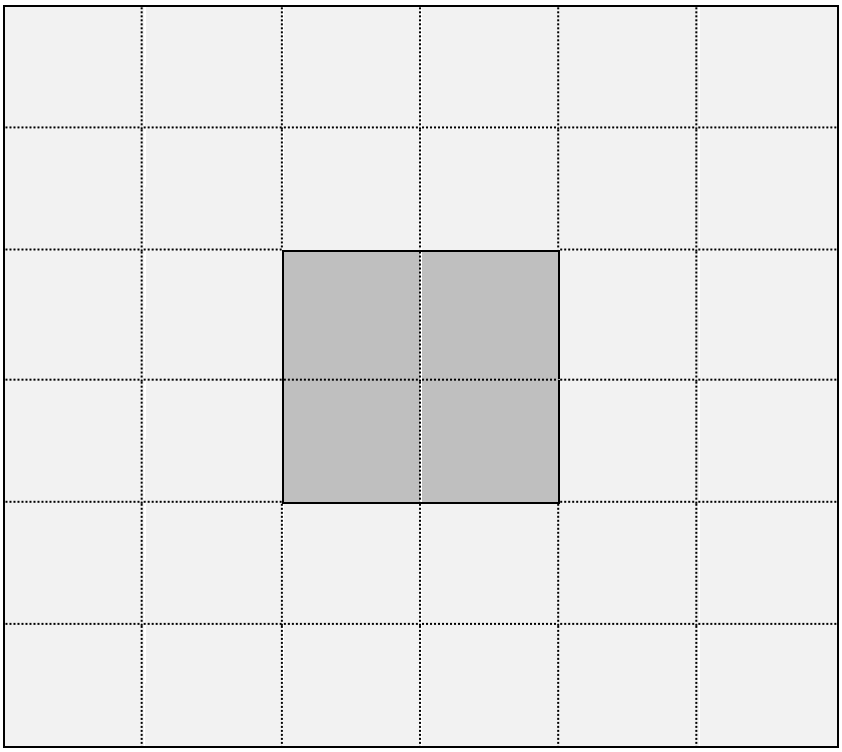


Figure 2.1: An adapted illustration of the ocular grid used to determine percentage parasitaemia. The square area indicated in dark grey is approximately 10% of the larger square (Kirsten *et al.*, 2008).

2.1.2.4 Synchronisation of *P. falciparum* 3D7 strain

Parasite cultures were synchronised using sorbitol according to modified methods reported by Lambros and Vanderberg (Lambros and Vanderberg, 1979). Contents of a culture flask, with parasites at the late ring stage (20 hour post invasion) were decanted into a 50 ml tube and centrifuged at 2000 *g* for 5 minutes. The supernatant was discarded and the pellet was suspended in 15 ml of a 5% working solution of sorbitol. The cultures were then placed in a water bath set at 37°C for 15 minutes. The contents of the 50 ml tube were centrifuged again at 2000 *g* for 5 minutes. The supernatant was discarded and the pellet was washed with 10 ml of incomplete culture medium followed by centrifugation at 2000 *g* for 10 minutes. The supernatant was discarded and a volume of 500 µl of packed RBCs was added to the pellet to compensate for the RBCs lost during the synchronisation process. This was followed by re-suspension of the pellet in complete medium. Culture flasks were washed out with a volume of incomplete culture medium before the cultures were placed back into

the culture flask. This was done to ensure that there were no late-stage parasites present in the culture flask. The cultures were then gassed for 30 seconds and placed in a rotary incubator at 37°C. The synchronisation process was carried out three consecutive times. Time zero of invasion was established after late schizonts were added to new cultures. Synchronisation began at 20 hours post invasion when cultures were at the late ring stage, then again after 30 hours when newly formed rings were present and finally the third synchronisation was carried out again at the 20 hour post invasion time point. This was done to ensure a narrow window of synchronised cultures. After this synchronisation sequence, cultures were synchronised again every second day at the early ring stage.

2.1.2.5 Purification of parasitised RBCs at the late trophozoite stage

All the isolation procedures were performed in a laminar flow unit to ensure sterility. Purification of late stage trophozoites was carried out using a Vario Magnetic Cell Sorter (VarioMacs) and cell separating (CS^+) columns that were purchased from MACS Miltenyl Biotec (Johannesburg, South Africa). It was imperative that cultures were at the same stage of the life cycle or more specifically at the late trophozoite stage when magnetic separation was carried out therefore cultures were only isolated after the synchronisation process described in Section 2.1.2.4 was completed. Figure 2.2 is an image of the VarioMacs apparatus used to magnetically isolate parasitised RBCs. The VarioMacs was assembled according to the manufacturer's supplied manual. The CS^+ column was prepared by using 50 ml of absolute EtOH (99.9%) to wash out the column and then repeating the wash step with 50 ml of deionised water. The CS^+ column was then preconditioned by running 50 ml of incomplete medium through the column. A parasite culture at a haematocrit of 5% and a parasitaemia of 10 - 15% was transferred to a 50 ml falcon tube and was centrifuged at 2000 *g* for 5 minutes. The supernatant was discarded and the pellet was re-suspended in a volume of 25 ml of incomplete culture medium. Cultures were then passed through the CS^+ column while it was attached to the VarioMacs. The flow through which consisted of non-parasitised RBCs and early stage parasites was discarded or placed back into culture if needed. The CS^+ column was then washed with incomplete medium until the effluent was colourless. Following this, The CS^+ column was removed from the VarioMacs and clamped into a burette stand. The parasitised RBCs that were captured in the column were then eluted from the column using incomplete medium. The parasitised RBCs were collected and

either fixed for microscopic analysis or prepared for the isolation of RBC ghosts for downstream proteomic analysis.

Figure 2.2: An image of the VarioMacs separating system that was used to obtain purified parasitised RBCs at the late trophozoite stage.

2.2 Visualisation of non-parasitised and parasitised red blood cell surfaces

2.2.1 Materials

2.2.1.1 Reagents

I. Ethanol (EtOH)

Absolute EtOH (99.9%) was obtained from Merck (Darmstadt, Germany). EtOH was used undiluted as well as diluted with deionised water to produce 30%, 50%, 70% and 90% v/v EtOH solutions.

II. Glutaraldehyde

Glutaraldehyde was obtained from Sigma-Aldrich (St Louis, USA) and a 2.5% glutaraldehyde solution was prepared by mixing 1 ml of a 25% glutaraldehyde solution, 1 ml of a 25% formaldehyde solution, 5 ml of PBS and 3 ml of deionised water.

III. Hexamethyldisilazane

Hexamethyldisilazane was purchased from Sigma-Aldrich (St Louis, USA) and used undiluted.

IV. Osmium tetroxide (OsO_4)

OsO_4 was procured from Sigma-Aldrich (St Louis, USA). A 0.5% working solution was made by diluting the reagent with deionised water. The reagent was stored at room temperature in a fume hood.

V. Phosphate buffered Saline (PBS)

As described in Section 2.1.1.1.

2.2.2 Methods

2.2.2.1 Preparation of samples for scanning electron microscopy (SEM)

Prior to sample preparation for SEM, *P. falciparum* parasites were synchronised to the late trophozoite stage (35 - 40 hours post invasion) and isolated as described in Sections 2.1.2.4 and 2.1.2.5 respectively. A volume of 200 µl of parasitised RBCs was transferred to Eppendorf tubes and fixed in 2.5% glutaraldehyde at a pH of 7.4 for 1 h at room temperature. Samples were then washed with PBS three times for 10 minutes for each wash step. Parasitised RBCs were fixed with 0.5% OsO_4 for 1 hour. A glass Pasteur pipette was used to add a drop of OsO_4 to cover the cells. This was done in a fume hood as OsO_4 is considered to be highly toxic. After fixation, cells were washed three times with PBS. Cells were then dehydrated serially in 30%, 50%, 70%, 90% and thrice in 100% with ethanol for ten minutes for each dehydration step. The sample preparation for SEM was completed by drying the samples in hexamethyldisilazane before mounting and coating with carbon.

Visualisation of samples was done using a ZEISS ULTRA plus FEG-SEM. The described method was also applied to non-parasitised RBCs.

2.3 Ghost membrane preparation from non- parasitised and parasitised RBCs

The method for RBC ghost preparation was based on the method described by Dodge *et.al*, (1963)

2.3.1 Materials

2.3.1.1 Reagents

I. Disodium Ethylenediaminetetraacetic acid (EDTA-Na_2)

EDTA was obtained from Sigma-Aldrich (St Louis, USA). A 1 mM solution of EDTA was prepared by dissolving 372 mg of EDTA powder in 1 litre of PBS at a pH of 8. The reagent was stored at 4°C.

II. PBS

As described in Section 2.1.1.1.

III. Phenylmethylsulfonyl fluoride (PMSF)

PMSF was purchased from Sigma Aldrich (St Louis, USA). A 100 mM stock solution was made by dissolving 174 mg in 10 ml of EtOH. A final 1 mM working solution was used for sample preparation. The solution was dispensed into 500 µl aliquots in Eppendorf tubes and stored at – 70°C.

2.3.2 Methods

2.3.2.1 Preparation of ghosts from non-parasitised RBCs

Venous blood was collected by venipuncture from healthy volunteers and freshly prepared to obtain RBC membrane fragments or ghosts. A total volume of 4 ml of whole blood was centrifuged at 1800 *g* for 10 minutes and the supernatant and buffy coat were removed. The RBC pellet was washed three times with PBS that was supplemented with 1 mM EDTA and a final concentration of 1 mM PMSF. Falcon tubes containing RBCs were centrifuged at

1800 *g* for 10 minutes for each wash step and the supernatant was discarded. The remaining RBC pellet was transferred to a 50 ml Falcon tube and lysed by addition of 50 ml deionised water that was supplemented with 1mM PMSF. RBCs were centrifuged at 3000 *g* for 10 minutes and the supernatant was removed and added to a new 50 ml Falcon tube. Deionised water was added to the RBCs as well as the removed supernatant and the lysis step was repeated. The supernatant was discarded and the remaining pellet of RBCs from each Falcon tube were combined. Samples were lysed for the third time with 30 ml of deionised water and centrifuged at 3000 *g* for 10 minutes. The supernatant was discarded and the RBC pellet was transferred to Eppendorf tubes. The samples were then lysed for a final time by adding deionised water and using micro-centrifugation at 16 000 *g* for 10 minutes. The haemoglobin from the lysed cells was washed away using PBS until a white pellet of RBC membrane ghosts was obtained. A small aliquot of the sample was used for protein quantification while the remainder of the sample was stored at -70°C until downstream proteomic analysis was carried out.

2.3.2.2 Preparation of ghosts from parasitised RBCs

P. falciparum parasites were synchronised to the late trophozoite stage as described in Section 2.1.2.4 and isolated using magnetic fractionation as described in Section 2.1.2.5. Following these two procedures, RBC membrane ghosts were prepared from the parasitised RBCs. Parasitised RBCs that were suspended in culture medium post magnetic isolation were centrifuged at 1800 *g* and washed three times with PBS that was supplemented with 1 mM EDTA and a final concentration of 1 mM PMSF. Parasitised RBCs were centrifuged at 1800 *g* for 10 minutes and the supernatant was removed and discarded after each wash step. The pellet was then transferred to an Eppendorf tube and centrifuged at 1000 *g* for 5 minutes. Deionised water that contained 1 mM PMSF was added to the parasitised RBCs to lyse the cells and the samples were vortex mixed every two minutes for 10 minutes to improve lysis. The cells were then subsequently collected by micro-centrifugation at 16 000 *g* for 15 minutes. The supernatant was removed and the ghost fraction of the pellet was added to another Eppendorf tube followed by centrifugation at 16 000 *g* for 10 minutes. The pellet was then washed with PBS three times. The lysis procedure for the parasitised RBCs differed to that of the non-parasitised RBCs as it was important to isolate the ghost fraction from parasite debri which would be a source of contamination and thus affect

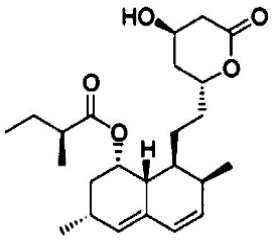
downstream proteomic analysis. An aliquot of the sample was used to determine the quantity of protein and the remainder of the sample was stored at -70°C for downstream proteomic analysis.

2.4 Treatment of non-parasitised and parasitised RBCs with statins

2.4.1 Materials

2.4.1.1 Reagents

I. Lovastatin



Lovastatin (mevinolin from Aspergillus sp.)
Molecular formula: $C_{24}H_{36}O_5$
Molecular weight: 407.54 g/mol

Lovastatin was purchased from Sigma-Aldrich (St Louis, USA) in the lactone form. Lovastatin was converted to the active form by dissolving 1.2 mg of the lactone form in 100 µl of absolute EtOH, 200 µl of a 200 mM potassium hydroxide solution (Sigma-Aldrich, St Louis, USA) and finally neutralised using 200 µl of a 200 mM hydrochloric acid solution (Sigma-Aldrich, St Loius, USA). The solution was then added to 2.5 ml of PBS to achieve a final stock concentration of 1 mM. The stock solution was distributed into aliquots and stored at -20°C. A final concentration of 10 µM was used for the assay.

2.4.2 Method

2.4.2.1 Treatment of non-parasitised and parasitised RBCs with lovastatin

A total volume of 2 ml of packed RBCs were pre-treated with a final concentration of 10 µM of lovastatin and incubated for 48 hours in a gaseous environment of 90% nitrogen, 5% carbon dioxide and 5% oxygen in an airtight rotary incubator at 37°C. RBCs ghosts were then prepared from the statin treated RBCs as described in Section 2.3.2.1. Separate to this, after parasitised RBCs were synchronised and isolated, parasitised RBCs were put back into

culture at a 5% parasitaemia by re-suspending the pellet in a final volume of 2 ml of packed RBCs that were pre-treated with 10 µM of lovastatin for 24 hours. The cultures were maintained as described in Section 2.1.2.2. After 24 hours, a blood smear was made to assess the viability of the cultures. Following an additional 24 hours, the parasitised RBCs were isolated, using the VarioMacs as described in Section 2.1.2.5, at the late trophozoite stage. RBC membrane ghosts were then prepared from treated non-parasitised RBCs as described in Section 2.3.2.2.

2.5 Protein quantitation using the Bicinchoninic acid (BCA) protein assay

2.5.1 Materials

2.5.1.1 Reagents

I. Reagent A

Reagent A consisted of the following reagents that were weighed out and dissolved in 100 ml of deionised water:

- 1 g Bicinchoninic acid (Sigma-Aldrich, St Louis, USA)
- 2 g Sodium carbonate (Sigma-Aldrich, St Louis, USA)
- 160 mg Sodium tartrate (Sigma-Aldrich, St Louis, USA)
- 400 mg Sodium hydroxide (Sigma-Aldrich, St Louis, USA)
- 950 mg Sodium bicarbonate (Sigma-Aldrich, St Louis, USA)

The pH of the above mixture was adjusted to 11.25 with a 10 M NaOH solution.

II. Reagent B

A mass of 400 mg of cupric sulphate powder, obtained from Sigma-Aldrich (St Louis, USA), was dissolved in 10 ml of deionised water and stored at 4°C.

III. Standard BCA working solution

A standard working solution was made by mixing reagent A and B in a 50:1 ratio. The working solution was stable for 1 week and stored at 4°C.

IV. Albumin (Human)

Human serum albumin was procured from Sigma-Aldrich (St Louis, USA). A 2.5 mg/ml stock solution of Albumin was used as the standard for the assay.

2.5.2 Method

2.5.2.1 Bicinchoninic acid (BCA) protein assay

The BCA protein assay was used to determine the quantity of protein in each sample. Dilutions were made from the prepared albumin standard solution to give concentrations between 0.1 - 2.5 mg/ml to generate an eight point standard curve. A volume of 5 µl of each albumin standard and 5 µl of each sample was added to wells of a 96-well plate. A volume of 250 µl of the BCA working solution was then added to each well with a final well volume of 255 µl. The 96-well plate was covered with foil to prevent light exposure and placed on a shaker for 2 minutes to allow for the mixing of the samples with the BCA reagent. The samples were then incubated at 60°C for 30 minutes. After the incubation period, samples were cooled to room temperature and the plate was analysed using an ELx800 UV universal microplate reader (Bio-Tek Instruments Inc. Vermount, USA) using a wavelength of 562 nm. Graphpad Prism 4-0 software (Graphpad software, San Diego California, USA, www.graphpad.com) was used to perform linear regression analysis and to determine the protein concentration of each sample.

2.6 Protein separation and mass fingerprinting

2.6.1 Materials

2.6.1.1 Reagents

I. Laemmli buffer

Laemmli sample buffer was prepared by combining the following reagents to make up a final volume of 15 ml:

- 6.4 ml of 1 M Tris-Cl (Sigma-Aldrich, St Louis, USA)
- 3 ml of 20% SDS (Sigma-Aldrich, St Louis, USA)
- 3 ml of 100% Glycerol (Sigma-Aldrich, St Louis, USA)

- 1.6 ml of β-mercaptoethanol (Sigma-Aldrich, St Louis, USA)
- 6 mg of Bromophenol blue (Sigma-Aldrich, St Louis, USA)

The pH was adjusted to 6.8 and the stock solution was dispensed in 500 µl aliquots and stored at -70°C.

II. Sodium dodecyl sulphate (SDS) running buffer

SDS running buffer was prepared by adding 30.3 g of Tris-base that was obtained from Sigma-Aldrich (St Louis, USA), 144 g of glycine that was purchased from Sigma-Aldrich (St Louis, USA) and 10 g of SDS to deionised water making up a final volume of 1 litre. The SDS buffer was stored at room temperature.

III. Protein mass standards

Precision Plus^{TM} Protein Standards Unstained were purchased from Bio-Rad Laboratories, Inc. (Berkeley USA). The protein standards covered a reference mass range from 10 kiloDaltons (kDa) to 250 kDa. The proteins standards were stored at -20°C.

IV. Coomassie brilliant blue G-250 stain (CBB)

CBB stain was obtained from Bio-Rad Laboratories Inc (California, USA). The reagent was used undiluted as per the instructions and stored at room temperature.

V. Silver stain

The silver staining method was based on the method of Dzandu *et. al. (*1984).

The following reagents were prepared for the silver stain procedure

- Fixative solution: 40% MeOH, 10% acetic acid and 50% distilled water
- Wash solution: 30% EtOH in distilled water
- Reductant solution: A mass of 200 mg of sodium thiosulfate was dissolved in 1 L of distilled water
- Silver stain: A mass of 2 g of silver nitrate was dissolved in 1 L of distilled water that contained 200 µl of formaldehyde

- Developer solution: A mass of 30 g of sodium carbonate and 5 mg of sodium thiosulfate was dissolved in 1 L of distilled water that contained 500 µl of formaldehyde
- Stop solution: 5% acetic acid in distilled water

Gels were fixed in fixative solution for 30 minutes and then washed 3 times in wash solution for 20 minutes for each wash step. Gels were then placed in the reductant solution for 1 minute followed by 3 wash steps for 30 seconds each. After the reduction step, gels were stained with silver stain for 15 minutes which was again followed by 3 wash steps for 1 minute each. Developing solution was added to gels and the reaction was allowed to proceed until band visualisation was achieved. Stop solution was finally added to stop the reaction and gels were stored at room temperature in fixative solution.

2.6.2 Methods

2.6.2.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated with SDS-PAGE using 10% or 4-20% mini Proteon TGX Stain Free pre-cast gels supplied by Bio-Rad Laboratories Inc. (California, USA). Prior to loading of samples onto the gels, samples were diluted at a 2:1 v/v ratio with Laemmli sample buffer and boiled for 5 minutes. Gel electrophoresis was carried out at a constant 60 volts (V) with SDS running buffer and a Bio-Rad electrophoresis system as shown in Figure 2.3. A 10 - 250 kDa Precision Plus™ protein standard ladder was loaded onto the gels as a molecular marker and equal amounts of protein (20 µg) for each sample was loaded into each well. The protein bands were visualised with Stain Free using a Gel Doc EZ imager and analysed using Image Lab software (Bio-Rad Laboratories Inc., California, USA) and then subsequently stained and visualised with either CBB or silver stain.

Figure 2.3: Illustration of the pre-cast gels and the apparatus used for SDS-PAGE (Bio-Rad Laboratories Inc.,California,USA) (with permission).

2.7 In-gel trypsinisation of proteins

The following in-gel trypsinisation method was based on the protocol that was described in Shevchenko *et. al.* (2006).

2.7.1 Materials

2.7.1.1 Reagents

I. Acetonitrile (ACN)

ACN was purchased from Romil, (Cambridge, UK) and a 75% solution of ACN was prepared by adding deionised water to a volume of 7.5 ml to make 10 ml. The solution was stored at 4°C and used within 2 weeks.

II. Ammonium bicarbonate (NH_4HCO_3)

NH_4HCO_3 was obtained from Sigma-Aldrich (St Louis, USA). A 50 mM solution was prepared by dissolving 0.2 g of NH_4HCO_3 in 50 ml of deionised water at a pH range of 7.5 - 8.5. A 25 mM solution of NH_4HCO_3 was made by adding 1 ml of the 50 mM NH_4HCO_3 solution to 1 ml of deionised water. The solutions were stored at 4°C and used within two weeks.

