Plasmodium falciparum infected erythrocyte induced modulation of host endothelial cells

Thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

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DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Basim Ahmed Othman

This thesis is a product of my own work which has been carried out during my PhD study in the Department of Parasitology, Liverpool School of Tropical Medicine, University of Liverpool, between November 2013 and September 2017. All the experiments presented in the result chapters were performed by me under the supervision of my supervisors, Professor Alister Craig, Dr Britta Urban and Dr Simon Wagstaff. The thesis was written by me with their guidance.

DEDICATION

To my Mum and Dad

To my lovely wife

To my sons

For everything

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May Allah protect me and all my family from the troubled times, the hardships, trials, tribulations. May Allah give me, my parent, my wife, my kids, my brothers and my sisters health, prosperity and goods.

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ABBREVIATIONS

ACTs	Artemisinin-based combination therapies
AGEs	Advanced glycation end products
ALPL	Alkaline phosphatase, liver/bone/kidney
Ang-1	Angiopoitin-1
ANO9	Anoctamin 9
AP1	Activator protein 1
AQP1	Aquaporin 1
ASK1	Apoptosis signal regulation kinase 1
BBB	Blood brain barrier
BSA	Bovine serum albumin
C3	Component 3
CIDRα	Cysteine-rich interdomain region
CSA	Chondroitin sulphate A
cm^2	centimetre squared
CM	Cerebral malaria
CMTM1	CKLF like MARVEL transmembrane domain containing 1
CO_2	Carbon dioxide
CPM	Carboxypeptidase M
CR1	Complement receptor 1
CSF2	Colony stimulating factor 2
CSF	Cerebrospinal fluid
CYP1A1	Cytochrome P450 family 1 subfamily A member 1
CYP1B1	Cytochrome P450 family 1 subfamily B member 1
DAVID	Database for Annotation Visualization and Integrated Discovery
DBLα	Duffy binding like domains
DCs	Dendritic cells
dl	decilitre
DNA	Deoxyribonucleic acid
DUSP1	Dual-specificity phosphatase-1
ECM	Extracellular matrix
ECs	Endothelial cells
EPCR	Endothelial protein C receptor
ERK1/2	Extracellular-signal-regulated kinase1/2
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FC	Fold change
FDR	False discovery rate
FPKM	*Fragments Per Kilobase of exon per Million fragments mapped*
g	gram
GADD45B	Growth arrest and DNA damage inducible beta
GO	Gene Ontology
GROα	Growth-regulated gene-alpha
H2O	Water
HBB	Haemoglobin subunit beta

HBMEC	Human brain microvascular endothelial cells
HDMEC	Human dermal microvascular endothelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HIV	Human immunodeficiency virus
HLH	Helix-loop-helix
HO-1	Haem oxygenase-1
HRVs	Human rhinoviruses
HSV-2	Herpes simplex virus type 2
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
ID1	Inhibitor of DNA binding 1
ID2	Inhibitor of DNA binding 2
ID3	Inhibitor of DNA-binding/differentiation 3
IFNγ	Interferon γ
IL-1β	Interleukin 1 β
IL-8	Interleukin-8
iNOS	inducible NOS
iRBC	infected red blood cell
JNK	c-Jun N-terminal kinase
KAUST	King Abdullah University for Science and Technology
kDa	kilodalton
LAD1	Ladinin 1
LFA-1	Leukocyte function-associated antigen
Log2FC	Log2 fold change
LSTM	Liverpool School of Tropical Medicine
LU	Lutheran molecule
M	Molar
mAb	monoclonal antibody
Mac-1	Macrophage-1 antigen
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractants protein-1
mg	milligram
ml	millilitre
mm^2	millimetre squared
mM	millimolar
MORN1	MORN repeat containing 1
mRNA	messenger ribonucleic acid
MS	Multiple sclerosis
NCAM	Neural cell adhesion molecule
NF-κB	Nuclear factor kappaB
ng	nanogram
NGS	Next generation sequencing
NO	Nitric oxide
NOG	Noggin
NPTX1	Neuronal pentraxin 1
°C	Degrees celsius
P	Passage

P_1	Counted parasitemia
P_2	Required parasitemia
PAM	Pregnancy associated malaria
PAR1	Protease-activated receptor 1
PARPBP	PARP1 binding protein
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PECAM-1	Platelet EC adhesion molecule 1
PfEMP-1	*P. falciparum* erythrocyte membrane protein
PfGPI	Glycosylphosphatidylinositol
PH	Power of hydronium
PLA2G4A	Phospholipase A2 group IVA
PRBC	*P. falciparum* infected erythrocyte
PV	Parasitophorous vacuole
qRT-PCR	Real-time reverse transcription polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RBC	Red blood cell
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per min
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
SAM	Sequence alignment map
sCAMs	soluble cell adhesion molecules
SHBG	Sex hormone binding globulin
SM	Severe malaria
sRBC	sickle cell RBC
SREBF	Sterol Regulatory Element Binding Transcription Factor
TCA	Citric acid
TGF- β	Transforming growth factor- β
TJPs	Tight-junctional proteins
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNS	Trypsin Neutralization Solution
TRX	Thioredoxin
TSP	Thrombospondin
TSS	Transcription start site
TXNIP	Thioredoxin interacting protein
UCP3	Uncoupling protein 3
UK	United Kingdome
UM	Uncomplicated malaria
ups	upstream sequence
USA	United State of America
V_1	Evaluated culture volume
V_2	Required volume
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

VIPR1	Vasoactive intestinal peptide receptor 1
WHO	World Health Organization
wRBC	Washed red blood cell
xg	Times gravity
YM	Incomplete parasite culture media
ZNF345	Zinc finger protein 345
μg	Microgram
μl	microliter
μm	Micormeter
μM	Micromolar
%	Percentage
/	Per

Thesis title: *Plasmodium falciparum* infected erythrocyte induced modulation of host endothelial cells

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ABSTRACT

Cerebral malaria is one of the complications of the broad clinical syndrome named 'severe malaria'. The pathogenesis of cerebral malaria is thought to be due to the ability of infected red blood cell (iRBC) to sequester in the blood microvasculature of vital organs. Sequestration, resulting from the interaction (often called cytoadherence) between parasite proteins expressed on the surface of iRBC such as *PfEMP-1,* and host endothelial cell adhesion molecules such as ICAM-1, CD36 and EPCR, can modulate the downstream effects of several biological processes in the endothelial cells such as EC activation, intracellular signalling, endothelial cell permeability and endothelial apoptosis.

The aim of this thesis is to gain a better understanding of how the *P. falciparum* parasite can modulate the gene expression of the host endothelial cells response to cytoadherence using the RNA-seq technique. We used an *in vitro* co-culture model of IT4var14 strain with HBMEC in the presence of 10ng/ml TNF. RNA-seq analysis of HBMEC transcriptome following co-culture showed significant differential regulation of genes which defined gene ontologies such as 'immune system' at 2 hours, 'immune response', 'arachidonic acid', 'positive and negative apoptotic pathways' at 6 hours, and 'immune response' and 'cell cycle pathways' at 20 hours of co-incubation. However, another co-culture model using IT4var37 isolate with HBMEC in the presence of TNF found that exposure of HBMEC to iRBC expressed genes in brain EC often in opposite levels compared to the IT4var14 strain under the same condition. These findings emphasize the ability of different *PfEMP-1* variants from the same genetic background (IT4) to modulate different gene expression in host cells, and also the potential ability of the malaria parasite to use *PfEMP-1 var* gene switching technique to protect itself from the host defensive system.

We conducted further co-culture experiments to assess the ability of IT4var14 parasite to modulate the transcriptional levels of different endothelial cells (HDMEC and HBMEC) that differentially express ICAM-1 and CD36 receptors; we demonstrated that there are similarities and differences in gene expression between TNF-stimulated HDMEC and HBMEC exposed to IT4var14 strain, which might be due to ICAM-1 expression in both ECs but the lack of CD36 on HBMEC, leading to the transduction of different signalling pathways in HDMEC and HBMEC. The ability of TNF stimulation in modulating the expression of genes in HBMEC exposed to IT4var14 parasite isolate, and uninfected erythrocytes on endothelium has also been examined in this study. It was found that the TNF plays a role in mediating the gene expression in human brain EC incubated with IT4var14 strain and uninfected RBC, this might be due to stimulation of TNF to HBMEC to induce expression of ICAM-1. These results are presented and discussed in the thesis with an aim to better understanding of the CM syndrome and the potential to lead to the discovery and development of new therapies for cerebral malaria.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview:

Malaria is an ancient vector-borne infectious disease transmitted via female *Anopheles* mosquitos and is caused by protozoa of the genus *Plasmodium*. It is one of the main tropical diseases distributed across many parts of the world. It remains one of the leading causes of morbidity and mortality in tropical areas especially in countries with limited resources (Bruce-Chwatt, 1987, Murray et al., 2012, Guerin et al., 2002).

Human malaria can be caused by various *Plasmodium* species including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* (Sutherland et al., 2010) and, lastly, *Plasmodium knowlesi* that has been recently found to infect humans, particularly in Malaysia (William et al., 2013).

This chapter reviews knowledge about malaria disease, parasite life cycle, clinical manifestation of malaria and malaria pathogenesis.

1.2 Malaria as a disease:

1.2.1 Malaria history:

Historically, malaria disease has been noted for more than 4000 years. It was initially described in ancient Chinese medical document by *Nei Ching* from around 2700 BC. The disease was widely recognized by the Greeks in the 400 BC where Hippocrates wrote about the characteristic symptoms of malaria (Cox, 2010). The word malaria derives from the Italian "mal ' aria" which means "bad air"; However, this was controversial until significant developments in research on malaria that allowed the identification of the main causative agent and the transmission vector at the end of the 19th century. In 1880, a French army surgeon called Charles Laveran discovered

the malaria parasites, whereas the initial evidence that mosquitos transferred *Plasmodium* parasite to humans was identified by Ronald Ross in 1897 (Cox, 2010).

1.2.2 Malaria epidemiology:

In 2013, an estimated 3.3 billion people around the world were at risk of malaria infection (WHO, 2014), with varying percentages of at risk populations between countries (Figure 1.1). The latest report from the World Health Organization (WHO) estimated number of worldwide malaria cases at 214 million and estimated malaria death numbers at 438.000 people (WHO, 2015). This represents a 37% reduction in malaria reported cases and a 60% reduction in mortality rate since 2000, due to widespread malaria eradication programmes. Most of the malaria cases and deaths take place in African regions with an average of 88% and 90% respectively, and more than two-thirds of these cases occur in children under five years old (WHO, 2015). The vast majority of morbidity and mortality for malaria globally is linked to *P. falciparum*, but mainly in tropical and subtropical areas in Africa (Snow et al., 2005).

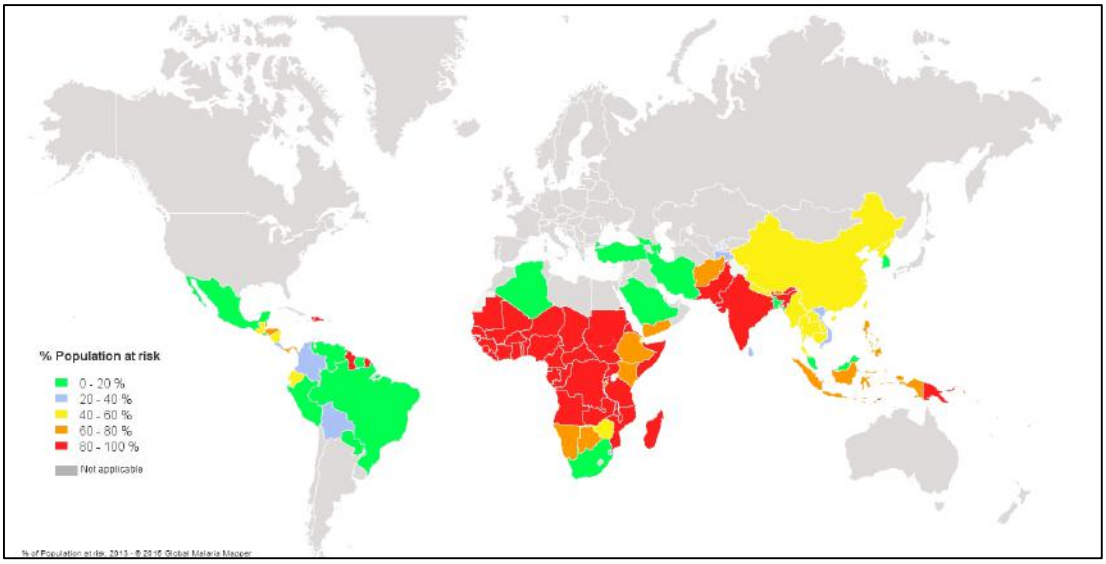


Figure 1.1: Global population at risk of malaria infection, 2013.
Global map shows classification of countries by percentages of population at risk of malaria infection in 2013 (Adapted from WHO, 2014).

1.2.3 Malaria control and prevention

There are several approaches that can be used to control and prevent distribution of malaria disease that include transmission interruption, vector control, treatment and vaccines (Chambers, 2012). To reduce the transmission of malaria disease, insecticide spraying and treated bed nets are largely used as vector control techniques that preventing bites from mosquitos. Although these techniques are reliable and have some effectiveness, mosquitos may develop resistance against insecticide and bed nets must to be replaced regularly (Liu, 2015). Artemisinin-based combination therapies (ACTs) are a standard treatment for the clinical malaria cases, which has an enhanced effect on reducing severe malaria (SM) and death (Dondorp et al., 2010). Of these strategies, the most successful approach has been the insecticide-treated bed nets with 68% decline in cases between 2000 and 2015 (Bhatt et al., 2015).

Implementation of insecticide spraying and ACT at a larger scale and continued use of insecticide-treated bed nets will be required to further reduce malaria prevalence. However, there is an increase in threats of using these programmes with the emergence of insecticide resistance (Ranson and Lissenden, 2016) and growing resistance against the artemisinin in South East Asia (Fairhurst, 2015). New insecticides and drugs are required but new strategies such as the development of effective vaccines will be required to induce the likelihood of malaria control and elimination (Hemingway et al., 2016).

1.3 *Plasmodium falciparum* life cycle:

The malaria parasite has a complex life cycle requiring two hosts (mosquito and human) (Figure 1.2). In the female *Anopheles* mosquitos, sexual reproduction of the parasite takes place, while asexual replication occurs in human host. Infection of the malaria begins when an infected mosquito vector injects sporozoites which are stored

in its salivary glands into the skin of the human host during feeding. Then, sporozoites migrate via blood vessels to the liver where they mature within the hepatic cells over a short period around (5-14 days) including multiple rounds of asexual multiplication to produce thousands of merozoites (Hansen et al., 2014). Merozoites are released after rupturing the hepatocyte in vesicles termed merosomes (Sturm et al., 2006), which protect merozoites until they reach into bloodstream (Baer et al., 2007).

In the bloodstream, within seconds of releasing merozoites, they invade the erythrocytes in a complex process involving multiple parasite-host protein interactions that can be divided into four stages: primary attachment and reorientation, formation of irreversible attachment tight junction, merozoite invasion by an actin-myosin motor and completion by resealing of the RBC membrane and shedding of merozoite surface proteins (Beeson et al., 2016). Within the erythrocyte, the parasite starts an asexual intra-erythrocytic development cycle within the parasitophorous vacuole (PV) involving an early ring trophozoite, late trophozoite, developing schizont and developed schizont stages (Delves et al., 2012). Rupture of the infected red blood cell (iRBC) and release of merozoites takes place in synchronisation after every 24 hours for *P. knowlesi*, 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, and 72 hours for *P. malariae*, and contributes to inflammatory response and clinical signs and symptoms observed (White et al., 2014). However, a small proportion of iRBC differentiate into male and female gametocytes that circulate in the bloodstream.

The mechanism of development of the parasite into gametocytes remains not clear, but may involve parasite density (Liu et al., 2011). During the blood feed, the gametocytes move to the mosquito midgut where iRBCs are digested and

gametocytes released, and sexual development occurs. Both male and female gametes are fertilized to form diploid zygotes that then develop into motile forms called ookinetes, which then travel into the mosquito midgut epithelial wall and differentiate into oocysts which produce thousands of sporozoites (Miller et al., 2013). Once the oocysts rupture, sporozoites are released and invaded the mosquito body cavity and migrate to the salivary glands where they reside until the mosquito takes a human blood meal. This completes the transmission cycle of the malaria parasite to the next human host (Smith et al., 2014).

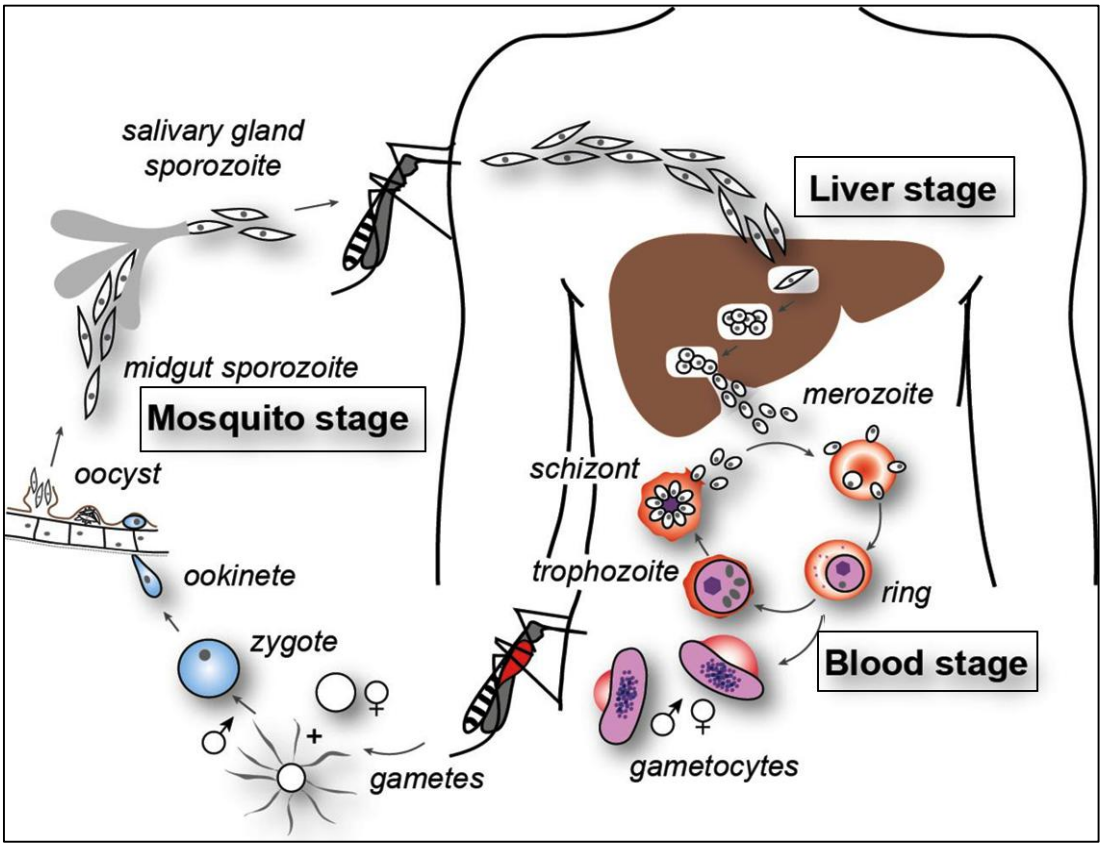


Figure 1.2: The life cycle of P. falciparum in mosquito and human.
Life cycle stages in the mosquito and human hosts. See text above for a detailed description. Figure adapted from (Cowman et al., 2012).

1.4 Clinical manifestations of malaria:

Individuals infected by malaria have a range of clinical outcomes. In an area of exposure to malaria infection, in early life, it starts usually with severe manifestations; around 10% of children develop severe malaria symptoms with different pathological impacts. Then, immunity is developed to severe symptoms in those individuals and they suffer only from uncomplicated malaria (UM), which is also referred to as mild malaria, particularly in areas with highly endemic such as Sub-Saharan Africa. As immunity develops further, clinical disease is rarely seen and cases are referred to as asymptomatic.

1.4.1 Uncomplicated malaria:

Generally, UM is characterised with several signs range from headaches, fatigue, muscle aches and abdominal discomfort, followed by vomiting, nausea orthostatic hypotension (White et al., 2014).

1.4.2 Severe malaria:

The vast majority of severe malaria cases caused by *P. falciparum* are associated with age dependent symptoms, particularly in high-transmission areas. SM in children consists three overlapping syndromes: severe malarial anaemia, metabolic acidosis/ respiratory distress and cerebral malaria (CM). In adults, in addition to CM and acidosis, acute pulmonary oedema, jaundice and acute renal injury are also classified as most common features of SM (White et al., 2014).

1.4.2.1 Severe anaemia:

Malaria is considered as one of the major cause of severe anaemia in young children, especially in areas with high transmission where repeat infection often occurs. Patients with severe anaemia are defined as haemoglobin concentration < 5 g/dl of blood in the presence of *P. falciparum* parasitaemia (Calis et al., 2008). Severe

malarial anaemia results from several ways; destruction of infected erythrocytes to release new generations of merozoites, lysis of uninfected erythrocytes by unknown mechanisms and minimised erythrocyte production. These are often followed by fever, and the typical description of fever is linked to the parasite cycle (White et al., 2014).

1.4.2.2 Acidosis and hypoglycaemia:

Metabolic acidosis is one of the lethal symptoms associated with severe malaria. In severe malaria patients, acidotic breathing usually is considered as a bad prognosis indicator for the disease, and is thought to be caused by the accumulation of lactic acid. Usually, lactic acidosis is linked to hypoglycaemia, especially in pregnant women and children (White et al., 2014).

1.4.2.3 Cerebral malaria:

One of the complications of severe malaria is cerebral malaria. It is defined as a coma, *P. falciparum* parasitaemia and Blantyre coma score ≤ 2 in children with the absence of other causes of coma (Molyneux et al., 1989). However, ruling out other causes of coma could be difficult and a study by Taylor and her colleagues demonstrated that in 23% of patients with a clinical definition of CM, the main reason of death was not CM (Taylor et al., 2004).

Coma is the hallmark of CM. It suddenly develops following seizures, and consciousness is usually very rapidly regained (within 24-48h) among children in malaria endemic areas of Sub-Saharan Africa (Idro et al., 2005). Systemic features reported among children with CM include hyponatremia (>50%), severe anaemia (20-50%), hypoglycaemia (30%), jaundice (8%) and metabolic acidosis exhibited as respiratory distress (English et al., 1996, English et al., 1997, English et al., 1998, Molyneux et al., 1989). Neurological deficits are another complication that affected

6-29% of children with CM at the time of discharge (Idro et al., 2004). One of the most common complications of CM in children is retinopathy. It occurred in >60% of children with CM and has three major characteristic features: retinal whitening, retinal vessel discoloration to white or pink-orange (Beare et al., 2004) and retinal haemorrhages (Olumese et al., 1997).

Endothelial activation is well characterised by expression of several adhesion molecules on the vessel surface such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin (Armah et al., 2005b), breakdown of endothelial cells tight-junctional proteins (TJPs) (Pino et al., 2005) and by a variety of other biomarkers like angiopoitin-1 (Ang-1), soluble cell adhesion molecules (sCAMs), vascular endothelial growth factor (VEGF) (Turner et al., 1998) and endothelial micro particles (Schindler et al., 2014).

Blood brain barrier (BBB) is defined as a physiological barrier which separates the cerebrospinal fluid (CSF) and the brain tissue from the blood circulation and has a vital role in brain function (Persidsky et al., 2006). Breakdown of this barrier is commonly associated with the central nervous system diseases. In CM, there were evidences that support the disruption of BBB due to sequestration of parasites in the brain microvascular endothelial cells, leading to upregulation of ICAM-1 receptor and reduction in cell junction proteins such as ZO-1, occluding and vinculin (Medana and Turner, 2006, Brown et al., 2001a).

Nitric oxide (NO) is considered as a natural gaseous molecule which may be involved in CM pathogenesis. During CM, several studies hypothesized that upregulated inducible NOS (iNOS) activity by inflammatory cytokines leading to increase NO production kills the malaria parasites as a part of innate immunity (Cramer et al., 2005, Pino et al., 2004). On the other hand, NO might diffuse across

the BBB into tissue of brain and kill neurons (Brown, 2001). Furthermore, it can also dysregulate the DNA gene expression of neurons and be responsible for reversible coma in patients with CM (Pacher et al., 2007).

The platelets play an important role in the pathogenesis of CM. An autopsy study by Grau *et al*. showed that platelet accumulation is observed at the site of parasite sequestration (Grau et al., 2003). Release of transforming growth factor- β (TGF- β) from α-granules of platelets in the brain microvascular cells is suggested to be one of the factors that mediates platelet-induced apoptosis of the brain endothelial cells (Wassmer et al., 2006c). Moreover, in an *in vitro* parasite co-culture study, iRBC incubated with platelets and tumour necrosis factor alpha (TNFα) cytokine had been shown to induce gene expression changes involved in inflammation and apoptosis in human brain microvascular endothelial cells (HBMEC) (Barbier et al., 2011).

Several reports have indicated the existence of high levels of pro-inflammatory cytokines in human CM for over two decades (Grau et al., 1989, Kern et al., 1989), and confirmed by different groups (Riley et al., 2010), however; other investigators suggested that there is no association between the severity of the disease and the pro-inflammatory cytokines effects (Conroy et al., 2010). Pro-inflammatory cytokines such as TNFα and interleukin 1 β (IL-1β) have been identified as potential mediators of CM disease (Miranda et al., 2013).

1.5 Malaria pathogenesis:

1.5.1.1.1 *P. falciparum* cytoadherence:

Infected red blood cells at trophozoite and schizont stages disappear from the peripheral circulation and adhere to other cells via specific parasite ligand and host-cell receptor interactions that result in sequestration of the parasite (Rowe et al., 2009). The parasites sequester in microvascular beds of various vital tissues and

organs such as placenta, subcutaneous tissue, kidney, lungs, liver and the brain, which avoids removal by the spleen (Buffet et al., 2011). Sequestration of iRBC is a result of cytoadherence of iRBC to the endothelial cells (ECs) which line the blood vessels. ECs express several receptors on their surfaces. Some of these receptors increase their expression in response to stimulation by inflammatory cytokines such as TNFα, which is known to be up-regulated in malaria (Chakravorty et al., 2008) and plays a vital role in severe malaria disease (Taylor et al., 2013, Manning et al., 2012). Several reports have showed that ECs receptors serve as ligands for iRBC adhesion (Figure 1.3). Adhesion of iRBC to EC receptors can be mediated by *P. falciparum* erythrocyte membrane protein 1 (*PfEMP-1*), which is a protein displayed on the iRBC surface and responsible for the adhesion to various host cells (Pasternak and Dzikowski, 2009). More details regarding some of the ECs receptors involved in cytoadherence, *PfEMP-1* and the impact of the cytoadherence on ECs are described briefly in sections below.

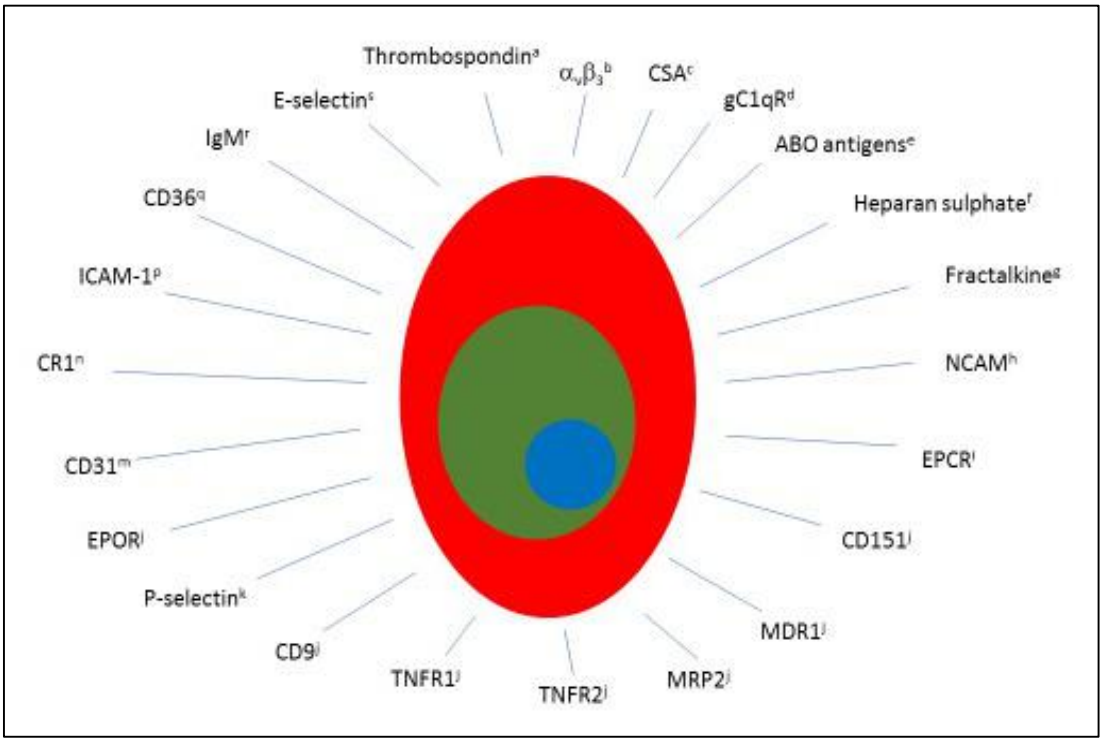


Figure 1.3: Summary of the human receptors that interact with the *Plasmodium falciparum*-infected erythrocyte.