III. 50 mM NH_4HCO_3/ 50% MeOH

A mass of 0.2 g NH_4HCO_3 was added to 25 ml of deionised water and 25 ml of MeOH added to produce a final concentration of 50 mM of NH_4HCO_3. The prepared solution was stored at 4°C and used within 2 weeks.

IV. Dithiothreitol (DTT)

A 1 M stock solution of DTT, (Sigma-Aldrich, St Louis, USA), was freshly prepared by weighing out a mass of 154 mg DTT powder and dissolving it in 1 ml deionised water. A 10 mM solution of DTT was prepared by adding 10 µl of the 1 M DTT solution to 495 µl of 50 mM NH_4HCO_3 and 495 µl of deionised water.

V. Iodoacetamide (IAA)

IAA was purchased from Sigma-Aldrich (St Louis, USA) and a 55 mM solution was prepared freshly by adding a mass of 10.2 mg IAA powder to 500 µl of 50 mM NH_4HCO_3 and 500 µl of deionised water. The light sensitive reagent was stored in a dark container prior to use.

VI. Trypsin

Sequence grade modified trypsin (Porcine) was supplied by Promega (Madison, USA). Trypsin stock solution was prepared by re-suspending 20 µg of trypsin in a volume of 200 µl of the supplied trypsin buffer. The prepared stock solution was dispensed into 20 µl aliquots in 1.5 ml Eppendorf tubes. Aliquots were stored at -20°C. A final working solution concentration of 10 ng/µl trypsin was made by adding 20 µl of the trypsin stock solution to 90 µl of a 50 mM NH_4HCO_3 and 90 µl of deionised water.

2.7.2 Methods

2.7.2.1 Cutting of gel bands and de-staining

Gels were carefully handled from the time of removal from the frame to avoid any protein contamination. New plastic containers were used for all staining and storage of gels. Relevant identified bands were carefully excised with a new sterile scalpel and diced into about 1 - 2 mm^3 pieces. The gel pieces were transferred into 0.5 ml Eppendorf tubes. A volume of 200 µl or enough to cover the gel pieces, of 50 mM NH_4HCO_3 in 50% MeOH was added. The gel pieces were then vortex mixed for 20 minute to destain the gel. The supernatant was removed, discarded and the step was repeated. Following this, 100 µl of 75% ACN was added to the gel pieces which were then vortex mixed for 20 minutes. The supernatant was removed, discarded and the gel pieces were dried in a vacuum centrifuge for 10 - 20 minutes.

2.7.2.2 Reduction and alkylation of proteins

Reduction of proteins was carried out by adding 25 µl of 10 mM DTT to the gel pieces. Following this, gel pieces were vortex mixed, centrifuged briefly and the reaction allowed to proceed at 60°C for 1 hour. Samples were then cooled to room temperature (22 - 23°C), before adding 500 µl of ACN which was followed by an incubation period of 10 minutes. The samples were centrifuged briefly and the supernatant was discarded. Proteins were then alkylated by adding a volume of 25 µl of 55 mM IAA to the gel pieces. The samples were incubated in the dark for 20 minutes at room temperature. Eppendorf tubes that contained the gel pieces were covered with foil, vortex mixed and centrifuged to allow for the gel pieces to settle to the bottom of the Eppendorf tube. The supernatant was removed, discarded and the gel pieces were washed with 100 µl of 50 mM NH_4HCO_3. The gel pieces were once again vortex mixed, centrifuged for 10 minutes and the supernatant discarded. Dehydration of the gel pieces was then achieved by adding 100 µl of 25 mM NH_4HCO_3 in 50% ACN. Samples were vortex mixed and centrifuged for 5 minutes. The supernatant was discarded and the dehydration step was repeated. The gel pieces were dried for 20 minutes in a vacuum centrifuge. At this point, gel plugs could be stored at - 20°C for several weeks.

2.7.2.3 Trypsinisation of proteins

Eppendorf tubes that contained the gel plugs were placed on ice at all times during the preparation for digestion of proteins. A volume of 5 - 50 µl of freshly prepared trypsin solution (10 ng/ml) was added to the gel pieces with a volume to just cover the gel pieces which was followed by an incubation period of 30 minutes. After the incubation period, gel pieces were rehydrated with trypsin for a further 60 minutes on ice. Following this, samples were centrifuged briefly allowing the gel pieces to settle to the bottom of the Eppendorf tube. Samples were then kept hydrated during enzyme cleavage by adding 25 – 50 µl of 25 mM NH_4HCO_3 to cover the gel pieces. Samples were incubated at 37°C overnight for protein digestion to proceed.

2.7.2.4 Extraction of peptides

Following trypsinisation, gel pieces were centrifuged briefly and the peptide digest solution transferred to a 0.5 ml Eppendorf protein low-bind tube. A volume of 50 µl, or just enough to cover the gel pieces, of 50% ACN/5% formic acid (FA) solution was added. Samples were vortex mixed for 20 - 30 minutes and then sonicated for 5 minutes. The aqueous peptide extract was removed and added to the first peptide extract solution. This step was repeated again before the combined peptide extracts were dried using the vacuum centrifuge. At this point, digests could be stored at -20°C for a few months before analysis.

2.8 Sample clean-up for mass spectrometry

2.8.1 Materials

2.8.1.1 Reagents

I. Pre-conditioned solvent

A pre-conditioned solvent was prepared by mixing 1 ml of MeOH with 100 µl of mass spectrometer grade formic acid (FA) that was purchased from Fluka Analytical (Basel, Switzerland) and 900 µl of MS grade water that was purchased from Sigma-Aldrich (St Louis, USA).

II. Equilibration solvent

An equilibration solvent was made by adding 100 µl of FA to 1.9 ml of MS grade water.

III. Elution solvent

An elution solvent was prepared by mixing 1.4 ml of ACN, 2 µl of FA and 598 µl of MS grade water.

2.8.2 Methods

2.8.2.1 Peptide desalting of trypsin digested samples

After trypsin digestion, samples were re-suspended in 20 µl of equilibration solvent, vortex mixed for 30 seconds and then sonicated in an ice bath for 5 minutes. Following this step, peptide desalting was carried out using Stage Tip Columns that were procured from Proxeon. The Stage Tip Column was pre-conditioned by adding a volume of 50 µl of the pre-conditioned solvent to the top of the column and forced through using a 2.5 ml Eppendorf combiTip. After this step, the column was equilibrated by adding a volume of 50 µl of the equilibration solvent to the top of the Stage Tip Column and the excess solvent was removed from the column with an Eppendorf combiTip. A volume of 20 µl of a trypsin digest was then loaded onto the column and the samples were washed with 20 µl of the equilibration solvent. Samples were then eluted from the column with 20 µl of the elution solvent and collected into 0.5 ml low bind Eppendorf tubes supplied by Sigma. Collected samples were vacuum centrifuge dried and stored at -20°C until mass spectrometric analysis was carried out.

2.9 Mass spectrometry

2.9.1 Parameters and method for mass spectrometric analysis

After in-gel trypsinisation and sample clean-up for mass spectrometry, peptide digests were re-suspended in 35 µl of a 2% ACN/0.2% FA solution. Samples were analysed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 4000 QTRAP or an AB Sciex 6600

TripleTOF mass spectrometers. Peptides were first captured and de-salted on an Acclaim PepMap C18 trapping column (75 µm × 2 cm) for 8 min at 5 µl/min using 2% ACN/0.2% FA, then separated on an Acclaim PepMap C18 RSLC analytical column (75 µm × 15 cm, 2 µm particle size). Peptide elution was attained using a flow-rate of 500 nl/min with a gradient from 4 - 60% B in 30 min (A: 0.1% FA; B: 80% ACN/0.1% FA). Nano-spray was achieved using a MicroIonSpray source assembled with a silica New Objective, PicoTip emitter. An electrospray voltage of 2.5-2.8 kV was applied to the emitter. The 4000 QTRAP and 6660 TripleTOF mass spectrometers were operated in Information Dependant Acquisition mode. Enhanced MS scans were acquired from m/z 400-1500 and three, in the case of 4000 QTRAP, or 30, in the case of 6600 TripleTOF, most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were automatically adjusted as a function of precursor ion mass of the 2^+, 3^+ and 4^+ charged peptide ions. The obtained MS/MS spectra provided peptide sequences information that was submitted to Protein Pilot v4.0.8085 software running the Paragon search engine (AB Sciex) and compared to a custom database including Uniprot *H. Sapiens*, Plasmo Db *Plasmodium falciparum* sequences as well as a list of common contaminats. The search was performed using the following parameters: trypsin was set as clevage enzyme with a single missed cleavage allowed, carbomedomethylation of cysteine was set as a constant modification whilst oxidation of methionine was set as a variable modification. A false discovery rate (FDR) analysis was performed using a reversed version of the custom sequence database described above. An FDR of 1 % on the protein level was used as a confidence cut-off.

Chapter 3: Results and Discussion

3.1 Culturing and maintenance of *P.falciparum* 3D7 strain

The *P. falciparum* 3D7 strain was utilised in this study where potential protein drug targets for severe malaria were identified and the effects of lovastatin on the expression of these parasite proteins were investigated. The evolution and adaptation of the malaria parasite over the years has resulted in a vast number of subtly different malaria parasite strains and essentially these inter-strain differences relate to drug resistance and infectious characteristics (Llinas *et al.*, 2006). The sequencing of the *P. falciparum* 3D7 strain genome was completed in 2002. Gene expression profiling methods have facilitated an intricate assessment of the unique expression and functional features of strain specific proteins that are expressed by the *P. falciparum* parasite (Gardner *et al.*, 2002). As a result of genetic profiling, the expression of fundamental components of parasitic knob formation has been confirmed in the laboratory *P. falciparum* 3D7 strain. Additionally, it has been established that the *P. falciparum* 3D7 strain positively expresses a variety of surface protein products from the *var*, *stevor* and *rif* gene families. The expression of these gene products play a key role in evasion of the host immune system and more importantly, a number of these expressed proteins induce alterations to the surface of host cells that facilitate cytoadhesion. (Llinas *et al.*, 2006). Hence, the 3D7 strain of the *P. falciparum* species was specifically selected for this study as the relevant proteome profile in addition to the cytoadhesive features that were under investigation in this research project were reported to be present in 3D7.

The *P. falciparum* parasite was cultured and cultivated according to standard operating procedures. Daily monitoring of parasite growth was achieved by preparing a blood smear of cultures. A thin monolayer of cells that was prepared from an unsynchronised culture, stained with Giemsa stain and visualised using light microscopy is illustrated in Figure 3.1. Non-parasitised RBCs and parasitised RBCs that contain parasites at the ring and trophozoite stages are visible and indicated with black arrows.

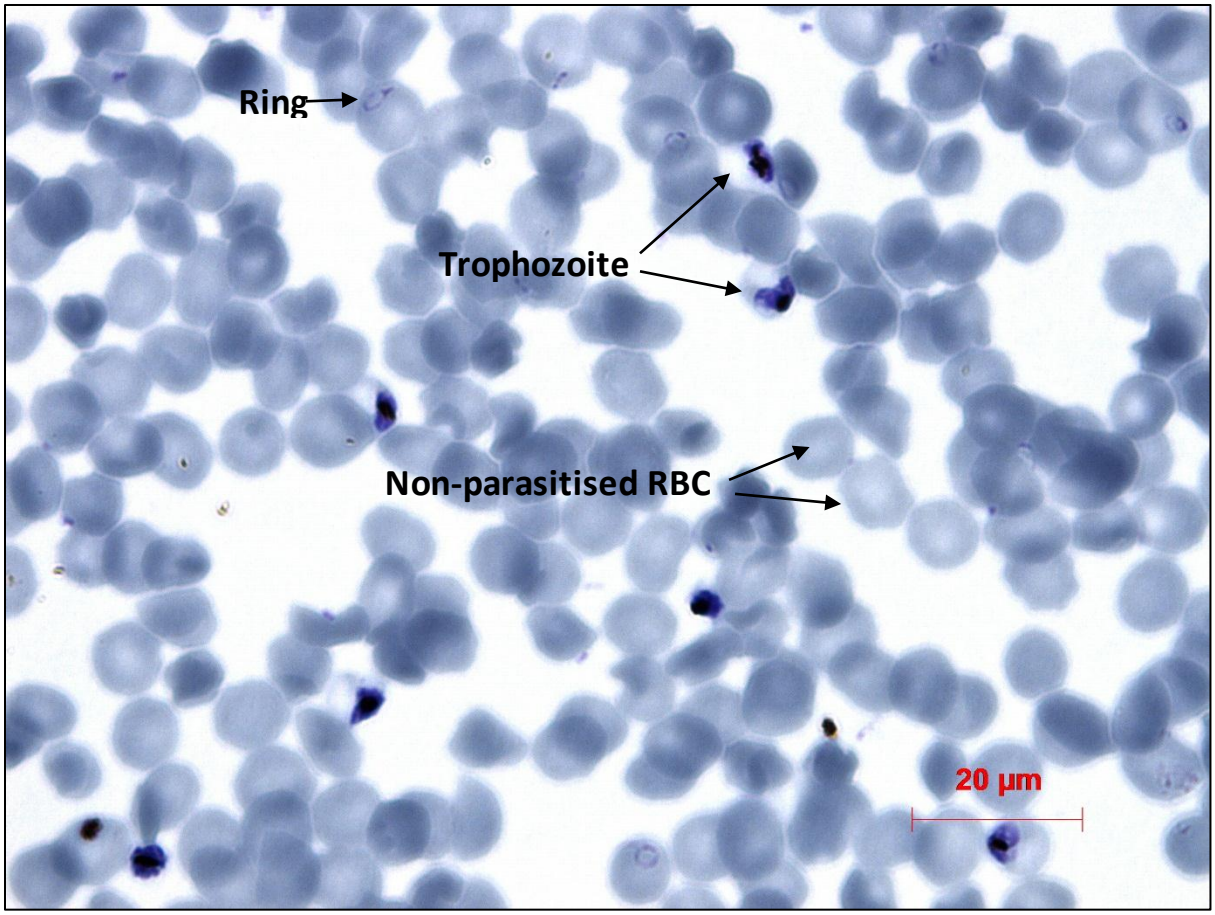


Figure 3.1: A thin blood film prepared from an unsynchronised *P. falciparum* 3D7 culture. Cells were fixed with methanol, stained with Giemsa for 4 minutes and viewed at a 100 x magnification. Non-parasitised RBCs, ring and trophozoite forms of the parasites are indicated with black arrows. (Scale: 20 µm)

General maintenance of cultures involved the replenishing of nutrients for the malaria parasite by changing culture medium daily and assessing the percentage parasitaemia present in the cultures. Anaerobic glycolysis is the energy pathway utilised by the parasite during the intra-erythrocytic stage of the life cycle (Mehta *et al.*, 2006). Parasitised RBCs reportedly produce significantly higher amounts of the glycolytic metabolite, lactic acid, than non-parasitised RBCs. It has also been shown that the level of lactic acid production depends on the intra-erythrocytic stage of the parasite's life cycle as trophozoite forms of the parasite produce relatively higher amounts of lactic acid compared to the other asexual blood stages. The accumulation of lactic acid has a negative effect on the continuous *in vitro* growth of the parasite and therefore it was imperative that culture medium was changed frequently to prevent toxic acidification to the malaria parasite (Zolg *et al.*, 1984). In addition to Roswell Park Memorial Institute Medium (RPMI) 1640 supplemented medium,

the malaria parasite primarily sustains its nutritional needs by consuming intracellular nutrients that are scavenged from the host RBCs. Venous blood was obtained from healthy individuals and collected into blood bags that contained citrate-phosphate anticoagulant solution as an alternative to heparin anticoagulant. This is due to the fact that heparin-like molecules has been shown to retard invasion pathways during the asexual blood stage of the malaria parasite life cycle (Boyle *et al.*, 2010). Donors differed with regards to blood type and this did not appear to affect the *in vitro* development of the malaria parasite. This was expected as all blood groups are suitable for the culturing of *P. falciparum* and only becomes relevant when considering compatibility with supplemented serum in culture medium (Jensen, 2002). However, this was not problematic as RPMI medium was supplemented with commercially available lipid rich bovine albumin based serum, Albumax II, which is compatible with all blood groups (Schuster, 2002).

The Giemsa stain was initially developed for the staining of the malaria parasite and still remains the “gold standard” for differentially staining malaria parasites for clinical diagnosis and laboratory research (Giemsa, 1902). A Giemsa stained slide was used to assess the parasitaemia of a culture flask and the parasitaemia was not allowed to exceed 8% with a 5% haematocrit (30 mL RPMI medium), as this was found to be the maximum percentage parasitaemia at which the parasites grew optimally. Cultures were split once they reached an 8% parasitaemia or diluted with fresh RBCs. However, downstream proteomic experiments required a significant amount of protein and therefore culturing of high volumes of parasites was necessary. Consequently, parasites were allowed to multiply to an approximate range of 10 – 16% parasitaemia prior to experiments. The high parasitaemia level required larger volumes of RPMI medium and strict monitoring in order to prevent any strain on the parasite as well as to ensure successful growth. Cyclic re-invasion of RBCs indicated that parasites maintained a consistent growth pattern with an exponential multiplication rate and displayed no signs of stress at these high percentage parasitaemia prior to further experimental procedures.

3.2 Synchronisation and isolation of parasitised RBCs

As mentioned previously, the aims of this study were directed towards the late trophozoite stage of the life cycle of the *P. falciparum* parasite as the factors contributing to the pathology of severe malaria are prominent during this particular stage. Figure 3.2 is a blood smear of a culture that was both sorbitol-synchronised and magnetically isolated. Parasite cultures were synchronised to the late trophozoite stage. The period of synchronisation ranged from 20 hours post invasion, again after a 30 hour interval and finally again at the 20 hour post second invasion time point. After this, maintenance of synchrony was achieved by synchronising cultures at a 48 hour interval when cultures were at the early ring stage. A blood smear was prepared after every synchronisation procedure and this frequency of synchronisation was found to be sufficient with regards to maintaining a high level of synchrony as late stage parasites were absent when cultures were visualised under the microscope. A narrow window of synchronisation was obtained and parasites were magnetically isolated and harvested at the late trophozoite stage (35 – 40 hours post invasion). Parasitised RBCs at the late trophozoite stage and non-parasitised RBCs are indicated with black arrows in Figure 3.2.

Once the *Plasmodium* parasite has infected its host, the malaria parasite is able to grow in synchrony in an infected individual. This coordinated growth pattern is reportedly attributed to temperature and circadian rhythm of the host's body (Garcia *et al.*, 2001). However, these host factors are obviously not present during the *in vitro* cultivation of the parasite and synchronised growth has to be artificially induced. A common and easy method to achieve this was developed in 1979 by Lambros *et al* and is known as the sorbitol synchronisation method (Lambros and Vanderberg, 1979). The mechanism of action of sorbitol relies on the degree of membrane permeability of parasitised RBCs during the intra-erythrocytic development of the parasite. Sorbitol selectively lyses mature parasitised RBCs, as the fragile membranes of RBCs at late stages of the life cycle are more susceptible to the osmotic action of sorbitol, thus yielding a culture predominantly consisting of early ring stage parasites free of late stage parasites (Lambros and Vanderberg, 1979). Cultures were adjusted to 5% sorbitol by addition of a sorbitol solution.