1.5.1.2 Host adhesion receptors:

The most common EC receptors that have been reported to mediate the sequestration of iRBC are CD36 (Barnwell et al., 1989, Oquendo et al., 1989), ICAM-1 (Berendt et al., 1989), endothelial protein C receptor (EPCR) (Turner et al., 2013), platelet EC adhesion molecule 1)/CD31 (PECAM-1) (Rowe et al., 2009), and chondroitin sulphate A (CSA) (Walter et al., 1982). In addition, there are more than ten other molecules have been identified as receptors play role in the cytoadhesion.

1.5.1.2.1 CD36:

CD36 is an integral protein expressed on a cell surface of a variety of host cells including endothelial, epithelial cells, monocytes, macrophages and platelets (Rowe et al., 2009). CD36 is involved in the regulation of membrane transport systems, platelet adhesion (McGilvray et al., 2000) and immune responses in humans

(Febbraio et al., 2001, Greenwalt et al., 1992). It has been revealed that CD36 is a common receptor that binds to most patient isolates (Chakravorty et al., 2008). However, there is no particular role for CD36 in the pathogenesis of the malaria (Rowe et al., 2009). Some studies carried out in Africa showed no difference in the ability of CD36-binding between parasite strains from patients with severe and uncomplicated malaria (Newbold et al., 1997, Rogerson et al., 1999). On the other hand, other evidence demonstrated that it is more associated to uncomplicated malaria strains (Ochola et al., 2011). In contrast, a study conducted on patients from Benin showed that CD36 bind to isolates from CM patients more than UM isolates (Almelli et al., 2014). A study by Wassmer and colleagues found that platelet-expressed CD36 could act as a bridge mediating binding of endothelial cells lacking in CD36 (such as in the brain) to iRBC (Wassmer et al., 2004).

1.5.1.2.2 ICAM-1:

ICAM-1 is an another commonly used receptor, which is expressed on endothelial cells and leukocytes (Rowe et al., 2009). The key role of ICAM-1 on ECs is allowing transmigration of the leukocyte from blood circulation to the target tissues in inflammatory sites induced by cytokine activation. This is mediated by the adhesion to specific leukocyte receptors such as macrophage-1 antigen (Mac-1) or leukocyte function-associated antigen (LFA-1). Also, ICAM-1 can mediate the adhesion to pathogenic organisms, such as human rhinoviruses (HRVs) (Staunton et al., 1989, Greve et al., 1989), and *Plasmodium falciparum* infected erythrocytes (Berendt et al., 1989). Involvement of ICAM-1 in the pathogenesis of malaria remains unclear. However, there are several evidences supporting the involvement of ICAM-1 in severe malaria diseases, specifically CM. Post-mortem studies demonstrated induced sequestration of the parasite in the brain of SM patients, who also showed increased

in expression of ICAM-1 (Turner et al., 1994). Additionally, *in vitro* studies have also shown the ability of ICAM-1 to adhere to different isolates from CM patients (Newbold et al., 1997, Ochola et al., 2011). ICAM-1 expression levels on various types of ECs can be stimulated by inflammatory cytokines, including IL-1β and TNFα which are over-stimulated during malaria infection (Chakravorty et al., 2008). Moreover, the expression levels of ICAM-1 can be also activated directly by iRBC (Tripathi et al., 2006, Viebig et al., 2005b).

1.5.1.2.3 EPCR:

The expression of EPCR in microvascular endothelial of several organs involving brain is quite low (Moxon et al., 2013). EPCR has many functions including anti-inflammatory responses via modifying the response of the signalling receptor protease-activated receptor 1 (PAR1) to thrombin, it also acts as a receptor for protein C in the process of producing activated protein C, which regulates coagulopathy protection, as well as it works to adhere the monocyte Mac-1 (CD11b/CD18) to vascular EC. This interaction might play a role in associating vascular inflammation and coagulation in acute vascular inflammatory disease (Fink et al., 2013). As both ICAM-1 and EPCR have the ability to bind to Mac-1 and *PfEMP-1*, it was suggested that similarities might exist between Mac-1 and *PfEMP-1* surfaces (Aird et al., 2014).

Two different studies conducted in CM showed the role of EPCR in the disease. Moxon and his colleagues demonstrated in their research that the EPCR had a role in children with cerebral malaria. The study found that loss of EPCR co-localized with sequestered iRBC at endothelial sites in the brains of children dying from CM (Moxon et al., 2013). They also revealed that levels of soluble EPCR in CSF in children with CM were high; however, the level in the plasma was not altered

compared to other malaria complications, which suggests that loss of EPCR linked cerebral coagulation and inflammation to parasite sequestration. Another study by Turner *et al* examined the binding of EPCR to particular *PfEMP-1* variants that have been associated with SM (Turner et al., 2013). It was found that adherence of the ECs of several organs, including brain to DC8 containing *PfEMP-1* was mediated by EPCR. The finding of this study was confirmed by examined the binding of parasites isolated from patients with severe malaria to EPCR, which was higher compared to the parasites isolated from patients with uncomplicated malaria.

1.5.1.2.4 PECAM-1:

This receptor is expressed widely on platelets, ECs, granulocytes and monocytes. It was reported that around 50% of malaria isolates from Kenya bound to PECAM-1 but there was no significant correlation between PECAM-1 and SM. Moreover, studies carried out in Africa showed no protection role for PECAM-1 against SM (Heddini et al., 2001). In contrast, expression of this receptor increased the risk of CM in Thailand (Rowe et al., 2009). Binding of PECAM-1 to *PfEMP-1* containing DC5 was assigned, and associated with SM in children from Tanzania, although the sample numbers were limited. Thus, it was suggested that SM can be mediated by DC5-*PfEMP-1* expressing parasites (Berger et al., 2013).

1.5.1.2.5 CSA:

Accumulation of *P. falciparum* iRBCs and monocytes in the placenta is known as a specific characteristic of pregnancy associated malaria (PAM) (Walter et al., 1982). Sequestration of iRBC in the human placenta is mediated by CSA (Duffy and Fried, 2003) and var2CSA *PfEMP-1* members (Viebig et al., 2005a, Avril et al., 2006, Bir et al., 2006, Buffet et al., 1999, Salanti et al., 2003). Then, antibodies that recognize

placental iRBCs develop and associate with protection against PAM in the subsequent pregnancies (Duffy and Fried, 2003, Staalsoe et al., 2001).

1.5.1.2.6 Other cytoadherence receptors:

Other EC receptors that have been implicated in cytoadherence of iRBC include: Thrombospondin (TSP) (Rowe et al., 2009), VCAM-1 (Ockenhouse et al., 1992b), neural cell adhesion molecule (NCAM) (Pouvelle et al., 2007), E-selectin (Schofield et al., 1996), P-selectin (Yipp et al., 2007) integrin αvβ3 (Siano et al., 1998) and fibronectin (Eda and Sherman, 2004). However, the role of these receptors (and others) in the pathogenesis of malaria is unknown (Rowe et al., 2009).

1.5.1.3 PfEMP-1:

P. falciparum causes more mortality and morbidity than any of the other species, and this might be associated with the expression of *PfEMP-1*. *PfEMP-1* is a variable protein with high molecular weight (200 to 350 kDa) (Aley et al., 1986) which is displayed on the surface of the iRBC and localized to structures known as knobs. The knob is visible on the iRBC surface from about 16-18 hours after invasion at the same time as sequestration takes place (Gardner et al., 1996). The presence of knobs is an important factor for optimal display of *PfEMP-1* for cell-cell interactions (Rug et al., 2006, Horrocks et al., 2005). *PfEMP-1* is implicated in cytoadherence to many adhesion receptors on the host endothelial cells. This protein is encoded by 50-60 different *var* genes per parasite genome of which only one *var* gene is expressed in a parasite at any particular time (Kirchgatter and Del Portillo, 2005). The majority of these *var* genes are located in subtelomeric regions of all 14 parasite chromosomes (Gardner et al., 2002).

P. falciparum uses a mechanism to evade from the host immune system, this is called an antigenic variation, which is defined as “the changes of the molecules of

the parasites exposed to the immune system over the course of an infection" (Deitsch et al., 2009). In this case, only a single gene is expressed while all the other genes are silenced; in other meaning, antigenic variation involves switching expression to an alternative *var* gene, resulting in transcription of an antigenically distinct *PfEMP-1* protein (Chakravorty et al., 2008, Smith et al., 2001). Switching takes place at an average of up to 2% per generation (Roberts et al., 1992), although this average differs between parasite variants (Horrocks et al., 2002).

The ability of parasites to interact with host cells and to mediate sequestration in several tissues can be determined by the variation in different *PfEMP-1* proteins (Montgomery et al., 2007). PfEMP-1 is structured from one domain in the intracellular exon, and multi-domains in the extracellular exon composed of Duffy binding like domains (DBLα) and cysteine-rich interdomain region (CIDRα) as the head structure, and followed by different numbers and classifications of DBL and CIDR domains (Gardner et al., 2002, Smith, 2014) (Figure 1.4). Many adhesive properties have been mapped to DBLs such as DBLβ type domain which is known, in some cases, to mediate binding to ICAM-1 (Springer et al., 2004), DBLα type which can mediate rosetting, a process of interacting with uninfected erythrocytes via complement receptor 1 (CR1) (Rowe et al., 1997), and CIDRα type domain which is responsible for binding to CD36 receptor (Miller et al., 2002) and EPCR (Turner et al., 2013).

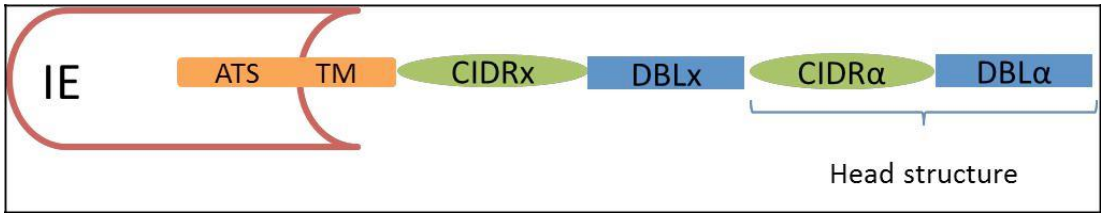


Figure 1.4: *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) structure.

The figure shows the typical arrangement of PfEMP-1 protein domains. The figure modified from (Smith, 2014).

1.5.1.4 Effects of cytoadherence on endothelial cells:

1.5.1.4.1 Direct activation of ECs:

Many studies on post-mortem tissue have showed alteration of the EC in malaria associated with cytoadherence. Up-regulation of ICAM-1 and E-selectin adhesion receptors in cerebral micro-vessels has supported the view of endothelial stimulation in fatal malaria (Turner et al., 1994), also a subsequent study that showed a positive association between malaria disease severity and increase of ICAM-1, VCAM-1, P-selectin and E-selectin, indicating widespread systemic activation of EC (Garcia et al., 1999).

Studies on post-mortem brain tissue have also demonstrated evidence of BBB disruption. Haemorrhages are seen in most cases, but they are small and not usually correlated with necrosis. Thus, it is supported that the processes regulating the integrity of BBB are altered, leading to accumulation of fluids and some plasma proteins into brain parenchyma and perivascular space, and contributing to cerebral oedema which can lead to death (Newton et al., 1998, Adams et al., 2002). However, it is also clear that the amount of vessel leakage is small and this supports the role of sequestered parasite as a major contributor to brain swelling. Recently, *in vitro* evidence suggests that the EC is activated during the malaria infection via β-catenin, which is released from ruptured iRBC and this can lead to alterations in EC junctions in HBMEC, leading to induced the permeability of the endothelial barrier (Gallego-Delgado et al., 2016).

The last decade has seen interest in investigating how the parasite sequestration can modulate the phenotype and function of EC directly. Several groups are focusing on

investigating the changes in EC that follow interaction with iRBC, using *in vitro* co-culture systems. It has become apparent that the alterations induced by iRBC, while they can increase the EC permeability, at the same time, they can protect EC from generalized cell death. Although malaria infection has been closely linked to widespread EC activation, this phase involves several changes seen in ECs, including functional changes and signal transduction. A number of i*n vitro* studies have showed over-expression of adhesion receptor (ICAM-1) (Viebig et al., 2005b, Tripathi et al., 2006). However, this response was specific to conditions of the cultures, source of the EC and also presence of TNF. In contrast to Viebig *et al*, Chakravorty and colleagues did not observe any increase of ICAM-1 expression by iRBC as there was a critical requirement for levels of TNF (Chakravorty et al., 2007). This was also seen in a different study showing direct activation of brain EC but not human umbilical vein endothelial cells (HUVEC) by iRBC (Tripathi et al., 2006). Although different types of ECs might respond differentially, the observation in all these studies is the ability of iRBC to induce alterations to ECs.

1.5.1.4.2 Intracellular signalling:

EC surface-adhesion receptors usually work as mediators of cell-cell adhesion but, in addition to this, they can also mediate intracellular signalling in EC. Two common cytoadhesion receptors in EC are ICAM-1 and CD36. Several studies have revealed that the interaction between CD36 and *PfEMP-1* can mediate extracellular-signal-regulated kinase1/2 (ERK1/2) phosphorylation in ECs. Furthermore, it was demonstrated that ERK1/2 activation was essential in supporting adhesion of iRBC via an ecto-alkaline phosphatase that dephosphorylates CD36 and then induces the CD36 binding affinity (Ho et al., 2005, Yipp et al., 2003). Indeed, a randomized

clinical study showed reduction of iRBC sequestration in patients with UM by using the alkaline phosphatase inhibitor, levamisole (Dondorp et al., 2007).

Similarly, it has been established that the interaction between iRBC and ICAM-1 can lead to intracellular signalling in ECs, particularly in leucocyte adhesion in inflammation. Activation of mitogen-activated protein kinases (MAPKs) following adhesion of surface ICAM-1 can lead to multiple functional and phenotypic changes, involving tyrosine phosphorylation of cytoskeletal proteins, such as paxillin, focal adhesion kinase (FAK) and p130Cas (CAS), resulting in rearrangement of cytoskeletal mediated by Rho activation (Etienne et al., 1998), modulation of tight protein junctions, movement of neutrophils towards junctions for transendothelial migration, over-expression of VCAM-1, mediated by activator protein 1 (AP1) and ERK1 (Lawson et al., 1999). There is evidence to suggest that intracellular domain of ICAM-1 is essential for T lymphocyte mediating signalling and migration following ICAM-1 adhesion (Greenwood et al., 2003). ICAM-1 signalling is complex and involves activities such as transcription factor activation MAPK, protein expression, oxidative stress via stimulation of xanthine oxidase, cytokine production and subsequent reactive oxygen species (ROS) production (Wang and Doerschuk, 2000). ICAM-1 adhesion was also shown to induce the expression of ICAM-1 and affect the ligation of leucocytes (Clayton et al., 1998). Moreover, the interaction between ECs and iRBC can lead to stimulation of p38 MAPKs and ERK1/2, c-Jun N-terminal kinase (JNK) in ECs, which was dependent on the avidity of the adhesion between ICAM-1 and *PfEMP-1* (Jenkins et al., 2007), and, in another study, it showed over-expression of ICAM-1 on HUVEC following adhesion with iRBC in the presence of TNF (Chakravorty et al., 2007) (Figure 1.5). Tripathi and colleagues observed nuclear translocation of the transcription factor nuclear factor

κB (NF-κB) as part of the intracellular signalling cascade that results in over-expression of ICAM-1, following adhesion with iRBC (Tripathi et al., 2006). Interestingly, they also observed reduction in ICAM-1 expression using ROS inhibitors.

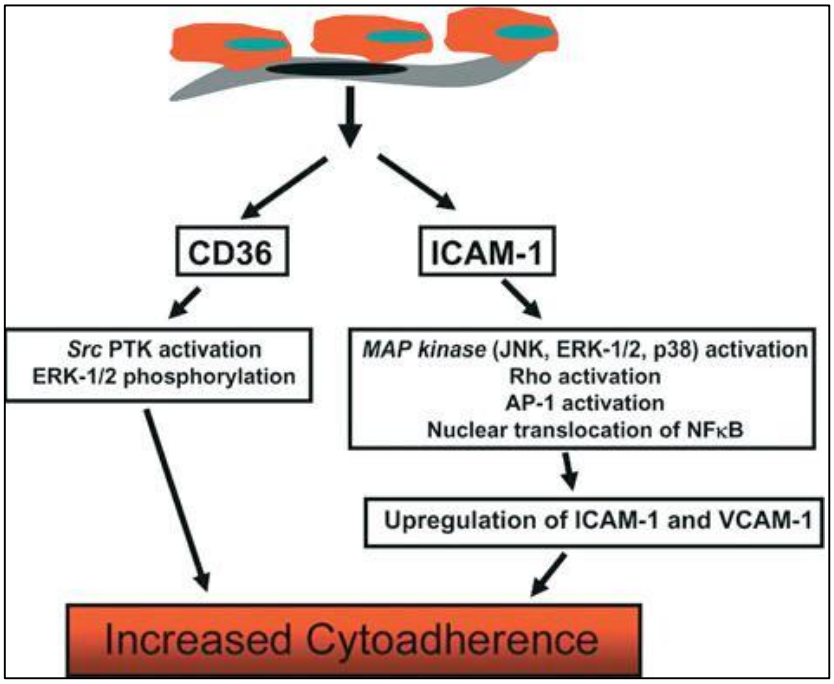


Figure 1.5: Summary of intracellular signalling pathways results from interaction iRBC to ECs.
The figure shows the intracellular signalling pathways activated in response to interaction iRBC vascular ECs via CD36 and ICAM-1. Figure adapted from (Chakravorty et al., 2007).

1.5.1.4.3 Indirect activation of ECs:

Although evidence supports a direct interaction between iRBC and ECs in the modulation of endothelium, the parasite produces proteins which can adhere to ECs or macrophages to initiate process of signalling cascades that can modulate these cells without direct interaction. For instance, the parasite-derived factor *P. falciparum* glycosylphosphatidylinositol (PfGPI) which is released during rupture of the mature schizont, can increase release of TNF from macrophages (Lu et al., 2006). *P. falciparum* merozoite proteins were also demonstrated to increase disruption of

endothelial barriers in a Src-family kinase-dependent manner, but not intact iRBC (Gillrie et al., 2007). A further mechanism for modulation of the ECs was identified by Treeratanapiboon and colleagues, in which endothelial permeability was induced following exposure to peripheral blood mononuclear cells (PBMCs) which were previously exposed to *P. falciparum* proteins (Treeratanapiboon et al., 2005).

1.5.1.4.4 Modulation of endothelial permeability:

Sequestration of iRBC in cerebral and pulmonary microvascular endothelial cells can be implicated in the development of two of the major symptoms of SM; CM and pulmonary oedema respectively. Breakdown of EC TJPs such as ZO-1, vinculin and occludin which maintain the integrity of vascular endothelial, was observed by analysis of the immunohistochemistry in vessels containing sequestered iRBC in post-mortem tissue from Malawian children and Vietnamese adults with CM (Brown et al., 2001a, Brown et al., 1999a). A study in Thailand, demonstrated a reduction in the expression of the TJPs vinculin, ZO-1 and occludin when iRBC isolated from CM patients were incubated with ECs (Susomboon et al., 2006).

In vitro studies have also shown an induction in the permeability of brain and lung ECs mediated by interaction with iRBC. Resistance of the BBB was significantly decreased by up to 70% following exposure to intact iRBC and also contact with iRBC-derived soluble factors from the culture supernatant, prepared by precipitation with around 40% ammonium sulphate (Tripathi et al., 2007). Further study using lung ECs also showed induced endothelial permeability following contact to iRBC (Pino et al., 2003, Essone et al., 2017). Reductions in the resistance of the EC have also been observed in platelet-mediated cytoadherence (Wassmer et al., 2006b).

1.5.1.4.5 Modulation of endothelial apoptosis:

Apoptosis is a complex biological process of cell death, and this process could be mediated by several stimuli, including cytokines, hormones, growth factors, viral or bacterial infection and immune responses (Thompson, 1995). Several *in vitro* studies have suggested that EC apoptosis is increased by sequestered iRBC in HBMEC. Although caspase 3 activity, which is an apoptosis marker, has been showed in 40% of CM patients, there was no evidence of particular damage in CM brain (Medana and Turner, 2006). Thus, EC apoptosis is not a specific feature of CM and could not be attributed an essential role in the BBB disruption. Similarly, pulmonary oedema may result from iRBC -induced lung capillaries permeability, and is considered to be a common feature of SM (Maguire et al., 2005), but it is not thought to be associated with host apoptosis.

The basis of malaria pathophysiology remains unclear, and there is considerable variance in the evidence for iRBC mediated cell death in vascular ECs (Figure 1.6). Some evidences suggested that there is a direct induction of apoptosis by iRBC, mediated through activation of caspase 3. On the other hand, other evidence has suggested that vascular endothelial apoptosis is an indirect process of oxidative stress due to iRBC -increased generation of ROS. Indeed, some studies used superoxide dismutase or other antioxidants which have shown protection of ECs in the presence of physical interaction with iRBC (Pino et al., 2003, Taoufiq et al., 2006). Other studies have revealed an increase of EC apoptosis following contact with iRBC. For example, apoptosis was increased in ECs when co-incubated with neutrophils in the presence of sera from malaria patients, which was mediated by products of neutrophil secretion (Hemmer et al., 2005).

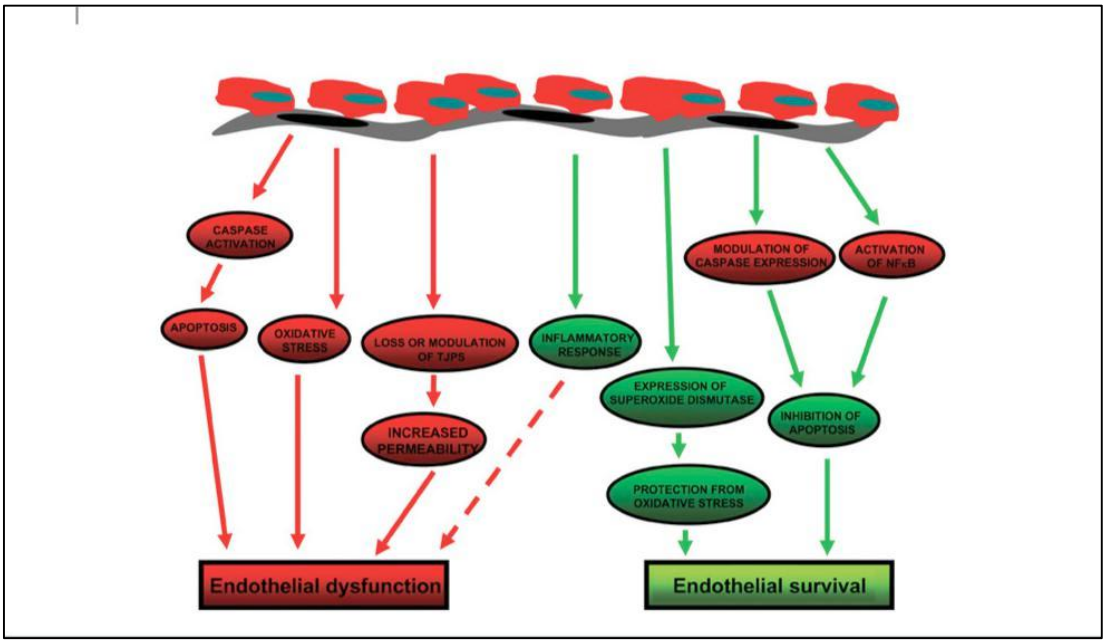


Figure 1.6: Effects of the iRBC cytoadherence to vascular endothelial cells.
The figure demonstrates the direct and indirect effects of interaction iRBC to ECs that can mediate endothelial dysfunction and survival. Figure adapted from (Chakravorty et al., 2007).

1.5.1.4.6 Modulation of the endothelial inflammatory response:

The interaction between ECs and iRBC leads to endothelial activation and induce a range of inflammatory cytokines. Indeed, there are many evidences observing induction of pro-inflammatory cytokines such as TNF (Kwiatkowski et al., 1990), IL-1β, interleukin-8 (IL-8) (Burgmann et al., 1995), interferon γ (IFNγ) and IL-6 in serum levels of SM patients. Moreover, TNF, TGF-β and IL-1β have been shown in post-mortem brain tissue in human CM (Brown et al., 1999a). A study by Clark and colleagues on post-mortem tissue from children with CM with no pathological diagnosis except malaria, showed high expression level of haem oxygenase-1 (HO-1), an inflammatory mediator, in liver, lung and brain tissues, suggesting widespread systemic inflammation (Clark et al., 2003). Endothelial vesiculation and the generation of microparticles are considered to be an additional result of EC activation in CM disease. These can contact other host cells, such as platelets and

leucocytes, to increase cytokine release, thus maintaining an inflammatory environment (Coltel et al., 2006).

There is a belief that inflammatory response being increased in the host following an infection with malaria is a key factor in mediating malaria pathogenesis (Clark et al., 2006). It is possible that systemic activation plays an important role in malaria pathology, as demonstrated by a study using a synthetic transcriptional inhibitor of TNF which demonstrated inhibition of ICAM-1 expression and also inhibition of iRBC interaction to HBMEC (Wassmer et al., 2006a). However, localized cytoadhesion between ECs and iRBC might also contribute directly to malaria disease, specifically during the early stages of malaria infection.

1.5.1.4.7 Modulation of endothelial survival:

Microarray analysis study of transcriptional changes in HUVEC following co-culture with iRBC in the presence of TNF by Chakravorty and colleagues have showed modulation of multiple genes that can mediate cell survival (Chakravorty et al., 2007). This involves up-regulation of uncoupling protein 3 (UCP3), which detoxifies ROS to protect ECs from oxidative stress (Cannon et al., 2006), and down-regulation of caspase 3 expression, which is a late stage marker of apoptosis. The protection of the ECs might be a mechanism to protect the *P. falciparum* parasite and enhance survival of the transmissible phenotypes. Thus, it is possible that, the adhesion of iRBC to ECs can suppress several genes that are mediating apoptosis and up-regulate genes that have a role in protection and survival of the ECs, in addition to inducing damage.

1.6 Thesis objectives:

In the last two decades, several studies have been conducted to understand malaria pathogenesis. Better understanding of this syndrome might lead to the discovery and

development of new therapies for SM. To do that, it is important to understand how the *P. falciparum* is modulating the host response to infection. This thesis aims to:

- Determine the transcriptional response to iRBC adhesion to brain endothelial cells in co-culture at 2, 6 and 20 hours.
- Compare this profile between two different *PfEMP-1* variants, ITvar14 (upsB) and ITvar37 (upsC).
- Identify differences in the transcriptional response to cytoadherence in brain (HBMEC) and dermal (HDMEC) endothelial cells.
- Examine the role of the uninfected erythrocyte and systemic TNF in endothelial modulation during cytoadherence.

These were mainly achieved using the RNA-seq technique. Summary of experiments in this thesis can be seen in Figure 1.7.

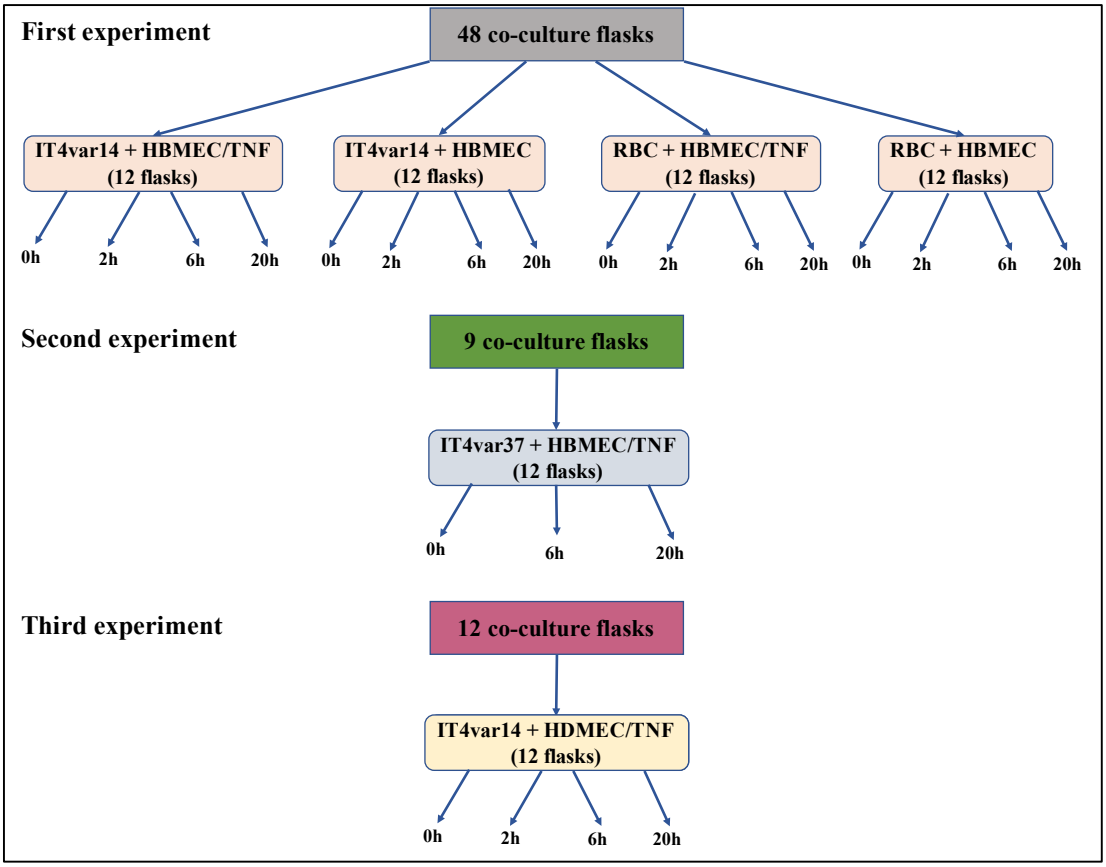


Figure 1.7: Summary of experiments in this study.

CHAPTER 2

MATERIALS AND METHODS

2.1 Malaria parasite culture:

Lab adapted IT4var14 (A4) (Ockenhouse et al., 1992a) and IT4var37 (4E12) (Janes et al., 2011) *P. falciparum* isolates were used in this study. These isolates were gift from Prof Alister Craig (Liverpool School of Tropical Medicine, UK) and Prof Joseph Smith (University of Washington, USA). They were grown and maintained in culture with some modification as described previously by Trager and Jensen (Trager and Jensen, 1976), with some modifications. The parasites were cultured at 1% haematocrit in O+ human erythrocytes and grown at 37°C in complete RPMI 1640 medium (supplemented with 10% human serum, 37.5 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 6 mM NaOH, 7 mM D-glucose, 25 mg/ml gentamicin sulfate, 2 mM L-glutamine) and at a pH of 7.2 in a plastic tissue culture flask which was filled with a gas mixture of 96% nitrogen, 3% carbon dioxide, and 1% oxygen prior to sealing.

2.1.1 Growth and washing media preparation:

For washing and growing the parasite, the materials below were mixed with the particular quantities and filtered in a laminar flow hood under aseptic conditions.

2.1.1.1 Washing medium:

- 500 ml of RPMI (R0883, Sigma, USA) stored at 4°C.
- 18.75 ml of HEPES Buffer (1 M, H0887, Sigma) stored at 4°C.
- 5 ml of 20% Glucose solution (7 mM, Sigma powder) kept at room temperature.
- 5 ml of L- Glutamine solution (200 mM, G7513, Sigma) stored at -20°C.
- 3 ml 1 M of Sodium Hydroxide solution kept at room temperature.
- 1.25 ml Gentamicin Sulphate solution to give 25 ng/µl (10 mg, G1272, Sigma) stored at 4°C.

2.1.1.2 Growth medium:

Only 100 ml of the mixture was used as parasite washing medium (incomplete medium) and stored at 4°C. 45 ml of human pooled serum was added to the remaining mixture then filtered and stored at 4°C as growth medium (complete medium). In parasite culture, all solutions must be warmed in an incubator or water bath at 37°C prior to use.

2.1.1.3 Human pooled serum:

Blood bags were collected from the Royal Hospital (Liverpool, UK) in non-anticoagulant blood collection bags and stored overnight at 4°C. Then, they were centrifuged for 20 minutes at 3000 rpm. After that, the serum was carefully taken, mixed and stored in 45 ml aliquots at -20°C.

2.1.2 Red Blood cells separation:

Non-red blood cells components were taken and removed from the whole blood prior to using it in culture of the parasite. First of all, 12.5 ml of Histopaque (10771, Sigma) was put in four 50 ml Falcon tubes (BD Falcon Conical Tube, BD Biosciences, UK). 25 ml of parasite washing medium was added to 25 ml of whole blood cells and mixed well in another 50 ml Falcon tube. After that, 12.5 ml of the mixture of blood was added to the four tubes containing Histopaque dropwise. The solutions were centrifuged for 15 minutes at 3000 rpm and the supernatants were aspirated prior to adding about 3 volumes of the washing medium. These were mixed and centrifuged again for 5 minutes at 3000 rpm. The supernatants were removed and the pellets of the RBC were re-suspended in an equal volume of washing medium and stored at 4°C. The RBCs have a haematocrit of about 50% and are known as washed RBC (wRBC).

2.1.3 Parasites thawing:

An appropriate volume of growth media, washing media, 12% NaCl, 1.8% NaCl and 0.9% with 0.2% glucose were warmed at 37°C prior to use. According to LSTM policy, the parasite vial was removed carefully from liquid nitrogen following the code of practice. The stabilate was rapidly warmed at 37°C prior to transferring it into a 50 ml Falcon tube. Relying on the pellet volume, one-fifth of its volume of 12% NaCl was added slowly dropwise and mixed gently with the pellet. Simply, 200 µl of 12% NaCl was added if the pellet was 1000 µl, then incubated for 5 minutes at room temperature. This was followed by the addition of five volumes of 1.8% NaCl to the pellet volume dropwise and incubation at room temperature for 5 minutes. Thereafter, five volumes of 0.9% NaCl containing 0.2% glucose was added dropwise and incubated at room temperature for 5 minutes. The mixture was centrifuged for 5 minutes at 1800 rpm. The supernatant was aspirated and the pellet was washed with washing medium and centrifuged for 5 minutes at 1800 rpm. The pellet then was re-suspended in an appropriate volume of complete medium, transferred to T25 cm^2 culture flask (BD Falcon, USA), gassed for 30 seconds and incubated in a 37°C incubator.

2.1.4 Evaluating parasite growth and maintenance of continuous culture:

The parasitemia was evaluated by using Giemsa thin smear and according to the results, an appropriate volume of wRBC was added. It was assessed by making a Giemsa thin smear which was then examined under a light microscope. 500 RBCs were counted; the number and stages of any iRBCs were recorded. The smears of cultures were obtained by taking a drop from parasite culture, spreading it smoothly on a labelled glass slid and air-dried at room temperature. The smear was fixed for few seconds with absolute methanol prior staining with 10X diluted Giemsa (stock of

the Giemsa stain solution was diluted with 10% of phosphate-buffered water [4 mM KH2PO4 and 20 mM Na2HPO4 at pH 7.2]) for approximately 20 minutes at room temperature. After that, the stain was washed off with tap water and the slide air-dried prior to examining it under a binocular light microscope by using (100x) oil immersion objective lens.

Most parasite cultures were adjusted at 1% haematocrit and 1% parasitemia. The formula that was used to adjust the parasitemia for continuous culturing was as follows: $P_1 V_1 = P_2 V_2$, where P_1 is the counted parasitemia for the evaluated culture, V_1 is the evaluated culture volume, P_2 is the required parasitemia to continue culturing the parasite, which is commonly 1-1.5%, and V_2 is the required volume of parasite culture to maintain the growth of the parasite at given the parasitemia. To keep the haematocrit at 1%, 10 µl of packed RBCs (100%) was required for 1 ml of parasite culture. But, because the wRBC stock was diluted to 50%, the volume is doubled. A coulter counter was used to assess the haematocrit of the parasite culture and wRBC.

2.1.5 Parasites synchronisation:

2.1.5.1 Plasmion flotation:

The parasite culture was transferred to a 50 ml Falcon tube and centrifuged for 5 minutes at 1800 rpm, then the supernatant discarded. The pellet was re-suspended in washing medium in 1.5X of pellet volume, and transferred into a 15 ml Falcon tube (BD Falcon Conical Tube, BD Biosciences, UK). Thereafter, the suspension was mixed with Plasmion in 2.5X of pellet volume and allowed to settle at 37°C for 20 - 30 minutes. In the top layer of the suspension, the trophozoite stage knobby iRBCs might be seen. This layer was transferred carefully to a new 15 ml Falcon tube. The tube after that was centrifuged for 5 minutes at 1800 rpm and the supernatant

removed. The pellet was re-suspended gently in 10 ml washing media and centrifuged for 5 minutes at 1800 rpm. Then, the supernatant was discarded and a thin smear of the pellet was prepared prior adding the appropriate volume of growth medium and fresh wRBC, and gassed as described earlier. Greater than 50% of iRBCs at mature stages were usually seen in the smear after this procedure. This kind of selection was performed routinely to make sure that knobby populations were kept for adhesion and co-culturing assays as described earlier by (Jensen, 1978).

2.1.5.2 Sorbitol:

Occasionally, parasite culture was synchronised by 5% of D-sorbitol (S3889, Sigma) which acts to lyse the iRBCs with mature stages of trophozoites (Lambros and Vanderberg, 1979). It was used when the parasite culture contained higher trophozoite parasitemia. The parasite culture was centrifuged at 1800 rpm for 5 minutes and pelleted, then treated with 10X of 5% D-sorbitol for 20 minutes at 37°C. After that, it was centrifuged for 5 minutes at 1800 rpm, the supernatant discarded and the pellet washed with 10 ml of incomplete medium. The pellet then was re-suspended in an appropriate volume of complete medium, transferred to a new culture flask, gassed and incubated at 37°C. The 5% of D-sorbitol was prepared as the following: 25 g of D-sorbitol (Sigma Co, UK) was dissolved in 500 ml of distilled water, the solution filtered and kept at 4°C.

2.1.6 Selection of IT4var14 parasite on BC6 antibody:

The selection of IT4var14 isolate from other var genes was performed using BC6 monoclonal antibody (mAb) (Oxford University) that specifically recognises IT4var14 (Smith et al., 1995). 50 µl of Protein G Dynabeads (10003D, Invitrogen) were purified on the magnet and washed 3 times with 500 µl of 1% BSA/PBS (A8327, 30% Bovine Serum Albumin and D8537, Dulbecco's phosphate buffer

saline, Sigma), then re-suspended in 366 µl of 1% BSA/PBS and 34 µl of BC6 original stock 235 µg/ml. The mixture was rotated at 15 rpm for 60 minutes at room temperature, thereafter washed 3 times with 500 µl of 1% BSA/PBS and re-suspended in 200 µl 1% BSA/PBS. Parasite culture was enriched for trophozoites stages by using plasmion as described above. The iRBC were re-suspended in 200 µl 1% BSA/PBS and incubated with BC6 labelled Dynabeads suspension, and rotated at 15 rpm at room temperature for 45 minutes then washed 3 times with 500 µl of 1% BSA/PBS very gently to remove unbound parasites. iRBCs were re-suspended in appropriate volume of growth media with fresh washed red blood cells and cultured as standard.

Parasites selected on BC6 were cryopreserved to provide sufficient material for adhesion and co-culturing assays using the same populations. This was made to minimize the impact of antigenic switching. Usually, the parasite was used in binding and co-culturing assays for up to 3 weeks post-selection.

2.1.7 Cryopreservation of parasites:

The parasite culture of around 5-8% parasitemia at ring stage was pelleted and cryopreserved by re-suspending in a glycerolyte freezing medium. Cryopreservation was performed according to the following calculation: 3X of the pellet volume was re-suspended in 5X volumes of the cryopreservation solution. Two steps were carried to add the cryopreservation media; first, the pellet was re-suspended with one-fifth volume of the required cryopreservation media dropwise then allowed to stand at room temperature for 5 minutes. Second, the remaining volume of the cryopreservation media was gently added and mixed completely prior transferring to properly labelled cryovials. Keeping the cryovials in a rack and covering with tissues to allow freezing at -80°C slowly for overnight. On the day after, Vials were

transferred to liquid nitrogen cryostorage. An example is given below for the calculation of cryopreservation: if the pellet was 1000 µl, then 1666 µl of the cryosolution was required. (1666 µl / 5) = 333 µl, this volume was considered as first volume that was suspended with the pellet. Thereafter, (1666-333) = 1333 µl, this remaining volume was the second re-suspension.

2.2 Endothelial Cells culturing:

HBMEC passage 3 (P3) (ACBRI 376) were obtained from Cell Systems (USA). Human dermal microvascular endothelial cells (HDMEC) (C-12210) were obtained from Promocell in 1ml of cryopreserved cells at (P1).

2.2.1 Endothelial medium preparation:

HBMEC and HDMEC medium were cultured in Endothelial Cell Growth Medium MV (ready-to-use) (C-22020) that was supplemented with Endothelial Cell Growth Medium MV SupplementMix (C-39225). To store and re-use the medium, it was kept at 4°C and aliquots were warmed in a water bath prior to use.

2.2.2 ECs thawing:

Sterile filtered plastic tissue culture flask (T25 cm^2) (BD Falcon, USA) was coated with 2 ml of 1% of Gelatin solution (G1393, type B, 2% in H2O, Sigma) for around 1 hour in a humidified tissue culture incubator at 37°C and 5% carbon dioxide (CO_2), then the solution was aspirated. Cryopreserved cells were warmed at 37°C prior to transferring the cells into the coated flask containing 5 ml of warmed endothelial medium, and allowed to attach at 37°C, 5% CO_2 incubator for 2-3 hours. After that, the endothelial medium was replaced by the same amount of warmed medium and incubated for 48 hours. Cells were examined for confluence and medium was replaced every 48 hours. According to the standard protocol and manufacturer's instructions, once the cells are confluent (80-90%), sub-culturing must be carried out.

2.2.3 ECs sub-culturing:

Sub-culturing of the ECs was done by Promocell detech kit (C-41220) which contains HEPES-buffered Balanced Salt Solution (HEPES-BSS), Trypsin/EDTA solution and Trypsin Neutralization Solution (TNS). These solutions were aliquoted and warmed at 37°C before using. Medium was aspirated from the flask prior to adding 1.5 ml of HEPES-BSS to wash off the remaining medium. HEPES-BSS then was aspirated before detaching the cells by 1.5 ml of trypsin. The detached cells were observed by an inverted microscope. Thereafter, similar amount of TNS was immediately added as soon as the cells have been detached to minimise the possible impact of trypsin. The mixture was transferred into 15 ml Falcon tube and centrifuged for 3 minutes at 300 xg. The cells after that were gently re-suspended into warmed fresh medium and distributed into three T25 cm^2 gelatin coated filtered tissue culture flasks. At this stage, the cells were considered at the next passage (P4) and expanded until P5 and then cryopreserved for assays at P6, P7 and P8.

2.2.4 ECs cryopreservation:

For cryopreservation, cells were detached as described for sub-culturing but then they were re-suspended at 5-7.5 $X10^5$ cells/ml in promocell Cryo-SFM (C-29910) instead of suspension in media. The Vials were gradually frozen at -20°C for few minutes to -80°C overnight, prior to storing them in the liquid nitrogen cryostore.

2.3 Detection of selected IT4var14 parasite on BC6 antibody by flow cytometry:

Fluorescence-activated cell sorting (FACS) was used to evaluate the IT4var14 parasite that was selected on BC6 mAb. Parasites were cultured and maintained following standard laboratory conditions. Mature pigmented trophozoites, at 3-8% parasitemia and a haematocrit of 1-2%, were enriched using plasmion floatation. In

terms of the plasmion flotation, the parasites were transferred to 50 ml falcon tube and centrifuged at 1800 rpm for 5 minutes. The pellets were suspended with 1250 µl warmed plasmion as (500 µl pellets × 2.5 plasmion solution) and 750 µl incomplete parasite culture media (YM) as (500 µl pellets × 1.5 YM) then incubated at 37°C for 20 minutes. After incubation, the supernatant was aspirated into a new 15 ml falcon tube then centrifuged at 1800 rpm for 5 minutes. The pellets were washed with 5 ml YM and centrifuged again at 1800 rpm for 5 minutes, 10 µl of enriched pellets (containing 50-60% of trophozoites-stage iRBCs, at 20% haematocrit) was re-suspended with 100 µl of 1% BSA/PBS in two 1.5 ml eppendorf tubes. The eppendorf tubes were centrifuged at 2500 rpm for 2 minutes, one of them was re-suspended with mAb BC6 diluted 1:10 in 1% BSA/PBS (8.5 µl of mAb BC6 + 91.5 µl of BPS) and the other re-suspended with 100 µl of 1% BSA/PBS as control sample, then they were incubated for 30-60 minutes in 37°C incubator.

In the meantime, the compatible secondary detecting antibody (goat anti-mouse IgM antibodies-APC conjugated (Southern Biotech, 1020-11L, 0.5 mg) was prepared as 1:100 (Ab: 1% BSA/PBS) (2 µl of APC + 200 µl of 1% BSA/PBS + 0.2 µl of Ethidium Bromide to stain the nuclei). After incubation, the tubes were centrifuged at 2500 rpm for 2 minutes then washed with 100 µl of 1% BSA/PBS and centrifuged at 2500 rpm for 2 minutes. The cells were re-suspended with 100 µl of the prepared secondary antibody and incubated at 37°C for 60 minutes in darkness. Tubes were centrifuged at 2500 rpm for 2 minutes then washed with 100 µl of 1% BSA/PBS and centrifuged again at 2500 rpm for 2 minutes to remove excess antibody. Cells were re-suspended in small glass tube containing 500 µl cell wash (FACS buffer) and analysed by using a Bickton-Dickinson FACSCalibur flow cytometer (BD LSR11).

Data were obtained by using FACSCalibur flow cytometer, then FlowJo software (TriStar, San Carlos, CA, USA) was used to collect and analyse 50.000 events.

BC6, a monoclonal antibody that recognise the surface of live iRBCs infected with IT4var14 *P. falciparum* strain, was included in the experiments as a positive control. Negative controls include the fluorescent reactivity of iRBCs with either an irrelevant mouse IgM or with secondary antibody and Ethidium bromide stain alone.

2.4 Mycoplasma detection test:

Mycoplasmas are small parasitic bacteria and considered as famous cell culture contaminants and could have deep impacts on host cell biology for survival (Rottem, 2003) and dysregulate host gene expression (Hopfe et al., 2013). Preventing contamination by mycoplasma is difficult for many reasons. Firstly, mycoplasma cells are small in diameter (0.3-0.8 μm) (Razin, 2006), and have the ability to escape through standard filtration membranes. Secondly, mycoplasmas are resistant to most commonly used antibiotics because they lack cell walls (Olarerin-George and Hogenesch, 2015). Thirdly, mycoplasmas have the ability to reach to high concentration in infected cells media without leaving turbidity (Young et al., 2010). Lastly, although the essential source of mycoplasma contamination is possibly other cell cultures, lab personnel are considered to be a likely source of contamination as well (Drexler and Uphoff, 2002).

The parasite strains, ECs cultures, washed blood cells and parasite culture media were regularly monitored for mycoplasma contamination using the Universal Mycoplasma Detection Kit (ATCC® 30-1012K™).

2.5 Detection of microvascular endothelial receptors by flow cytometry:

Endothelial cells were grown in 12-well plates until they become confluent at 37°C in a 5% CO_2 incubator. Cells in six wells were then, stimulated with 10ng/ml TNF overnight. The ECs were washed with 500 µl HEPES buffer, trypsinized with 500 µl of trypsin and neutralised with 500 µl of TNS. Cells were transferred to 1.5 ml eppendorf tubes and centrifuged at 5000 rpm for 5 minutes then the pellets were washed with 500 µl of 1% BSA/PBS and centrifuged at 5000 rpm for 5 minutes prior to adding conjugated monoclonal antibody APC- mouse anti human ICAM-1, FITC mouse anti human CD31, FITC mouse anti human CD36 and APC- mouse anti human-EPCR for both stimulated and non-stimulated cells as (10 µl of each antibody + 490 µl of 1% BSA/PBS in each eppendorf tube) (BD Biosciences Ltd). They were incubated for 60 minutes at 37°C in the dark. In parallel, isotype matched antibodies were used as a control for non-specific labelling (BD Biosciences Ltd). The cells were centrifuged at 5000 rpm for 5 minutes then washed with 500 µl of 1% BSA/PBS prior transferring to small glass tube that contained 500 µl of FACS buffer. The receptors expression levels were analysed by using a Bickton-Dickinson FACSCalibur flow cytometer.

2.6 Static Protein Adhesion assays:

Static protein-binding assays were performed as described previously (Patil et al., 2011). Two 60 mm Plastic petri dishes (Falcon code 1007; Becton Dickinson, Oxford, UK) were spotted with 2 µl of ICAM-1 and CD36 proteins at concentration of 50 µg/ml in triplicate in a radial pattern and incubated in a humidified chamber for a maximum of two hours at 37°C, to permit the proteins to adsorb to the surface,

after which the proteins spots were aspirated off using a fine tip, and the plates were blocked with 1% BSA/PBS overnight at 4°C. The plastic petri dishes were warmed at 37°C for one hour prior to the assay. In the meanwhile, the parasites from cultures containing mostly mature trophozoites were prepared at 3% parasitemia and 1% haematocrit in sterile binding buffer (RPMI 1640 R4130 (Sigma, Dorset, UK) in 1l distilled H2O with HEPES, glutamine and 2% glucose at pH 7.2). The blocking buffer was discarded from the plates, and 1.25 ml of the parasite suspension was incubated in each plate at 37°C for 60 minutes with gentle rotation of the plates, to re-suspend the parasites, every 10 minutes. The dishes were carefully washed with binding buffer medium (4-6 washes) until almost no background was seen, and then the adherent cells were fixed in 1% glutaraldehyde in PBS for one hour. The plates were stained with 10% Giemsa for about 20-30 minutes, rinsed with water and dried overnight.

Six images of each spot were captured under x200 magnification by using software HC Image (Sewickley, USA). The images were analysed by Image-Pro version 7 (Rockville MD, USA). Binding for each protein was expressed as the mean number of the bound iRBC per mm^2 in all spots for that protein in at least three independent experiments.

2.7 Preparation of endothelial cells for co-culturing:

ECs between passages 4-8 were used in the experiments. Methodologies of the endothelial cell culture have been described in detail above. When cells reached to 80-90% confluence, half of the EC flasks were stimulated with the addition of 10ng/ml TNF overnight.

2.8 Preparation of malaria parasites for co-culture:

P. falciparum strains were cultured as described above. Parasites were cultured until they reached 5-8% parasitemia (trophozoite or schizont stages), and then they were enriched by using plasmion in order to obtain 50-60% parasitemia.

2.9 Co-culture of ECs with malaria parasite:

The concentrated parasites were re-suspended in EC medium to a parasitemia of 50% and 1% haematocrit. Endothelial medium was removed from EC flasks and the cells then were re-suspended with 5 ml of parasites suspension for 0, 2, 6 and 20 hours at 37°C, in a 5% CO_2 incubator. In the control group, uninfected red blood cells were re-suspended in 5 ml endothelial cell medium to obtain 1% haematocrit and applied onto EC monolayers for 0, 2, 6 and 20 hours at 37°C, 5% CO_2 in a humidified incubator. At each time point, the co-culture medium was aspirated from each flask and the cells were washed 1X with EC medium to remove unbound parasites or uninfected RBC. 1.5 ml of EC medium was added to each flask prior harvesting the cells by cell-scraper. The cells were then transferred to 1.5 ml labelled sterile eppendorf tubes.

2.9.1 RNA extraction from co-culture assays:

In this study, extraction and purification of high quality RNA free from any other contaminants was an impotent step. The RNA was extracted from each co-cultured flask according to the RNeasy Mini Kit (Qiagen, 74104) protocol instructions.

Samples were centrifuged at 13.000 rpm for 1 minute and the supernatant discarded. 350 µl of buffer RTL was added to the pellet and homogenized for 1 minute with vortex. Then, 350 µl of 70% ethanol was added to the homogenate and transferred to RNeasy mini spin column in 2 ml collection tube. After that, the tube was

centrifuged at 8000 xg for 15 seconds and the flow-through removed. 700 µl of RW1 buffer was added to the spin column, centrifuged for 15 seconds at 8000 xg and the filtrate discarded. 500 µl of buffer RPE was added to the column, and then centrifuged at 8000 xg for 15 seconds and the flow-through discarded. This step was repeated but with centrifugation 2 minutes instead of 15 seconds. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute to dry the membrane. The column was then placed in a new 1.5 ml collection tube and 50 µl of RNase-free water was directly added and centrifuged for 1 minute at 8000 xg to elute the RNA. This step was repeated using another 50 µl RNase-free water. The RNA sample was stored at -80°C. High precautions were taken to avoid any RNase contamination when preparing and handling the RNA.

2.9.2 Quality assessment of RNA samples:

The quality of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (RNA 6000 Nano Kit, 5067-1511, Agilent technologies) and following its protocol instructions. The Agilent Bioanalyzer 2100 separates the RNA according to their sizes and quantifies each molecule. The RNA quality test was repeated twice to ensure the RNA products were at high quality. 2100 Expert software (Syngene, Cambridge, UK) was used to analyse the outputs. RNA integrity number (RIN) which reflects degradation level of RNA samples was the very important outcome value of the software (Schroeder et al., 2006).

2.9.3 RNA sequencing:

RNA samples were sent to King Abdullah University for Science and Technology (KAUST) (Kingdom of Saudi Arabia) for mRNA deep sequencing. Agilent 2100 Bioanalyzer was used to check the quality of RNA samples and only samples with

RIN of greater than 7 were used for sequencing. The raw sequencing data was received by FTP transfer in FASTQ format.

2.9.4 Bioinformatics for RNA sequencing data:

Although the next generation sequencing (NGS) machines generated the great amount of data, the correct interpretation of data using bioinformatics tools is critical. The machines of NGS can amplify and sequence a fragment of DNA on a fixed spot, thus photos with high-resolution could be taken to record changes in colour of a spot after each pyro-sequencing round. Hence, raw reads of sequencing output might be considered as images instead of readable text files. Manufacturers always supply software that acts as sequencing platforms to transfer these images into sequencing data. Thus, the starting point of most bioinformatics analysis is the sequence reads.

2.9.4.1 Quality assessment of raw sequencing reads:

The quality control of the raw sequencing data is the first step. The high-throughput NGS machine produces the RNA-seq data. These data contain millions of short reads and their quality control are complicated. FastQC (Andrews) software is a quality control tool for high throughput sequence data. It is easy to use, allows checking of the quality of reads and includes several useful statistics describing the quality of data: Total Number of Sequences contained in the results of sequencing, Per Base Sequence Quality, Per Sequence Quality Score, Per Base Sequence Content, Per Sequence GC Content and Sequence Length Distribution.

The most important quality indicator is the Total Number of Sequence, reads with few numbers in the results of RNA sequencing causes lack of detection of genes that have lower expression, thereby reducing the integrity of transcriptome. Per Base Sequence Quality detects the fraction of bases with low quality at each read position

and represents the accuracy of the reads. Per Base Quality Score exhibits the subset of reads that have universally low-quality values and a general loss of quality within the sequencing run. Per Base Sequence Content demonstrates base content of each sequence position of all sequences, irregular distribution of content means the library of sequencing is not random. There were two possibilities for this; either containing sequences with over representation or biased fragmentation that may happen when the library was generated using hexamer random priming (Hansen et al., 2010). Per Sequence GC Content measures the content of the GC across the whole length of each read in random library. Unusual shaped distribution indicates a problem with the library because of specific contaminantion, such as adapter dimer. Sequence Length Distribution displays if all sequences are the same length.

2.9.4.2 Mapping of RNA sequencing reads:

In the last decade, several mapping software tools had been developed to map short sequences to human reference genome. ELAND (Cox, 2007) and Maq (Li et al., 2008a) were the earliest aligners. Although they had advantages, they were slow and require more memory compared to aligners using Burrows-Wheeler transformation, Bowtie and BWA (Li and Durbin, 2009).

In this study, the sequences were aligned to UCSC *Homo sapiens* reference genome hg38 using TopHat v2.1.0 (Trapnell et al., 2009), which is integrated with Bowtie v2.2.6.0 (Langmead et al., 2009). TopHat eliminates a few number of reads based on quality scoring of each read and thereafter maps the reads to a provided human reference genome. The pre-built UCSC *homo sapien*s hg38 bowtie index was downloaded from the UCSC Genome Browser site (http://hgdownload.soe.ucsc.edu/downloads.html#human). Less than 40 alignments per read, with up to 2 mismatches per alignment were used as the TopHat default

settings. TopHat builds its database in a two-round mapping procedure. First, it tries to map sequences to the human reference genome and identifies potential splice junctions from the initial mapping. Second, it maps the sequences that were not aligned to the first round to the junctions of the exons.

2.9.4.3 Quality assessment of sequence alignment:

Quality of the sequence alignment to human reference genome for each sample was assessed by Samtools-flagstat (Li et al., 2009), which collects statistics about sequence reads based on their sequence alignment map (SAM) flags.

2.9.4.4 Identification of gene expression changes:

After mapping sequences to the human reference genome, many analyses could be carried out with existing software. In theory, the number of sequences aligned to a gene is proportional to the gene abundance. Software uses counts of reads mapped to each gene as a starting point to identify differentially expressed genes. Cuffdiff software (Trapnell et al., 2012) was used to determine differential expression of genes between Control and examined groups. The expression of those genes was reported in *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) (Mortazavi et al., 2008). A strict criterial was used to select differentially expressed genes: normalized expression >1 FPKM, Log2FC (log2 fold change) >1 (up-regulated) or Log2FC <-1 (down-regulated), P-value <0.05 and FDR (false discovery rate) <0.05 (calculated as described (Subramanian et al., 2005)) as threshold to control the number of false positives. Analysis of the Cuffdiff were performed by using the human reference genome comparing the examined samples to the control samples. For those analyses, the biological replicates were pooled together.

2.9.4.5 Visualization of different expression genes:

Gene expression analysis is considered one of the most popular analysis in the biomedical science field. In the last decade, high-throughput genomics have been developed until the introduction of RNA sequencing. With regard to the RNA-seq as a high-throughput gene expression profiling assay, the heatmap is one of the most popular methods of presenting the data of gene expression (Zhao et al., 2014). R software has been used as a tool to generate the heatmap function (Eisen et al., 1998). This hierarchical clustering method was used to identify genes with high and low expression over different time points.