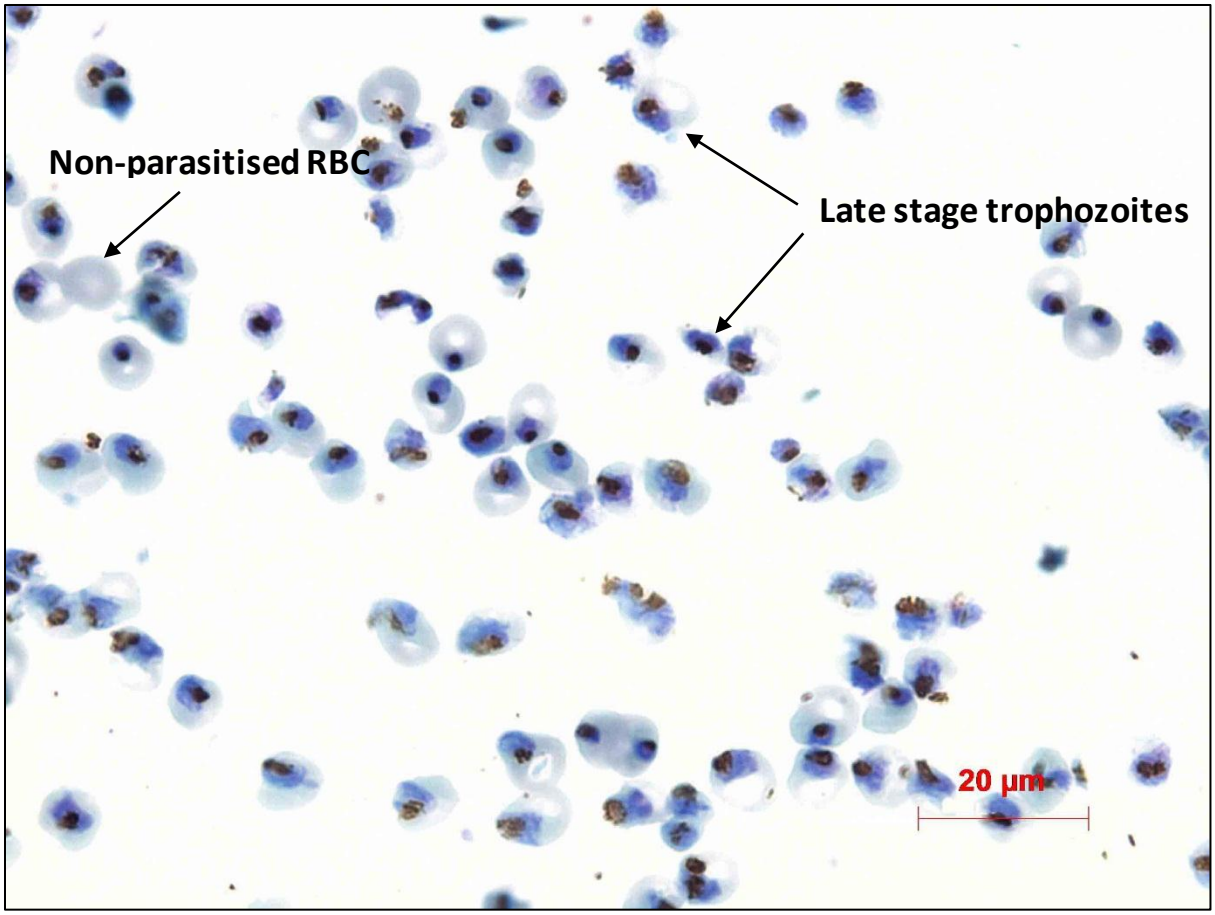


Figure 3.2: A thin blood film prepared from a tightly synchronised *P. falciparum* 3D7 culture consisting of purified late staged trophozoites. Cells were synchronised with sorbitol three times followed by timed harvesting by magnetic separation with the VarioMacs resulting in > 90% stage specific infected RBC isolates. Cells were fixed with methanol, stained with Giemsa for 2 minutes and viewed at a 100 x magnification. Non-parasitised RBCs and parasitised RBCs at the late trophozoite stage (35 – 40 hours post invasion) of the *P. falciparum* cycle are indicated with black arrows. (Scale: 20 µm)

After cell lysis of parasitised RBCs hosting advanced stages of the parasite the cells were harvested, resuspended in appropriate media and further cultured to achieve relatively narrow synchronisation to the late trophozoite stage. In order to maintain synchrony and ensure tightly synchronised cultures, the procedure had to be repeated at least twice a week as cultures were inclined to grow out of synchrony after a period of time. Synchronisation was a vital step in experimental procedures as downstream proteomic analysis involved the investigation of stage specific proteins that are implicated in cytoadhesion.

Following synchronisation, magnetic isolation was used to separate parasitised RBCs from non-parasitised RBCs. This was done to ensure that samples consisted of a population of greater than 90% parasitised RBCs at the late trophozoite stage, essentially free of non-parasitised RBCs or other life cycle stages of the parasite. The *Plasmodium* parasite degrades and consumes RBC cytosolic haemoglobin resulting in the proteolytic breakdown of haemoglobin into amino acids and haeme. Free haeme is oxidised and polymerised into a haemazoin crystal that possesses paramagnetic properties. As haeme polymerisation is maximal during the late trophozoite stage, paramagnetism was exploited to isolate parasitised RBCs at this particular stage (Francis *et al.*, 1997; Ribaut *et al.*, 2008). A blood smear was prepared to assess cultures post isolation and the attainment of a high fold increase of parasitised RBCs at the late trophozoite stage was confirmed. The recovery of parasitised RBCs was greater than 90% and this was consistent with previous studies (Karl *et al.*, 2010; Mata-Cantero *et al.*, 2014). It was observed that exposure of cells to a magnetic field did not affect the morphology or development of parasitised RBCs, thus the integrity of cultures was not compromised prior to further experiments (Figure 3.2). However, when running cultures through the VarioMACs column it was found that the recovery of parasitised RBCs was inversely proportional to increased flow rates. This was an indication that the magnetic force of attraction between cells and the VarioMACs column was not of a very strong nature (Karl *et al.*, 2010). Consequently, the flow rate had to be adjusted and lowered for optimal recovery rates. Additionally, non-parasitised RBCs were still present in a very small proportion of the final isolated fraction. Possible explanations for this is that there may be non-selective binding of the uninfected red blood cells to the isolation column or certain biological factors such as RBCs rosetting, which is a distinct feature of the *P. falciparum* 3D7 adhesive strain, may not be easily disrupted by magnetic separation and may affect the purity of a culture post magnetic isolation (Karl *et al.*, 2010). An alternative reason for the presence of non-parasitised RBCs could also be attributed to technical faults such as insufficient washing of the VarioMACs column before it was removed from the magnet and cells were captured. The use of magnetic separation was a highly efficient method to isolate parasitised RBCs at the late trophozoite stage as the percentage parasitaemia of parasitised RBCs post magnetic isolation was greater than 90% and the presences of non-parasitised RBCs in the final fraction was found to be low enough to not

warrant further purification steps that introduces further sample loss which becomes a concern.

3.3 Morphological differences between the outer surfaces of non-parasitised and parasitised RBCs

Concern has been expressed that long term culturing under laboratory conditions may alter the surface expression of some parasite proteins resulting in diminished knobs and potentially less surface expressed adhesion molecules of parasite origin. To assess the surface morphology and confirm that the cultures still exhibited knobs structures, scanning electron microscopy (SEM) was utilised to compare the cell surfaces of non-parasitised and late trophozoite stage parasitised RBCs. Samples from synchronised cultures that were magnetically isolated were prepared for SEM analysis and micrographs that were obtained are displayed in Figure 3.3. Distinct differences regarding surface morphology between uninfected RBCs and parasitised RBCs were observed and these results were comparable to previous studies (Gruenberg *et al.*, 1983). Figure 3.3 (A and B) represents single micrographs of both non-parasitised RBCs and parasitised RBCs at the late trophozoite stage. Sample preparation for SEM analysis could be excluded as the cause of these differences, as RBCs and parasitised RBC samples were prepared in exactly the same way and this was confirmed by visualising both samples in a single micrograph. It was therefore established that the observed alterations were in fact induced by the presence of the *Plasmodium* parasite within the RBC. Furthermore, SEM provided enhanced resolution that was necessary to capture the three dimensional ultrastructure of RBCs and also confirmed the preservation of cell morphology and structure during culturing and post magnetic isolation.

Figure 3.4 (A) and Figure 3.5 (A) illustrate the typical biconcave shape and smooth surface of a healthy RBC respectively and these images are consistent with studies done on surface morphology of RBCs from healthy individuals (Clarke *et al.*, 1971; Longster *et al.*, 1972). In contrast, micrographs in Figure 3.4 (B) and Figure 3.5 (B) represent the altered surface of late trophozoite stage parasitised RBCs that exhibit elevated electron dense structures, commonly known as parasitic knobs, that are widely distributed on the surface of

parasitised RBCs. Additionally, the presence of the intracellular parasite is evidently responsible for distorting the discoid shape of a normal RBC. The knob formation on the surface of the RBC at the trophozoite stage development of the parasite has been reported in several studies and therefore these results do not present anything new (Aikawa *et al.*, 1983; Rug *et al.*, 2006). However, the reason for confirming that these parasitic modifications were present was that literature sources have indicated that certain malaria culture strains, that initially test positive for the expression of parasitic knob structures prior to *in vitro* culturing, may lose their ability to exhibit surface knob elevations after prolonged periods of laboratory sub-culturing (Langreth *et al.*, 1979; Quadt *et al.*, 2012). Hence, before any further experimentation was done it was imperative to verify the expression of knob-like structures in the laboratory culture strain that was used in this study. The importance of this was based on the knowledge that key proteins that play a fundamental role in cytoadhesion are associated or located on parasitic knobs (Waller *et al.*, 1999; Horrocks *et al.*, 2005). Additionally, parasitic knobs predominantly occur at the late trophozoite stage of the parasite's life cycle and SEM provided secondary confirmation that the parasitic cultures were indeed synchronised to the specific stage of interest for this study (Gruenberg *et al.*, 1983).

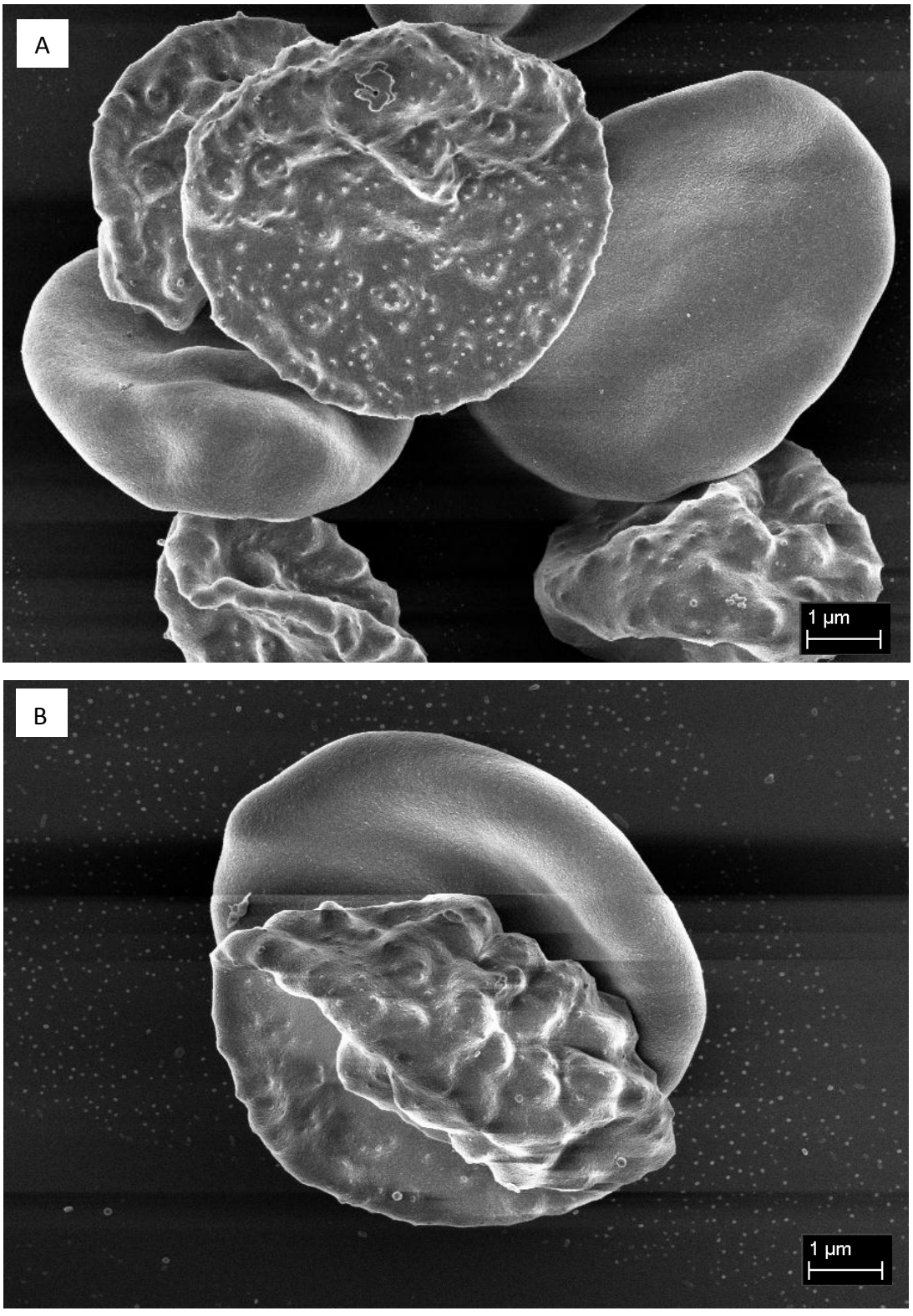


Figure 3.3: SEM micrographs of non-parasitised and parasitised RBCs at the late trophozoite stage. (A and B) Cells were suspended in 2.5% glutaraldehyde for 30 minutes, washed with phosphate buffered saline (PBS), fixed in 0.5% osmium tetra oxide ($Os0_4$) and sequentially dehydrated with ethanol (EtOH). Samples were then dried in hexamethyldisilazane (HMDS), followed by mounting and coating with carbon. Visualisation of samples was done using a ZEISS ULTRA Plus FEG-SEM. (Scale = 1 µm)

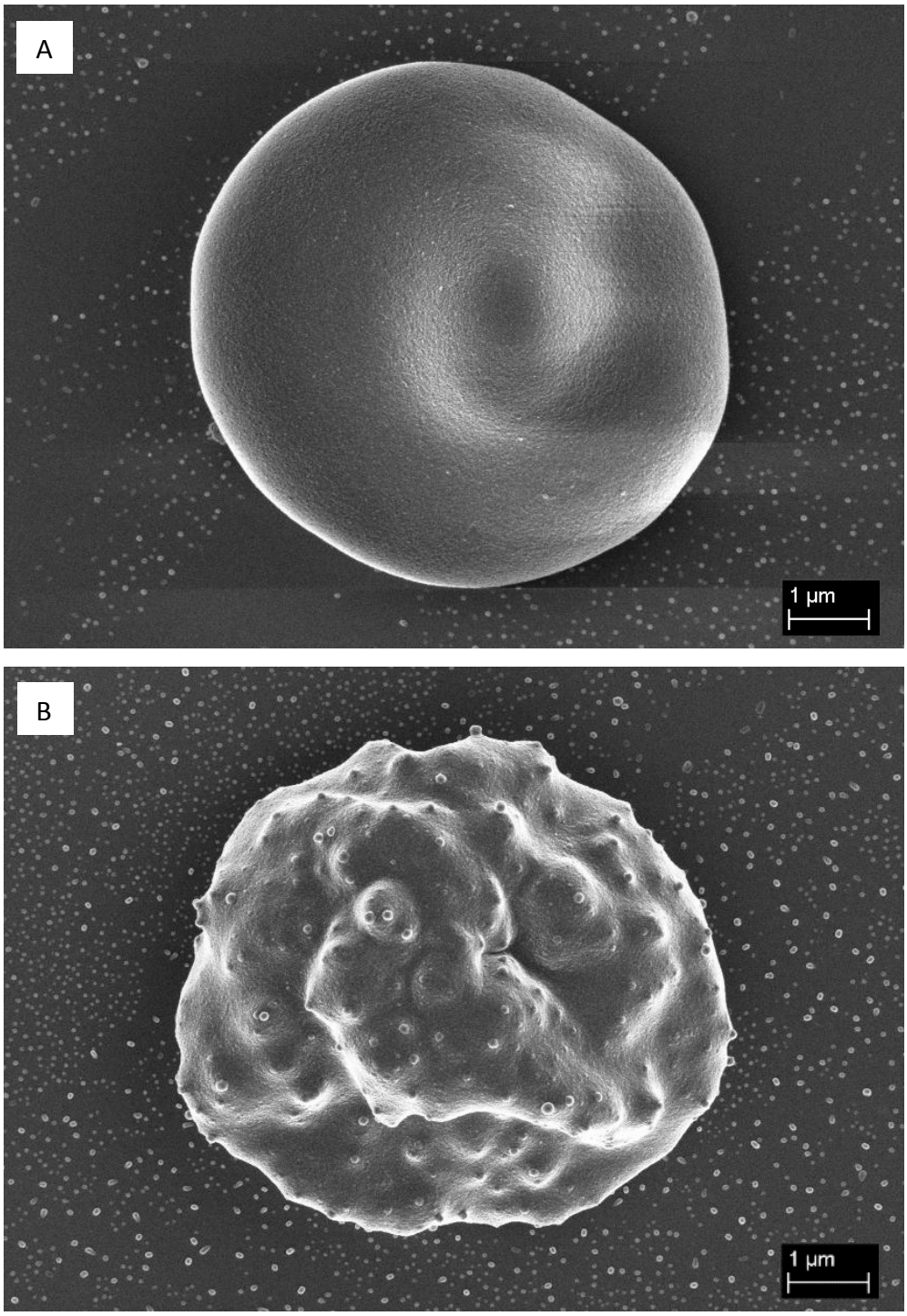


Figure 3.4: SEM micrographs highlighting the morphological differences between a non-parasitised RBC and a parasitised RBC. (A) Shows the normal morphology, which includes the smooth biconcave disc shape, of a RBC from a healthy individual. (B) Represents a parasitised RBC at the late trophozoite stage with knob protrusions distributed on the surface of the parasitised in addition to the distorted shape and structure of the RBC that is caused by the intracellular *Plasmodium* parasite. (Scale = 1 µm)

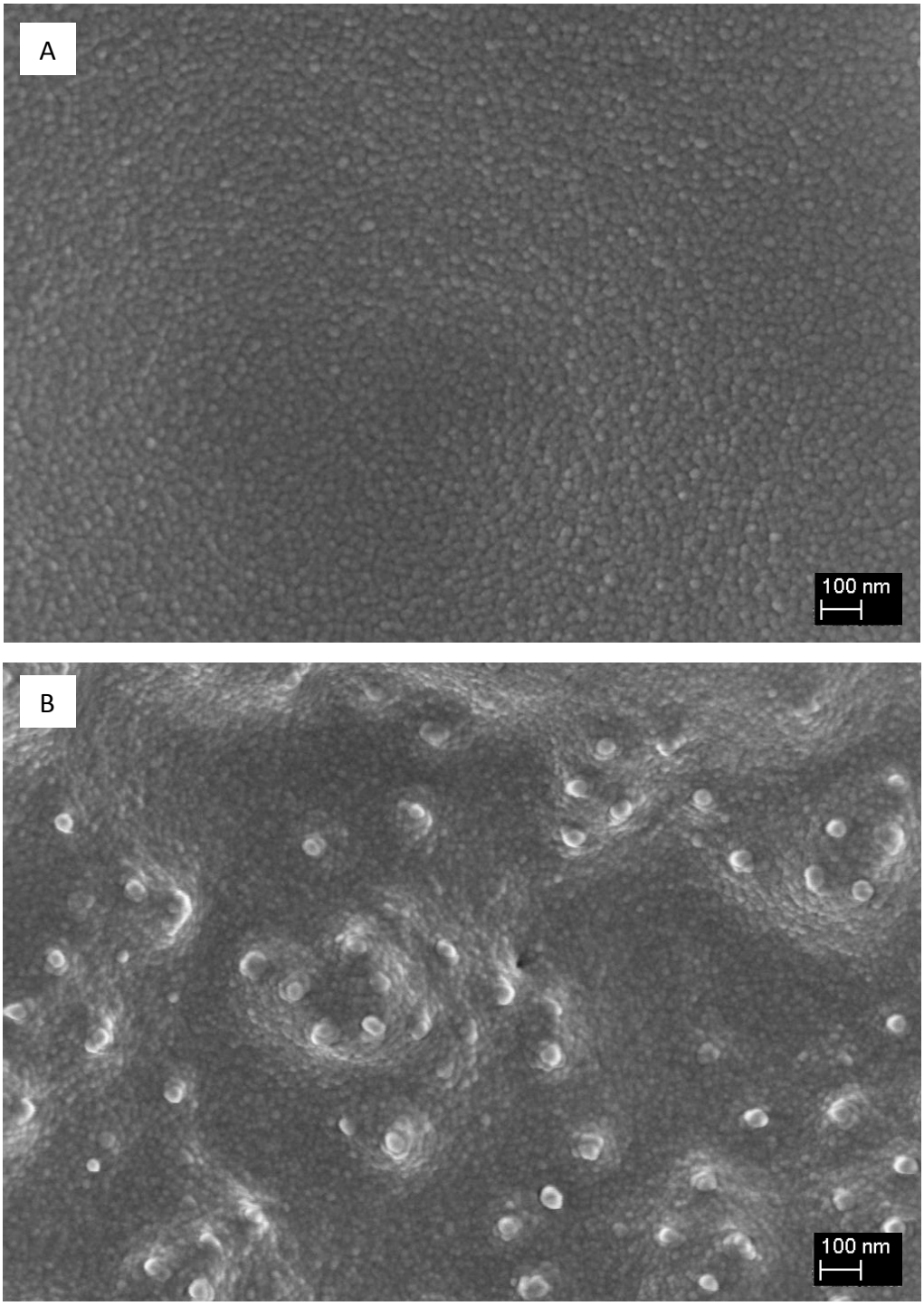


Figure 3.5: SEM micrograph of the surface of a non-parasitised and a parasitised RBC at the late trophozoite stage. (A) Represents the smooth surface of a RBC from a healthy individual with no elevated knob like protrusions. (B) Illustrates the electron dense particles known as knob protrusions that are induced by the *Plasmodium* parasite at the late trophozoite stage. (Scale = 100 nm)

3.4 Proteomic Analysis

Proteomic analysis was carried out after successfully optimising culturing conditions for the *P. falciparum* parasite and the isolation of tightly synchronised cultures was achieved. Four cm^2 sized culture flasks containing parasite cultures at the late trophozoite stage (35 – 40 hours post invasion) with a parasitemia that ranged between 12-16% on the day of isolation was passed through the VarioMACS at least twice. The high parasitemia level required a large volume of culture medium to sustain the nutritional requirements for the increased number of parasites in a single culture flask. Therefore the volume of culture medium was increased from 40 ml to a final volume of 100 ml per a culture flask a day prior to isolation with a final haematocrit of 2%. Isolated samples were combined from which parasitised RBC ghosts were prepared and protein quantitation, using the Bicinchoninic acid (BCA) protein assay, indicated that a final protein concentration of 1.2 mg/ml for the final parasitised RBC pellet was harvested. Further experimental procedures included the fractionation of isolated membrane proteins from non-parasitised and parasitised RBCs using SDS-PAGE. SDS-PAGE was performed using a Laemmli Tris-glycine system and a Bio-Rad Laboratories, Inc gel electrophoresis system. SDS-PAGE gels were imaged using Stain Free technology (Gel Doc EZ imager from Bio-Rad Laboratories, Inc) where no protein stain is required for band identification and sequential Coomassie brilliant blue (CBB) staining is needed as the protein bands cannot be seen in the Stain Free gels for band cutting. Protein bands that were considered to be relevant or of high importance were then carefully excised and processed for mass spectral proteomic analysis. Destaining, in-gel reduction, alkylation and tryptic digestion of proteins were performed prior to mass spectrometric analysis using nano LC-MS/MS. Peptide fragment spectra obtained from nano LC-MS/MS analysis were searched using a custom database including Uniprot *H. Sapiens*, Plasmo Db *Plasmodium falciparum* sequences as well as a list of common contaminants. A confidence cut-off of 1 % FDR, on the protein level, was selected.

3.4.1 Isolation of non-parasitised RBC ghosts

Proteomic based studies encounter a number of technical challenges that are commonly associated with proteome complexity. Less abundant proteins are often overshadowed or completely concealed by higher abundant proteins thus negatively affecting the sensitivity

of the protein detection methods used. In order to avoid reduced detection of less abundant but potentially highly relevant proteins, membrane ghosts from non-parasitised RBCs were effectively produced by hypotonic osmotic lysis. This resulted in the reduction or total elimination of abundant intracellular RBC contents such as cytosolic haemoglobin. During RBC ghost isolation, 1 mM disodium ethylenediamine tetraacetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to buffers. PMSF is a non-specific protease inhibitor that interacts with cysteine proteases such as papain as well as serine proteases such as trypsin, chymotrypsin and thrombin. The susceptibility of human RBCs to proteolytic degradation was reported by Fairbanks *et al* who suggested that the main source of proteinases can be attributed to contaminating leukocytes during the ghost RBC preparation. During RBC ghost preparation, cells are centrifuged and washed several times followed by the removal of the buffy coat layer which comprises of leukocytes. However, a small proportion of leukocytes including aggregated platelets may remain behind in the layer of ghost cells where they are able to exert their proteolytic activity (Fairbanks *et al.*, 1971). The use of a protease inhibitor such as PMSF, EDTA anticoagulant and also keeping the sample preparation time to a minimum successfully prevented the proteolytic degradation of RBC membrane proteins during sample preparation.

3.4.2 Separation of non-parasitised RBC ghost proteins using SDS-PAGE

After RBC ghosts were successfully isolated, SDS-PAGE was used to separate membrane proteins and the protein mass fingerprint is shown in Figure 3.6. The BCA assay was utilised to determine the protein concentration in prepared samples where a final protein concentration of 3.1 mg/ml was obtained and it was established that loading 20 µg of protein for each sample onto the gel was sufficient to produce a good quality and reproducible protein mass fingerprint. Samples were loaded into triplicate wells on a 4-20% gradient gel that was run at a constant 60 volts (V). A gradient gel consisting of 4-20% polyacrylamide gradient was chosen to fractionate membrane proteins as it allowed for the separation of membrane proteins over a broad range of molecular weights. The reason for choosing SDS-PAGE over two dimensional gel electrophoresis (2-DE), even though 2-DE is considered to be a more sensitive approach and is known for its higher resolving ability over SDS-PAGE, was that several studies have highlighted various limitations of 2-DE to separate membrane proteins (Adessi *et al.*, 1997; Rabilloud *et al.*, 2008; Fontaine *et al.*, 2012). Cited

studies confirmed that hydrophobic, large sized membrane proteins are often under represented and poorly separated on 2-DE gels thus supporting the use of SDS-PAGE for the separation of RBC ghost proteins.

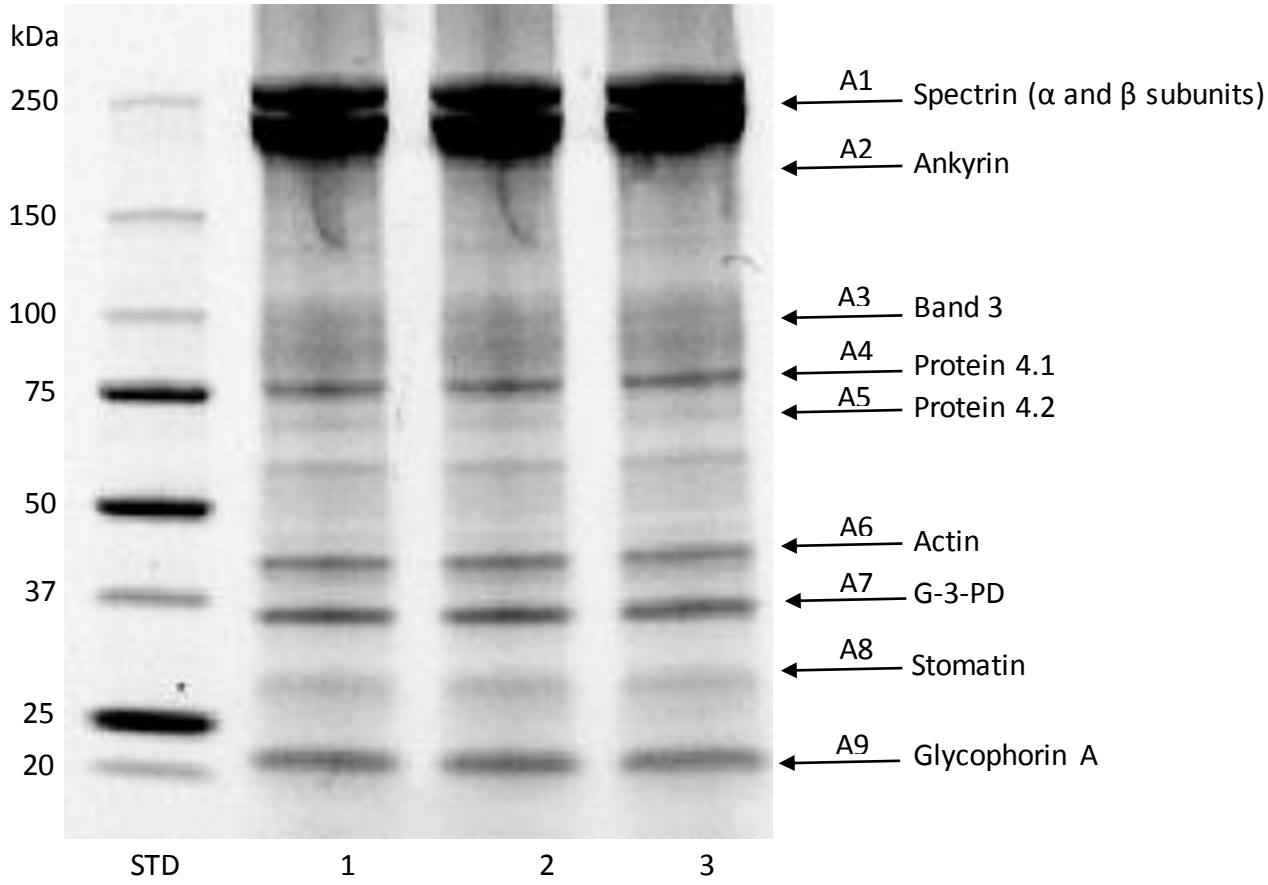


Figure 3.6: Stain Free imaging of a SDS-PAGE protein mass fingerprint of normal RBC ghosts on a 4-20% gradient gel. (Lanes 1-3) Cells were washed with PBS supplemented with disodium ethylenediamine tetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) followed by lysis of RBCs by osmotic shock and washed several times to obtain a white ghost pellet. Samples were treated with Laemmli buffer and SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V. The left lane represents molecular mass standards ranging between 20-250 kilodaltons (kDa). Bands labelled A1-A9 were cut out, in-gel trypsinised and analysed using mass spectrometry (MS). Annotation of gel is according to MS data analysis. G-3-PD is glyceraldehyde-3-phosphate dehydrogenase. A summary of identified proteins are listed in Table 3.1.

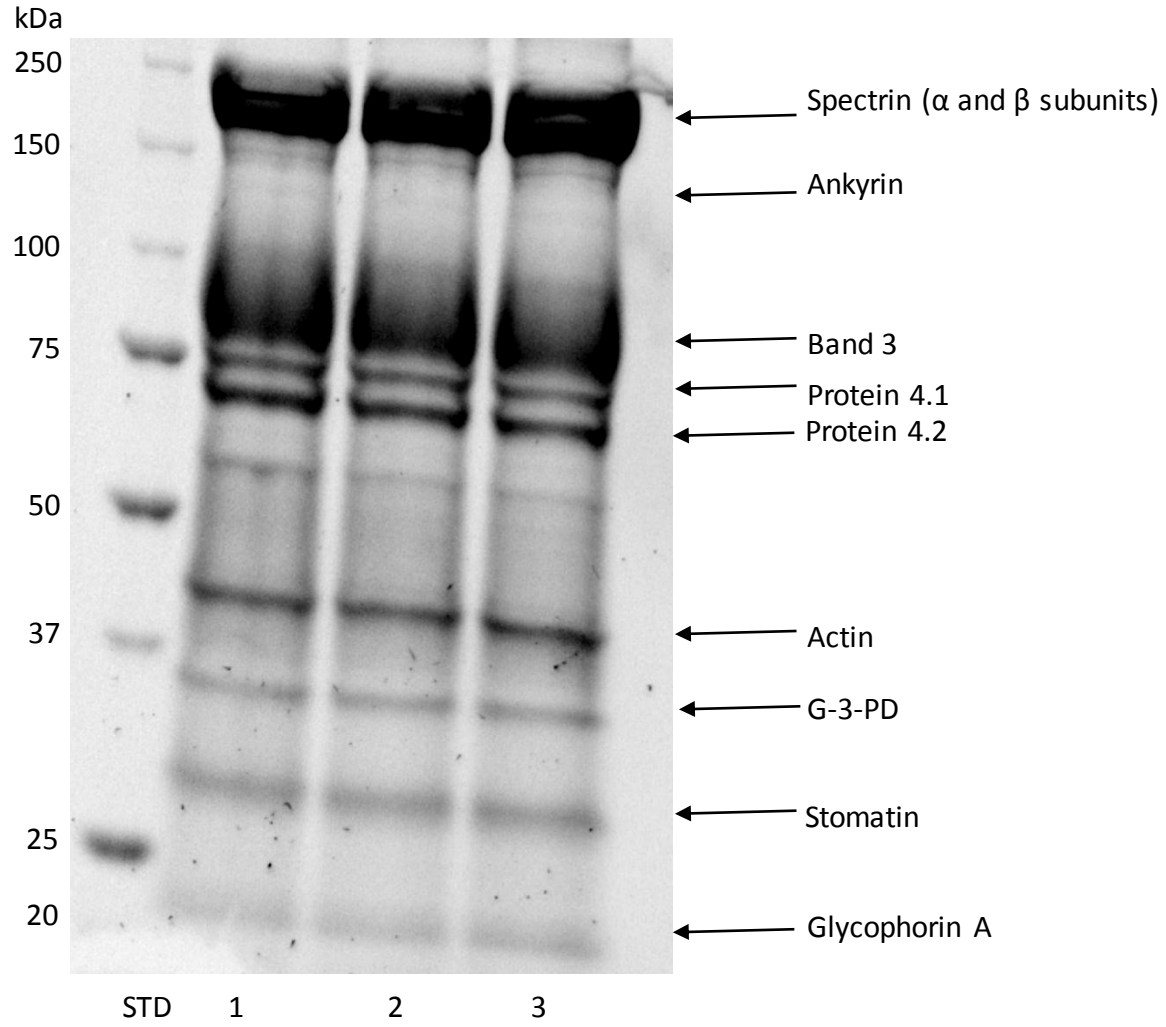


Figure 3.7: Stain Free imaging of a SDS-PAGE protein mass fingerprint of normal RBC ghosts on a 10% fixed concentration gel. (Lanes 1-3) Cells were washed with PBS supplemented with disodium ethylenediamine tetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) followed by lysis of RBCs by osmotic shock and washed several times to obtain a white ghost pellet. Samples were treated with Laemmli buffer and SDS-PAGE of proteins was carried out on a 10% Criterion gel at a constant 60 V. The left lane represents molecular mass standards ranging between 20-250 kDa. Major RBC membrane proteins are annotated according to their expected mass but were not confirmed using LC-MS/MS. G-3-PD is glyceraldehyde-3-phosphate dehydrogenase.

Despite the fact that fixed concentration gels are usually used to separate RBC ghost proteins, the protein mass fingerprint that was obtained in Figure 3.6 was found to be comparable to previous studies even though a gradient gel and Stain Free technology were used (Fairbanks *et al.*, 1971; Barker, 1991; Low *et al.*, 2002). This was confirmed when comparing the mass fingerprint obtained on a 10% fixed concentration gel in Figure 3.7 to that of Figure 3.6, where minor differences with regards to positioning and relative spacing of bands were observed. However a distinguishable difference between the two gels was the change in appearance of Band 3, as a sharp dark band was observed on the 10% fixed

concentration gel where the intensity of Band 3 was similar to that of spectrin. This was in contrast to the less intense Band 3 that was spread over a wider region on the 4-20% gel in Figure 3.6. Band 4.1 and 4.2 were also found to be more distinct and more intense on the gel in Figure 3.7 compared to Figure 3.6. As sample preparation and running conditions were kept constant, it was assumed that the reason for these band variations was associated with the percentage of polyacrylamide and consequently pore sizes within the different gels which ultimately altered the migration pattern of proteins. Although an improved mass fingerprint was obtained when using a 10% fixed concentration gel, it was decided that the identification of parasite proteins was the main focus of this study and so 4-20% gradient gels were chosen for protein separation as it allowed for the fractionation of proteins over a broader molecular mass range. This was found to be advantageous in this study, where the untargeted identification of *P.falciparum* proteins over a wide mass range was carried out.

Table 3.1: A summary of identified RBC membrane proteins with associated accession numbers, mass in kDa, no. of identified peptides and % of sequence coverage. Band no. correlates to labelled bands in Figure 3.6 that were excised and analysed using LC-MS/MS for protein identification.

Accession number	Protein	Band No.	Mass (kDa)	No. of identified peptides	% of sequence coverage
P60709	Actin	A6	41.71	39	73.07
P16157	Ankyrin-1	A2	206.14	169	64.49
P02730	Band 3	A3	101.73	139	64.98
P11171	Band 4.1	A4	96.96	44	45.95
P16452	Band 4.2	A5	76.96	57	66.28
P27105	Stomatin	A8	31.71	21	69.44
P04406	Glyceraldehyde-3-phosphate dehydrogenase	A7	36.03	20	67.46
P02724	Glycophorin A	A9	16.32	9	35.33
P02549	Spectrin alpha subunit	A1	279.84	195	63.54
P11277	Spectrin beta subunit	A1	246.32	180	71.64

*Number of peptides and percentage of sequence coverage with a greater than or equal to 95% confidence was taken from the PeptideShaker analysis of the Protein Pilot data.

Membrane proteins were first named according to their position on electrophoretic gels and as a result of this, they were initially known as the 'Band' proteins. (Fairbanks *et al.*, 1971). In this study the majority of these 'band' proteins, both cytoskeletal and membrane

associated, were identified from their molecular mass and then confirmed by nano LC-MS/MS techniques using the gel illustrated in Figure 3.6. A summary of identified proteins are listed in Table 3.1. Band 1 and 2 proteins represent spectrin α and β chains respectively. Spectrin α and β are the main components of the RBC membrane skeleton and underlie the lipid bilayer and integral membrane proteins. Spectrin α and β are characteristically one of the most abundant proteins found on RBC membrane electrophoresis gels and are situated in the 200-250 kDa region in Figure 3.6 (annotated as band A1). Spectrin is a heterodimer that plays a fundamental role in cell strength and flexibility by associating with adducin, actin and protein Band 4.1 (Cohen *et al.*, 1980; Mische *et al.*, 1987). Additionally, spectrin binds to Band 3 which is a prominent intergral transmembrane transporter protein (Figure 3.6, Band A3). The binding of spectrin to Band 3 is facilitated by ankyrin (Figure 3.6, Band A2) and the association between these three membrane proteins functions as a bridge between the skeletal membrane and the lipid bilayer of the RBC membrane (Bennett and Stenbuck, 1979). In addition to the mechanical and stabilising properties of Band 3, this highly abundant protein also acts as an anion transporter which is involved in carbon dioxide transport from tissues into RBCs (Wang, 1994). Glycophorin belongs to the sialoglycoprotein family and is a transmembrane protein. Glycophorin C specifically plays a key role in RBC shape by binding to Band 4.1 protein. Maintenance of RBC membrane integrity is further achieved by the binding of protein Band 4.2 to Band 3, ankyrin and protein Band 4.1 (Anderson and Lovrien, 1984). The formation and functionality of the RBC membrane structure is largely dependent on numerous protein interactions that span the RBC membrane. In this study, major RBC membrane proteins that are involved in these protein interactions were successfully represented on a SDS-PAGE gel. SDS-PAGE was followed by LC-MS/MS in order to confirm the identity and characterisation of these proteins. It was established that membrane proteins were resolved over a wide molecular range that was found to be highly reproducible. The chosen method in turn provided a protein mass fingerprint that served as a reference to which membrane proteins of parasitised RBCs were compared.

3.4.3 Identification of non-parasitised RBC ghost proteins using mass spectrometry

After SDS-PAGE, gels were imaged using Stain Free technology and subsequently stained with CBB stain for band visualisation. Bands of interest that were visually apparent on CBB

stained gels were excised and analysed using LC-MS/MS. Coomassie stain has been used to stain proteins on polyacrylamide gels since 1965 and is widely used today for proteomic experiments due to its easy detection, high sensitivity and more importantly, its compatibility with down line LC-MS/MS analysis due to no analytical interference resulting from non-covalent or reversible binding (Meyer and Lamberts, 1965; Dyballa and Metzger, 2012). CBB is a triphenylmethane dye that forms a blue colour complex with proteins by forming both hydrophobic bonds with aromatic residues and binding to positively charged basic amino acids such as histidine, lysine, and arginine via electrostatic interactions. CBB stain has a high specificity for proteins but the dye is also able to penetrate the gel matrix and to bind weakly to polyacrylamide to a small degree so gels must be destained prior to protein band visualisation (Steinberg, 2009).

In any proteomic experiment sample preparation plays a critical role in obtaining high-quality results. LC-MS/MS provides the mass accuracy as well as the sensitivity that is required for proteomic applications, however, the quality of LC-MS/MS data is largely dependent on sample preparation. Therefore careful consideration was taken with regards to sample handling, technical procedures and the quality of reagents utilised during the preparation of peptides for LC-MS/MS analysis. Whole proteins cannot be extracted efficiently from gels and also tend to ionised poorly during LC-MS/MS analysis, therefore in-gel digestion of proteins and extraction of peptides were carried out prior to LC-MS/MS analysis. The first step involved the destaining of proteins after bands of interest were excised from gels. Destaining of proteins was carried out using ammonium bicarbonate (NH_4HCO_3), methanol (MEOH) and acetonitrile (ACN). The use of an organic solvent such as ACN disrupts the interaction between CBB stain and proteins, whereas the use of NH_4HCO_3 weakens the electrostatic interaction between the stain and positively charged amino acids (Gundry *et al.*, 2009). Destaining was followed by reduction and alkylation of proteins to irreversibly disrupt disulphide bonds between cysteine amino acids. Unfolding of the tertiary structure of a protein leads to the exposure of an increased number of cleavage sites to the proteolytic activity of trypsin (Gundry *et al.*, 2009). Sequence grade porcine trypsin was used to digest proteins into peptides by hydrolysing peptide bonds at the carboxyl terminal of all lysine and arginine residues in the proteins (Olsen *et al.*, 2004). To prevent trypsin autolysis, a minimum concentration (10 ng/µl) of trypsin was used for each

band. Trypsin is itself a protein and high amounts of trypsin can lead to self-digestion. This autolysis can result in additional precursor peaks and increases the background signal which may have a negative effect on the detection of low abundant proteins during analysis (Hustoft *et al.*, 2012).

Following in-gel trypsinisation, the resulting peptides were extracted from gel pieces and analysed using nano LC-MS/MS. During LC-MS/MS analysis, a significant level of ion suppression and background signal in the MS/MS spectra was initially observed. Factors that contributed to the diminished signal of peptides were presumed to be contaminating buffers, detergents and salts that were carried over from sample preparation. This required that samples had to be desalted off-line by passing the extracted peptide digests through clean-up Stage-Tip C18 columns. Samples were successfully desalted and high quality MS/MS spectra for peptides were then obtained after re-analysing the samples following this clean-up step. The tandem MS/MS spectra provided peptide fragmentation information that was analysed using Protein Pilot software and matched to *in silico* spectra from a protein database. Figures 3.8, 3.9, and 3.10 represent the mass spectrometer data analysis results that were obtained for the proteins in the gel section labelled A3 in Figure 3.6 representing 78 – 110 kDa proteins and the region where Band 3 anion transporter would be expected to be found.