2.9.4.6 Functional and pathway analysis of differentially expressed genes:

Lists of differently expressed genes are often the results from the RNA sequencing. To extend these into the underlying biology requires functional and pathway analysis. Several databases and software of gene functions have been developed for this purpose, covering simple to more advanced pathway approaches (Khatri et al., 2012). These usually depend on existing knowledge and their protein products. Several tools commonly used include the Database for Annotation Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009) and Reactome pathway database v56 (Fabregat et al., 2016). DAVID was used under default setting to identify genes types that were differentially expressed. It has the ability to cluster genes into similar groups and functions, using data such as Gene Ontology (GO) term categories (biological process, molecular function and cellular component). Genes with more/less than 1 log2 fold change of expression were uploaded to the applications.

CHAPTER 3

GLOBAL TRANSCRIPTIONAL PROFILES OF HUMAN BRAIN MICROVASCULAR ENDOTHELIAL CELL RESPONSES TO MALARIA PARASITE INTERACTIONS

3.1 Introduction:

Cerebral malaria is considered as a part of severe complicated malaria, with mortality rate in adults from 15% in southeast Asia (Dondorp et al., 2005) to 8.5% in African children (Dondorp et al., 2010). The standard clinical definition of CM is a diffuse encephalopathy that is characterized by seizures and loss of consciousness (coma) (Newton et al., 2000). The clinical manifestations of CM differ from adults and children, and the causes of these differences are still not entirely clear but might be associated with age and immunity of patients (Olliaro, 2008).

Cerebral malaria is a complicated clinical syndrome. Starting from infection by the *Plasmodium falciparum* parasite, then followed by parasite asexual multiplication and cytoadherence in brain endothelial microcapillaries. Cytoadhesion of iRBC on microvessels is a form of sequestration that has important roles in CM pathogenesis, either; directly by blockage microvessels that supply nutrients and oxygen to the brain, therefore causing blood flow obstruction resulting in tissue hypoxia and avoidance of splenic clearance; or indirectly by coagulation defects, inflammation mediation and endothelium dysfunction (Storm and Craig, 2014). Binding of iRBC multiple human brain receptors such as ICAM-1 (Berendt et al., 1989) and EPCR (Turner et al., 2013) is mediated by a specific parasite surface molecule (*PfEMP-1*).

There are several key events following sequestration of iRBC on brain endothelial cells that can play roles in CM pathogenesis. These include endothelial cell activation (Kim et al., 2011), breakdown BBB (Medana and Turner, 2006), oxidative stress (Percario et al., 2012), platelet activation and apoptosis (van der Heyde et al., 2006), and neuroinflammation (Miranda et al., 2013) (Figure 3.1).

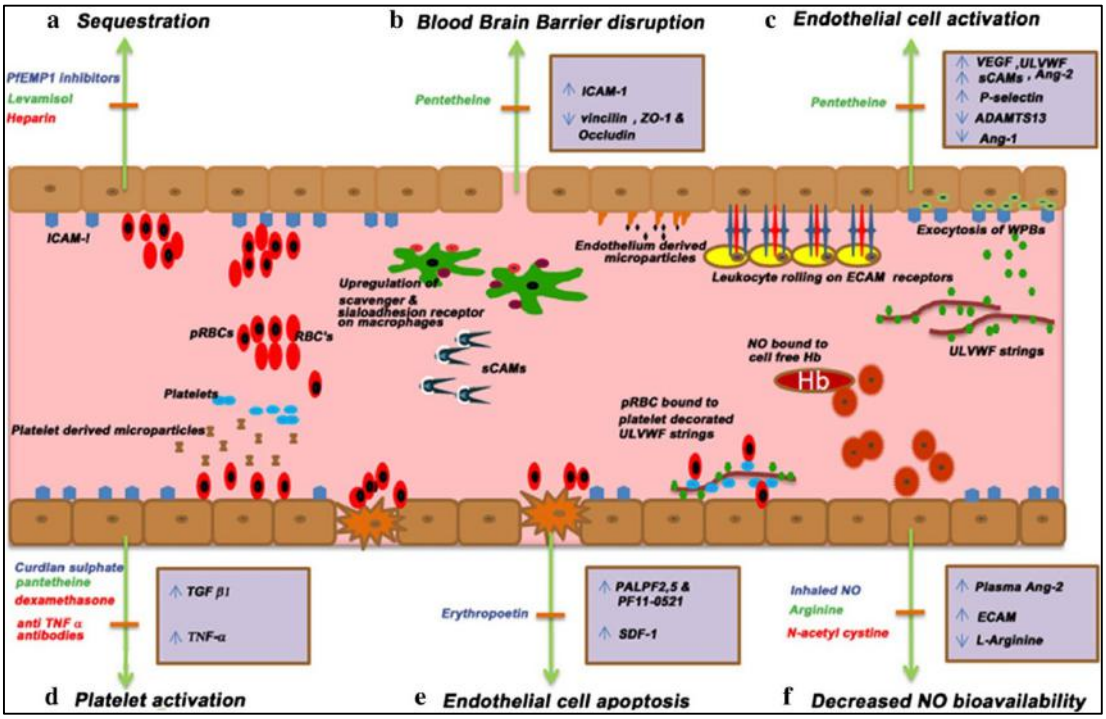


Figure 3.1: Mechanisms of CM pathogenesis.
a) *P. falciparum* infected erythrocyte (pRBCs) sequestration, **b)** Blood brain barrier disruption, **c)** Endothelial cell activation, **d)** Platelet activation, **e)** Endothelial cell apoptosis and **f)** Decreased NO bioavailability. Grey boxes represent molecules with changed levels (induce shown by upward arrow; reduce shown by downward arrow) as an outcome of each mechanism. This figure is adapted from (Hora et al., 2016).

In recent years, several applications have been developed to study the gene expression and regulation of the genome such as microarray and RNA sequencing. Although microarray is reliable and sometimes more cost effective than RNA-seq, it suffers from number of limitations, such as limited detection range (Wang et al., 2009), relatively low resolution and high background noise (Casneuf et al., 2007). RNA-seq measures the expression of the gene in a more sensitive manner and can also measure other aspects of RNA molecules, including splicing patterns (Pan et al., 2008, Sultan et al., 2008), changes of expression of individual transcript variants (Trapnell et al., 2010), allelic specific expression (Heap et al., 2010), sequencing variants (Barbazuk et al., 2007, Medvedev et al., 2009, Montgomery et al., 2010, Li et al., 2011), RNA editing events (Picardi et al., 2010, Peng et al., 2012) and accurate

transcription start site (TSS) mapping (Sultan et al., 2008). Moreover, RNA-seq can detect 25% more genes than the microarray does (Sultan et al., 2008).

In the last two decades, several cerebral malarial studies have investigated the mechanisms of the pathogenesis using cDNA microarray technology, most of them concentrating on the role of the malaria parasite in the disease (Claessens et al., 2012, Subudhi et al., 2015b, Goel et al., 2014, Subudhi et al., 2015a). However, limited studies have focused on responses of the host cells to the malaria parasite interactions in CM disease (Chakravorty et al., 2007, Barbier et al., 2011). Up to now, there has no RNA sequencing study carried out to investigate the response of HBMEC to the malaria iRBC co-adhesion interaction.

The aim of this chapter is to investigate the ability of the IT4var14 parasite variant to modulate gene expression in HBMEC in the presence of the inflammatory cytokine TNFα, at different time points in a co-culture system, using the RNA sequencing technology. Gene functional analysis and pathways analysis were also investigated under the same conditions.

3.2 Methods:

3.2.1 Parasite culture:

IT4var14 lab isolate was cultured as described in the general methods chapter using standard culturing techniques in O+ human erythrocytes at 1% haematocrit (Trager and Jensen, 1976).

3.2.2 ECs culture:

HBMEC was cultured in endothelial cells culture media in a humidified tissue culture incubator at 37°C and 5% CO_2 as explained in the general methods chapter.

3.2.3 Detection of mycoplasma infection in parasite isolate and HBMEC:

Mycoplasma infection was examined in IT4var14 parasite and HBMEC as described in the general methods chapter.

3.2.4 Characterization of the IT4var14 malaria parasite and HBMEC:

FACS was used to detect the population representation of the IT4var14 parasite culture, which selected on BC6 mAb. Also, it was used to confirm the expression of ICAM-1, EPCR and CD31 protein receptors in the presence and absence of TNFα on HBMEC. Static protein adhesion assays were performed to assess the binding of iRBC to ICAM-1 and CD36 proteins. All these procedures were described in the general methods chapter.

3.2.5 Co-culture ECs with malaria parasite:

HBMEC were co-cultured with the IT4var14 parasite in the presence of TNFα stimulation at 0, 2, 6 and 20 hours in a humidified incubator at 37°C, 5% CO_2 as described in the general method chapter.

3.2.5.1 RNA extraction and quality control:

As described in the general methods chapter, RNA from each co-cultured flask was extracted and purified from any other contaminations. Quality of the RNA samples

then was assessed using an Agilent Bioanalyzer 2100.

3.2.5.2 Overview of the workflow for RNAseq data analysis:

Although there are several freely available software packages used for the analysis of RNA-seq, selection of software of the bioinformatics workflow is critical and the choice depends on the aim of the analysis. For our RNA sequencing study, the purpose was to reveal differences of gene transcription levels of the TNFα activated HBMEC after incubation with the IT4var14 *P. falciparum* strain at 2, 6 and 20 hours. As described in the general methods chapter, the software tools which were used in our pipeline are demonstrated in Figure 3.2.

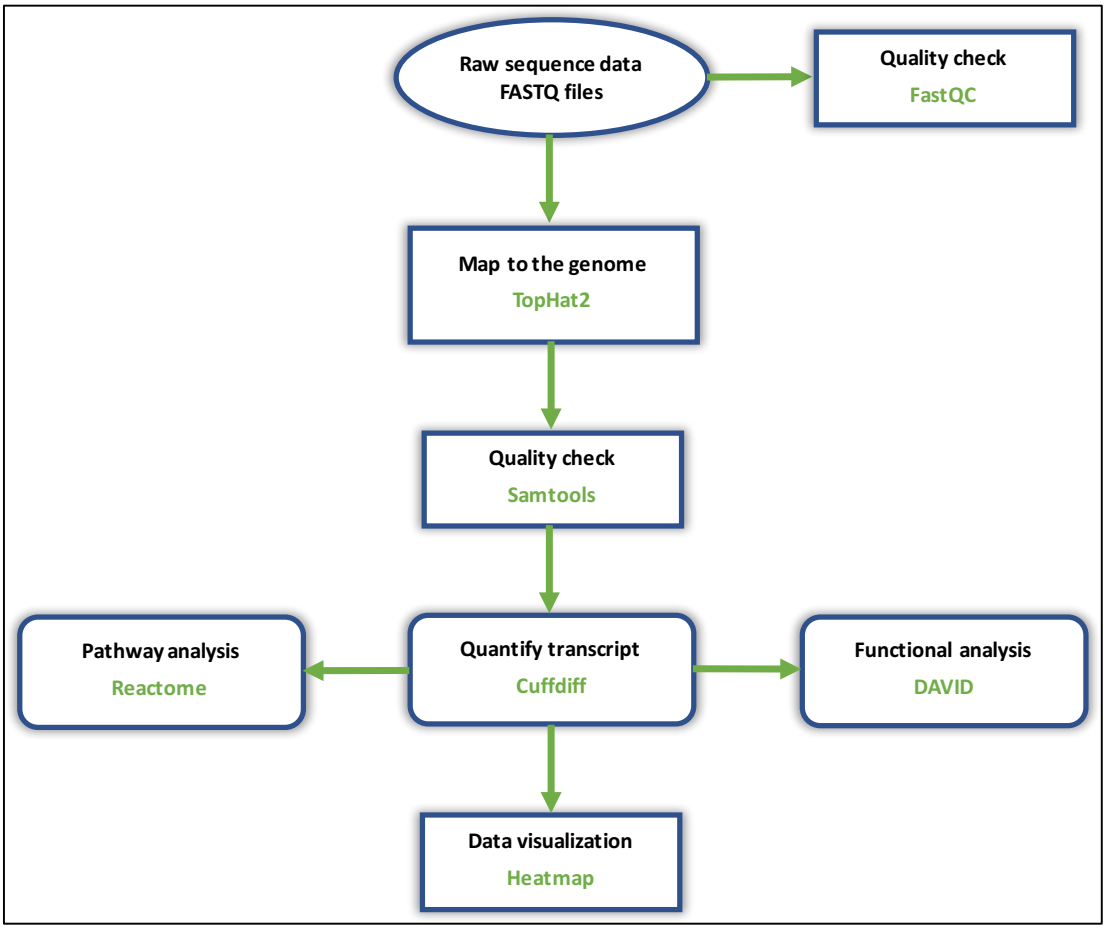


Figure 3.2: The workflow of the RNAseq analysis and software.
FastQC was used to check the quality of the fastq files. Quality of the filtered sequences were then mapped to UCSC *Homo sapiens* reference genome hg38 with TopHat2. Samtools was used to examine the quality of the alignments. Cuffdiff was used to quantify differentially expressed transcripts. Gene functional analysis was investigated using the DAVID tool. Reactome was also used to test the gene pathway analysis. Genes with differential expression levels at 2, 6 and 20 hours of incubation compared to 0 hour of iRBC with TNFα activated HBMEC were visualized using Heatmap.

3.2.5.3 Reverse transcription of mRNA (cDNA synthesis):

Prior to cDNA synthesis, each RNA sample was checked using an Agilent 2100 Bioanalyzer as described in the general methods chapter to ensure each sample had an accurate concentration measurement. cDNA synthesis was performed using RT^2 first strand kit (QIAGEN Cat #330401) according to the manufacturer's instructions. It was carried out in 20 µl of reaction volume which is appropriate for 25 ng-5 µg of total RNA. In a sterile 0.5 ml eppendorf tube, 2 µl buffer GE was mixed with 0.6 µg

RNA, RNase-free water to give a final volume of 10 µl of genomic DNA elimination mix. This was heated for 5 minutes at 42°C, and then immediately placed on ice for at least 1 minute. A reverse-transcription mix was then prepared to add to each tube containing 4 µl 5x buffer BC3, 1 µl control P2, 2 µl RE3 Reverse Transcriptase Mix and 3 µl RNase-free water. 10 µl of the reverse-transcription mix was added to each tube containing 10 µl genomic DNA elimination mix, mixed gently and incubated for exactly 15 minutes at 42°C. Then the reaction was inactivated immediately by heating at 95°C for 5 minutes and 91 µl RNase-free water added to each reaction tube.

3.2.5.4 Primer design for q-RT-PCR validated genes:

The following genes were selected to validate the RNA-seq results at 6 hour of incubation IT4var14 parasite with HBMEC/TNF (Table 3.1). The primers were requested from Sigma-Aldrich company at 100 micro molar (µM), then diluted to a concentration of 0.5 µM by nuclease-free distilled water (Sigma Cat #W4502) prior to use in qRT-PCR reactions.

Table 3.1: Primer sequences table.

Gene name	Forward primer sequence	Reverse primer sequence	Primer publication
LAMC2	ACATTCCTGCCTCAGACCAC	TCCCTTGTCAGTTGCTCCAT	(Zhang et al., 2014)
PLA2G4A	CGTGATGTGCCTGTGGTAGC	TCTGGAAAATCAGGGTGAGAATAC	(Bickford et al., 2013)
VCAM-1	CATGGAATTCGAACCCAAACA	GGCTGACCAAGACGGTTGTATC	(Koo et al., 2003)
ID2	TCAGCCTGCATCACCAGAGA	CTGCAAGGACAGGATGCTGATA	(Khan et al., 2011)
NFKBIA	ACAGAGGACGAGCTGCCCTA	CCTTTGCGCTCATAACGTCA	(van der Pouw Kraan et al., 2014)
HBA2	CCGGTCAACTTCAAGCTCCT	CGGGCAGGAGGAACGGCT	(Qadah et al., 2015)
AQP1	TGGACACCTCCTGGCTATTG	GGGCCAGGATGAAGTCGTAG	(Motulsky et al., 2014)
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	(Minsky and Roeder, 2015)

3.2.5.5 Real-Time PCR:

All pipette tips used in the qRT-PCR were DNase, RNase free, sterile filter tips and work was performed on a clean bench. Q-RT-PCR reactions were set up by mixing 1 µl cDNA template and 7.5 µl of nuclease-free distilled water (Sigma Cat #W4502), 12.5 µl 2x RT^2 SYBR Green Mastermix (QIAGEN Cat #330500), 2 µl forward primer and 2 µl reverse primer with a final concentration 0.5 µM and in a final volume of 25 µl for each reaction. qRT-PCR reactions were mixed by vortexing prior to loading in a Mx3000P 96-well PCR plate (Agilent Technologies Cat #401334) and sealed with optical caps (Agilent Technologies Cat #401425). The plate was then run on a Stratagene Mx3005P device (Agilent Technologies, Santa Clara, California, U.S.A). The following cycling parameters in the Mx3005P software were applied: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

3.2.5.6 Interpreting qRT-PCR results:

The expression of the gene of interest was calculated using the comparative C_t method for relative measurement of gene expression. The mean of the replicate sample outputs was calculated as a first stage; then for the second stage, the ΔCt value was calculated by subtracting the Ct value of the target gene from the reference gene as shown in this equation below:

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ housekeeping gene}$$

For the third stage, the ΔΔCt value was calculated by subtracting ΔCt of the stimulated samples from the basal samples as exhibited below:

$$\Delta\Delta Ct = \Delta Ct \text{ stimulated samples} - \Delta Ct \text{ basal samples}$$

Finally, the range in fold expression was calculated as following: $2^{-(\Delta\Delta Ct)}$.

GAPDH was used in this study as a housekeeping gene.

3.3 Results:

3.3.1 Detection of mycoplasma infection in parasite isolate and HBMEC:

Harvesting the parasite and EC free from the mycoplasma infection was the first challenge in this study. The IT4var14 parasite and HBMEC cultures were tested and showed no mycoplasma contamination (Appendix 1).

3.3.2 Characterisation of the IT4var14 malaria parasite and HBMEC:

3.3.2.1 Static adhesion of IT4var14 parasite on ICAM-1 and CD36 proteins:

Based on the level of binding to ICAM-1 and CD36, IT4var14 was characterised as a good binder for both proteins in agreement with a previous study (Madkhali et al., 2014) (Appendix 2).

3.3.2.2 Detection of HBMEC receptor expression:

The expression of the endothelial cell markers CD31, ICAM-1 and EPCR, with and without TNFα stimulation, were confirmed using FACS (Figure 3.3). ICAM-1 expression was higher with TNFα stimulation. However, with TNFα activation, EPCR was slightly reduced. These results are consistent with previous studies (Madkhali et al., 2014).

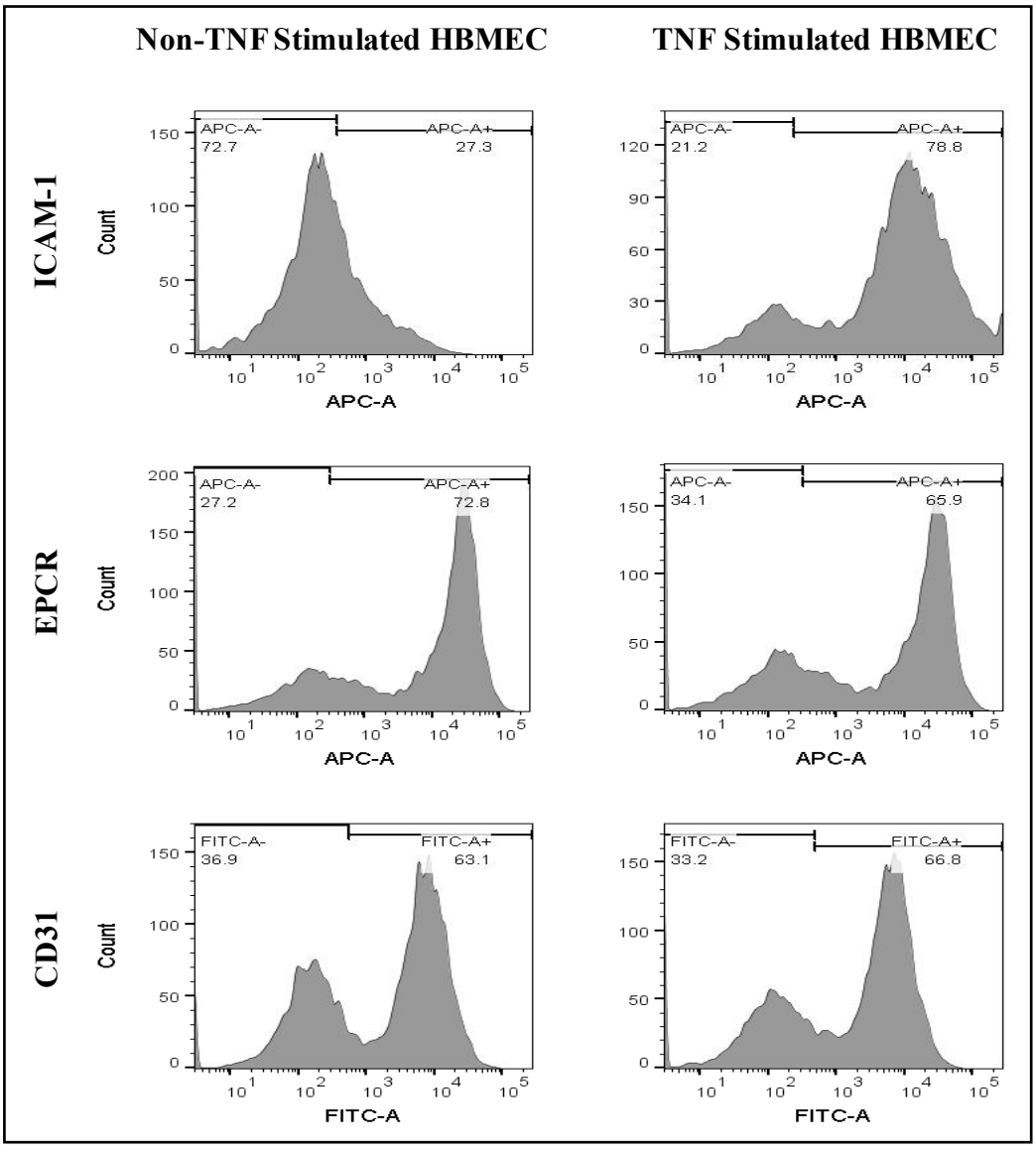


Figure 3.3: Detection of HBMEC receptor expression.
FACS analysis of ICAM-1, EPCR and CD31 expression on HBMEC without (left panel) and with (right panel) TNF stimulation. Stimulation was for 24 hrs using 10 ng/ml TNF. ICAM-1, EPCR and an endothelial marker (CD31) are shown at the upper, the middle and the bottom panels respectively.

3.3.3 Quality control of RNA samples:

In order to identify transcriptional changes within TNFα-stimulated HBMEC as a result of interaction with erythrocytes infected with the IT4var14 parasite, three independent co-culture experiments were carried out at 0,2,6 and 20 hours, and the RNA was isolated. An Agilent 2100 Bioanalyzer was used to determine the quality of the RNA samples. This machine measures the quality of the RNA using RIN, which compares the intensities of the 18S and 28S ribosomal RNA (rRNA). Samples with RIN of higher than 7 are deemed to be of minimal degradation and good quality. In this study, all the samples showed the RIN more than 7 (Table 3.2).

Table 3.2: RIN calculated by Agilent 2100 Bioanalyzer.

First Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
1	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	9.7	106
2	Parasite co-cultured with HBMEC at 2 hours (TNF +ve)	10	163
3	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	10	273
4	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	10	209
Second Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
5	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	9.9	124
6	Parasite co-cultured with HBMEC at 2 hours (TNF +ve)	10	149
7	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	10	246
8	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	10	143
Third Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
9	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	10	277
10	Parasite co-cultured with HBMEC at 2 hours (TNF +ve)	9.6	396
11	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	8.8	279
12	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	9.1	428

The table presents the RIN and the RNA concentration of each sample. The high RIN calculated by the 2100 Bioanalyzer indicated the very high quality of the isolated RNA.

3.3.4 Quality control of the raw reads:

Quality of the raw sequences was checked with FastQC. Several aspects of the sequences' quality were demonstrated as figures, including: Per Base Sequence Quality, Per Sequence Quality Score and Per Sequence GC Content (Figure 3.4). Although scores of the quality reduce along with positions of the sequences, the quality bases on the 3' end of reads in all the samples were observed to be above a score of 20. On the basis of quality score per read, quality of the most frequently mean in all samples were shown at score 37. In order to investigate the quality of the GC distribution over all reads, GC content across the whole length of each read in all samples were roughly similar to the modal of normal distribution of GC content.

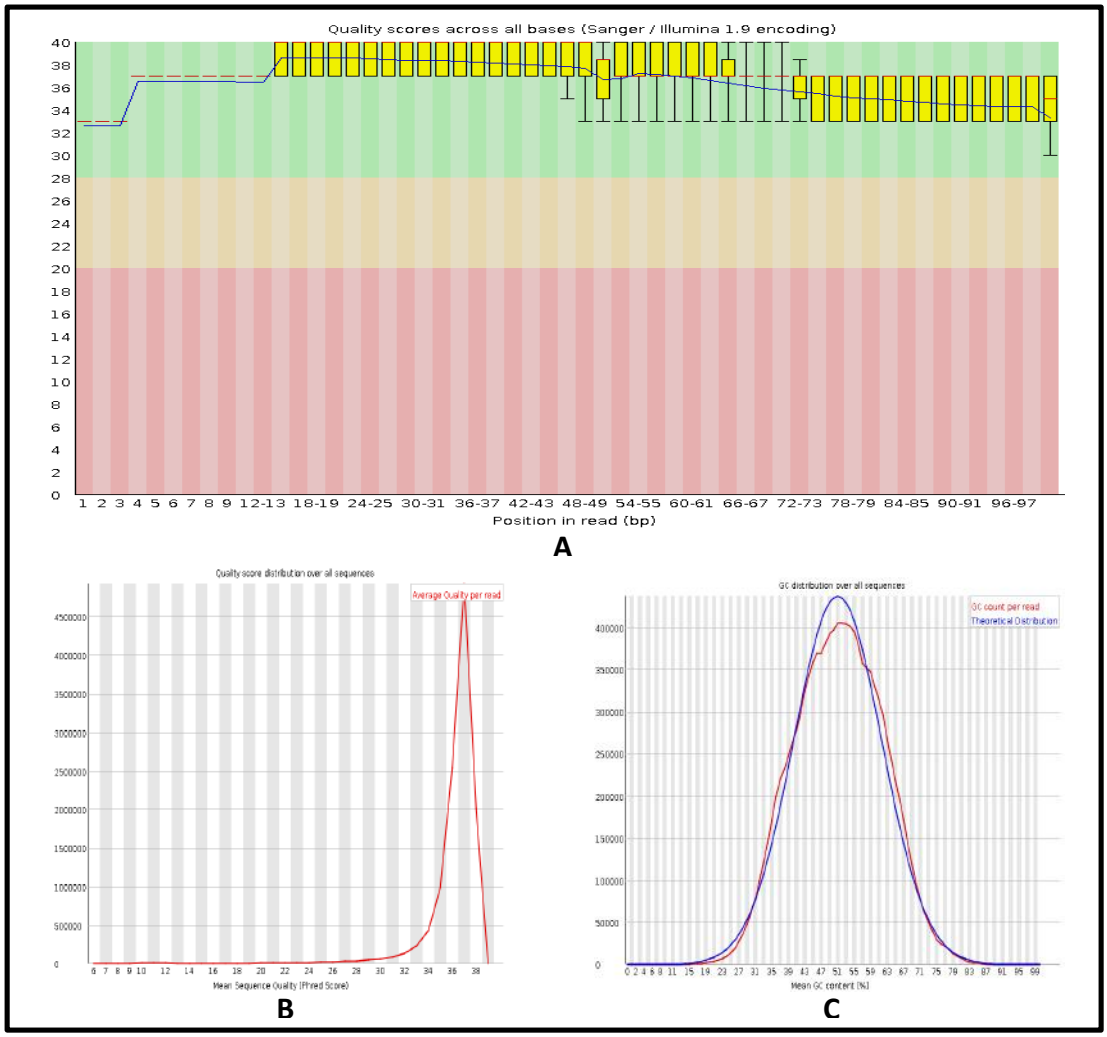


Figure 3.4: Quality control of the raw reads.

A: Box plots of base quality scores of read positions across all reads in a sample. The x-axis shows the position of the reads. The y-axis is the quality scores of the bases. **B**: Quality score distribution over all sequences in a sample. The x-axis is the quality of mean sequence. The y-axis shows the number of sequences. **C:** Quality of the GC distribution over all reads. The x-axis demonstrates the mean GC content. The y-axis represents the number of reads.

3.3.5 Mapping of RNA sequencing reads:

After checking the quality of the raw sequence data FASTQ files, TopHat v2.1.0 was used to map sequences to UCSC *Homo sapiens* reference genome hg38. The total number of sequences produced from each sample in this study across all time points was between 9,637,117 and 43,077,924, with an average across all samples of 27,198,576. Before aligning to the human reference genome, between 2.8% and 5.9% of the reads were removed due to low quality. On average, 92.1% of the sequences mapped to the human genome (Table 3.3).

Table 3.3: Summary of reads mapping to the human reference genome (UCSC hg38) using TopHat v2.1.0.