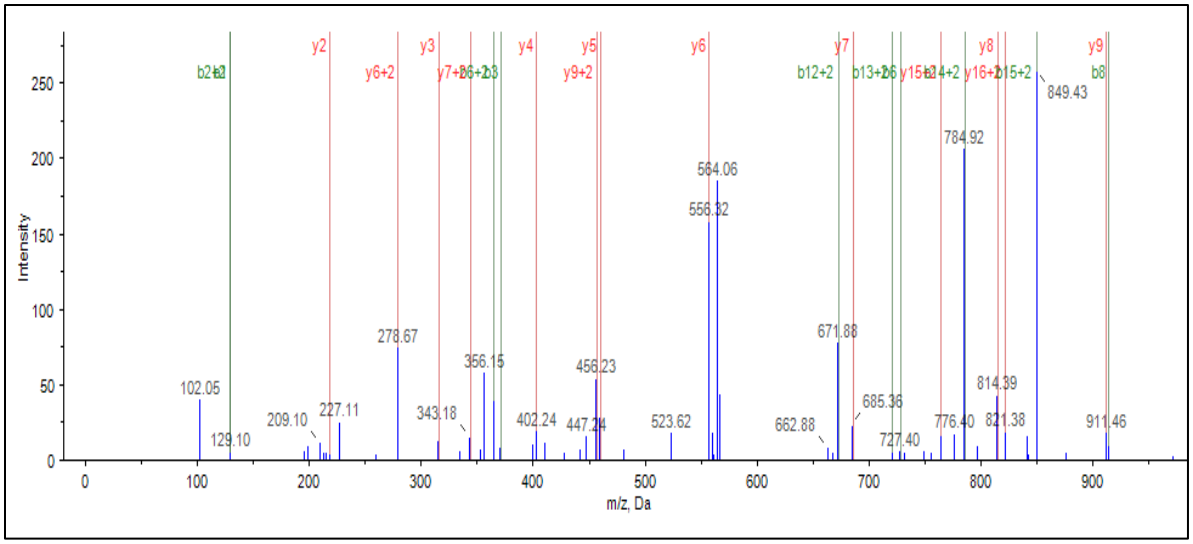


Figure 3.8: MS/MS spectrum for a selected doubly charged peptide (KKLELDGEKETAPEEPGSPAK) derived from RBC membrane protein Band 3 represented by a series of b and y ions. Following in-gel protein digestion, peptides are separated using liquid chromatography through a C18 column and then fragmented using Collision Induced Dissociation (CID) resulting in the above MS/MS spectrum that consists of a series of b and y ions. Fragment peaks that are generated from the amino terminus of a peptide are designated as 'b ions' whereas peaks extending from the carboxyl terminus are designated as 'y ions'. Peptides are sequenced from both the amino and carboxyl terminus and the MS/MS spectra obtained is analysed by Protein Pilot software against a protein sequence database for protein identification. *'m/z'* is mass per charge and 'Da' is Daltons.

Fragmentation Evidence for Peptide

KKLELDGEKETAPEEPGSPAK

Residue	b	b+2	y	y+2
K	129.1022	65.0548	2253.1609	1127.0841
K	257.1972	129.1022	2125.0659	1063.0366
L	370.2813	185.6443	1996.9710	998.9891
E	499.3239	250.1656	1883.8869	942.4471
L	612.4079	306.7076	1754.8443	877.9258
D	727.4349	364.2211	1641.7602	821.3838
G	784.4563	392.7318	1526.7333	763.8703
E	913.4989	457.2531	1469.7118	735.3596
K	1041.5939	521.3006	1340.6692	670.8383
E	1170.6365	585.8219	1212.5743	606.7908
T	1271.6842	636.3457	1083.5317	542.2695
A	1342.7213	671.8643	982.4840	491.7456
P	1439.7740	720.3907	911.4469	456.2271
E	1568.8166	784.9120	814.3941	407.7007
E	1697.8592	849.4332	685.3515	343.1794
P	1794.9120	897.9596	556.3089	278.6581

Figure 3.9: Table representing b and y ions that correspond to identified fragment masses corresponding to the amino acid sequence of a single peptide matched to the Band 3 protein. Ions that were positively identified with an equal or greater than 95% confidence are indicated in the green blocks.

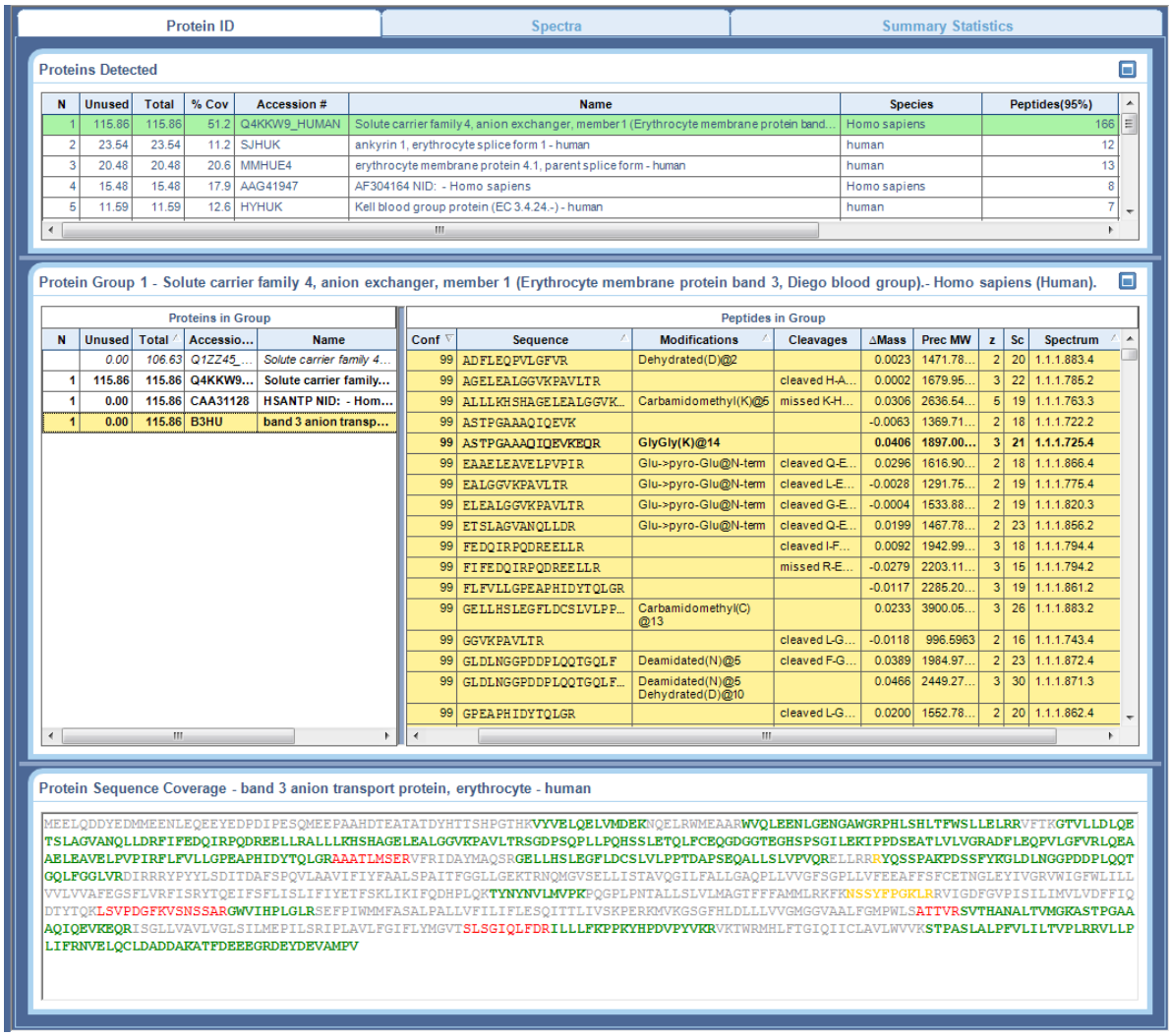


Figure 3.10: Example of Protein Pilot software analysis indicating protein characterisation and sequence coverage for RBC membrane protein Band 3. The gel band annotated as A3 on the gel in Figure 3.6 was cut out, processed to block disulphide bridge formation then in-gel trypsinised and analysed by nano-LC-MS/MS. RBC membrane protein, Band 3, was successfully identified by matching peptide fragment spectra from 166 peptides to *in-silico* spectra from a protein database and the above information was generated by the Protein Pilot software. Characteristics such as percentage of sequence coverage, accession number, species, name of proteins and the corresponding number of sequenced peptides are presented by the software. All proteins that are identified in the sample are ranked according to the number of uniquely matched peptide sequences for each protein. Listed proteins 2-5 were also positively identified in the sample but found fewer peptides due to lower abundance. The Protein Sequence Coverage block represents the protein sequence of identified protein Band 3. Sequence coverage that is highlighted in green- indicates a high confidence, yellow- represents a moderate confidence, red- indicates a relatively low confidence and grey- shows the areas where no sequence coverage for the protein was obtained.

3.4.4 Separation of parasitised RBC ghost proteins

Preparation of ghosts from parasitised RBCs was optimised and ghost proteins were separated using SDS-PAGE as described for the uninfected RBCs. SDS-PAGE running

conditions were identical to the conditions for non-parasitised RBCs and 20 µg protein for each sample was loaded onto a 4-20 % Criterion gel. The results are shown in Figures 3.11, 3.12 and 3.13. Each protein mass fingerprint was compared to the fingerprint obtained from non-parasitised RBCs that served as a control (Figure 3.6). When analysing the gel fingerprint in Figure 3.11, it was observed that major RBC membrane and cytoskeletal proteins were either absent or under represented on the gel. Gel bands that should have contained highly abundant spectrin subunits were distinctly missing in addition to the absence of Band 3 as well as protein Bands 4.1 and 4.2. Although proteomic differences between non-parasitised and parasitised RBCs were expected, it was highly unlikely that major host membrane proteins were not expressed post-infection. Therefore the most plausible explanation for this occurrence was that proteins were susceptible to protease activity in the absence of a protease inhibitor during sample preparation. This was supported by the increased number of bands at the bottom of the gel as a result of the proteolytic degradation of larger molecular weight proteins. The *P.falciparum* parasite possesses a range of proteases, the most studied and prominent proteolytic enzymes belonging to the cysteine family known as the falcipains. Falcipains cleave the carbonyl carbon of a peptide bond and this reaction is catalysed by a cysteine residue. The main function of falcipains is to hydrolyse haemoglobin into amino acids for the nutritional needs of the parasite. Additionally, these cysteine proteases also aid the entry and exit of the parasite from the host cell by cleaving membrane proteins (Rosenthal, 2004). Falcipains are categorised into four main papain enzymes, however the most relevant proteases to this study would be the falcipain-2 and falcipain-3 enzymes as both proteases are reported to be highly expressed during the trophozoite stage. It has been observed that falcipain-2 tends to cleave RBC membrane proteins such as ankyrin and protein Band 4.1 during the late trophozoite and schizont stage (Dahl and Rosenthal, 2005). Moreover, it has been reported that Plasmepsin-II (which is an aspartic protease) is responsible for the degradation of spectrin, protein Band 4.1 and actin in prepared RBC ghosts (Le Bonniec *et al.*, 1999). It is evident that the *Plasmodium* parasite is capable of extensive proteolytic activity and this has a significant effect on the protein mass fingerprint of RBC membrane proteins using SDS-PAGE. Furthermore these results highlight the crucial role of a protease inhibitor during sample preparation for proteomic experiments, as proteolysis can lead to inaccurate

assumptions about the mass and relative abundance of various proteins when analysing a SDS-PAGE protein mass fingerprint.

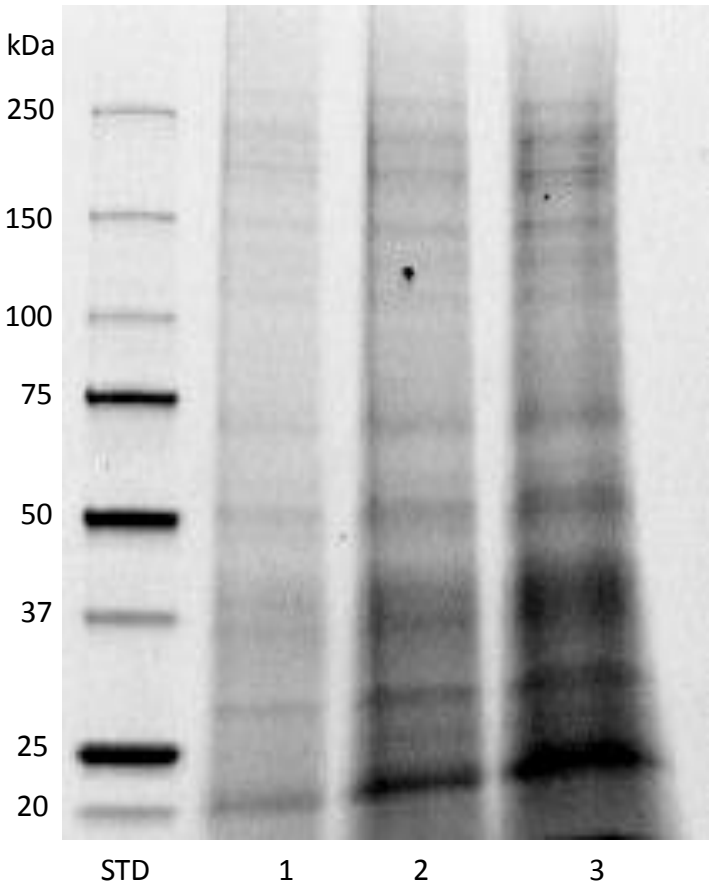


Figure 3.11: Stain Free imaging of a SDS-PAGE protein mass fingerprint of ghost proteins from parasitised RBCs. (Lanes 1-3) Cells were lysed with deionised water, washed with PBS before Laemmli sample buffer was added to the membrane pellet. SDS-PAGE of ghost proteins was carried out on a 4-20% Criterion gel at a constant 60 V.

Sample preparation for parasitised RBC ghosts was adjusted to include PMSF, a non-selective protease inhibitor, and the results for the RBC membrane proteins using the modified method are illustrated in Figure 3.12. It can be seen that the addition of a protease inhibitor made a significant difference to the protein mass fingerprint obtained. Protein bands containing expected major membrane proteins were now visible and comparable to the mass fingerprint obtained in Figure 3.6. However, a visible wave-like pattern on the gel in Figure 3.12 was present and this was presumed to be a result from the high salt content from the sample buffers that were used during sample preparation. An attempt to prevent the formation of the pattern, samples that were already reconstituted in Laemmli sample

buffer were further diluted with distilled water and homogenised. This proved to be an effective method as the resulting gel fingerprint that was obtained thereafter is illustrated in Figure 3.13.

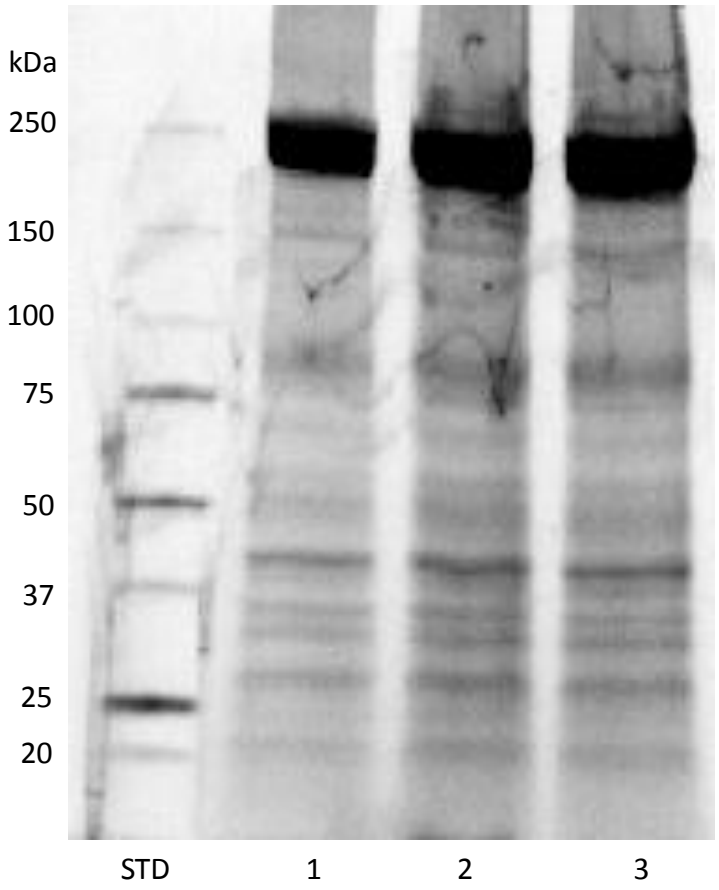


Figure 3.12: Stain Free imaging of a SDS-PAGE protein mass fingerprint of ghost proteins from parasitised RBCs. (Lanes 1-3) Cells were lysed with deionised water, washed with PBS that was supplemented with 1 M EDTA and 1 M PMSF. Laemmli sample buffer was added to samples at a 1:2 v/v ratio and SDS-PAGE of proteins was carried out on a 4-20% criterion gel at a constant 60 V.

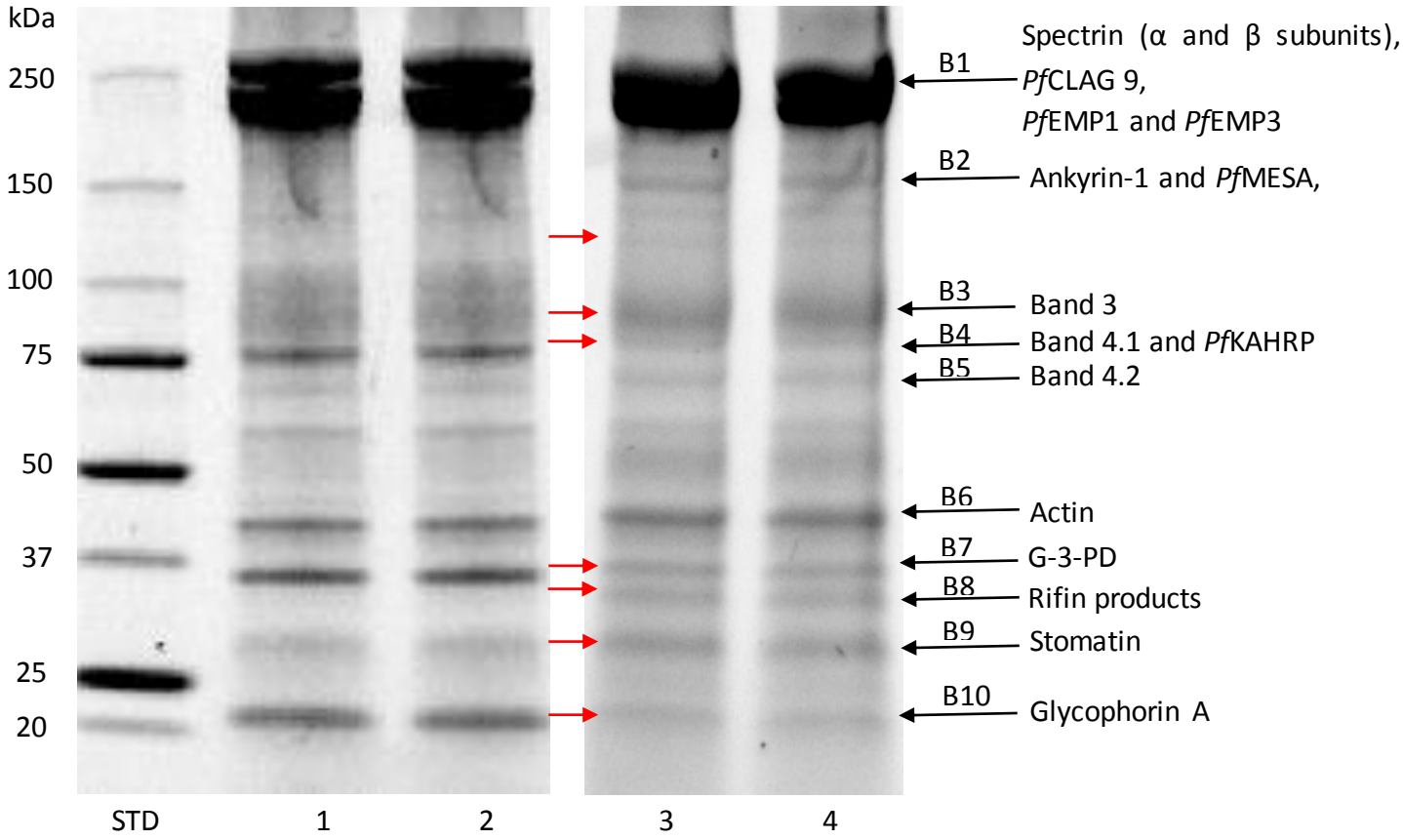


Figure 3.13: Stain Free imaging of a protein mass fingerprint of ghost proteins from non-parasitised (Lanes 1-2) and parasitised at the late trophozoite stage RBCs (Lanes 3-4). Parasitised RBCs were washed with PBS supplemented with 1mM EDTA and 1 mM PMSF followed by lysis with deionised water. Laemmli sample buffer was added to samples followed by a further 1:1 dilution with deionised water and homogenisation using a Model 3000 Ultrasonic Homogeniser. SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V. Notable visual band differences are indicated by red arrows. Bands labelled B1-B10 were cut out, in-gel trypsinised and analysed using mass spectrometry. Protein identities are annotated according to mass spectrometry data analysis. G-3-PD is glyceraldehyde-3-phosphate dehydrogenase. Only relevant parasite proteins that are associated with parasitic cytoadhesion or play a supporting role are annotated, where *Pf*CLAG9 is *Plasmodium falciparum* cytoadherence linked asexual gene 9 product, *Pf*MESA is *Plasmodium falciparum* mature parasite infected erythrocyte surface antigen ; *Pf*EMP1 is *Plasmodium falciparum* erythrocyte membrane protein1, *Pf*EMP3= *Plasmodium falciparum* erythrocyte membrane protein 3; *Pf*KAHRP= knob associated histidine rich protein and Rifin is a product of the *rif* gene family. A summary of identified proteins are listed in Table 3.2.