	PRBC TNF+ 0h			
	1	**2**	**3**	**Average**
Total reads	35,185,811	33,174,010	43,077,924	37,145,915
Reads removed	4.7%	4.0%	4.1%	4.3%
Read aligned to reference genome	93.1%	95.6%	94.9%	94.53%
	PRBC TNF+ 2h			
	1	**2**	**3**	**Average**
Total reads	18,776,296	10,569,872	9,637,117	12,994,428
Reads removed	5.9%	3.5%	3.1%	4.6%
Read aligned to reference genome	89.9%	90.6%	90.8%	90.4%
	PRBC TNF+ 6h			
	1	**2**	**3**	**Average**

Total reads	51,236,486	26,479,076	22,343,112	33,352,891
Reads removed	4.2%	2.8%	2.9%	3.3%
Read aligned to reference genome	91.8%	91.0%	91.0%	91.27%
	PRBC TNF+ 20h			
	1	**2**	**3**	**Average**
Total reads	22,577,870	27,386,295	25,939,044	25,301,069
Reads removed	3.8%	3.3%	3.4%	3.5%
Read aligned to reference genome	93.6%	89.0%	93.9%	92.2%

Total reads and the percentages of those reads removed due to low quality and aligned to hg38 by TopHat. PRBC indicates the *P. falciparum* infected erythrocyte.

3.3.6 Identification of differentially expressed genes:

After mapping the sequencing reads to the human reference genome with TopHat, differential expression on a gene level was determined with Cuffdiff in FPKM. In this study, gene expression changes of HBMEC after incubation with *P. falciparum* parasite and TNFα were calculated as following: the ratio of examined groups FPKM (2, 6 and 20 hours) to the control group FPKM (0 hour). The results then were converted to Log2FC.

3.3.6.1 Differentially expressed genes in HBMEC at 2 hours incubation:

From the analysis, 109 genes were identified as significantly differentially expressed with an adjusted FDR < 0.05 and at least 1Log2-fold change, of them 33 genes were found up regulated and 76 genes were down regulated. Top ten up and down regulated genes in TNFα-stimulated HBMEC co-cultured with IT4var14 parasite at 2 hours are presented in (Figure 3.5). In the up regulated genes, anoctamin 9 (ANO9) has the highest fold change around 19-fold, followed by alkaline phosphatase, liver/bone/kidney (ALPL) as a next highest fold change with 7-fold. Whereas, the

most down regulated genes at this time point were CKLF like MARVEL transmembrane domain containing 1 (CMTM1) and aquaporin 1 (AQP1) with fold changes of about -14 and -12 respectively. A full description about the top ten up and down regulated genes is given in appendix 3.

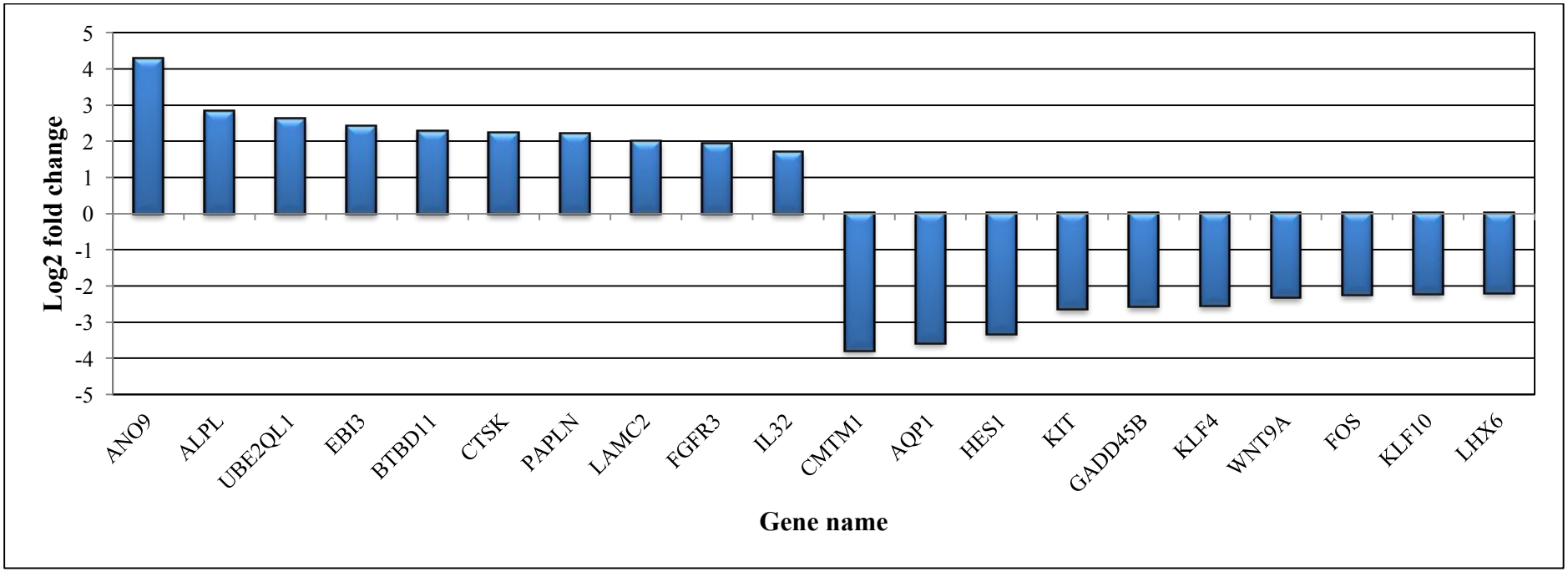


Figure 3.5: Top 10 up/down regulated genes in TNF-stimulated HBMEC incubated with IT4var14 parasite at 2 hours co-culture.
The differentially expressed genes in TNF-stimulated HBMEC co-cultured with IT4var14 parasite isolate at 2 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of those genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

3.3.6.2 Differentially expressed genes in HBMEC at 6 hours incubation:

Based on the level of gene expression in TNFα-stimulated HBMEC incubated with IT4var14 parasite at 6 hours co-culture, there were 88 significantly expressed genes were detected in this current study. Of these genes 17% were identified as up regulated genes and 83% were down regulated genes. Among the top ten up and down regulated genes (Figure 3.6), the greatest fold change was observed with the complement component 3 (C3) in up regulated genes with approximately 121-fold change, while the lowest fold change in the same group was phospholipase A2 group IVA (PLA2G4A) with about 2.5-fold change. On the other hand, inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2) and AQP1 stand out as the top two most down regulated genes, respectively. ID2 was down regulated by -18-fold change, whereas AQP1 was down regulated by -14-fold change. Appendix 4 shows more information about the top ten up and down regulated genes.

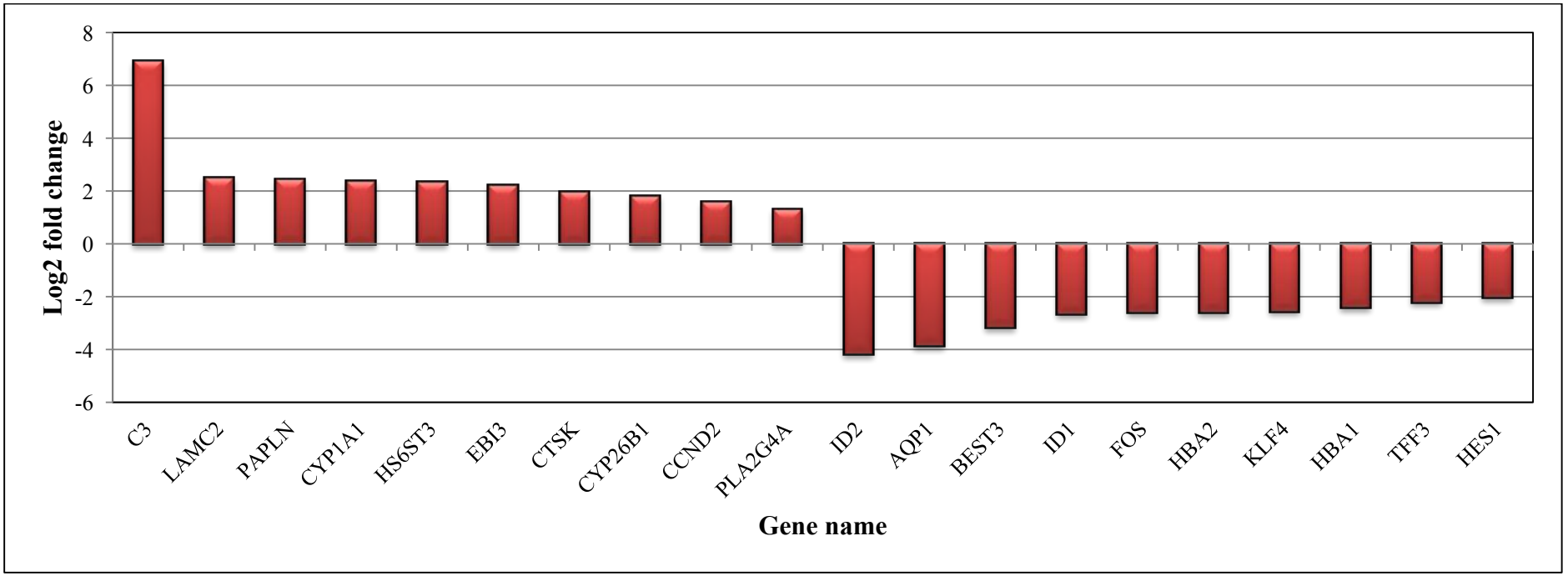


Figure 3.6: Top 10 up/down regulated genes in TNF-stimulated HBMEC incubated with IT4var14 parasite at 6 hours co-culture.
The differentially expressed genes in TNF-stimulated HBMEC co-cultured with IT4var14 parasite isolate at 6 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of those genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

3.3.6.3 Differentially expressed genes in HBMEC at 20 hours incubation:

In total of 226 differentially expressed genes were identified (log2 fold change >1, FDR value <0.05) in TNFα-stimulated HBMEC after co-culturing with IT4var14 parasite at 20 hours co-culture. These included 25% up regulated and 75% down regulated genes. Also, 10% of genes showed a >2 log2 fold change of expression level. The fold change expression among the top ten up regulated genes varies from 5 to 12, and the most robust up regulated gene was observed for cytochrome P450 family 1 subfamily A member 1 (CYP1A1) which showed 12-fold change more expression (Figure 3.7). The fold change among the top ten down regulated genes varies from -4 to -41, and consistent with the finding that noggin (NOG) and ID2 are the top two regulated genes (Figure 3.7). More details about the top ten up and down regulated genes are shown in appendix 5.

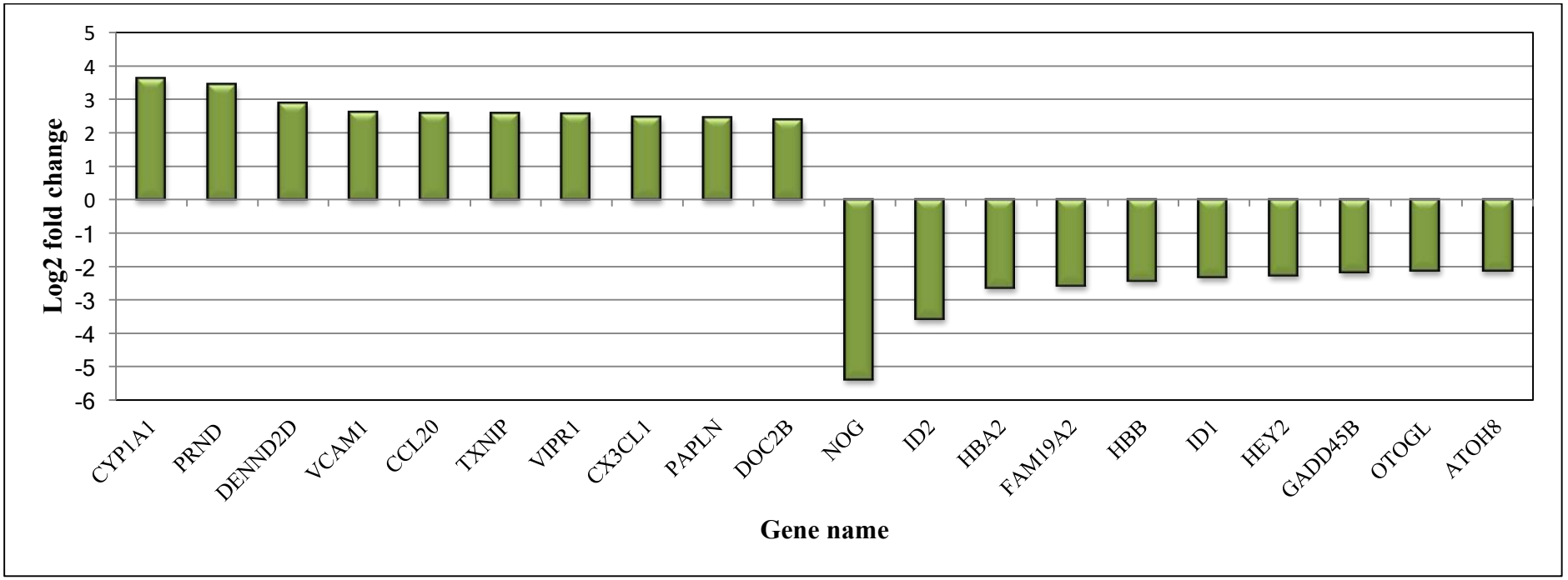


Figure 3.7: Top 10 up/down regulated genes in TNF-stimulated HBMEC incubated with IT4var14 parasite at 20 hours co-culture.
The differentially expressed genes in TNF-stimulated HBMEC co-cultured with IT4var14 parasite isolate at 20 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of those genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

3.3.7 Visualization of different expression genes:

A heatmap was used in this study to present the genes of HBMEC/TNF with high and low expression levels over different time points (2, 6 and 20 hours) that were incubated with the IT4var14 parasite. Several genes were differentially expressed over the time of co-culture from 2 to 20 hours, either increased such as CYP1A1 and DUSP1 or decreased like EBI3 and HEY2 (Figure 3.8).

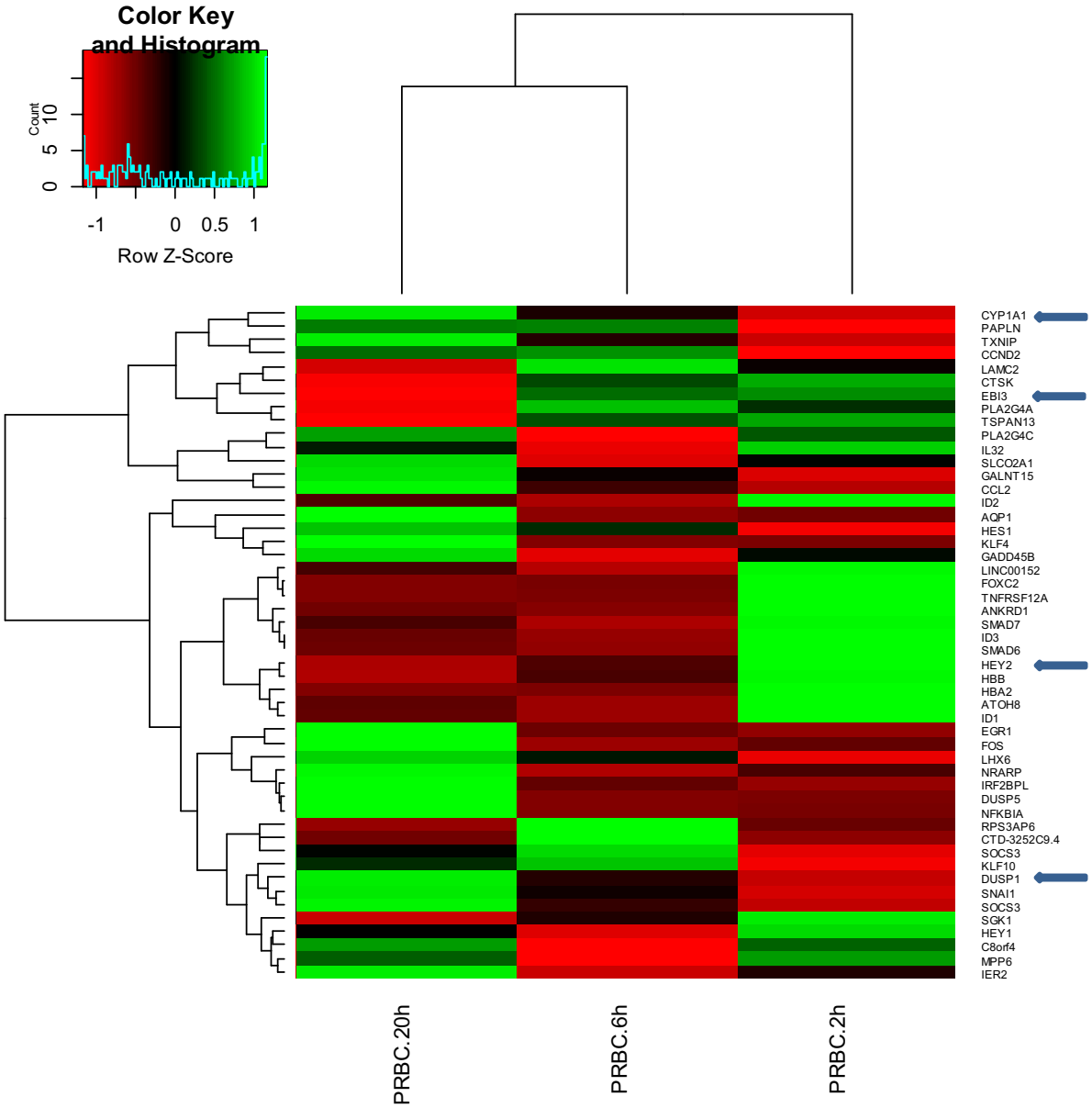


Figure 3.8: Heatmap of differentially expressed genes of HBMEC/TNF co-cultured with IT4var14 parasite at 2, 6 and 20 hours.

Each column represents the incubation time of TNF-stimulated HBMEC with IT4var14parasite and each row represents 1 gene. PRBC indicates the *P. falciparum* infected

erythrocyte. Green and red indicate expression levels above and below the mean for each gene, respectively.

3.3.8 Functional analysis of differentially expressed genes:

To functionally categorise differentially expressed genes of HBMEC/TNF in response to IT4var14 parasite at 2, 6 and 20 hours, GO analysis was performed on both up and down regulated genes separately. The GO data were categorised based on biological process, molecular function and cellular component categorise with *P* value <0.05.

At 2 hours of incubation, the biological process ontology found that positive regulation of cell migration was enriched in the up regulated genes list, while regulation of transcription from RNA polymerase II promoter were enriched in down regulated genes. With regards to molecular function, the results indicate increased cytokine activity and decreased protein binding and transcription factor binding. In terms of the cellular component, extracellular space (a space that separates one cell membrane from another) was found associated with up regulated genes. Four terms are specific to down regulated genes, which are nucleus, cytoplasm, nucleoplasm and extracellular space (Table 3.4).

Table 3.4: Functions analysis enrichment result at 2 hours incubation.

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Positive regulation of cell migration	3	2.5E-2	2.8E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Positive regulation of transcription from RNA polymerase II promoter	16	7.5E-8	1.2E-4
Negative regulation of transcription from RNA polymerase II promoter	12	6.4E-6	9.8E-3
Response to muscle stretch	4	1.5E-5	2.4E-2
Ventricular septum morphogenesis	4	8.1E-5	1.2E-1
Transforming growth factor beta receptor	5	1.5E-4	2.4E-1

signaling pathway			
Cellular response to calcium ion	4	4.4E-4	6.7E-1
Peptidyl-threonine dephosphorylation	3	4.5E-4	6.9E-1
Endoderm formation	3	5.4E-4	8.2E-1
Negative regulation of ERK1 and ERK2 cascade	4	6.4E-4	9.8E-1
Somatic stem cell population maintenance	4	9.0E-4	1.4E0
MAPK cascade	6	9.5E-4	1.5E0
Intracellular signal transduction	7	1.1E-3	1.7E0
Cellular response to retinoic acid	4	1.2E-3	1.8E0
Response to lipopolysaccharide	5	1.3E-3	2.0E0
Negative regulation of myeloid cell differentiation	3	1.2E-3	1.9E0
Cellular response to glucocorticoid stimulus	3	1.5E-3	2.3E0
Regulation of sequence-specific DNA binding transcription factor activity	3	2.4E-3	3.6E0
Inactivation of MAPK activity	3	2.4E-3	3.6E0
Negative regulation of DNA binding	3	3.0E-3	4.5E0
Negative regulation of transcription, DNA-templated	7	3.2E-3	4.7E0
Negative regulation of Notch signaling pathway	3	3.2E-3	4.8E0
Cell maturation	3	4.9E-3	7.3E0
Positive regulation of cell differentiation	3	5.2E-3	7.7E0
Positive regulation of tyrosine phosphorylation of Stat3 protein	3	5.5E-3	8.1E0
Cellular response to hormone stimulus	3	7.6E-3	1.1E1
Outflow tract morphogenesis	3	8.0E-3	1.2E1
Response to camp	3	8.0E-3	1.2E1
Skeletal muscle cell differentiation	3	9.0E-3	1.3E1
Response to progesterone	3	9.4E-3	1.3E1
Positive regulation of fibroblast proliferation	3	1.1E-2	1.5E1
Positive regulation of smooth muscle cell proliferation	3	1.3E-2	1.8E1
Cell migration	4	1.4E-2	1.9E1
Response to cytokine	3	1.2E-2	1.7E1

Cell chemotaxis	3	1.5E-2	2.1E1
Response to insulin	3	1.6E-2	2.2E1
Transcription from RNA polymerase II promoter	6	1.6E-2	2.2E1
Regulation of cell proliferation	4	1.7E-2	2.3E1
Positive regulation of transcription, DNA-templated	6	1.7E-2	2.3E1
Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	3	1.7E-2	2.3E1
Liver development	3	2.0E-2	2.6E1
Palate development	3	2.1E-2	2.7E1
Negative regulation of inflammatory response	3	2.2E-2	2.9E1
Dephosphorylation	3	2.6E-2	3.3E1
Angiogenesis	4	2.7E-2	3.4E1
Negative regulation of cell proliferation	5	2.8E-2	3.5E1
Negative regulation of protein kinase activity	3	3.4E-2	4.1E1
Peptidyl-tyrosine dephosphorylation	3	3.4E-2	4.1E1
Cell-cell signaling	4	3.8E-2	4.5E1
Activation of MAPK activity	3	3.9E-2	4.6E1
Cellular response to tumor necrosis factor	3	4.1E-2	4.7E1
Chemotaxis	3	4.9E-2	5.4E1

Cellular Component – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Extracellular space	9	2.4E-4	2.2E-1
Cell cortex	3	1.2E-2	1.1E1
Extracellular region	7	1.6E-2	1.4E1

Cellular Component – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Nucleus	30	1.9E-5	2.0E-2
Nucleoplasm	16	5.1E-3	5.4E0
Extracellular space	10	9.6E-3	9.9E0
Cytoplasm	23	1.2E-2	1.2E1

Molecular Function – Up Regulated Genes: Go term	Size	P-value	FDR
cytokine activity	3	1.9E-2	1.8E1

Molecular Function – Down Regulated Genes: Go term	Size	P-value	FDR
Transcription factor binding	7	2.0E-4	3.1E-2
Transcription factor activity, sequence-specific DNA binding	11	4.9E-4	3.8E-2
MAP kinase tyrosine/serine/threonine phosphatase activity	3	6.8E-4	3.5E-2
Sequence-specific DNA binding	8	8.5E-4	3.4E-2
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	3	2.3E-3	7.2E-2
Protein binding	29	4.5E-3	1.1E-1
Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	4	3.3E-2	4.9E-1
Protein tyrosine phosphatase activity	3	3.6E-2	4.8E-1
Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	3	4.4E-2	5.1E-1

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

For the HBMEC/TNF response to iRBC exposure at 6 hours, the biological process of up regulated genes demonstrated that members belonging to cell proliferation and proteolysis were more abundant. On the other hand, among down regulated genes in the same category, genes involved mostly in regulation of transcription, regulation of apoptotic process and regulation of Notch signalling pathway were in greater number. In the molecular function category, the majority of low expressed genes were involved in protein and DNA binding function. With respect to the cellular component, extracellular space was associated with up regulated genes. However,

cytoplasm, nucleus, nucleoplasm and cytosol were found as the most abundant terms in down regulated genes (Table 3.5).

Table 3.5: Functions analysis enrichment result at 6 hours incubation.

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Positive regulation of cell proliferation	3	3.8E-2	3.7E1
Proteolysis	3	3.8E-2	3.7E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Negative regulation of transcription from RNA polymerase II promoter	16	1.1E-10	1.7E-7
Positive regulation of transcription from RNA polymerase II promoter	14	4.8E-7	1.6E-4
Negative regulation of Notch signaling pathway	5	7.7E-7	1.7E-4
Ventricular septum morphogenesis	4	5.1E-5	8.2E-3
Transforming growth factor beta receptor signaling pathway	5	8.4E-5	1.1E-2
Bicarbonate transport	4	1.8E-4	1.9E-2
Notch signaling pathway	5	1.9E-4	1.8E-2
Negative regulation of transcription, DNA-templated	8	2.2E-4	1.7E-2
Notch signaling involved in heart development	3	2.2E-4	1.6E-2
Pulmonary valve morphogenesis	3	2.7E-4	1.7E-2
Response to hydrogen peroxide	4	3.7E-4	2.1E-2
Negative regulation of sequence-specific DNA binding transcription factor activity	4	5.2E-4	2.8E-2
Oxygen transport	3	6.3E-4	3.1E-2
Response to muscle stretch	3	8.1E-4	3.7E-2
Labyrinthine layer blood vessel development	3	9.1E-4	3.9E-2
BMP signaling pathway	4	9.4E-4	3.7E-2
Negative regulation of apoptotic process	7	1.0E-3	3.7E-2
Hydrogen peroxide catabolic process	3	1.1E-3	4.0E-2
Artery morphogenesis	3	1.4E-3	4.6E-2
Positive regulation of transcription, DNA-templated	7	1.7E-3	5.3E-2
Metanephros development	3	2.1E-3	6.2E-2

Positive regulation of cell death	3	2.7E-3	7.7E-2
Ureteric bud development	3	4.1E-3	1.1E-1
Negative regulation of osteoblast differentiation	3	4.7E-3	1.2E-1
Negative regulation of BMP signaling pathway	3	5.7E-3	1.3E-1
Skeletal muscle cell differentiation	3	6.9E-3	1.5E-1
Response to progesterone	3	6.9E-3	1.5E-1
Positive regulation of apoptotic process	5	7.6E-3	1.6E-1
Response to cytokine	3	8.9E-3	1.8E-1
Transcription from RNA polymerase II promoter	6	9.0E-3	1.7E-1
SMAD protein signal transduction	3	1.1E-2	1.9E-1
Cellular response to organic cyclic compound	3	1.1E-2	1.9E-1
Cellular oxidant detoxification	3	1.2E-2	2.0E-1
Cellular response to retinoic acid	3	1.4E-2	2.2E-1
Protein heterooligomerization	3	1.4E-2	2.2E-1
Regulation of apoptotic process	4	1.6E-2	2.4E-1
Cellular response to interleukin-1	3	1.8E-2	2.5E-1
Angiogenesis	4	1.9E-2	2.5E-1
Transcription, DNA-templated	11	2.0E-2	2.6E-1
Neuron differentiation	3	2.6E-2	3.0E-1
Cellular response to tumor necrosis factor	3	3.1E-2	3.4E-1
Nervous system development	4	3.5E-2	3.5E-1
Regulation of cell cycle	3	3.8E-2	3.7E-1
Negative regulation of gene expression	3	4.7E-2	4.2E-1

Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Transcription factor activity, sequence-specific DNA binding	14	1.9E-7	2.4E-5
Transcription factor binding	9	2.9E-7	1.9E-5
Haptoglobin binding	3	1.7E-5	7.1E-4
Transcription regulatory region DNA binding	6	1.5E-4	4.6E-3
Transcriptional activator activity, RNA	6	2.2E-4	5.6E-3

polymerase II core promoter proximal region sequence-specific binding			
Oxygen transporter activity	3	5.0E-4	1.1E-2
R-SMAD binding	3	1.1E-3	2.1E-2
Peroxidase activity	3	1.2E-3	2.0E-2
Sequence-specific DNA binding	7	1.3E-3	1.9E-2
Histone deacetylase binding	4	2.0E-3	2.5E-2
Protein binding	25	2.2E-3	2.5E-2
Protein dimerization activity	4	5.4E-3	5.2E-2
Oxygen binding	3	5.6E-3	5.0E-2
Heme binding	3	4.2E-2	2.3E-1

Cellular Component – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Extracellular space	4	4.4E-2	3.2E1

Cellular Component – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Haptoglobin-hemoglobin complex	3	3.1E-5	2.0E-3
Cytoplasm	25	1.1E-4	3.6E-3
Nucleus	25	2.1E-4	4.5E-3
Nucleoplasm	17	2.7E-4	4.4E-3
Hemoglobin complex	3	3.4E-4	4.4E-3
Endocytic vesicle lumen	3	6.1E-4	6.6E-3
Transcription factor complex	5	1.1E-3	1.0E-2
Blood microparticle	3	4.4E-2	2.8E-1
Cytosol	13	7.8E-2	4.1E-1

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

With regards to co-culturing the HBMEC/TNF with the IT4var14 parasite at 20 hours, several GO terms were explored with both up and down regulated genes. In

the biological process category, highly expressed genes were found involved in cell chemotaxis, inflammatory response, immune response and cell adhesion. Whereas, low expressed genes were strongly associated with regulation of transcription, DNA-templet, regulation of transcription from RNA polymerase II promoter and cell division. Molecular function terms demonstrated that chemokine activity was the only term identified in the up regulated genes. Most of the down regulated genes were involved in protein binding and DNA binding. In terms of the cellular component category, only 3 terms were specific to up regulated genes, which were plasma membrane, extracellular space and extracellular region. However, down regulated genes were found mostly associated with nucleus, cytoplasm, nucleoplasm, cytosol and membrane (Table 3.6).

Table 3.6: Functions analysis enrichment result at 20 hours incubation.

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Cell chemotaxis	5	1.5E-5	6.0E-3
Chemokine-mediated signaling pathway	5	2.2E-5	4.3E-3
Chemotaxis	5	1.8E-4	2.4E-2
Lymphocyte chemotaxis	3	1.9E-3	1.7E-1
Cellular response to tumor necrosis factor	4	2.1E-3	1.5E-1
Monocyte chemotaxis	3	4.3E-3	2.5E-1
Response to hypoxia	4	7.4E-3	3.4E-1
Cellular response to interferon-gamma	3	7.7E-3	3.2E-1
Response to mechanical stimulus	3	8.3E-3	3.1E-1
Positive regulation of gtpase activity	6	9.5E-3	3.2E-1
Regulation of blood pressure	3	1.0E-2	3.0E-1
Neutrophil chemotaxis	3	1.0E-2	2.9E-1
Cellular response to drug	3	1.1E-2	2.9E-1
Inflammatory response	5	1.1E-2	2.8E-1
Cellular response to interleukin-1	3	1.2E-2	2.7E-1

Positive regulation of inflammatory response	3	1.2E-2	2.7E-1
Immune response	5	1.6E-2	3.0E-1
Cell adhesion	5	2.1E-2	3.5E-1

Biological Process – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Cell division	16	6.8E-9	1.1E-5
Negative regulation of transcription from RNA polymerase II promoter	20	1.5E-7	2.3E-4
Negative regulation of transcription, DNA-templated	15	3.8E-6	6.0E-3
Mitotic nuclear division	11	4.5E-6	7.1E-3
Ventricular septum morphogenesis	5	3.4E-5	5.4E-2
Positive regulation of transcription from RNA polymerase II promoter	19	5.1E-5	8.0E-2
Sister chromatid cohesion	7	5.3E-5	8.4E-2
Negative regulation of pathway-restricted SMAD protein phosphorylation	4	5.5E-5	8.6E-2
Chromosome segregation	6	7.6E-5	1.2E-1
Positive regulation of transcription, DNA-templated	13	1.2E-4	1.9E-1
Negative regulation of BMP signaling pathway	5	2.0E-4	3.1E-1
Transforming growth factor beta receptor signaling pathway	6	3.2E-4	5.0E-1
G1/S transition of mitotic cell cycle	6	5.1E-4	8.0E-1
Negative regulation of sequence-specific DNA binding transcription factor activity	5	6.0E-4	9.4E-1
Somatic stem cell population maintenance	5	8.1E-4	1.3E0
Mitotic spindle organization	4	9.3E-4	1.5E0
Notochord development	3	1.4E-3	2.2E0
Notch signaling involved in heart development	3	1.4E-3	2.2E0
Regulation of mitotic metaphase/anaphase transition	3	1.4E-3	2.2E0
BMP signaling pathway	5	1.5E-3	2.3E0
Mitotic metaphase plate congression	4	1.7E-3	2.7E0
Protein localization to kinetochore	3	1.8E-3	2.8E0
Pulmonary valve morphogenesis	3	1.8E-3	2.8E0
Ureteric bud development	4	1.9E-3	2.9E0
Negative regulation of osteoblast differentiation	4	2.0E-3	3.1E0
Cellular response to peptide	3	2.2E-3	3.4E0

Positive regulation of astrocyte differentiation	3	2.2E-3	3.4E0
Cell proliferation	9	2.5E-3	3.9E0
Forebrain development	4	3.2E-3	5.0E0
Positive regulation of cellular protein metabolic process	3	4.1E-3	6.3E0
Regulation of G1/S transition of mitotic cell cycle	3	4.6E-3	7.1E0
Labyrinthine layer blood vessel development	3	6.5E-3	9.8E0
Positive regulation of gene expression	7	6.9E-3	1.0E1
Artery morphogenesis	3	7.9E-3	1.2E1
Peptidyl-serine phosphorylation	5	8.6E-3	1.3E1
Positive regulation of cardiac muscle cell proliferation	3	8.7E-3	1.3E1
Negative regulation of apoptotic process	9	9.1E-3	1.3E1
Cellular response to drug	4	1.0E-2	1.5E1
Positive regulation of erythrocyte differentiation	3	1.0E-2	1.5E1
Transcription, DNA-templated	22	1.2E-2	1.8E1
Metanephros development	3	1.3E-2	1.9E1
Mesoderm formation	3	1.4E-2	2.0E1
Ventricular septum development	3	1.4E-2	2.0E1
Regulation of cell growth	4	1.5E-2	2.1E1
Negative regulation of Notch signaling pathway	3	1.5E-2	2.1E1
Microtubule-based movement	4	1.5E-2	2.2E1
DNA replication	5	1.8E-2	2.5E1
DNA replication initiation	3	1.8E-2	2.5E1
Cell maturation	3	2.2E-2	3.0E1
Male gonad development	4	2.3E-2	3.0E1
Positive regulation of cytokinesis	3	2.4E-2	3.1E1
Cellular response to hypoxia	4	2.4E-2	3.2E1
Peptidyl-threonine phosphorylation	3	2.5E-2	3.3E1
Osteoblast differentiation	4	3.0E-2	3.8E1
Positive regulation of nitric oxide biosynthetic process	3	3.1E-2	3.9E1
Outflow tract morphogenesis	3	3.5E-2	4.3E1
Notch signaling pathway	4	3.8E-2	4.6E1
Negative regulation of cell proliferation	7	4.3E-2	5.0E1

Positive regulation of apoptotic process	6	4.4E-2	5.1E1
Transcription from RNA polymerase II promoter	8	4.8E-2	5.4E1
Negative regulation of neuron differentiation	3	5.0E-2	5.6E1

Molecular Function – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Chemokine activity	4	2.0E-4	2.3E-1

Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Protein binding	83	6.2E-8	1.5E-5
Transcription factor activity, sequence-specific DNA binding	20	7.5E-6	9.2E-4
Transcription factor binding	10	7.5E-5	6.1E-3
Transcription regulatory region DNA binding	8	3.9E-4	2.4E-2
Chromatin binding	9	3.2E-3	1.5E-1
ATP-dependent microtubule motor activity, plus-end-directed	3	5.0E-3	1.8E-1
Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	5	5.3E-3	1.7E-1
Atpase activity	6	5.9E-3	1.7E-1
Histone binding	5	7.3E-3	1.8E-1
Peroxidase activity	3	8.3E-3	1.8E-1
Microtubule motor activity	4	1.4E-2	2.5E-1
Protein dimerization activity	5	1.5E-2	2.4E-1
Histone deacetylase binding	4	2.6E-2	3.5E-1
DNA binding	18	3.2E-2	3.7E-1
RNA polymerase II transcription factor binding	3	3.5E-2	3.9E-1
Transcription corepressor activity	5	3.9E-2	4.0E-1
Protein homodimerization activity	10	4.0E-2	3.9E-1
Microtubule binding	5	4.2E-2	3.9E-1
Protein domain specific binding	5	4.2E-2	3.9E-1
ATP binding	16	4.7E-2	4.1E-1
RNA polymerase II core promoter sequence-specific DNA binding	3	5.0E-2	4.2E-1

Cellular Component – Up Regulated Genes: Go term	Size	P-value	FDR
Extracellular space	11	5.1E-4	5.5E-1
Extracellular region	9	2.2E-2	2.1E1
Plasma membrane	16	2.4E-2	2.3E1

Cellular Component – Down Regulated Genes: Go term	Size	P-value	FDR
Nucleoplasm	48	4.3E-12	5.2E-9
Nucleus	64	1.6E-9	1.9E-6
Cytoplasm	52	5.8E-5	7.0E-2
Condensed chromosome outer kinetochore	3	9.9E-4	1.2E0
Midbody	6	1.1E-3	1.3E0
Nuclear chromatin	7	1.1E-3	1.3E0
Transcription factor complex	7	1.1E-3	1.3E0
Kinetochore	5	1.5E-3	1.7E0
Cytosol	34	1.7E-3	2.1E0
Condensed chromosome kinetochore	5	1.9E-3	2.3E0
Spindle microtubule	4	2.4E-3	2.8E0
Kinesin complex	4	4.0E-3	4.7E0
Transcriptional repressor complex	4	4.5E-3	5.2E0
Spindle midzone	3	5.8E-3	6.7E0
Protein complex	8	1.2E-2	1.4E1
Centrosome	8	1.5E-2	1.6E1
Membrane	22	2.2E-2	2.3E1
Spindle pole	4	2.8E-2	2.9E1
Chromosome, centromeric region	3	4.6E-2	4.4E1

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

3.3.9 Pathway analysis of differentially expressed genes:

The GO database does not cover every biological aspect of different regulated genes and several GO classes overlap with each other. The Reactome pathway enrichment analysis was used in order to illustrate selected sets of differentially expressed genes in HBMEC/TNF incubated with *P. falciparum* IT4var14 at 2, 6 and 20 hours in particular pathways.

The study identified 59 host response pathways that were significantly up regulated in response to IT4var14 parasite in HBMEC/TNF ($P < 0.05$ and at least 3 input genes) at 2 hours. The most affected pathways of the up regulated genes were immune system followed by cytokine signalling in the immune system (8 and 7 genes respectively (Figure 3.9). On the basis of pathways of down regulated genes, 100 host response pathways were identified for this time point of co-culture. The highly significant pathways in terms of numbers of genes were signal transduction and the immune system (Figure 3.9).

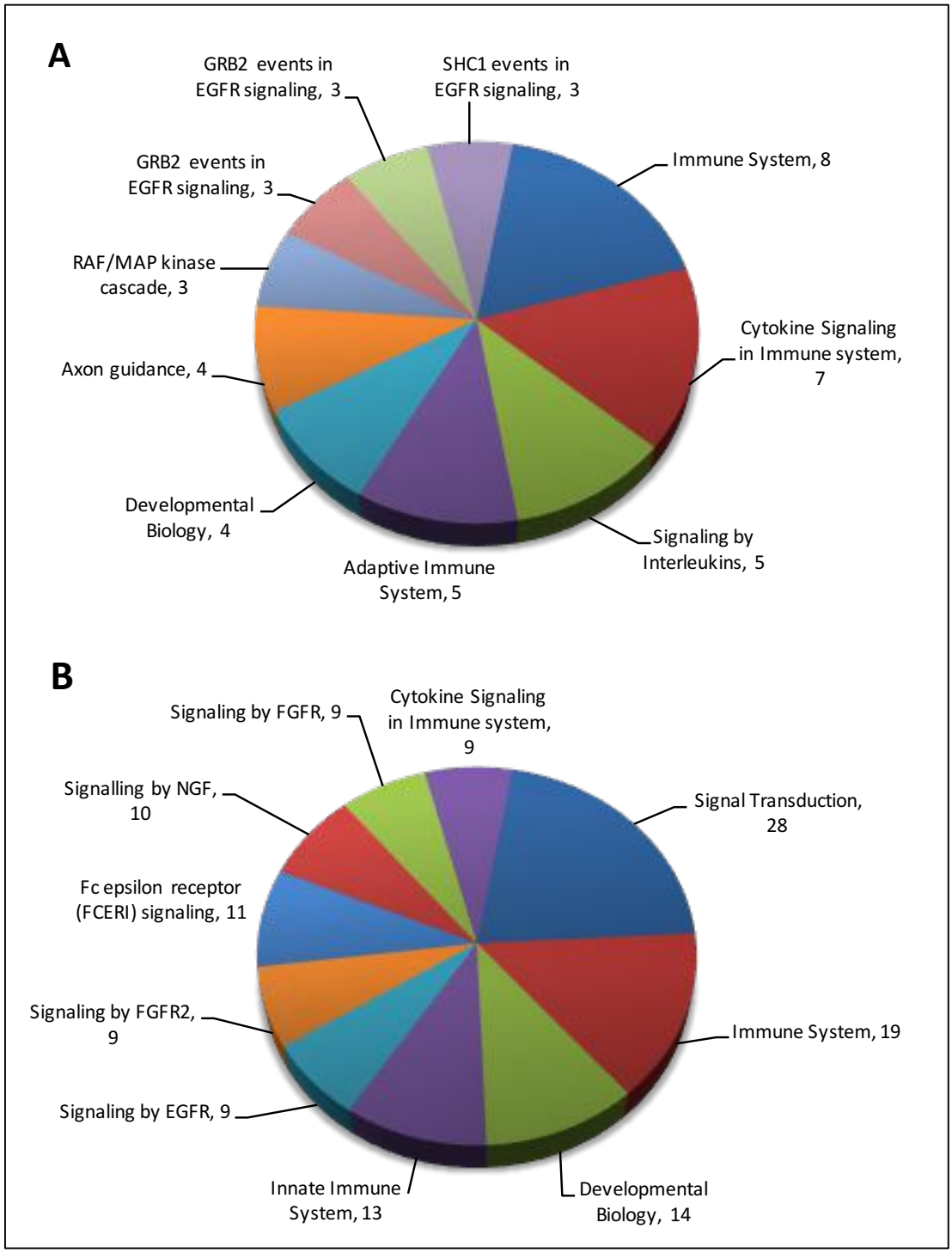


Figure 3.9: Top ten pathways of up and down regulated genes of HBMEC/TNF responding to iRBC at 2 hours.

A) pathways of up regulated genes and **B)** pathways of down regulated genes. The pathways were selected according to the number of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

At 6 hours of incubation, only 2 pathways were observed with highly expressed genes, both of which are associated with the immune system (Table 3.7). In contrast, 18 pathways were found associated with low expressed genes. The most significant pathways of the down regulated genes in terms of numbers of genes were signal transduction, the citric acid (TCA) cycle and respiratory electron transport and signalling by NOTCH1 (Figure 3.10).

Table 3.7: pathways of up regulated genes of HBMEC responded to *P. falciparum* and TNF exposure at 6 hours.

Rank	Pathway name	No. of genes	*P* value
1	Innate Immune System	4	1.87E-2
2	Immune System	5	3.91E-2

The pathways were selected based on number of genes and *P* value <0.05.

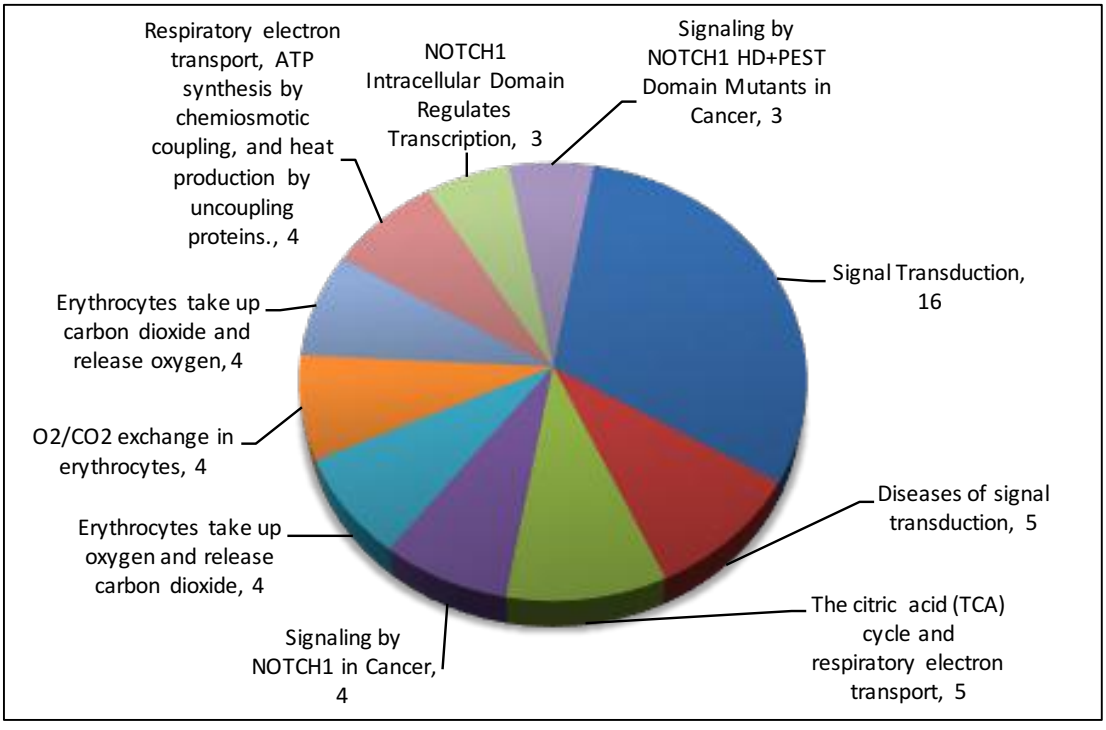


Figure 3.10: Top ten pathways of down regulated genes of HBMEC/TNF responding to iRBC at 6 hours.
The pathways were selected according to numbers of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

The pathway analysis identified 22 and 73 pathways of up regulated and down regulated genes respectively at 20 hours of co-culture. Extracellular matrix organisation and developmental biology were found as top pathways of up regulated genes (Figure 3.11). However, signal transduction and the cell cycle were the majority of down regulated genes at this time point, with 49 and 34 genes respectively (Figure 3.11).

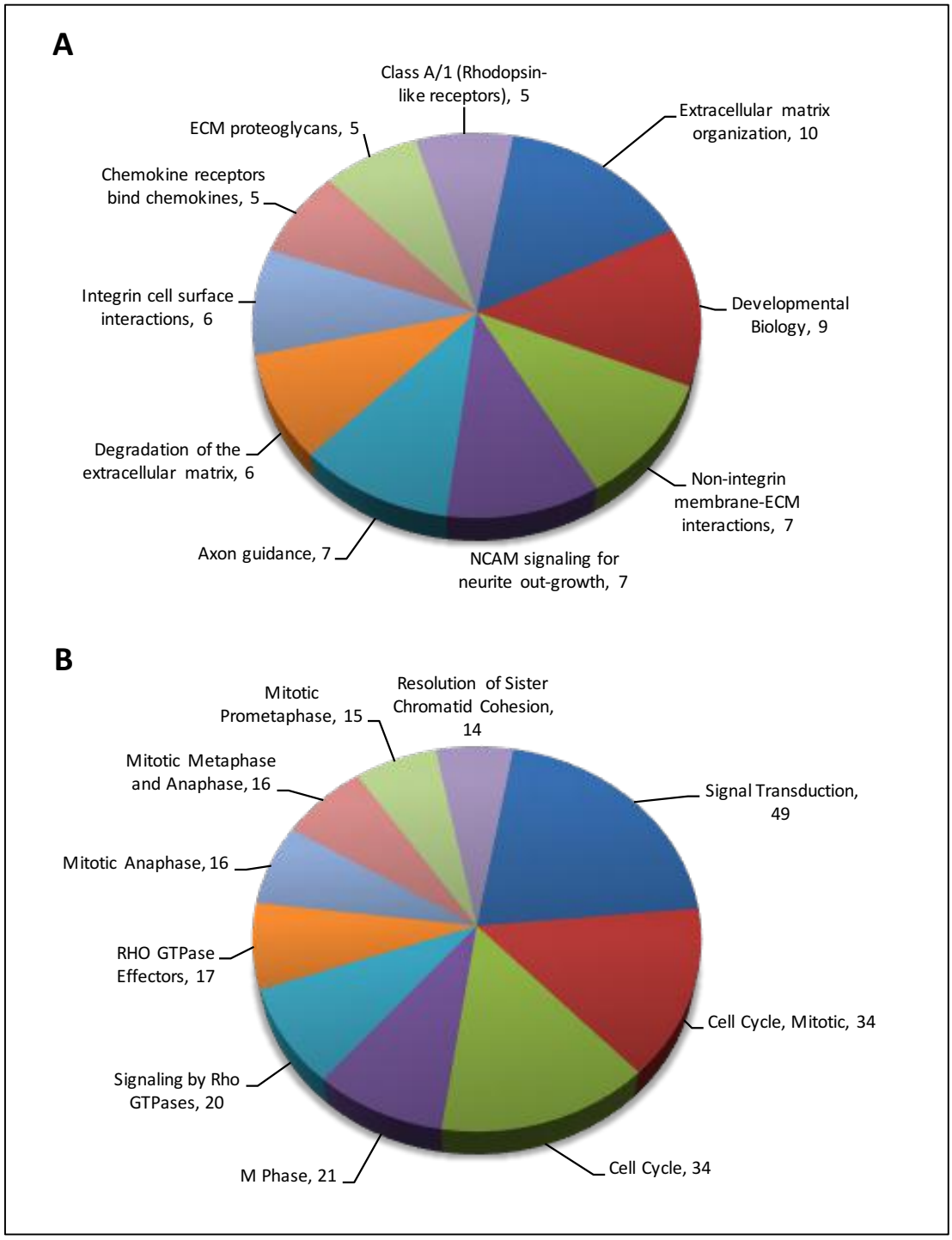


Figure 3.11: Top ten pathways of up and down regulated genes of HBMEC/TNF responding to iRBC at 20 hours.

A) pathways of up regulated genes and **B)** pathways of down regulated genes. The pathways were selected according to number of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

3.3.10 Validation of seven differentially regulated gene by qRT-PCR:

To validate the results of the RNA-seq in this study using an alternative method, we performed a qRT-PCR experiment to measure the expression of seven selected genes. In RNA-seq data, LAMC2, PLA2G4A and VCAM-1 were up regulated by 5.7, 2.48 and 1.26-fold change respectively, whereas; ID2, AQP1, HBA2 and NFKBIA were down regulated by-17.75, -14.44, -6 and -2.21-fold change respectively. In qRT-PCR data, these corresponding numbers in up regulated genes were 4.19, 1.82 and 1.43-fold change respectively, while; in down regulated genes were -16.69, -16.64, -3.5 and -1.95-fold change respectively (Figure 3.12). The results of these seven genes examined by RNA-seq and qRT-PCR were in a good agreement, which corroborates our RNA-seq outputs.

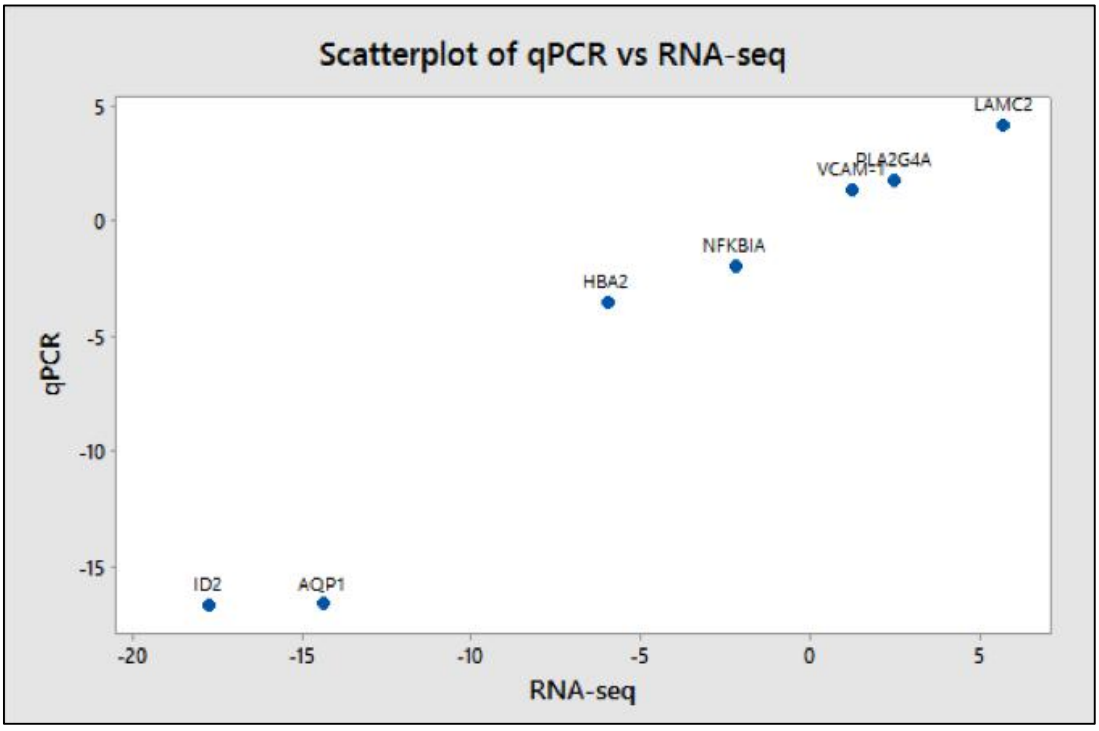


Figure 3.12: qRT-PCR validation of seven differentially expressed genes from HBMEC/TNF responding to IT4var14 iRBC at 6 hours co-culture.
The scatter plot shows the fold changes of the seven genes determined from the qRT-PCR were compared to those detected by RNA-seq. Replicates (n=3) of each sample were run in qRT-PCR.

3.4 Discussion:

Cerebral malaria pathogenesis is known to be associated with sequestration of *P. falciparum* iRBC in the brain microvasculature, resulting in immunomodulatory interactions and complex cellular responses, including co-regulators such as adhesion molecules and cytokines. The pathophysiological mechanisms of CM are not yet completely understood. The role of the endothelial cells in the CM pathogenicity was investigated previously using microarray technique (Chakravorty et al., 2007, Tripathi et al., 2009). However, there is still a need to further investigate the role of the brain endothelial cell in disease with more sensitive next generation sequencing techniques such as RNA-seq. To better understand the responses of the brain endothelium to interaction with *P. falciparum* iRBC after stimulation with the cytokine TNF, we performed the global transcriptional profile of the HBMEC after exposure with IT4var14 parasite isolate at 2, 6 and 20 hours. In addition, we investigated the functional and pathway analysis of the differentially expressed genes under the same conditions.

Our study provides the first comprehensive insight into the transcriptome of human brain microvascular endothelial cells co-cultured with an ICAM-1 binding parasite line using RNA-seq, a powerful next generation sequencing platform, since a literature search for PubMed database with key words of cerebral malaria, HBMEC and RNA-seq retrieves zero records as of March 2017.

In order to obtain parasite and EC at high quality for the co-culturing experiments, we monitored for the presence of mycoplasma contamination in parasite and brain endothelial cells, the binding of the IT4var14 parasite to ICAM-1 and CD36 proteins using static adhesion assays and the expression of ICAM-1 before and after TNF stimulation by FACS prior to setting up the co-cultures.

The first important step to obtain a good RNA sequencing is extraction of the RNA with high quality (RIN>8) (Bettscheider et al., 2011). Our RNA samples isolated from the co-culture experiments at 0, 2, 6 and 20 hours were observed to have RIN values more than (8.5), and that supports the robustness of our RNA-seq results. To investigate the quality of the RNA-seq raw data, we used the FastQC tool, and all the data of RNA-seq have passed all aspects of the sequence quality measures. The average of the total number of reads that were produced from each sample was 27,198,576, which is enough to deliver sufficient sequence coverage for genome-wide transcriptome profiling based to a pervious report by Sultan *et al*. (Sultan et al., 2008). Our average of 92.1% total sequences that mapped to human reference genome met the standards of the quality of the RNA-seq technique (Mortazavi et al., 2008).

In this section, genes identified by the RNA-seq data from human brain endothelial cells after TNF stimulation exposed to IT4var14 parasite for 2, 6 and 20 hours will be discussed according to their functions and pathways. There are numerous reasons for selecting these time points in our co-culture model: First, the expression of ICAM-1 protein on human brain endothelial cells is significantly increased after 1 hour of incubation with *P. falciparum* iRBC and TNF, thus increasing the probability of parasite sequestration to the host cell. Second, the maximum level of ICAM-1 expression was observed at 6 hours of co-culture between malaria parasites and TNF induced human brain endothelial cells (Tripathi et al., 2006). Third, we hypothesised that at 20 hours of co-incubation, the IT4var14 parasite will be at a mature trophozoite stage, and some of the iRBCs will be ruptured and release several soluble molecules that may affect the host cells genes expression. The discussion will mainly focus on major pathways including immune system at 2 hours of co-culture; immune

system, arachidonic acid, pro-apoptotic as well as anti-apoptotic processes at 6 hours of incubation; and briefly, immune system and cell cycle at 20 hours of exposure.

We observed significant up regulation of 33 HBMEC genes and down regulation of 76 genes in response to *P. falciparum* and TNF exposure at 2 hours. Functional and pathways analysis of differentially regulated genes revealed impact on several substantial biological pathways. Among the up regulated genes were molecules belonging to the immune system (Table 3.8); whereas, the down regulated genes also belonged to immune system (Table 3.9).

Table 3.8: Up regulated expression of selected genes representing specific pathways in HBMEC co-cultured with IT4var14 parasite and TNF at 2 hours, such as immune system.

GO term	Genes symbol	Fold change
Immune system	IL32	3.24
	EBI3	5.3
	CTSK	4.65

The table illustrates fold changes in HBMEC response to infected red blood cells and TNF, compare with control.

Table 3.9: Down regulated expression of selected genes representing specific pathways in HBMEC co-cultured with IT4var14 parasite and TNF at 2 hours, such as immune system.