Table 3.2: A summary of identified cytoadhesive related membrane proteins from parasitised RBCs with associated accession numbers, mass in kDa, number of identified peptides and % of sequence coverage. Band no. correlates to labelled bands in Figure 3.12 that were excised and analysed using LC-MS/MS for protein identification.

Accession number	Protein	Band No.	Mass (kDa)	No. of identified peptides	% of sequence coverage
*Pf*_3D7_0220800	*P. falciparum* cytoadherence linked asexual gene protein 2	B1	171.05	7	7.92
*Pf*_3D7_0302500	*P. falciparum* cytoadherence linked asexual gene protein 3.1	B1	167.13	52	42.41
*Pf*_3D7_0831600	*P. falciparum* cytoadherence linked asexual gene protein 8	B1	165.51	10	8.75
*Pf*_3D7_0935800	*P. falciparum* cytoadherence linked asexual gene protein 9	B1	160.31	7	6.27
*Pf*_3D7_0809100	*P. falciparum* erythrocyte membrane protein 1	B1	240.98	4	4.41
*Pf*_3D7_0201900	*P. falciparum* erythrocyte membrane protein 3	B1	273.49	5	33.84
*Pf*_3D7_0202000	*P. falciparum* knob associated histidine rich protein	B4	71.26	19	25.08
*Pf*_3D7_0500800	*P. falciparum* mature parasite infected erythrocyte surface antigen	B2	168.19	91	47.42

*Number of peptides and percentage of sequence coverage with a greater or equal to 95% confidence was taken from the PeptideShaker analysis of the Protein Pilot data.

Figure 3.13 shows the final protein mass fingerprint that was used to investigate the RBC membrane proteome of parasitised RBCs by making comparisons to the mass fingerprint shown in Figure 3.6. Bands that appeared to be more intense (darker), less intense (lighter), displaced or completely absent when compared to the control (Figure 3.6 and re-displayed in Figure 3.13) were noted as band differences and of relevance. Visual assessment of band intensity was adequate to identify band differences on gels, however it should be noted that band intensities can also be determined by making use of commercially available software to perform densitometric analysis of protein bands. Densitometry can be used to assess the intensity of specific bands which corresponds to protein expression levels however because a single gel band consists of numerous proteins, it is difficult to determine which specific protein is upregulated or downregulated. For example, the intensity of gel bands that

represent protein 4.1, G-3-PD and Glycophorin A on the protein fingerprint in Figure 3.13 were markedly reduced and this may be a possible indication of decreased or total inhibition of protein expression of these proteins when visualising the gel. However, LC-MS/MS confirmed the identity of these RBC membrane proteins along with other major cytoskeletal proteins at their expected mass on the protein fingerprint in Figure 3.13. These results highlight that although the *Plasmodium* parasite is able to induce significant changes to the proteome of the RBC membrane by inserting a number of proteins on the membrane of parasitised RBCs, these insertions do not affect the presence of essential host membrane proteins nor do they become the dominant proteins on the RBC surface. Thus confirming that host membrane proteins are preserved and remain the dominant features on the membranes of parasitised RBCS at the late trophozoite stage. This would be expected as the parasite requires the host cell to maintain some degree of structural and functional integrity after invasion to withstand the parasite's intra-erythrocytic development.

Band differences between the non-parasitised and parasitised RBC were observed on the gel (indicated by red arrows in Figure 3.13) and it was assumed that these visually apparent band differences were due to the presence of parasite proteins or the alteration of host proteins by the parasite. After gel bands (B1-B10) were analysed by LC-MS/MS, a large number of *P. falciparum* proteins were identified but only found proteins that have been previously reported to be relevant with regards to cytoadhesion or severe malaria are reported here and discussed further in this study.

Several *P. falciparum* cytoadherence linked asexual gene (*Pf*CLAG) products were identified in gel band B1 in Figure 3.12. Cytoadherence linked asexual gene product, *Pf*CLAG 9 protein, is a 160 kDa size protein that is expressed by late stage parasites and translocated to the surface of parasitised RBCs. Gene knockout studies have shown that the expression of the *Pf*CLAG 9 protein is necessary for the adherence of parasitised RBCs cells to endothelial host receptors, such as cluster determinant 36 (CD36), to facilitate platelet-mediated clumping which is a common feature of severe malaria (Trenholme *et al.*, 2000). Other *Pf*CLAG proteins that may play a role in cytoadhesion and that were identified in this study include *Pf*CLAG 2, *Pf*CLAG 3.1 and *Pf*CLAG 8 (not annotated on the gel). The molecular weights of these proteins also fall in the range of 160 – 170 kDa. Hence, the fact that these proteins

along with *Pf*CLAG 9 were found in the 200-250 kDa region of the gel suggests the possibility of cross linkage of these proteins to other proteins to impart higher molecular weight to the proteins. Identification of these proteins at a higher mass range than expected could also be due to incomplete migration to the theoretical molecular weight of these proteins on the SDS-PAGE gel which is a common occurrence with large sized proteins. Possible reasons for incomplete migration may be due to glycosylation of proteins as well as low binding to the SDS detergent thus leading to decreased movement of proteins through the gel. *Pf*EMP3, with a mass of 273 kDA was identified in excised band B1, however *Pf*EMP1, which is a significant antigenic determinant in cytoadhesion was identified in band B1 by LC-MS/MS with a sequence coverage of only less than 4.41% and a total of 4 identified peptides (Table 3.2). Possible reasons for this poor identification may include decreased solubility during SDS-PAGE where it is possible that a protein such as *Pf*EMP1 may have been too large to move through the gel or was insoluble at the bottom of the wells. Other factors for poor identification may include insufficient peptide extraction from the gel or coelution with higher abundance peptides during the chromatographic elution. Alternatively, excision of gel bands is not an automated procedure and is subjected to human error. It is possible that this high molecular weight protein was situated on the upper region of band B1 and that during the excision process, the *Pf*EMP1 was not collected. Therefore the protein may not have been present in the sample, thus suggesting that *Pf*EMP1 was probably identified as a false postive durig LC-MS/MS analysis.

P. falciparum mature parasite infected erythrocyte surface antigen (*Pf*MESA), also known as *P. falciparum* erythrocyte membrane protein 2 (*Pf*EMP2), was identified in excised gel band B2 with a mass of about 168 kDA. *Pf*MESA is located on the membrane of parasitised RBCs associated with Band 4.1 protein (Lustigman *et al.*, 1990). Although *Pf*MESA is not required for the formation of parasitic knobs or cytoadhesion, it has been proposed that the parasite protein may have regulatory effects with regards to the rate at which parasitised RBCs bind to endothelial cells (Magowan *et al.*, 1988). *P. falciparum* knob associated histidine rich protein (*Pf*KAHRP) was identified in excised gel band B4. As previously mentioned, *Pf*KAHRP is a well characterised 70-100 kDa size protein that induces knob formation on the surface of parasitised RBCs at the late trophozoite stage (Rug *et al.*, 2006). Due to its distinct role in cytoadhesion, *Pf*KAHRP is a potential drug target for severe malaria therapy and

consequently was one of the main proteins under investigation in this study. The successful identification of relevant membrane parasite proteins, that are reportedly associated with cytoadhesion and consequently severe malaria, provided confirmatory data that parasitic cultures were analysed at the relevant stage of the life cycle. Furthermore, the chosen experimental workflow was found to be efficient whereby three different isolated samples were analysed in triplicates using SDS-PAGE which produced a reproducible protein mass fingerprint that was analysed by LC-MS/MS. The acquired data was therefore assumed to be a reliable reference point that was used in the evaluation of the effects of lovastatin on RBC and parasitised RBC protein expression.

3.5 Assessment of the effects of lovastatin on membrane proteins of non-parasitised and parasitised RBCs at the late trophozoite stage

The effect of lovastatin on the proteome of RBCs and parasitised RBCs was assessed. Normal RBCs that were obtained from healthy individuals were treated with a final concentration of 10 µM of lovastatin. Lovastatin is normally used to treat dyslipidaemias as its main pharmacological action involves the lowering of cholesterol plasma levels by inhibiting HMG-COA reductase. The standard daily dosing of lovastatin ranges between 10-40 mg (Sirtori, 2014). The body of an average person contains 5 litres of blood, thus after taking an oral dose of 40 mg of lovastatin the approximate maximal plasma concentration of lovastatin is assumed to be 20 µM (molecular mass of lovastatin= 404.5 g/mol). Lovastatin is subjected to significant first pass metabolism resulting in a reduction of systemic bioavailability after oral administration (Sirtori, 2014). Thus taking into consideration the pharmacological profile of lovastatin, the final concentration that was presumed to be therapeutically relevant and the concentration at which normal RBCs and parasitised RBCs were exposed to was adjusted to 10 µM. After treatment, RBC ghost proteins were isolated according to previously described methods and analysed by SDS-PAGE. Separately, normal RBCs were pre-treated with 10 µM of lovastatin. Lovastatin was added to isolated parasitised RBCs at the late trophozoite stage. Resuspended cultures were incubated for 48 hours followed by the isolation of parasitised RBCs. Viability of cultures was assessed by blood smears of cultures at 24 hour intervals during the incubation period. The progression of cultures through successive stages of the life cycle in addition to increasing parasitaemia

percentages indicated that cell viability was retained after exposure to drug treatments. Isolated ghost cells from each sample were analysed using SDS-PAGE gels which were stained with CBB or separately stained with silver stain or imaged with Stain Free software as shown in Figure 3.14, 3.15 (A and B) and 3.16 respectively. Figure 3.15 (B) is an image generated by the Gel Doc EZ imager software from Bio-Rad laboratories, Inc. that allows for Stain Free images to be represented in silver stain colours.

CBB stained and Stain Free imaged gels were found to be comparable as no additional bands were visible when the gel images (Figure 3.14 and 3.16) from the same gel were compared. CBB staining allowed for the control of sample loading, as any variance with regards to the amount of protein that was loaded onto the gel would be reliably detected by the stain. Although CBB is the standard for gel staining in proteomics and is required for band visualisation before band excision, the sensitivity of the CBB stain has been matched by newer and improved technology such as Stain Free technology. Alternatively, an increased number of bands were visualised when gels were stained with silver stain (Figure 3.15). This was consistent with previous studies that showed silver stain has greater sensitivity for the detection of membrane proteins when compared to CBB stain. Silver stain has the ability to detect a number of other macromolecules which include glycoproteins, nucleic acids and lipoproteins (Dzandu *et al.*, 1984; Dzandu *et al.*, 1985). Advantages of silver stain include reduced amount of sample required and that protein loss, due to protein diffusion during gel storage, is minimised by fixation with formaldehyde (Wray *et al.*, 1981). However incompatibility with LC-MS/MS analysis due to crosslinking has limited the use of silver stain in the field of proteomics.

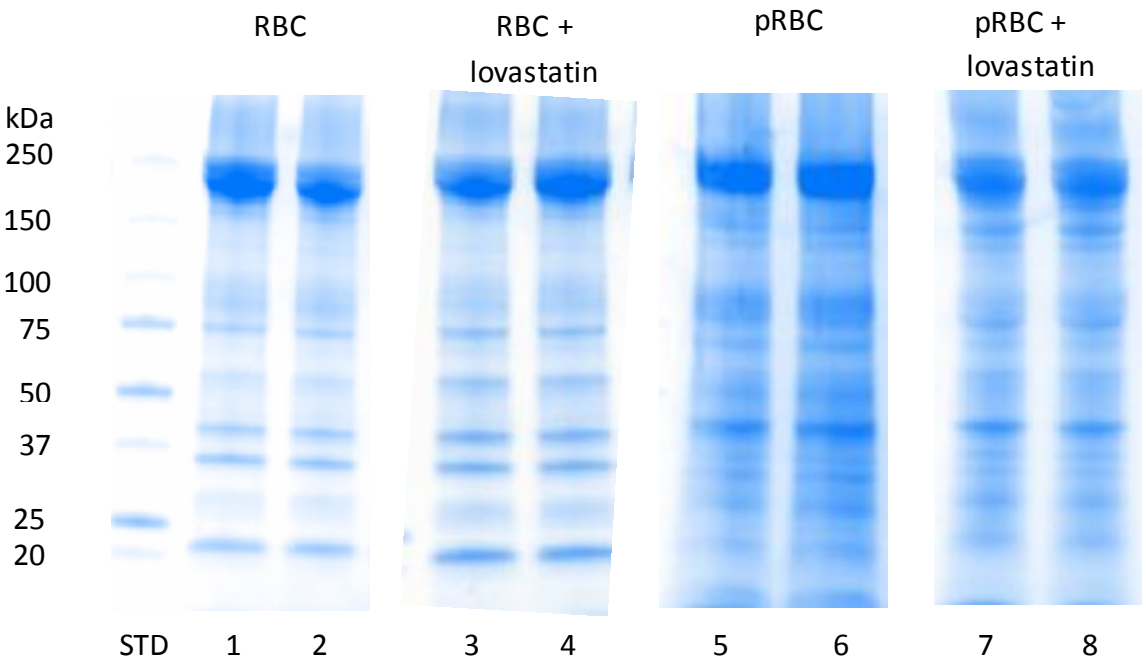


Figure 3.14: Coomassie stained gels representing ghost proteins from untreated/treated normal RBCs and parasitised RBCs (pRBC) with lovastatin. (Lanes 1-2) Fresh untreated RBCs, (Lane 3-4) RBCs treated with 10 µM of lovastatin for 24 hours, (lane 5-6) ghost proteins from parasitised RBCs at the late trophozoite stage and (Lane 7-8) ghost proteins from parasitised RBCs that were treated with 10 µm of lovastatin for 24 hours. Laemmli sample buffer was added to all samples and SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V.

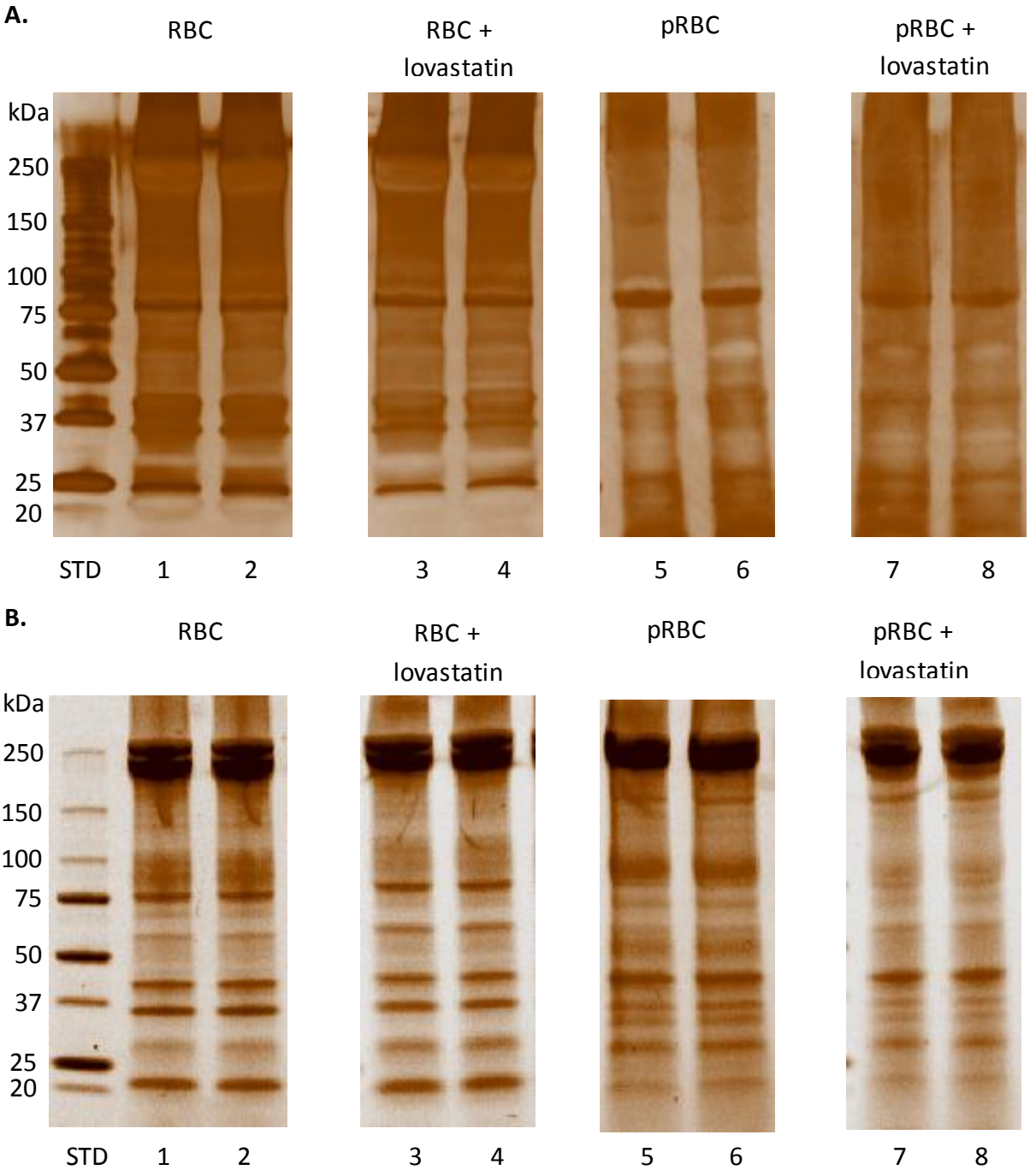


Figure 3.15: (A) Silver stained gels and (B) Stain Free gel images that are depicted in silver stain colours. Gels are a representation of ghost proteins from untreated/treated normal RBCs and parasitised RBCs (pRBCs) with lovastatin. (Lanes 1-2) Fresh untreated RBCs, (Lane 3-4) RBCs treated with 10 µm of lovastatin for 24 hours, (lane 5-6) ghost proteins from parasitised RBCs at the late trophozoite stage and (Lane 7-8) ghost proteins from parasitised RBCs that were treated with 10 µm of lovastatin for 24 hours. Laemmli sample buffer was added to all samples at a 1:2 v/v ratio and SDS-PAGE of proteins was carried out on a 4-20% criterion gel at a constant 60 V.

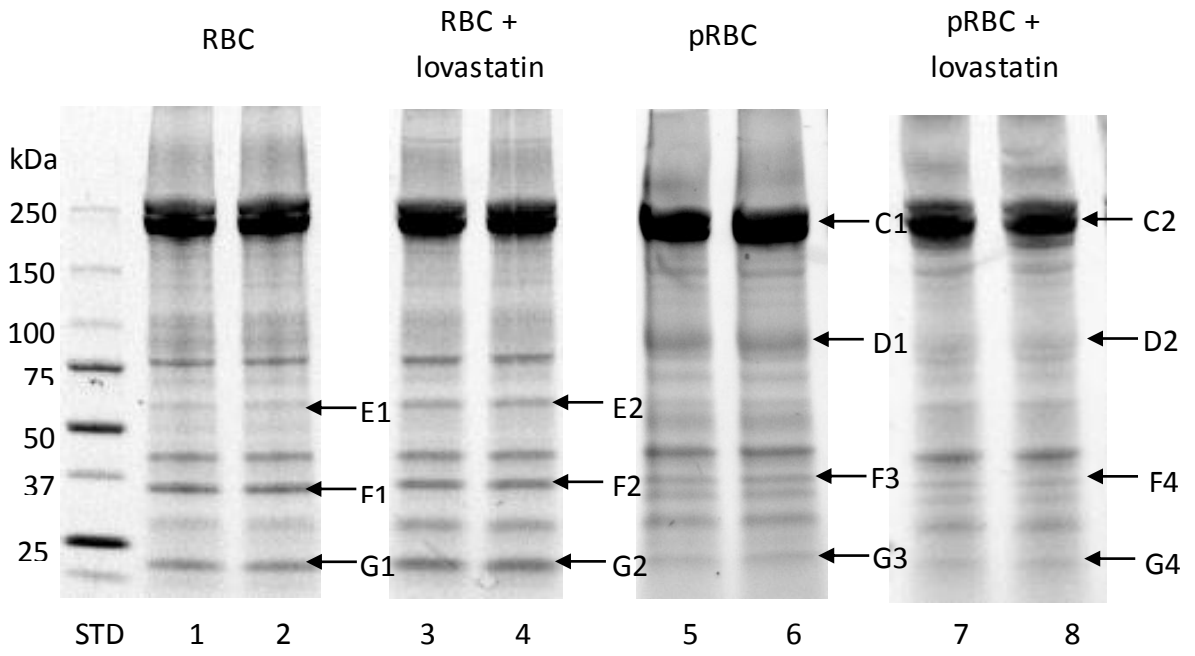


Figure 3.16: Stain Free imaging of a SDS-PAGE protein mass fingerprint of ghost proteins from lovastatin untreated/treated normal RBCs and parasitised RBCs (pRBCs). (Lanes 1-2) Fresh untreated RBCs, (Lane 3-4) RBCs treated with 10 µM of lovastatin, (lane 5-6) ghost proteins from pRBCs at the late trophozoite stage and (Lane 7-8) ghost proteins from pRBCs that were treated with 10 µM of lovastatin. Laemmli sample buffer was added to all samples and SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V.

Effects on the proteome of RBCs and parasitised RBCs after treatment with lovastatin were monitored by comparing protein mass fingerprints of treated samples to untreated controls. Bands of interest shown on the gel illustrated in Figure 3.16 were analysed to monitor the presence or absence of membrane associated proteins after treatment with lovastatin. Bands that are annotated C-G in Figure 3.16 were excised, destained, reduced and alkylated then in-gel trypsinised and extracted peptides were sequenced using nano LC-MS/MS for protein identification. The identified proteins were filtered to highlight only major RBC membrane proteins and parasite proteins that play a direct or supporting role in cytoadhesion and only these are reported. Bands C1 and C2 were analysed as relevant high molecular weight membrane proteins were expected to be present in the 250 kDa region on the gel. Bands C1 and C2 were analysed and it was found that spectrin (α and β subunits) and ankyrin-1 were present in each sample from these bands analysed, indicating that lovastatin did not suppress the expression of these host proteins. This was expected as mature RBCs can not synthesise new proteins and due to the tight association with the plasma membrane were not expected to have been lost to the surrounding environment.

Cytoadhesive membrane associated parasite proteins, *Pf*CLAG 2, *Pf*CLAG 3.1, *Pf*CLAG 8 and *Pf*CLAG 9, *Pf*EMP1 and *Pf*EMP3, were all found to be present in the lovastatin treated parasitised RBC sample. The presence of these proteins in the treated sample group suggested that the expression of these cytoadhesive related proteins were not significantly inhibited by the treatment with lovastatin. In Figure 3.16, visual band differences can be observed in the regions of 78 kDa (excised bands D1 and D2), 60 kDa (excised bands E1 and E2), 35 kDa (excised bands F1-F4) and 18 kDa (excised bands G1-G4). These regions were excised and processed for mass spectrometric analysis and following data analysis no significant difference with regards to the presence of major host proteins or cytoadhesive parasite proteins were identified within these bands. It should be noted that the data was analysed for protein identity specifically and not abundance, as what appears as a single band on a gel can represent many proteins when analysed by mass spectrometry. Host cell proteins such as Bands 3 and 4.1 were found in excised bands D1 and D2 from untreated and treated parasitised RBCs and this was also seen for *Pf*KAHRP. The fact that *Pf*KAHRP, which is considered to be a potential drug target for severe malaria, was expressed in the lovastatin treated sample of parasitised RBCs indicated that the expression of *Pf*KAHRP was not inhibited by lovastatin treatment and consequently the mechanism of action of lovastatin does not involve the inhibition of *Pf*KAHRP expression. However, possible lovastatin induced alteration to *Pf*KAHRP that could affect functionality, such as alkylation or truncation of the knob associated protein was not within the scope of this study and thus cannot be excluded. Protein composition was similar for excised bands E1 and E2 whereby actin was present in both bands. Glyceraldehyde 3-phosphatase dehydrogenase was found in bands numbered F1-F4 and glycophorin A was found in bands G1-G4 for all treatments and control samples while the parasite protein, rifin, was identified in both untreated and treated parasitised RBCs only. Visual band differences were observed between all 4 sample groups, however these band differences could not be associated with proteins related to cell adhesion which are of interest in this study, as all relevant proteins, originating both from the host and the parasite were identified in respective untreated and lovastatin treated samples. This suggests that band differences were then possibly a result of the presence or absence of proteins that were not pertinent to this study.

The fact that proteins associated with cytoadhesion were present on the membranes of lovastatin treated parasitised RBCs implies that lovastatin did not induce significant inhibition of parasite protein expression, thus suggesting an alternative mode of action for the drug's reported positive effect on patients with severe malaria. To further elucidate a possible mechanism of action, the effect of lovastatin on membrane protein abundance was assessed. It is proposed that a decrease in cytoadhesive related proteins may in turn result in a reduced rate at which parasitised RBCs adhere to host endothelium leading to a decrease in the sequestration of the parasitised RBCs into vital organs which would in turn potentially alleviate the severity of symptoms associated with severe malaria. Label-free methods for protein quantification are popular tools in the field of proteomics and was utilised in this study to determine which cytoadhesive related proteins were upregulated or downregulated in treated samples. Gel lanes from each sample group were cut out into five fractions that were pre-treated, then in-gel trypsinised and analysed using nano LC-MS/MS. After protein database searches of the individual excised gel bands were assessed, the results for individual bands per lane were combined to provide a global protein identification and quantification of the complete gel separated proteome of each sample. PeptideShaker software was used as a visualising tool for further protein analysis. PeptideShaker software is able to display the number of peptides, protein sequence coverage and number of spectra associated with a specific protein. Estimations are based on the peptides that have been validated for each protein. An example of data output by PeptideShaker is illustrated in Figure 3.17.

Figure 3.17: Example of Data generated by PeptideShaker software. Data generated by the software can be seen in the protein table at the top; the peptide and peptide to spectrum match (PSM) tables, both in the middle left; the spectrum view in the middle right; and the sequence coverage at the bottom. Sequence coverage that is highlighted in green-indicates peptides that were validated, yellow- represents peptides that were doubtful and red- indicates peptides that were not validated. Peptides in blue carry a variable modification such as oxidation.

In label-free protein experiments, protein quantitation and comparisons of protein abundance can be achieved by looking at three separate but related MS/MS parameters. These parameters are: the number of identified peptides, the protein sequence coverage and the spectral count for each identified protein during LC-MS/MS analysis. Quantitation is based on the fact that an increase in protein abundance would result in an increased number of fully sequenced peptides from the digest and consequently lead to greater overall confident protein sequence coverage. Additionally, amino acid sequence can be interpreted from higher intensity MS/MS spectra with greater confidence, therefore the higher the absolute concentration of individual peptides the more MS/MS spectra would be confidently sequenced leading to a higher spectral count for a protein present at higher concentration (Zhu *et al.*, 2009). Studies have shown, when comparing the three different quantitative parameters, that spectral count shows the greatest linear correlation to protein abundance (Old *et al.*, 2005; Zhang *et al.*, 2006). Spectral counting for protein abundance is

a relatively straightforward approach but normalisation of data is critical to account for sample and experimental variation so that accurate observations regarding proteomic changes can be made. Protein concentration assays were carried out to ensure that the same amount of protein for each sample was loaded onto the gel. Gels were also stained with CBB stain for quality control to confirm uniform loading. However slight variance in the amount of protein loaded onto the gel may affect the relative quantitation by MS/MS analysis by giving a skewed representation of the number of MS/MS spectral counts for each protein and consequently the accuracy of determining protein abundance may be compromised. MS/MS data was assessed and it was found that Ankyrin-1 was easily identified and showed greater than 50% coverage while showing limited variation in abundance between samples based on spectral counting. Therefore Ankyrin-1 was used to normalise for protein concentration variation in comparable samples from the same gel. MS/MS data was assessed and it was found that The normalised spectral counts for each cytoadhesive related proteins were reported (listed in Table 3.3) to give an indication of whether lovastatin has an effect on the level of protein expression.

The number of spectra was also used to give an indication of in which fraction a specific protein was detected. This was based on the confidence at which a peptide or protein was identified in a fraction within the context of the whole analysis. Examples of data that were acquired for actin are displayed in Figures 3.18. Actin has an approximate mass of 40 kDa and was expected to be in the protein band (annotated with red arrows in Figures 3.18) that is positioned just above the 37 kDa standard on the gel. The identification of actin in the annotated band was confirmed when individual fractions were excised from both control samples of RBCs and parasitised RBCs (see Figure 3.6 and 3.13).

Table 3.3: The number of spectra for *P. falciparum* proteins that are associated with cytoadhesion. Number of spectra was obtained for boh untreated and treated parasitised RBCs (pRBC) with lovastatin (lov). Data normalisation using Ankyrin-1 was performed. Number of spectra is an indication of the relative abundance of a protein within a specific sample group. *Pf*EMP1, *Pf*EMP3 and *Pf*CLAG3.1 were not validated in the untreated parasitised RBC sample therefore no spectral counts were obtained for these proteins. This also occurred with *Pf*CLAG8 in the treated parasitised RBC sample.

Accession no.	Protein	Spectra no.	
		pRBC	pRBC + lov
*Pf*_3D7_0302500	Cytoadherence linked asexual protein 3.1 (*Pf*CLAG3.1)	-	110.16
*Pf*_3D7_0831600	Cytoadherence linked asexual protein 8 (*Pf*CLAG8)	12.53	-
*Pf*_3D7_0935800	Cytoadherence linked asexual protein 9 (*Pf*CLAG9)	7.16	7.65
*Pf*_3D7_0809100	Erythrocyte membrane protein 1 (*Pf*EMP1)	-	7.65
*Pf*_3D7_0201900	Erythrocyte membrane protein 3 (*Pf*EMP3)	-	9.18
*Pf*_3D7_0202000	Knob associated histidine rich protein (*Pf*KAHRP)	12.53	42.84
*Pf*_3D7_0500800	Mature parasite erythrocyte infected surface antigen (*Pf*MESA)	12.53	84.0

According to the number of spectra confidently assigned for actin in Figure 3.18 (B), it can be seen that the highest number of spectra for actin is found in excised Fraction IV for all sample groups (RBCs; RBCs treated with lovastatin; parasitised RBCs and parasitised RBCs treated with lovastatin) and this correlated to the annotated band on the gel in Figure 3.18 (A). The bar graph in Figure 3.18 (B) also indicated that actin was found to be present in Fractions I, II, III and V, but at significantly lower abundances than in Fraction IV. Possible causes for this background signal may be due to incomplete migration of the protein through the gel during SDS-PAGE, non-specific binding to the gel or unreacted gel monomers and the fact that LC-MS/MS has the sensitivity to detect the presence of this protein at much lower levels in the other excised fractions. Further reasons could be carry over between LC-MS/MS runs or even that a small number of peptides from other proteins may have a region of overlapping sequence of amino acids that is incorrectly assigned to actin and then counted as an actin peptide spectrum giving a “background noise” of counts.

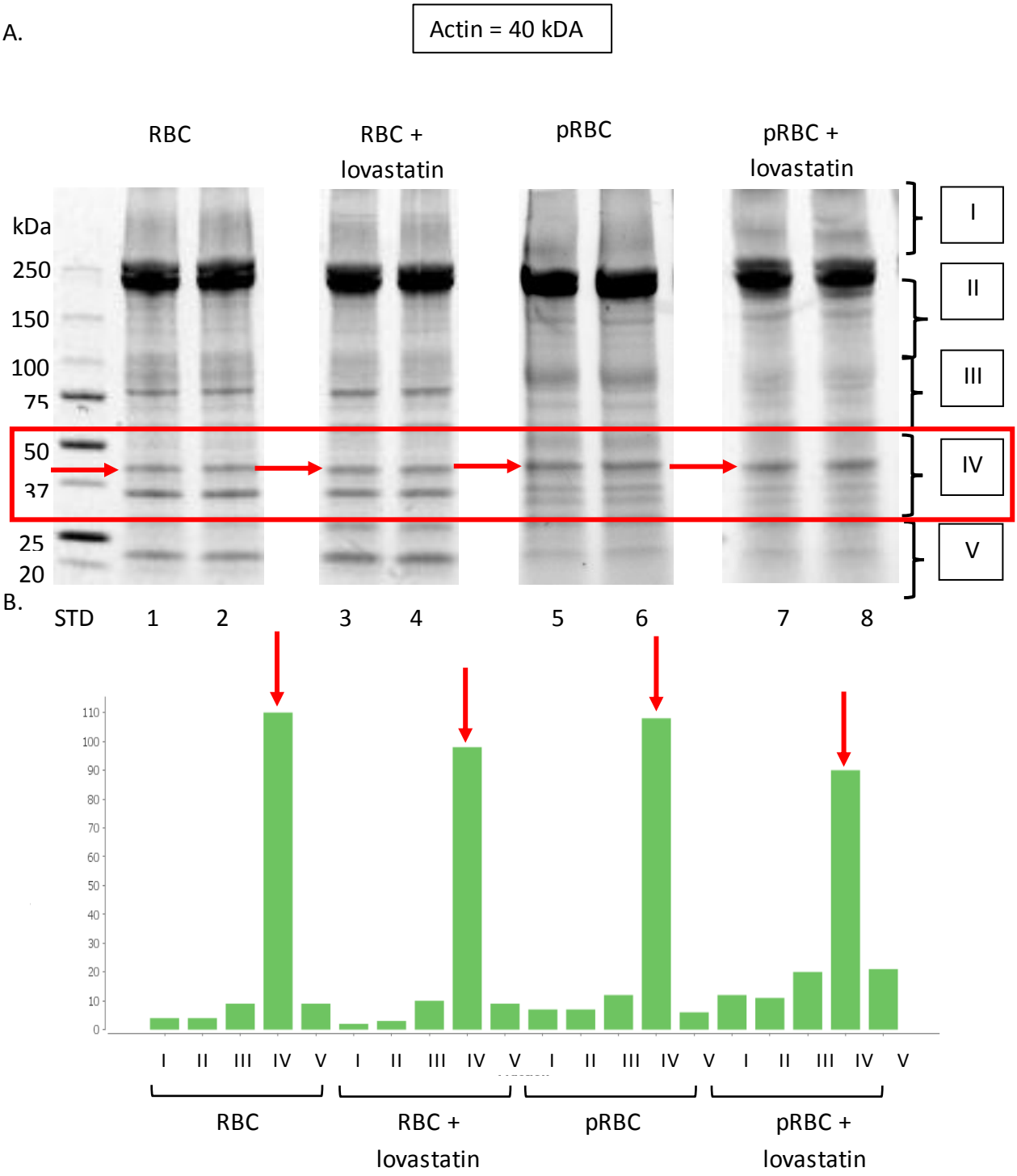


Figure 3.18: Stain Free imaging of a SDS-PAGE protein mass fingerprint of ghost proteins from untreated and treated normal red blood cells (RBCs) and parasitised RBCs (pRBCs) with lovastatin. The abundance of actin in all sample groups is indicated using the number of spectra. (A) Lanes 1-2: Fresh untreated RBCs; Lane 3-4: treated RBCs with 10 µM of lovastatin;, Lane 5-6: parasitised RBCs at the late trophozoite stage and Lane 7-8: parasitised RBCs at the late trophozoite stage that were treated with 10 µM of lovastatin. Laemmli sample buffer was added to all samples and SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V. Fractions (I-V) were excised for each sample group, in-gel trypsinised and analysed using nano LC-MS/MS. (B) Number of spectra for actin in each excised gel band as indicated on the right of the gels in (A) for the different sample groups. Numbered Fractions (I-V) are indicated on the bar graph for the different sample groups.

This type of data analysis proved to be useful in monitoring proteomic changes induced by treatment with lovastatin and was carried out for parasite proteins. An example of the acquired data for *Pf*KAHRP is shown in Figure 3.19 (A and B). Similarly, the abundance of *Pf*KAHRP was determined in each excised band of parasitised RBCs. The results showed that *Pf*KAHRP was predominantly found in Fraction III for both untreated and treated parasitised RBCs. This once again corresponded to the gel in Figure 3.19 (A), as *Pf*KAHRP has an approximate mass of 70 kDa and was detected (annotated with red arrows) at the expected mass range on the gel. The results showed that *Pf*KAHRP was still present on the membranes of lovastatin treated parasitised RBCs thus further confirming that the expression of this protein was not inhibited by lovastatin treatment. It can also be seen that the total amount of protein is significantly lower in the parasitised RBC sample when compared to the treated parasitised RBCs. This observation was also evident when looking at the number of spectra for *Pf*KAHRP in Table 3.3 whereby the spectral count was three times greater in the treated sample. These results would thus imply that lovastatin had a stimulating effect on the expression of *Pf*KAHRP as it can be seen that *Pf*KAHRP was present at a higher abundance on the membranes of treated parasitised RBCs when compared to the untreated control. This trend was also seen with cytoadhesive related parasite protein, *Pf*MESA, where the spectral count was found to be seven times higher in the treated group. The spectral count for *Pf*EMP1 and *Pf*EMP3 was also relatively low, the exception being *Pf*CLAG 9, when compared to the other cytoadhesive related proteins in the treated sample (Table 3.3). No conclusions can be made with regards to the changing levels of these adhesion molecules due to lovastatin treatment as the observed abundance of these proteins in the treated group were poorly identified in the untreated parasitised RBC group. Thus spectral count could not be reliably used to calculate the abundance of these proteins in the untreated control sample and therefore no comparisons could be made.

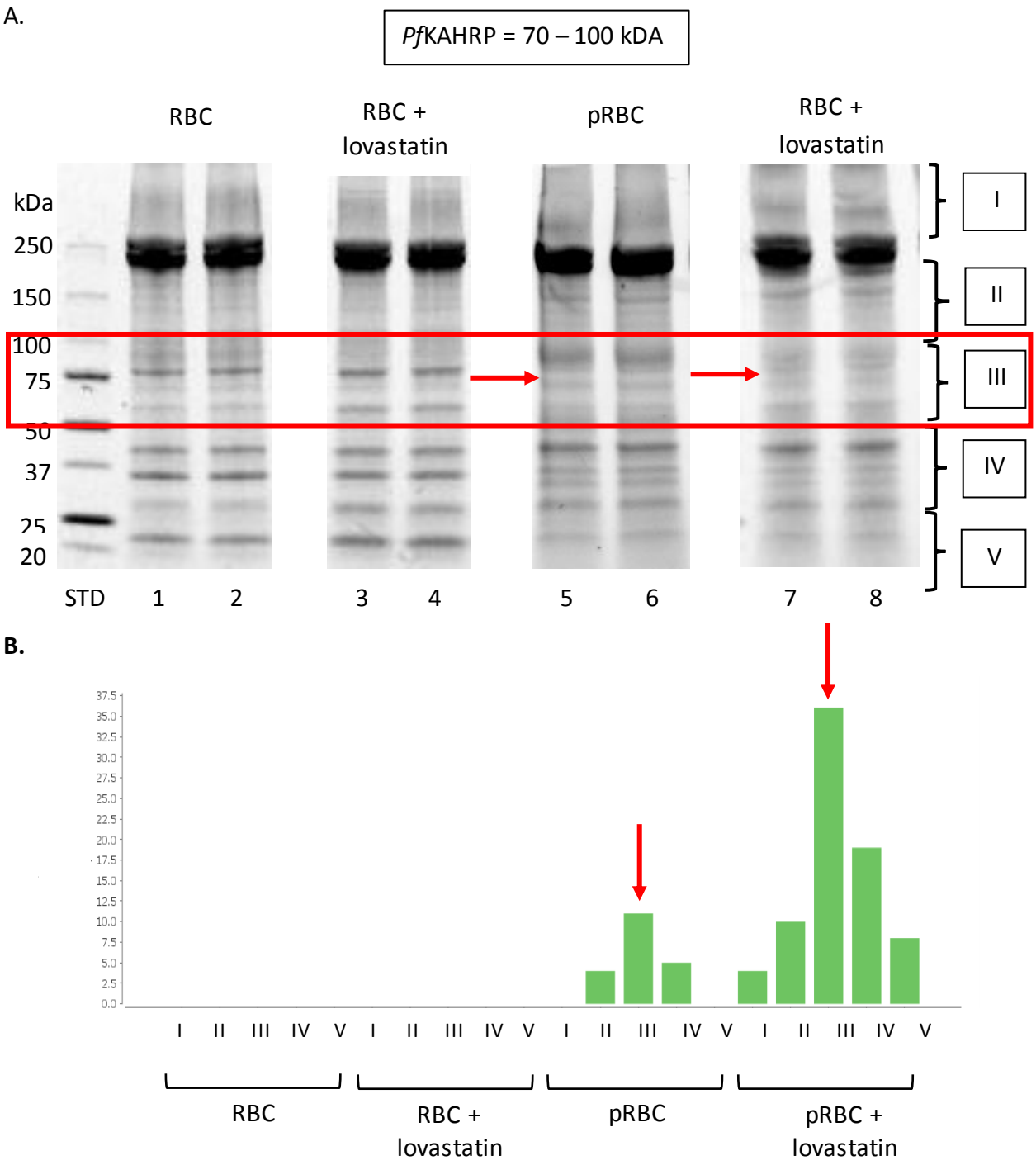


Figure 3.19: Stain Free imaging of a SDS-PAGE protein mass fingerprint of ghost proteins from untreated and treated normal red blood cells (RBCs) and parasitised RBCs (pRBCs) with lovastatin. The abundance of *Pf*KAHRP in all sample groups is indicated using the number of spectra. (A) Lanes 1-2: Fresh untreated RBCs; Lane 3-4: treated RBCs with 10 µM of lovastatin; Lane 5-6: parasitised RBCs at the late trophozoite stage and Lane 7-8: parasitised RBCs at the late trophozoite stage that were treated with 10 µM of lovastatin. Laemmli sample buffer was added to all samples and SDS-PAGE of proteins was carried out on a 4-20% Criterion gel at a constant 60 V. (B) Number of spectra for *Pf*KAHRP in each excised gel band as indicated on the right of the gels in (A) for the different sample groups. Numbered Fractions (I-V) are indicated on the bar graph for the different sample groups.