GO term	Genes symbol	Fold change
Immune system	CCL2	-4.1
	CXCL1	-2.1
	FOS	-4.71

The table illustrates fold changes in HBMEC response to infected red blood cells and TNF, compare with control.

Our study demonstrated an increase in mRNA for IL32, which was more than 3-fold change. IL32 is a cytokine that can induce proinflammatory mediators and further

contribute to autoimmune diseases (Heinhuis et al., 2012). A study by Netea *et al* found that stimulation of whole blood with *Mycobacterium tuberculosis* can induce the production of IL32 (Netea et al., 2006). Furthermore, it was reported that expression of IL32 increased in virus infections such as human immunodeficiency virus (HIV) (Rasool et al., 2008) and influenza virus (Li et al., 2008b). The role of the IL32 cytokine in CM is still unclear and needs to be investigated.

Over expression of EBI3 genes have been observed in this study at 2 hours of incubation. EBI3 is a soluble α-receptor that consists of 2 fibronectin-like domains and is a unit of formation of the IL27 cytokine, which has apro- and anti-inflammatory functions (Aparicio-Siegmund and Garbers, 2015). Compared with another report on endothelial response to malaria interaction, Tripathi *et al* found that EBI3 gene is a NF-kappaB target gene in brain endothelial cells, induced by *P. falciparum* and TNF exposure (Tripathi et al., 2009). Little is known about the effect of EBI3 in CM pathogenesis.

Another overexpressed gene that is involved in immune system pathways at 2 hours exposure is the CTSK gene, which was highly activated with around 5-fold change. Cathepsin K is a peptidase C1 protein family member that is known to be a lysosomal cysteine protease (Chen et al., 2007, Dodds et al., 2001). Several studies demonstrated the role of CTSK in the immune system. Work by Asagiri *et al* in mice suggested that CTSK is required in functions of Toll-like receptor (TLR) 9 in dendritic cells (DCs), which play a critical role in recognition of several microbial products and stimulation of defence responses (Asagiri et al., 2008). Furthermore, in terms of a role for CTSK in TLRs functions, TLRs are an essential part of the innate and adaptive immune system and have been suggested as driving the inflammation in the periodontitis (Beklen et al., 2008, Xie et al., 2011). Moreover, CTSK also has

important functions in the immune system during periapical diseases (Ma et al., 2013). The role of the CTSK in cerebral malaria requires further investigations.

In interacting of the IT4var14 malaria strain and TNF with brain endothelial cells at 2 hours, our findings suggested that the potential role of malaria parasite in activation of the immune system in HBMEC during overexpression of the IL32, EBI3 and CTSK genes.

The incubation of IT4var14 parasite isolate and TNF with human brain endothelial cells at 2 hours is also associated with reduction in mRNA expression level of several candidate genes involved in immune system (Table 3.9). We demonstrated that there is a reduction in expression of CCL2 gene by around -4-fold change. CCL2 or chemokine monocyte chemoattractants protein-1(MCP-1) is considered to be one of the strongest chemoattractants for monocytes in inflammation (Ransohoff, 2002, De Groot and Woodroofe, 2001). In the brain, it is produced by astrocytes and resident microglia, and to some extent by endothelial cells (Glabinski et al., 1996, Hanisch, 2002, Harkness et al., 2003).

In a study curried out by Stamatovic *et al* to clarify the influences of the CCL2 on BBB permeability, it was found that CCL2 induces a significant increase in the BBB permeability in *in vivo* conditions (Stamatovic et al., 2005). Moreover, in an *in vitro* study on mouse brain endothelial cells by Stamatovic *et al*, they showed that CCL2 increases the brain endothelial permeability through induction of tight junction proteins such as ZO-1, ZO-2, occludin and claudin-5 (Stamatovic et al., 2003). In terms of the impact of the CCL2 in malaria disease, Abrams *et al* showed that placental malaria infection associates with induction in expression of CCL2 (Abrams et al., 2003). The results from the above studies demonstrate an increase in CCL2 expression leads to dysfunction of the BBB and hence damaging of the brain cells,

whereas our outcomes showed a negative regulation in expression of CCL2 gene and that may play a protective role against the CM disease.

As demonstrated by GO and pathway analysis, we found that the expression of CXCL1 gene was decreased by approximately -2-fold change. CXCL1 (also known as keratinocyte-derived chemokine, KC) is a chemoattractant for neutrophils, which plays an essential role in inflammation (Baggiolini et al., 1989), and has also been identified in human as growth-regulated gene-alpha (GROα) (Semple et al., 2010). Several studies have revealed the role of CXCL1 in brain, Parasadaniantz and Rostene in their study in mouse brain showed that the involvement of CXCL1 in neurotransmitter release (Melik-Parsadaniantz and Rostene, 2008). Furthermore, CXCL1 seems to be involved in the pathology of human multiple sclerosis (MS) (Lund et al., 2004) and also involved in ischemic brain injury during neutrophil infiltration in ischemic brain tissue (Emerich et al., 2002).

Compared with other reports on malaria-endothelial cell responses, Tripathi *et al* observed that CXCL1 as a NF-kappaB target gene was up regulated consistent with the crucial role of the endothelial in enhancing leukocyte infiltration at the site of parasite sequestration or after sequestration (Tripathi et al., 2009). In contrast, our results showed a decrease in expression of this gene and emphasize the role of different malaria parasite variants in activation of the human brain endothelial cells and also giving a potential role of modulating host defence against the malaria parasite infection.

Another down regulated gene which was involved in immune system pathway in our co-culture study at 2 hours was the FOS gene. FOS is known as a metabolic indicator and it triggers the overexpression of downstream genes during hypoxia. (Mishra et al., 1998). Stimulation of FOS is important for the neuron survival, because it

indicates the susceptibility of a particular region of the brain specially when exposed to tissue hypoxia (Ness et al., 2008). A study by Ma *et al* on *P. berghei* cerebral malaria showed that an induction in the expression level of FOS was observed in experimental murine *P. berghei*-induced cerebral malaria (Ma et al., 1997). In addition, the first study that was directly linking the expression levels of FOS to human malaria disease was carried out by Sobota *et al* in children aged between 6 months and 5 years in Bandiagara and found that according to the microarray analysis, FOS gene was expressed at higher level in UM cases (Sobota et al., 2016). The author concluded that the possible reason for elevation this gene's expression in uncomplicated malaria cases is prolonged suppression of FOS gene following severe infection. On the other hand, inhibition of this gene is likely to correlate with neuron dysfunction. Further studies should be conducted to clarify the role of this gene in human CM disease.

As a result of interaction the IT4var14 parasite and TNF iRBC to modulate the gene expression pathways on host endothelium involved in the immune system as a protective role through induction of specific expression genes or pathogenic role during reduction of particular genes in HBMEC.

The most critical time point in our study is a 6 hours incubation, as at this time we would expect the highest level of *PfEMP-1*-dependent iRBC adhesion to ICAM-1 and thus increase the sequestration level in HBMEC. Our study observed that 15 and 75 up and down regulation genes respectively were expressed by HBMEC under this condition. Analysis of functions and pathways of genes with differential expression showed that the significant over-expressed genes were involved in immune system pathway and arachidonic acid pathway (Table 3.10), while; the significantly under-expressed genes were involved in several pathways such as immune system, positive

regulation of apoptotic process, negative regulation of apoptotic process (Table 3.11).

Table 3.10: Up regulated expression of selected genes representing specific pathways in HBMEC co-cultured with IT4var14 parasite and TNF at 6 hours, such as immune system and arachidonic acid pathway.

GO term	Genes symbol	Fold change
Immune system	C3	121.32
	TXNIP	2.14
	EBI3	4.67
	CTSK	3.9
Arachidonic acid pathway	PLA2G4A	2.45

The table illustrates fold changes in HBMEC response to infected red blood cells and TNF, compare with control.

Table 3.11: Down regulated expression of selected genes representing specific pathways in HBMEC co-cultured with IT4var14 parasite and TNF at 6 hours, such as immune system, Positive regulation of apoptotic process and Negative regulation of apoptotic process.

GO term	Genes symbol	Fold change
Immune system	RELB	-3.17
	EGR1	-3.7
	CCL2	-2.02
	FOS	-6.1
Positive regulation of apoptotic process	NFKBIA	-2.21
	GADD45B	-8.2
	ID1	-6.33
Negative regulation of apoptotic process	DUSP1	-2.4
	AQP1	-14.44
	ID3	-3.32

The table illustrates fold changes in HBMEC response to infected red blood cells and TNF, compare with control.

The immune response of the host is considered to be a critical factor in the CM pathogenesis. The most significant up regulated gene at 6 hours of co-culture was C3 with around 121-fold change. Complement component 3 is a central molecule in the complement system, it supports the activation of all three complement activation pathways; the classical, alternative and lectin pathways. C3 plays particular roles in the immune system, providing an immediate response to pathogens, also keeping the cascade alert, increasing the amplification of the complement response and assisting to coordinate downstream immune responses (Ricklin et al., 2016). Although, C3 is a common molecule which activates all three pathways of the complement system, it become the target for several viruses that have developed mechanisms against C3 such as, vaccinia, herpes simplex virus (HSV) type 1 and type 2 (HSV-2) (Sahu et al., 1998).

A study by Lackner *et al* on mice infected with *Plasmodium berghei* suggested that C1q and C5 as complement factors have roles in the neuropathology of CM, whereas C3 did not show any significant alterations in the brain (Lackner et al., 2008). On the other hand, our results provide evidence for a highly significant increase in expression of C3 on the brain endothelial cells and this may correlate with *P. falciparum* sequestration specifically when compared with the C3 expression at 2 hours of incubation with IT4var14 parasite and TNF cytokine. Also, the induction of C3 expression suggests the role of the host immune response in this neuro-infectious disease.

Another significantly up regulated gene, also involved in the immune response, is thioredoxin interacting protein (TXNIP). TXNIP is a vitamin D3-induced gene, an inhibitor of the thioredoxin (TRX), mediates inhibition of the proliferation of the cell and has a pro-apoptotic function through stimulation of apoptosis signal regulation

kinase 1 (ASK1) (Zhou and Chng, 2013). It has been reported its role also in increase the oxidative stress as a result of cellular apoptosis (Tobiume et al., 2001). Despite the expression of TNXIP in our study being high, it is still not known what the role of TXNIP is in the CM disease.

When comparing the expression of EBI3 and CTSK genes in 2 and 6 hours of incubation HBMEC, we observed that the expression of these genes at 6 hours of co-culture were slightly decreased and this may be explained with gradually increased expression of ICAM-1 and sequestration of iRBC on HBMEC, thus a little decrease of the immune system in response to increase *P. falciparum* infection.

One of the most important observations in our study is induction of the PLA2G4A gene. PLA2G4A is a metabolic enzyme which catalyses hydrolysis of membrane phospholipids to release arachidonic acid and prostanoid biosynthesis (Hua et al., 2015) (Figure 3.13). The expression of this gene is up regulated by the NF-kappaB pathway, which in turn is modulated by proinflammatory cytokine such as TNF (Lee and Burckart, 1998).

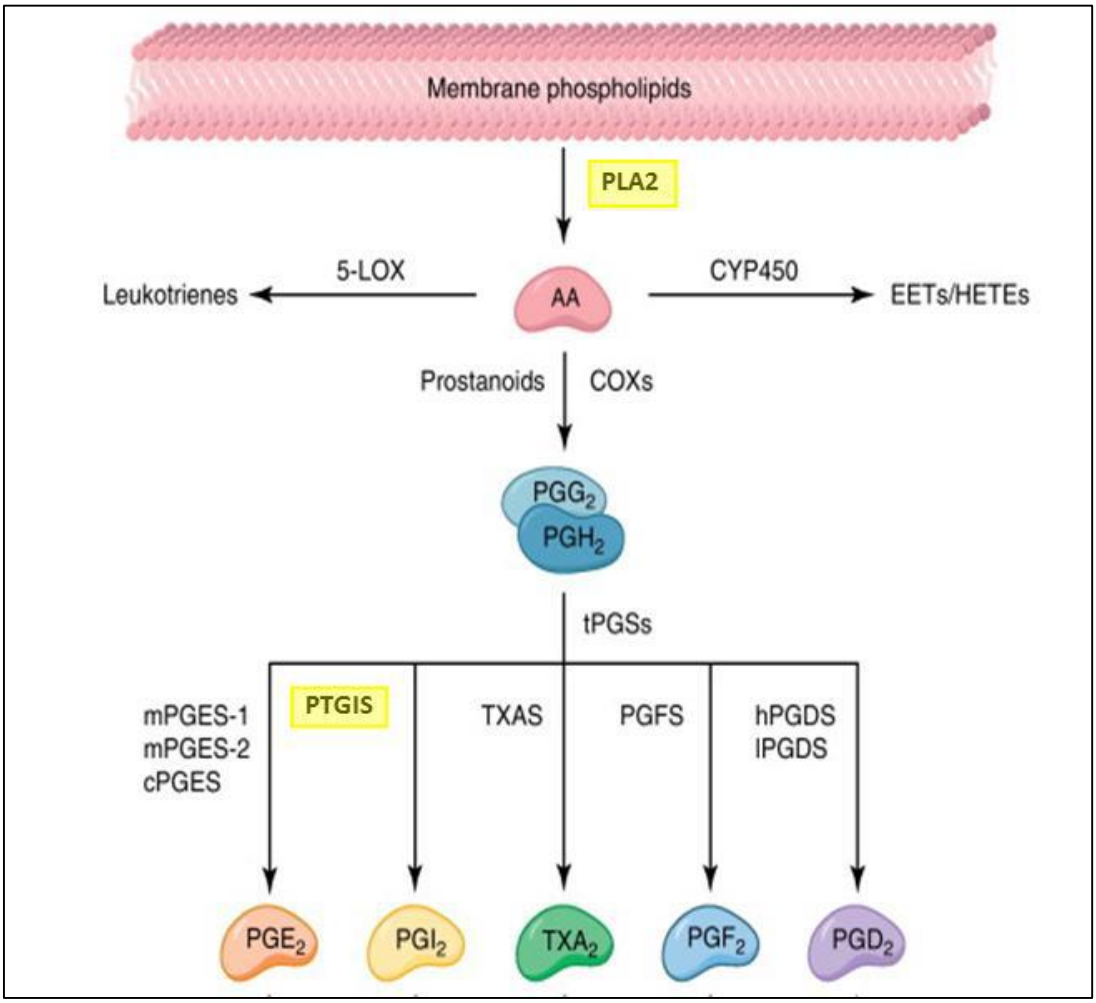


Figure 3.13: Prostanoid biosynthesis.

Arachidonic acid (AA) is released from membrane phospholipids by the phospholipases. AA is metabolized by COX enzymes to form prostanoids. The COX product PGH2 is further metabolized by specific synthases to yield prostaglandins and thromboxane, which bind prostanoid receptors to evoke a wide array of biological effects. This figure is adapted from (Salvado et al., 2012).

Pappa *et al* revealed that PLA2G4A is one of the mediators of brain swelling associated with CM disease in children (Pappa et al., 2015). Brain swelling has been shown to be the strongest predictor of mortality in paediatric CM (Seydel et al., 2015). Several mechanisms for increased brain volume might exist in clinical cases such as CM (Nag et al., 2009), including processes that affect the function of BBB (Seydel et al., 2015, Dorovini-Zis et al., 2011, Brown et al., 2001a). There is a positive correlation between brain volume and the expression of PLA2G4A.

Arachidonic acid, which is released due to activation of PLA2G4A can be a source of energy, signalling and possible mediator of inflammation (Pappa et al., 2015). Also, arachidonic acid can induce the permeability of HBMEC monolayers by prostaglandin E_2 stimulation of EP_3 and EP_4 receptors (Dalvi et al., 2015). The potential role of PLA2G4A in CM disease need to be more investigations in the future.

Co-culturing the iRBC with HBMEC and TNF at 6 hours also leads to reduction of the expression of multiple significant genes involved in the immune system (Table 3.11). At this time point we demonstrated reduction of RELB gene expression with approximately -3-fold change. RELB is known as a member of the NF-kappaB family, which plays a key role in multiple biological process such as the inflammatory and immune response, as well as cancer development and cell survival (Gerondakis and Siebenlist, 2010, Baldwin, 2012). Our study revealed down regulated expression of RELB gene compared with the up regulated expression of RELB in other reports (Baud and Jacque, 2008, Vogel et al., 2013, Yoza et al., 2006), the reason for this gene expression reduction and its role in CM is still unknown.

Another down regulated gene which involved in immune system was EGR1. EGR1 is described as a gene which participates in functions of cognition and memory (Jones et al., 2001, Bozon et al., 2002, Knapska and Kaczmarek, 2004). Gersten *et al* found that in their study on monkeys with SIV encephalitis (SIVE), EGR1 was down regulated in SIVE as a consequence of the host response to infection, leading to disruption in learning (Gersten et al., 2009). Moreover, Pouget *et al* found that reduction of EGR1 has been associated with autoimmune disease and schizophrenia (Pouget et al., 2016). Since EGR1 is down regulated gene in models of several

neurological diseases, we asked whether this gene reduction might be a consequence of brain cells response to IT4var14 parasite interaction, perhaps being responsible for some of the post-infection neurological deficits seen in CM survivors. Whether this gene has a role in CM disease is an important question to be addressed by future research.

Comparing our results of significantly down regulated genes at 2 and 6 hours of interaction with the IT4var14 parasite with brain endothelial cells in the presence of TNF cytokine, we observed that the decreased expression of CCL2 became less marked with time (-2.02 (6 hours) and -4.1 (2 hours) FC). The reason for this increase might be because of the induction of ICAM-1 expression and malaria parasite sequestration, and therefor increase the BBB permeability and the probability of CM disease. In contrast, at 6 hours of co-culture the FOS gene expression decreased with around (-6 FC) when compared with its expression at 2 hours (-4.7 FC). This reduction increases the question about whether the IT4var14 parasite sequestration on HBMEC may leads to neuron dysfunction in CM disease.

Exposure of IT4var14 parasite isolate to human brain endothelial cells in the presence of TNF at 6 hours can modulate the expression of genes in HBMEC. Of these genes identified in our study as differentially expressed, some work as activators for apoptosis and other inhibitors for apoptosis. Numerous studies have been conducted on CM and concluded that malaria parasite can mediate the expression of genes involved in pro-apoptotic process (Punsawad et al., 2013, Liu et al., 2016, N'Dilimabaka et al., 2014).

The expression level of NFKBIA gene in our study was low with about -2-fold change. NFKBIA is also known as (IκBα), and its function is as an inhibitor of NF-kappaB signalling. Activation of NFKBIA in most cells leads to anti-apoptotic

activity (Luo et al., 2005). A microarray study by Tripathi *et al* that evaluated the gene expression of HBMEC after exposure to 3D7 parasite isolate without TNF stimulation at 6 hours found that the expression level of the NFKBIA gene was high as NF-kappaB inhibitory protein (Tripathi et al., 2009). However, we observed a reduction in NFKBIA gene expression, and this may suggest that down regulation of this gene might activate the NF-kappaB pathway and thus increase the pro-apoptotic activity in human brain endothelial cells.

Our results in this study revealed that mRNA level of growth arrest and DNA damage inducible beta gene (GADD45B) was significantly decreased with roughly -8-fold change. GADD45B is recognized originally as an essential factor in apoptosis, DNA repair, growth arrest, cell survival and probably DNA demethylation (Liu et al., 2015). Overexpression of GADD45B gene inhibits apoptosis in ischemic stroke (Liu et al., 2015) and in other cell types such as NIH3T3 and INS-1Eb cells (Larsen et al., 2006, Engelmann et al., 2008). By contrast, GADD45B was found to increase apoptotic death in murine hepatocytes (Cho et al., 2010) and cardiomyocytes (Kim et al., 2010). Our research is the first in CM to find down regulation of expression of GADD45B, the mechanism of this reduction is still unknown but we suggest that this gene may involve in the pro-apoptotic death in CM, perhaps causing the phenomenon of ring haemorrhages (a neuropathological feature of CM disease) (Figure 3.14).

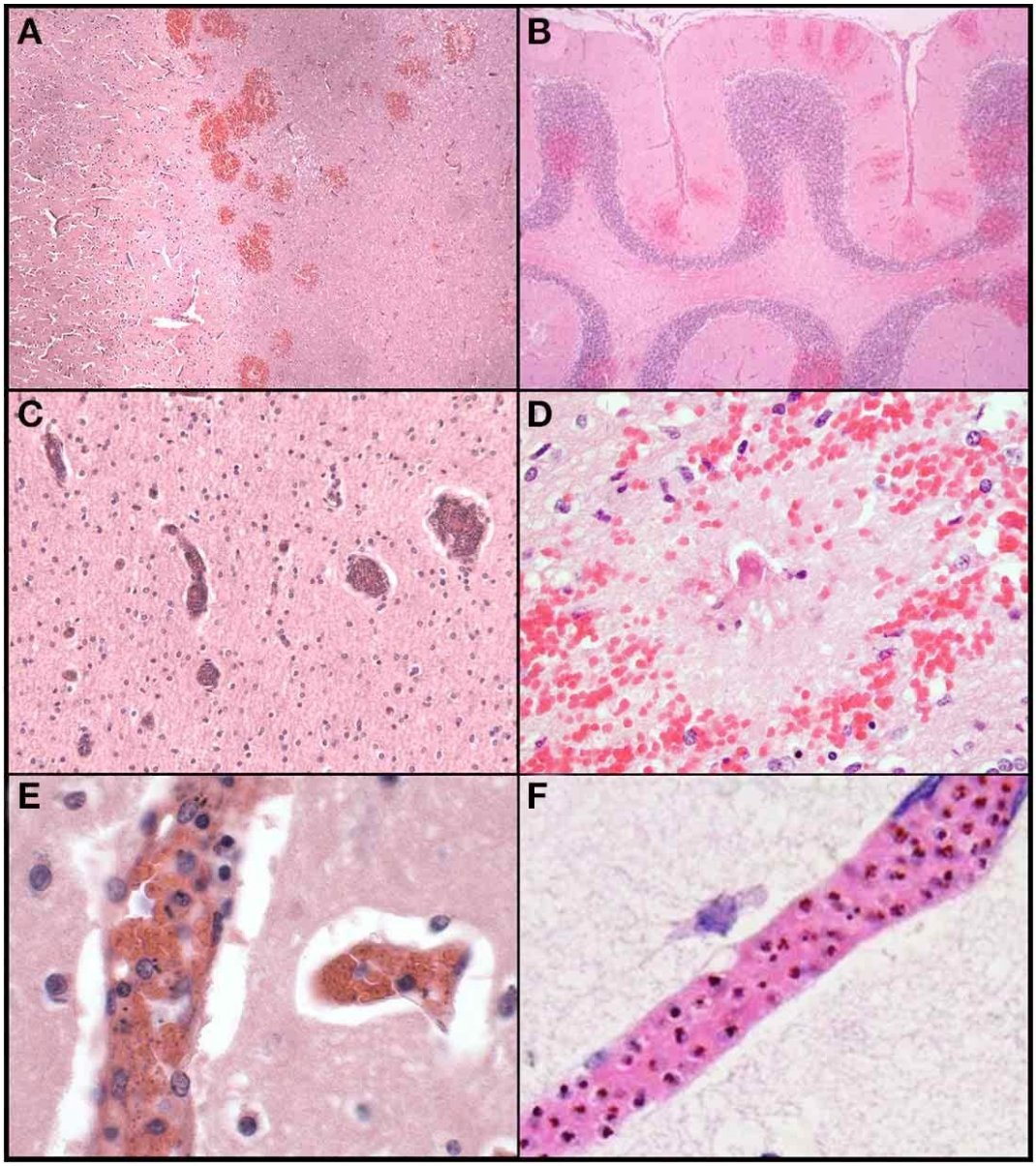


Figure 3.14: A summary of representative images from the histological examination of the brain in the autopsy series is shown for CM cases.
The abrupt transition from grey to white matter **(A)** and the presence of ring haemorrhages are demonstrated in this classic case of CM (CM2, 100X, H&E). The cerebellum **(B)** with ring haemorrhages in all levels including white and grey matter are shown (100x, H&E). Visibly congested blood vessels **(C)** even at low power may be the result of dense sequestration downstream; these vessels can contain both parasitized and uninfected red bloods (200X, H&E). The classic appearance of a ring haemorrhage with fibrin **(D)** is shown; these haemorrhages can also include pigmented parasites, free pigment, and admixed fibrin within the microvessel at the nexus of the lesion; uninfected erythrocytes constituting the surrounding haemorrhage are seen (400X, H&E). Two examples of sequestration showing predominantly early (less pigmented) parasites **(E)**, and late stage (more pigmented) parasites **(F)** densely packing vessels (1000X, H&E). The figure is adapted from (Milner et al., 2014).

Another highly down regulated gene at this time point is inhibitor of DNA binding 1 (ID1) with approximately -6-fold change. ID1 belongs to the helix-loop-helix (HLH) family of proteins, and plays a key role in different cellular processes, such as cellular differentiation, cell proliferation and apoptosis (Perk et al., 2005). Induction of this gene is linked to tumorigenesis in a number of cancers such as breast and cervical cancers (Lin et al., 2000, Schindl et al., 2001). Hao *et al* revealed that increased expression of ID1 promotes osteosarcoma cell proliferation and inhibits cell apoptosis (Hao et al., 2016). On the other hand, our outcomes demonstrated a reduction of this gene and this may induce the apoptosis in brain endothelial cells as a result of sequestration of IT4var14 parasite on HBMEC.

A reduced mRNA level of Dual-specificity phosphatase-1 (DUSP1) at 6 hours co-culture has been observed in our results. DUSP1 is a member of the threonine-tyrosine dual-specificity phosphatase family (Shen et al., 2016). It plays a role in a variety of biological processes including: cell proliferation, stress responses, differentiation and transformation, cycle arrest, inflammation and apoptosis mainly by regulation MAPK signalling (Owens and Keyse, 2007, Saxena and Mustelin, 2000, Keyse, 2000). The transcriptional level of DUSP1 was investigated in different human tumours, prostate, colon, gastric, breast, bladder and lung cancer (Bang et al., 1998, Loda et al., 1996, Manzano et al., 2002, Wang et al., 2003, Vicent et al., 2004). Gil-Araujo *et al* conducted research on DU145 prostate cells and found that overexpression of DUSP1 enhances apoptosis and decreases NF-kappaB activity (Gil-Araujo et al., 2014). According to the previous study we can suggest that incubation of IT4var14 parasite with brain endothelial cells and TNF may reduce the expression level of DUSP1 and thus decrease the pro-apoptotic activity as well as

increasing the expression level of ICAM-1, based on the induction of NF-kappaB level in HBMEC. However, further research is necessary.

In the present study, we demonstrated reduction in the expression level of AQP1 with around -14-fold change. AQP1 is known as a transmembrane protein responsible for water transport and is selectively expressed in several types of cells (Dong et al., 2012, Verkman et al., 2008, Esteva-Font et al., 2014). It has been reported that the AQP1 expression level is associated with apoptosis in different types of diseases such as osteoarthritis, myocardial infraction and various kinds of cancers (Gao et al., 2016, Li et al., 2015, Qin et al., 2016, Xia et al., 2013). When comparing the expression level of AQP1 at 6 hours of incubation with that at 2 hours in our study we found that the AQP1 slightly decreased from 2 to 6 hours in co-culture (-14 and -12 FC respectively). Furthermore, this study suggests the role of AQP1 as a negative regulator of apoptosis in HBMEC and may support the protective role of the malaria parasite in reducing cell death in endothelial cells.

The low expression levels of inhibitor of DNA-binding/differentiation 3 (ID3) have been detected in this research. ID3 is known as a member of the Id family of negative helix-loop-helix transcription factors, which function in the maturation and development of lymphocytes (Maruyama et al., 2011), vascular endothelial cell differentiation (Felty and Porther, 2008) and, in addition to oncogenes, in human cancers such as human lung adenocarcinoma, prostate cancer and breast cancer cells (Chen et al., 2015). The effect of ID3 on apoptosis of human lung adenocarcinoma A549 cell line has been examined by Chen *et al*, and they demonstrated that up regulation of ID3 significantly increased the rate of the apoptosis in A549 cell (Chen et al., 2016). To date, there is no research confirming the correlation between down regulation of ID3 and the apoptosis in the CM disease., but we suggest that there is a

role of ID3 in human CM disease by inhibition of the apoptosis in human brain endothelial cells.

We observed in our research that 57 genes were over-expressed and 168 were under-expressed in HBMEC incubated with IT4var14 malaria parasite and TNF at 20 hours co-culture. The functions and pathways analysis of significant differentially expressed genes showed that those with up regulated expression were involved in the immune system. While, down regulated genes were involved in cell cycle. At 20 hours of co-culture, we demonstrated that CCL20, CXCL2 and CCL2 genes expression were up-regulated with around 6, 2 and 3-fold changes respectively (Table 3.12).

Table 3.12: Up regulated expression of selected genes representing specific pathways in HBMEC co-cultured with IT4var14 parasite and TNF at 20 hours, such as immune system.

GO term	Genes symbol	Fold change
Immune system	CCL2	3.1
	CCL20	5.97
	CXCL2	2.4

The table illustrates fold changes in HBMEC response to infected red blood cells and TNF, compare with control.

Cytokines and chemokines have important roles in the pathogenesis of CM (Hunt and Grau, 2003, Armah et al., 2005a). Ochiel *et al* observed that there was an increase in the production level of CCL2 with severe *P. falciparum* infection (Ochiel et al., 2005). Compared with our result for CCL2 at 2, 6 and 20 hours of incubation, we observed an increase in the expression level of CCL2 at 20 hours, and this induction in CCL2 may increase the BBB dysfunction and promote pathogenesis of CM disease.