Label-free experiments need to be very tightly controlled for quantitative proteomics and a number of factors need to be taken into consideration before conclusions can be drawn from the above set of results. Label-free experiments may be adequate for total protein identification, however when looking at differential protein expression in four different samples, technical variation may play a significant role in data analysis. For example, the fact that a higher protein level was seen with proteins in the treatment group when compared to the untreated group may be attributed to the fact that SDS-PAGE gels for each comparison were run after different storage time intervals and some gels were stored for a longer period of time than other gels before in-gel trypsinisation and nano LC-MS/MS analysis were completed. This occurred because each of the four sample groups were prepared and collected at different times during the study. Protein loss, to which low abundant parasite proteins are more susceptible, during gel storage may be one of the reasons for the lower protein level in the untreated parasitised RBC sample group as this set of samples were isolated before treated samples and consequently stored for a longer period of time. It can therefore not be confidently claimed that lovastatin had a stimulatory effect on protein expression. Furthermore, even though spectral counting can be used to fairly accurately measure the overall level of protein abundance, as well as identify large protein differences between compared samples, it has been shown that spectral counting has limitations when attempting to measure relatively small changes between protein expression (Liu *et al.*, 2004). This suggests that the use of spectral counting would not be sensitive enough to measure small proteomic changes induced by the treatment with lovastatin. Lastly, samples were run in triplicates on SDS-PAGE gels but were only analysed in a single run by nano LC-MS/MS due to limited resources. It has been shown that improved coverage of lower abundant proteins, such as *Pf*EMP1 and *Pf*EMP3, can be achieved by increasing the number of LC-MS/MS analytical runs. Therefore because proteomic assessments were analysed using single LC-MS/MS runs per treatment, it cannot be established whether the reason for identifying *Pf*EMP1 and *Pf*EMP3 with a low confidence in the untreated parasitised RBCs sample group was due to technical or biological variance. It also needs to be taken into consideration that during LC-MS/MS analysis, parasite protein identification can be obscured by the presence of excessive host proteins which may potentially interfere with the identification of parasite analytes of interest. Furthermore the presence of highly abundant host proteins and associated

changes in the host proteome could not only limit the detection of the number of parasite proteins that can be found in the large dynamic range of host verses parasite protein composition but the difference in level of abundance may also potentially conceal any noticeable changes in the expression of low abundant parasite proteins such as *Pf*EMP1 and other parasite proteins.

Consequently, the effects of lovastatin on proteins such as *Pf*EMP1 and *Pf*EMP3 could not be determined. As a result of these technical variabilities, the spectral counting approach was not found to be robust enough for the monitoring of differential protein expression between sample groups. The effects of lovastatin on the upregulation or downregulation of cytoadhesive related proteins could therefore not be confidently reported or established in this study.

However, it can be stated that the chosen proteomics analysis method was sufficient to monitor proteomic changes with regards to total presence and absence of proteins on membranes of treated parasitised RBCs. It was established that lovastatin did not inhibit the expression of cytoadhesive related parasite proteins such as *Pf*CLAGs, *Pf*KAHRP, *Pf*EMP1, *Pf*EMP3, and *Pf*MESA as all of these proteins were identified on the membranes of parasitised RBCs that were treated with lovastatin. It may be speculated that lovastatin may not exert any effects on the parasite's ability to express these proteins on the membranes of parasitised RBCs and the inhibitory effects on cytoadhesion that have been reported in literature may be the result of the ability of lovastatin to downregulate receptors that are involved in cytoadhesion on the host endothelium. As protein abundance was not optimally assessed and that there were few replicates used in this study, the possibility of downregulation of parasite proteins that are involved in cytoadhesion cannot be excluded at this stage.

Chapter 4: Final Conclusions and Recommendations

4.1 Discussion and Conclusions

Optimised culturing methods along with microscopy analysis and mass spectrometric proteomic techniques were utilised to characterise some parasite induced proteomic changes that occur on membranes of parasitised RBCs at the late trophozoite stage. The primary aim of the study was to make use of advanced analytical techniques to identify *P. falciparum* proteins that are known to contribute to the pathogenesis of severe malaria by facilitating the cytoadherence of parasitised RBCs to the endothelium of the human host which ultimately results in the parasite evading an immune response and splenic clearance. The effects of physiologically relevant concentrations of lovastatin on the expression of cytoadhesion related proteins were then assessed with the goal of providing new insight and extending existing knowledge pertaining to the potential use of statins as an adjunctive therapy for severe malaria. The use of statins as an adjunctive therapy, particularly lovastatin, would be a vital tool in the treatment of severe malaria as it could lead to a decrease in the severity of symptoms as it has been shown that drugs from this drug class have the ability to impede features of the life threatening syndrome associated with severe malaria. This could consequently lead to a significant decrease in the high mortality rate among individuals who suffer from severe malaria.

Culturing of the *P. falciparum* 3D7 laboratory strain was a fundamental step in this study where culturing at high parasitaemia levels was optimised, tightly synchronised cultures were maintained and a high percentage enrichment of parasitised RBCs at the late trophozoite stage was successfully achieved. Attainment of sorbitol synchronised cultures that were magnetically isolated using the Vario Magnetic Cell Sorter (VarioMACS) was confirmed by assessing blood smears, which were prepared from parasite cultures, using light microscopy. Morphological differences between normal RBCs and parasitised RBCs were visualised using scanning electron microscopy (SEM) that confirmed that the reported features that systematically decrease during repeated laboratory culture were still observed. Erythrocyte ghost isolation from non-parasitised and parasitised RBCs was optimised and protein separation was carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Parasite induce alterations to the

membrane of parasitised RBCs were visualised as band differences when comparing protein mass fingerprints to a control mass fingerprint comprising of isolated ghost proteins from normal uninfected RBCs. Effects of lovastatin on both host and parasite protein expression were also visualised as band differences via comparative analysis of separate protein mass fingerprints from four different sample groups; RBCs, RBCs that were treated with lovastatin, parasitised RBCs and parasitised RBCs that were treated with lovastatin. Fractions that included protein bands of interest were excised, in-gel trypsinised and successful peptide sequencing was carried out using nano liquid chromatography tandem mass spectrometry (LC-MS/MS) to determine total presence and absence of proteins. The obtained sequence data was used to identify proteins by comparison to both human and *Plasmodium* protein sequence databases which positively identified many expected proteins despite being present in very low abundances. Monitoring proteomic changes in terms of protein abundance changes that occurred as a result of the parasite's presence within the RBC and also the susceptibility of protein expression to the treatment with lovastatin met with mixed success. Separate whole lane digestion of the four different sample groups was carried out and analysed by nano LC-MS/MS in an attempt to show quantitative proteomic differences utilising spectral counting.

The outer surface of parasitised RBCs was clearly visualised and compared to non-parasitised RBCs using SEM. Significant changes in cell shape and appearance, as a result of the intracellular parasite was observed. Additionally, a large number of distinct parasitic knob structures were observed to be distributed throughout the surface of parasitised RBC. This was a positive result as it implied an increased probability of the presence of a number of cytoadhesive relative parasite protein drug targets as these parasite antigens are known to be located on the surface of parasitic knobs.

Isolation of RBC ghost from both non-parasitised RBCs and parasitised RBCs for SDS-PAGE was successfully carried out. The preparation of RBC ghosts from whole blood and parasitised RBCs that were isolated from parasite cultures was optimised and this effectively minimised the presence of highly abundant intracellular content such as haemoglobin and parasite residues located within parasitised RBCs. Further reduction of sample complexity prior to LC-MS/MS analysis was achieved by separating ghost proteins using SDS-PAGE

where reproducible protein mass fingerprints of RBC surface and cytoskeletal proteins, originating from both the host and parasite, were obtained. After method optimisation, it was established that 20 µg of protein per a well for both non-parasitised and parasitised RBCs was sufficient to generate high resolution protein mass fingerprints that could be used for comparative analysis. The use of a non-selective protease inhibitor, such as phenyl methylsulfonyl fluoride (PMSF), was found to be very important and the requirement was clearly demonstrated when the inhibitor was excluded from the sample preparation of parasitised RBC ghosts. Significant absence of high molecular weight proteins indicated extensive protease activity which was assumed to be a result of proteases originating from the *Plasmodium* parasite. These findings indicated and further highlighted that the inclusion of a protease inhibitor is crucial when preparing samples for downstream proteomic analysis in order to avoid making inaccurate inferences with regards to total protein concentration or relative protein abundance. Gels were imaged using Stain Free software, Coomassie brilliant blue (CBB) stain and silver stain. Stain Free imaging was found to be comparable and as sensitive as CBB stain as no major visual band differences were observed when comparing the stained gels. Silver stain resulted in a greater number of stained bands as silver stain additionally stains both lipids and sialoglycoproteins however, due to MS/MS incompatibility, silver stained bands were not subsequently analysed with LC-MS/MS.

By using RBC ghosts, both the major RBC membrane and cytoskeletal proteins as well as the membrane inserted parasite proteins of parasitised RBCs were successfully identified on gels by visual inspection followed by protein identification of proteins in selectively excised fractions using in-gel trypsinisation of alkylated proteins followed by nano LC-MS/MS and database searching. The presence of all the key host RBC membrane proteins post infection confirmed that the presence of the intracellular parasite does not significantly affect the expression of host proteins and that host proteins remain the principal components of the RBC membrane even after invasion by the parasite. Most of the known proteins that play a role in cytoadhesion were identified in this study. Proteins that are involved in parasitic knob formation and that are associated with these knob structures were successfully identified and include *P. falciparum* knob associated histidine rich protein (*PfKAHRP*), *P. falciparum* mature parasite infected erythrocyte surface antigen (*Pf*MESA) as well as *P. falciparum* cytoadherence linked asexual gene (*Pf*CLAG) proteins. Most parasite proteins

were identified at their reported mass in the gels but *Pf*CLAG proteins were an exception as they were found at higher mass ranges than expected, which suggested incomplete gel migration of these proteins or even possible cross linkage with other proteins.

Both non-parasitised and parasitised RBCs were treated with 10 µM of lovastatin for 48 hours. After analysing protein mass fingerprints of isolated ghosts from both untreated and treated sample groups, band differences were visually observed on both Stain Free and CBB stained gels. However when analysing these band differences using LC-MS/MS it was established that all relevant RBC membrane host and parasite proteins were found in treated sample groups thus indicating that lovastatin did not inhibit the expression of these proteins. Moreover, the band differences were most likely a result of protein changes that did not involve the proteins that were under investigation in this study. It was not expected that lovastatin would have an effect on host RBC membrane protein expression, however the fact that no observed changes with regards to protein inhibition were seen for cytoadhesive related proteins, which included *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) which is a major determinant of cytoadhesion, indicated that although lovastatin has been shown to impede ligand-receptor interactions involved in cytoadhesion the mechanism involved does not include the total inhibition of parasite protein cytoadhesive ligands.

A limitation of excising bands of interest on SDS-PAGE that are only visually apparent on Stain Free gels is that protein bands, that may be present in a low abundance and consequently not be clearly visible on the gel, may be missed or excluded when assessing band differences. Therefore in order to prevent the biased analysis of only high abundant protein bands that were clearly visible on gels, whole lane digestion was carried out for each sample. This method ensured that global protein identification and characterisation was achieved. Fractions of whole lane digestions were analysed by nano LC-MS/MS and acquired data was successfully used for protein monitoring between samples. The results confirmed that inhibition of all cytoadhesive related proteins was not induced by treatment of lovastatin.

A general move from descriptive proteomics to that of quantitative proteomics has become a prime focus in the field of proteomics. The assessment of relative abundance of proteins was carried out by using spectral counting which correlated to protein abundance by using the number of peptide to spectrum matches (PSM) obtained from a single LC-MS/MS run. This label-free method to quantify protein abundance is a widely used technique, however the limitations of this approach in the absence of stringent experimental controls were clearly demonstrated in this study. The lack of sufficient biological and technical replicates as well as the degree of technical variability that was not controlled for affected the reliability of the data obtained from this method and in turn prevented the assessment of differential protein expression of low abundance proteins between sample groups. An alternative method is thus required to assess and validate whether treatment with lovastatin has a significant effect on the relative abundance of cytoadhesive related proteins, as a decrease in abundance of these proteins may potentially lead to a reduced rate of cytoadherence, however additional studies would be needed to confirm this hypothesis.

This research project consisted of distinct aims and well-defined study objectives that were each successfully completed by using an advanced research approach and powerful analytical tools. Alterations that occur on membranes of parasitised RBCs at the late trophozoite stage of the *P. falciparum* life cycle were successfully characterised by utilising three dimensional microscopy that resulted in great visual imaging. Proteomic changes on membranes of parasitised RBCs and assessement of therapeutic concentrations of lovastatin on the total expression of identified proteins was efficiently carried out by generating reproducible, high resolution protein mass fingerprints whereby distinct band differences were clearly observed and were consequently used to effectively and reliably monitor proteomic changes. These results were further confirmed by cutting-edge analytical equipment and software that generated high quality mass spectrometry data. The data obtained in this study illustrated the capabilities of advanced technology that is currently available in the rapidly growing field of proteomics, where a majority of the low abundant *P. falciparum* parasite proteins were successfully identified in the presence of high abundant host proteins. Furthermore, this study highlights the potential of integrating pharmacological based studies and proteomic techniques whereby changes in protein

expression and consequently biological changes due to drug action at physiologically relevant drug concentrations can be effectively assessed by making use of innovative proteomic techniques. The results from this study can be used to exclude the fact that lovastatin inhibits parasite proteins involved in certain pathological processes involved in severe malaria and therefore redirect research efforts towards exploring the possibility that lovastatin may exert its activity within the host by down-regulating host adhesion molecules. Also the possibility that lovastatin may decrease the relative protein abundance of parasite protein expression involved in cytoadhesion should not be excluded. Overall, this research has provided an improved insight in the assessment of the potential use of statins as an adjunctive therapy thus resulting in a step closer in developing or initiating a new therapeutic approach for patients who have been diagnosed with severe malaria with the end goal of alleviating the suffering caused by this life threatening disease.

4.2 Study Limitations and Recommendations

Experimental methods and workflows were optimised and implemented to successfully achieve the research aims and objectives of this study, however there are some aspects that have limitations and could be improved upon with the following recommendations.

One of the limitations found in this study was the loss of protein from bands on SDS-PAGE gels due to storage of gels for prolonged periods of time. This resulted in protein diffusion, potential crosslinking or decomposition from the gels where it appears that faint bands associated with low abundant proteins and even proteins with a low molecular mass were apparently highly susceptible. This in turn resulted in diminished peptide extraction and poor sequence coverage of key parasite proteins such as *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) during LC-MS/MS analysis. Low confidence of protein identification of specific proteins of interest prevented the relative quantitation of protein for comparative analysis between controls and treatments due to invalid or absent MS/MS data. Thus, the importance of preventing protein loss from gels following SDS-PAGE analysis cannot be emphasised enough and a recommendation would be to cut out and digest bands

immediately after electrophoresis or at least as soon as possible to prevent significant protein loss.

Silver stain was used as a staining technique in this study and was found to be superior to Stain Free and Coomassie brilliant blue in terms of staining a higher amount of protein bands. The silver stain is known for its higher sensitivity however due to incompatiblity with mass spectrometry, bands of interest were not subsequently analysed by LC-MS/MS. It is therefore proposed that the use of different stains such as Flamingo or SYPRO Ruby fluorescent gel stains should be used instead, as these fluorescent stains have a similar sensitivity to that of silver stain with an added advantage of being highly compatible with mass spectrometry.

Despite the fact that extensive proteomic data was obtained in this study, a targeted approach with regards to data analysis was carried out where focus was only placed on proteins that had a defined role in cytoadhesion according to literature sources. This proved to be an effective method as it meant that focus was placed on known drug targets for severe malaria. However because a large part of the data was excluded it may be suggested that this approach may impart some bias and potentially lead to an oversight in identifying unknown proteins that may play a part in cytoadhesion. Therefore an improvement of the study would be to re-analyse the obtained data by making use of advanced proteomic software tools to look at global proteomic changes and also to highlight potential biological pathways that may be upregulated or down-regulated during the late trophozoite stage. This would in turn provide a more comprehensive view of the parasite's ability to induce cytoadhesion. Furthermore it would provide insight into the protein expression state of the malaria parasite in response to drug treatment whereby a thorough assessment of the inhibitory effects of lovastatin on cytoadhesion related proteins would be achieved.

Spectral counting is a relatively easy method that can be used for protein quantitation, however it is also one of the least sensitive methods when compared to other label free techniques, whereby small changes in protein expression may not be efficiently detected. It is therefore recommended that different label free methods such as multiple reaction monitoring (MRM) or sequential window acquisition of all theoretical fragment ion spectra

(SWATH) be used for protein quantitation instead, as these methods have greater senstivity that is needed to successfully monitor differential protein expression. An added advantage of these approaches includes increased specificity as these techniques allow for targeted analysis of proteins of interest and avoids huge amounts of data that usually results from non-targeted analysis.

Alternatively, It is proposed that this study could be further improved upon by making use of a stable isotope labelling technique for the quantitative proteomic method instead of the label-free approach that was used in this study. As the *P. falciparum* parasite is cultured *in vitro*, a suitable labelling method known as stable isotope labelling with amino acids in cell culture (SILAC) would be an appropriate choice when compared to the above mentioned label free methods. Changes, with regards to abundance, in the proteome of parasitised RBCs at the late trophozoite stage followed by treatment with lovastatin could be successfully monitored by this metabolic labelling approach. The advantages of SILAC is that high accuracy and reproducibility are achievable as labelled and unlabelled samples are distinct from each other and can therefore be combined at a very early stage of sample preparation which may be at the cell culture level or at the latest before RBC ghost preparation step. This would in turn result in less technical variances being introduced in sample preparation before LC-MC/MS analysis. Therefore this labelled approach would be the ideal solution to the very experimental factors that limited successful protein quantitation in this study.

With regards to the assessment of statins as potential anti-cytoadhesion therapy for severe malaria, it is recommended that further studies include a wider range of therapeutic concentrations to be tested on parasite protein expression. As it may be the case that statins do in fact inhibit the expression of cytoadhesive related parasite proteins but this inhibition may only be noticeable at higher drug concentrations. Literature has identified other commonly used statins, such as atorvastatin, as a potential adjunct to antimalarial therapy and so it is suggested that future studies also include the assessment of other agents from this drug class as it may be possible that alternate statins might have a greater inhibitory potential on cytoadhesion when compared to lovastatin.

This study may be expanded by exposing laboratory cultured endothelial cells (e.g. Human lung endothelial cells (HLEC)), that positively express adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) which are specific ligands for *Pf*EMP1, to parasitised RBCs. This would provide an ideal platform to further investigate host-parasite interactions involved in cytoadhesion. Furthermore, the level of cytoadhesion could be assessed after pre-treating cells with statin therapy followed by exposure to parasite cultures. Proteins from host-parasite interactions could then be isolated and protein expression could subsequently be assessed by utilising the exact proteomic techniques employed in this study. In contrast to this study, proteomic assessment would be done after exposure of parasitised RBCs to host adhesion molecules thus providing a comprehensive experimental model of molecular mechanisms associated with cytoadhesion.

This study has provided a basic platform for the assessment of the statins as potential treatment to avoid the serious symptoms of severe malaria but failed to provide solid proof that the drug would work. However if this study is expanded upon, and if the above recommendations and improvements are taken into consideration it would undoubtedly provide a foundation for the definitive assessment of whether statins could be used as a new drug for adjunctive therapy for decreasing severe malaria symptoms. An improved study would further provide an experimental model on which existing or new drugs can be tested, thus ultimately leading to the development of an effective complimentary treatment for severe malaria.

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Appendix

Letter of Ethical Approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

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Faculty of Health Sciences Research Ethics Committee

12/09/2013

Approval Certificate
New Application

Ethics Reference No.: 304/2013

Title: Characterisation of Plasmodium falciparum proteins expressed on infected red blood cell surfaces as potential drug targets for severe malaria therapy.

Dear Mrs.C Pillay

The **New Application** as supported by documents specified in your cover letter for your research received on the 01/07/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 11/09/2013.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (304/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

DR R SOMMERS; MBChB; MMed(Int); MPharmMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee
University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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