CCL20 is expressed in mucosal sites, ectodermal tissue, peripheral blood mononuclear cells and to some extent in lymphoid tissues (Schutyser et al., 2003, Meissner et al., 2003). CCL20 stimulates the maturation and migration of leucocytes, as well as, together with CXCL2 participate in HBMEC protection during malaria parasite sequestration (Tripathi et al., 2009). We suggest that the CCL20 and CXCL2 genes might play a crucial protective role in HBMEC against CM disease.

Among the down regulated genes that significantly expressed at 20 hours of co-culture IT4var14 parasite with human brain endothelial cells and TNF, most of them involved in cell cycle (34 genes). At 20 hours of incubation, the expression of ICAM-1 on HBMEC is still at a significant level promoting sequestration (Tripathi et al., 2006), but this is in addition to rupturing iRBC and release of several molecules such as hemozoin (Tyberghein et al., 2014). The reasons of the involvement of most of the down regulated genes in cell cycle are not known and need to be investigated further.

In conclusion, the interaction of IT4var14 malaria parasite with HBMEC and TNF at 2, 6 and 20 hours can mediate the differential expressed genes involved in various biological pathways. Possible pathways implicated in the pathogenesis of the human CM disease include the immune system at 2 hours of incubation; the immune system, arachidonic acid pathway, positive and negative apoptotic pathways at 6 hours of incubation as previously discussed in this chapter. These findings support the idea that sequestration of the IT4var14 parasite on human brain endothelial cells in the presence of TNF stimulation within 20 hours may alter the behaviour of the host cell toward either protection or susceptibility to CM disease. However, further studies are required to elucidate the influence of interaction of malaria parasite on human brain endothelial cells in terms of the CM disease.

CHAPTER 4

MODULATION OF THE ENDOTHELIAL CELL TRANSCRIPTOME FOLLOWING CO-CULTURE WITH IT4VAR37

4.1 Introduction:

Sequestration of the malaria infected erythrocytes on blood microvasculature endothelial cells is a major virulence mechanism besides the antigenic variation that protects the parasitized erythrocyte from immune detection and destruction, as well as ensuring prolonged survival for parasites at a population level. This mechanism is largely mediated by an infected erythrocyte surface protein called *PfEMP-1* (Kraemer and Smith, 2006). Each malaria parasite strain has the potential to encode around 60 *PfEMP-1* proteins through their family of *var* genes (Frank and Deitsch, 2006). Switches in the expression of *var* gene allow infected erythrocytes to avoid host immunity and may alter disease manifestations (Smith et al., 1995).

It has been mentioned that despite of their gene diversity, *var* genes can be classified according to the upstream sequence (ups), direction of transcription and location in the chromosomes into three main groups (ups A, B and C) and two intermediate groups (B/A and B/C) (Lavstsen et al., 2003). UpsA and upsB genes are found characteristically in subtelomeric regions of the 14 chromosomes, while upsC genes are in four central regions (Kraemer et al., 2007, Kraemer and Smith, 2003, Lavstsen et al., 2003). Several studies have suggested robust association between upsA *PfEMP-1* and CM (Lavstsen et al., 2012, Turner et al., 2013, Avril et al., 2012). Furthermore, Almelli and his colleagues found that in their study on cerebral malaria patients, upsA and upsB *var* genes were associated with CM disease (Almelli et al., 2014). On the other hand, numerous studies found that upsC was associated with UM (Jensen et al., 2004, Kyriacou et al., 2006, Rottmann et al., 2006).

qRT-PCR studies on malaria infected individuals have suggested that these associations come from *var* genes expression (Mugasa et al., 2012, Rottmann et al., 2006, Kyriacou et al., 2006). In addition, Jensen *et al,* in their study on semi-immune

children from Ghana and Tanzania, showed that Group A and B/A *var* genes were up-regulated. However, members among group C *var* genes were down-regulated suggesting their correlation with UM (Jensen et al., 2004).

The variability of the *PfEMP-1* domains has been shown to mediate several interactions with human receptors such as DBLβ domains binding to ICAM-1 (Howell et al., 2008, Smith et al., 2000) and some CIDRα domains binding to CD36 (Baruch et al., 1997). IT4 strains from both upsB and upsC were CD36 binders, and this is more consistent with findings from previous studies that ups B and C *PfEMP-1* variants are more linked to CD36 (Cabrera et al., 2014, Kraemer and Smith, 2006, Robinson et al., 2003). The role of the CD36 in the pathogenesis in malaria disease is still not clear.

Madkhali and his group have tried to assess the ability of upsC isolate (IT4var37) to bind CD36 and ICAM-1 proteins under static and flow conditions and they found that IT4var37 strain was a very good binder for CD36 under both static and flow conditions, whereas the same isolate did not bind to ICAM-1 protein (Madkhali et al., 2014).

ICAM-1 is considered to be an important receptor that mediates the interaction of malaria parasite with the brain microvascular endothelial cells and plays a role in CM pathogenesis (Gray et al., 2003) as we discussed in the previous chapter. We aim in this chapter to assess the ability of IT4var37, an isogenic parasite variant to IT4var14 expressing a different *PfEMP-1*, as a good binder to CD36 receptor to modulate the mRNA expression in HBMEC in the presence of TNF stimulation at 6 and 20 hours of co-culturing model using the RNA-seq technology. Findings in this chapter will be compared with the previous chapter results in terms of the functional and pathways analysis.

4.2 Methods:

4.2.1 Parasite culture:

In this chapter, IT4var37 malaria parasite lab strain was cultured using standard culturing techniques as previously described in the general methods chapter (Trager and Jensen, 1976).

4.2.2 EC culture:

HBMEC was cultured and used in this part of our project as explained before in the general methods chapter.

4.2.3 Detection of mycoplasma infection in IT4var37 parasite strain and HBMEC:

Potential infection with mycoplasma in both IT4var37 and HBMEC was assessed as shown in the general methods chapter.

4.2.4 Characterization of the IT4var37 parasite and HBMEC:

Static protein adhesion assays were carried out to examine the binding of IT4var37 parasite to CD36 and ICAM-1 proteins and its procedure was described before in the general methods chapter.

4.2.5 Co-culture malaria parasite with ECs:

IT4var37 parasite was co-cultured with the HBMEC and TNF stimulation at 0, 6 and 20 hours as explained in the general methods chapter.

4.2.5.1 RNA extraction, quality control and RNA-seq data analysis:

RNA from each co-cultured flask used in this part of the study was extracted, purified from contamination and checked for its quality using an Aglient 2100 Bioanalyzer as shown previously in the general methods chapter. The workflow for RNA-seq data analysis was described in Figure 3.2.

4.3 Results:

4.3.1 Identification of mycoplasma infection in IT4var37 parasite and HBMEC:

As we mentioned before in the beginning of this project, obtaining the EC and the parasite free from the infection by mycoplasma was the first important step to ensure a good quality in our results. The IT4var37 and the HBMEC were free from the mycoplasma infection as shown in Appendix 6.

4.3.2 Static adhesion of IT4var37 parasite on CD36 and ICAM-1 proteins:

The binding level of IT4var37 isolate to CD36 was high, and this result is in agreement with Madkhali *et al* (Madkhali et al., 2014), including no binding observed with ICAM-1 protein (Appendix 7).

4.3.3 Quality control of RNA extraction samples:

RNA samples were isolated in this study from three independent interaction experiments of IT4var37 with HBMEC/TNF at 0, 6 and 20 hours. An Agilent Bioanalyzer 2100 was used to identify the RNA samples' quality. According to the RIN value that was measured in each RNA sample in this study, all samples showed a good RIN of more than 9 (Table 4.1).

Table 4.1: RIN calculated by Agilent 2100 Bioanalyzer.

First Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
1	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	9.6	124
2	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	9.3	131
3	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	9.4	139
Second Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
4	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	10	124
5	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	10	127
6	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	9.8	145
Third Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
7	Parasite co-cultured with HBMEC at 0 hour (TNF +ve)	9.7	120
8	Parasite co-cultured with HBMEC at 6 hours (TNF +ve)	10	132
9	Parasite co-cultured with HBMEC at 20 hours (TNF +ve)	9.4	129

The table presents the RIN and the RNA concentration of each sample. The high RIN calculated by the 2100 Bioanalyzer indicated the very high quality of the isolated RNA.

4.3.4 Identification of differentially expressed genes in HBMEC at 6 hours incubation:

Gene expressions changes of TNF-stimulated HBMEC exposed to IT4var37 were measured by Cuffdiff in FPKM as described in the previous chapter section 3.3.6. At 6 hours of incubation, 418 genes were seen as being significantly differentially expressed genes with an adjusted FDR < 0.05 and 1Log2-fold change. Among these, 140 genes were described as up-regulated genes and 278 genes were down-regulated genes. The top ten up and down-regulated genes are presented in Figure 4.1. The most up-regulated gene at this time point was the neuronal pentraxin 1 (NPTX1) with around 16-fold change. While, ladinin 1 (LAD1) was the highest down regulated

gene with about -36-fold change. A table with the full description of up and down-regulated genes is shown in appendix 8.

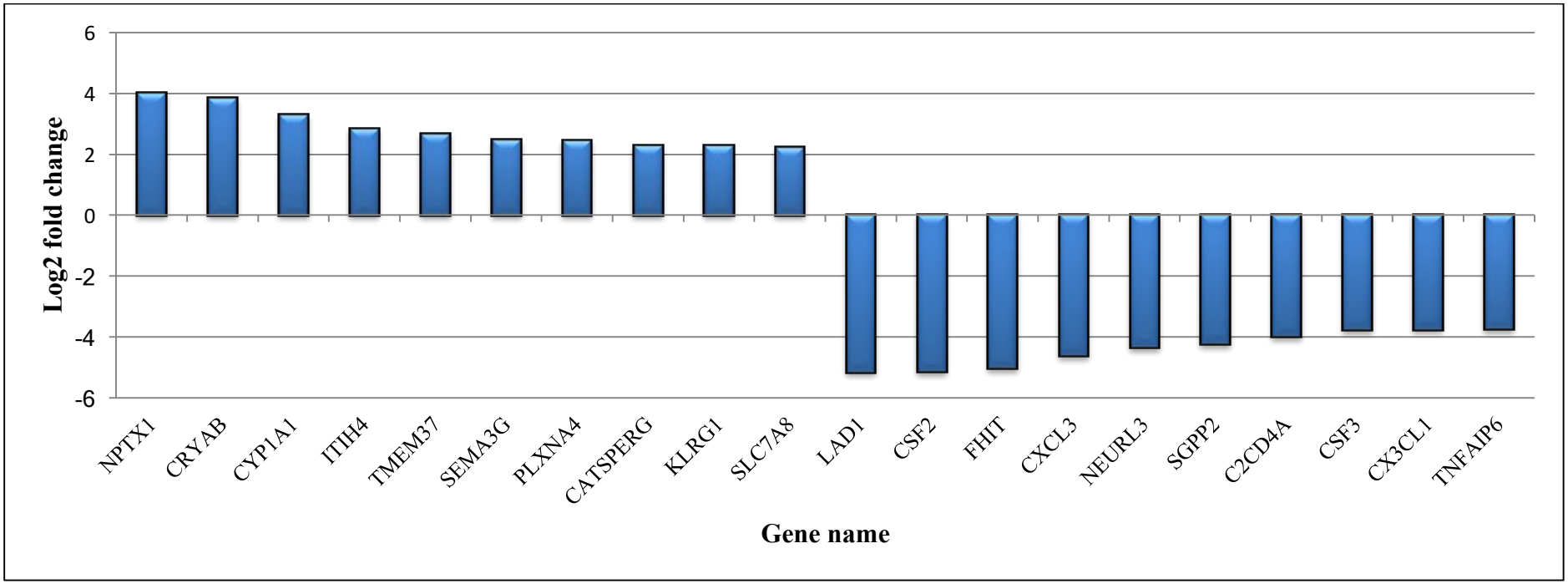


Figure 4.1: Top 10 up/down regulated genes in TNF-stimulated HBMEC incubated with IT4var37 parasite at 6 hours co-culture.
The differentially expressed genes in TNF-stimulated HBMEC co-cultured with IT4var37 parasite isolate at 6 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of the genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

4.3.5 Identification of differentially expressed genes in HBMEC at 20 hours incubation:

In total of 1312 genes with differential expression were identified in TNFα-stimulated HBMEC interacted with IT4var37 parasite at 20 hours, approximately 38% were found belonging to over-expressed genes, however; nearly 68% were shown belonging to lower-expressed genes. Analysis of the gene expression fold change among the top ten up-regulated genes demonstrated that the fold changes varied from 10 to 64, and the highest expressed gene was the sex hormone binding globulin (SHBG) (Figure 4.2). On the other hand, fold changes of the top ten down-regulated genes varied from -38 to -222, and the most robust down-regulated gene was observed with colony stimulating factor 2 (CSF2) (Figure 4.2). Further details about both top ten up and down-regulated genes are described in appendix 9.

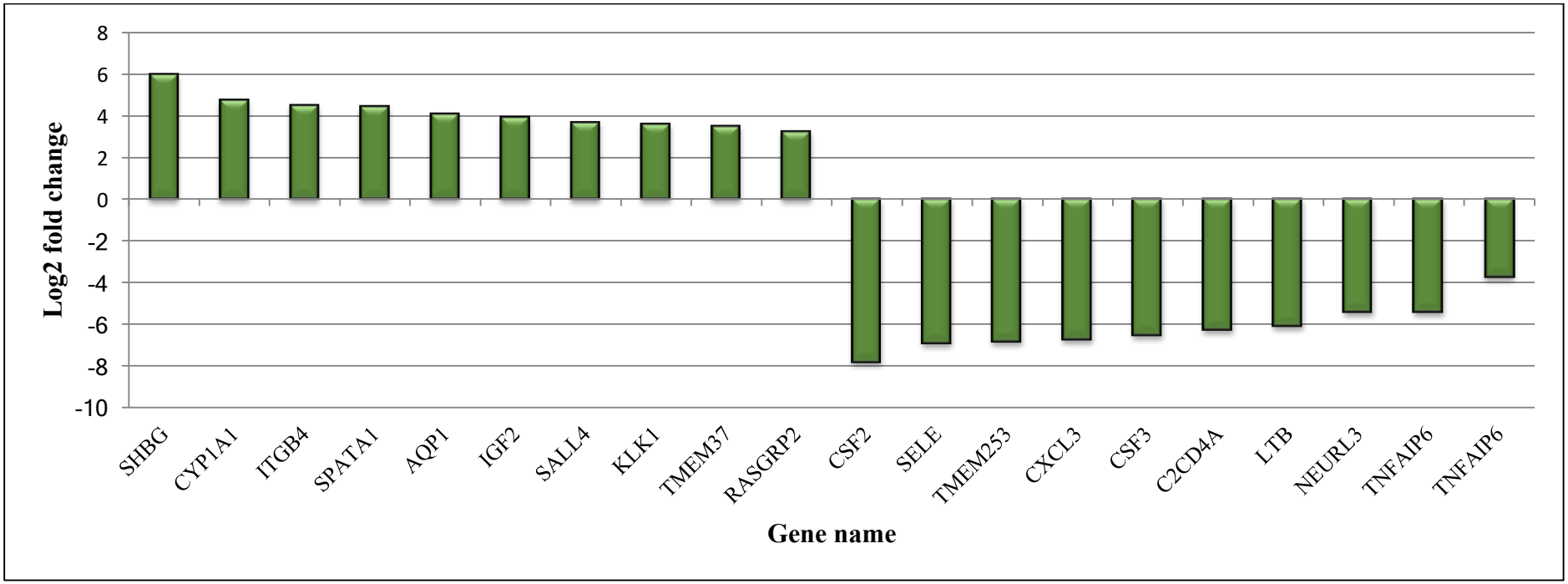


Figure 4.2: Top 10 up/down regulated genes in TNF-stimulated HBMEC incubated with IT4var37 parasite at 20 hours co-culture.
The differentially expressed genes in TNF-stimulated HBMEC co-cultured with IT4var37 parasite isolate at 20 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of the genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

4.3.6 Functional and Pathway analysis of differentially expressed genes:

GO analysis was carried out in this RNA-seq study on genes that were significantly up and down-regulated as a result of co-culturing IT4var37 iRBC with HBMEC/TNF at 6 and 20 hours based on the three categorisations; biological process, molecular function and cellular component. Tables which describe data of the GO analysis at 6 and 20 hours co-culture can be seen in appendices 10 and 11.

Reactome pathway enrichment analysis was used in this study amongst the over and under-expressed genes in TNF-stimulated HBMEC after exposure to *P. falciparum* IT4var37 at 6 and 20 hours. The research identified 15 host response pathways involved in genes with up-regulation ($P < 0.05$ and at least 3 input genes) from co-culturing IT4var37 parasite with HBMEC/TNF at 6 hours. Hemostasis and cell surface interactions at the vascular wall were the most affected pathways regarding the up-regulated genes (25 and 7 genes respectively) (Figure 4.3). In contrast, 82 host response pathways were shown in the same time point and conditions with significantly down-regulated genes. The highly significant pathways with respect to number of genes were the immune system, followed by cytokine signalling in immune system (162 and 101 genes respectively) (Figure 4.3).

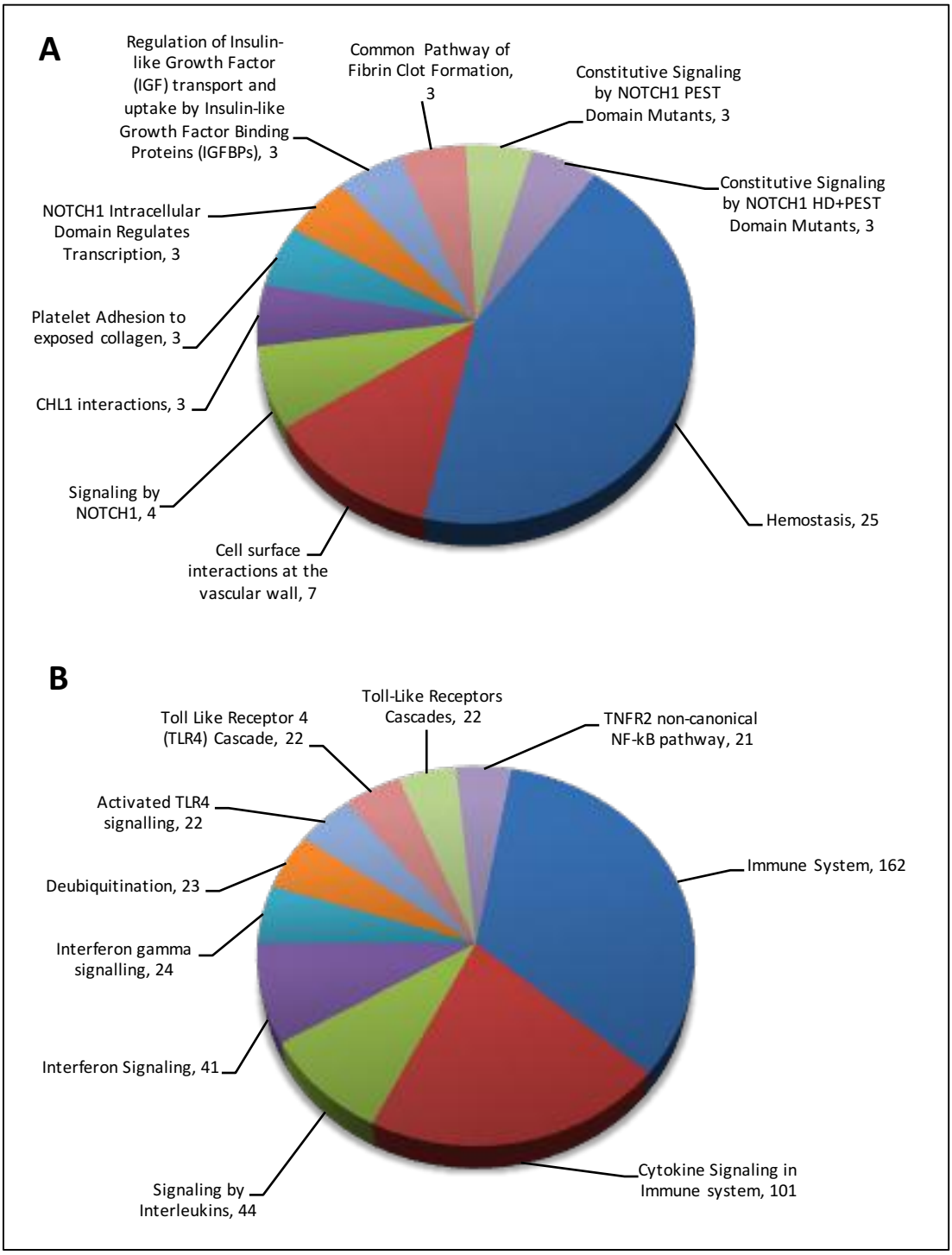


Figure 4.3: Top ten pathways of up and down regulated genes of HBMEC/TNF responding to IT4var37 at 6 hours.

A) pathways of up regulated genes and **B)** pathways of down regulated genes. The pathways were selected according to the number of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

The pathway analysis of significant genes at 20 hours of incubation HBMEC/TNF with IT4var37 identified 9 and 121 pathways in over and under-expressed genes respectively. With regard to the number of genes involved in each pathway, regulation of cholesterol biosynthesis by Sterol Regulatory Element Binding Transcription Factor (SREBF) and non-integrin membrane-ECM interactions were shown as top two pathways of over-expressed genes (Figure 4.4). On the other hand, cytokine signalling in immune system and cell cycle pathways were the majority of under-expressed genes at the same time point of co-culture with 124 and 97 genes respectively (Figure 4.4).

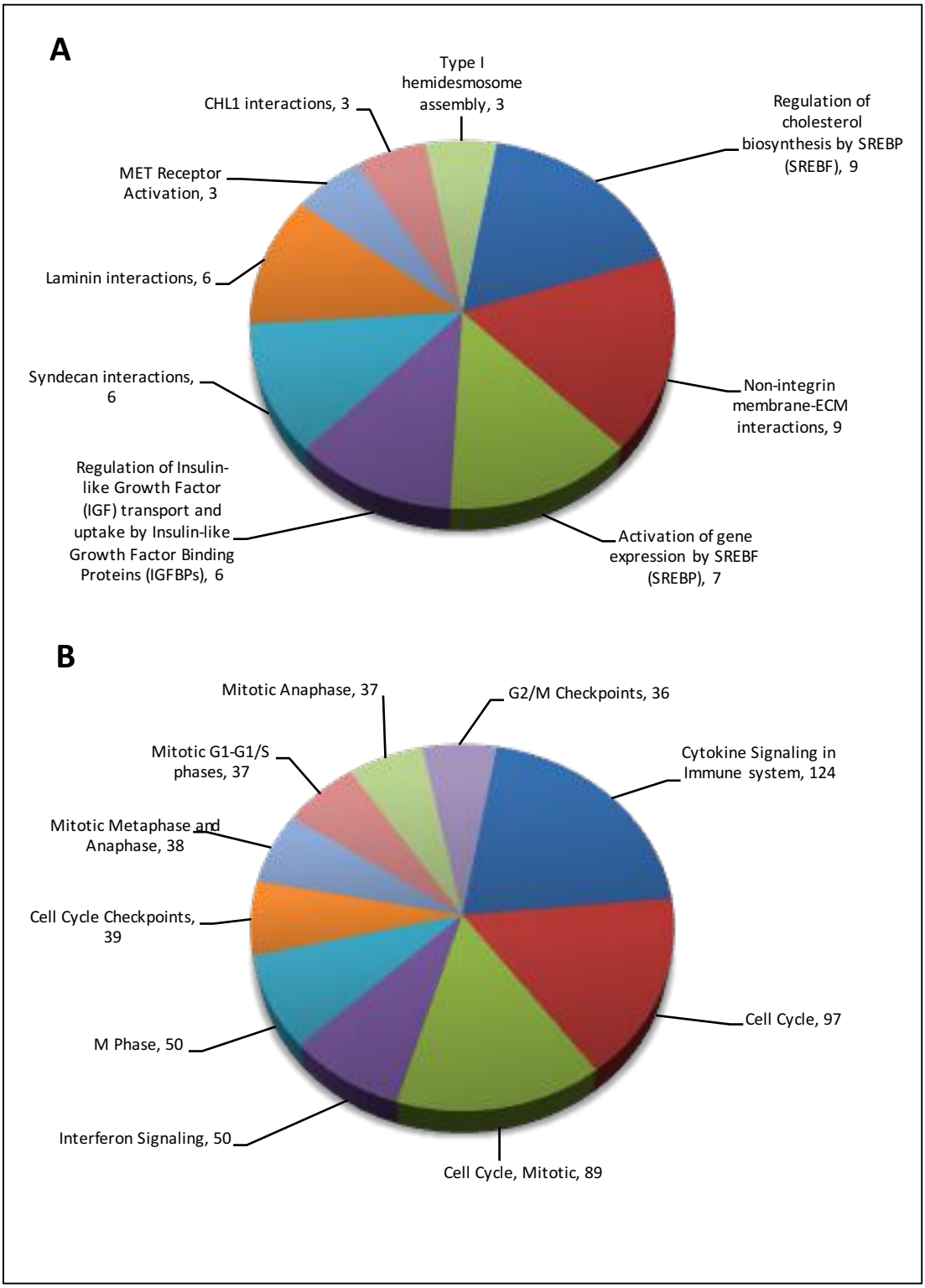


Figure 4.4: Top ten pathways of up and down regulated genes of HBMEC/TNF responding to IT4var37 at 20 hours.

A) pathways of up regulated genes and **B)** pathways of down regulated genes. The pathways were selected according to the number of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

4.4 Discussion:

One of the fundamental questions that has not been addressed yet is how malaria pathogenesis takes place and what are the roles of various protein receptors in the disease. Sequestration of the infected erythrocytes on the blood microvascular endothelial cells can protects the parasite from being destroyed by the host immune system (Newbold et al., 1999). This interaction can be mediated by proteins on the infected erythrocyte surface, for example *PfEMP-1* (Kraemer and Smith, 2006) and microvascular endothelial cells proteins such as, ICAM-1 (Howell et al., 2008), CD36 (Baruch et al., 1997) and EPCR (Turner et al., 2013).

Furthermore, cytoadherence can mediate the downstream effect on the host endothelial cells that can impact the susceptibility to the disease. From the previous chapter, the interaction of the IT4var14 parasite with ICAM-1 in the presence of TNF to modulate the downstream effects on mRNA expression levels in HBMEC was discussed. In this chapter, we aimed to examine the effect of the IT4var37 parasite strain to modulate the transcriptional levels in HBMEC using RNA-seq technique and comparing the outcomes with IT4var14 parasite co-culture from the previous chapter results.

IT4var37 parasite line had been identified as a good binder for CD36 protein (Madkhali et al., 2014). The role of CD36 in CM pathogenesis is still not understood. Almelli and his group found that in their binding study on children under 5 years of age with CM, CD36 is a common binding receptor in CM (Almelli et al., 2014). However, there are some concerns about the methods of adhesion that were used in this study due to the time of incubation and concentration of the proteins. In this thesis, it was unexpected to obtain the huge number of significantly differentially expressed genes after incubation IT4var37 parasite isolate with HBMEC/TNF at 6

and 20 hours, especially because HBMEC have not expressed CD36 to malaria parasite isolates (Newbold et al., 1997, Ochola et al., 2011).

The RNA-seq results of incubation IT4var37 with TNF-stimulated HBMEC at 6 hours demonstrated that 418 genes were shown as significantly differentially expressed genes, whereas the IT4var14 parasite incubation from the previous chapter on the same time point showed 88 significant genes. Comparison of the expressed genes in incubation IT4var14 and IT4var37 parasites with brain endothelial cells and TNF at 6 hours revealed differences in the results. There are 18 significant genes only detected in both outputs of RNA-seq at this time point of co-culture model. (Figure 4.5).

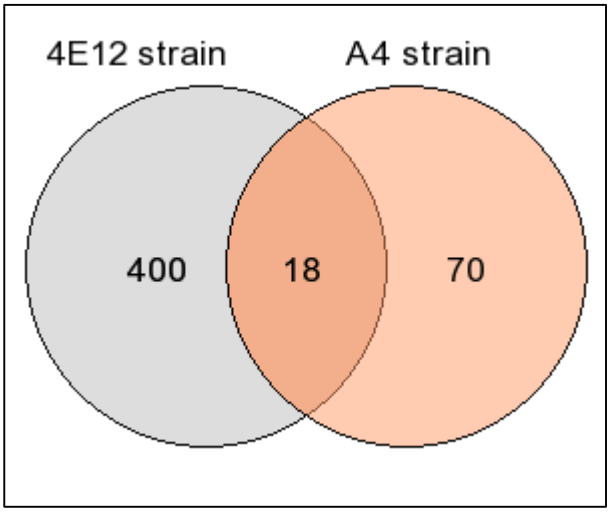


Figure 4.5: Comparison between number of significant genes in HBMEC/TNF co-cultured with IT4var14 and IT4var37 parasite at 6 hours.
The Venn diagram showing 18 genes were found expressed in both IT4var37 and IT4var14 iRBC incubated with TNF-stimulated HBMEC at 6 hours.

The number of genes showing differences and similarities in differentially expressed genes in IT4var37 and IT4var14 parasites incubated with human brain endothelial cells in the presence of TNF at 20 hours were also identified in Figure 4.6. 69 overlapping expressed genes were identified between the two RNA-seq results.

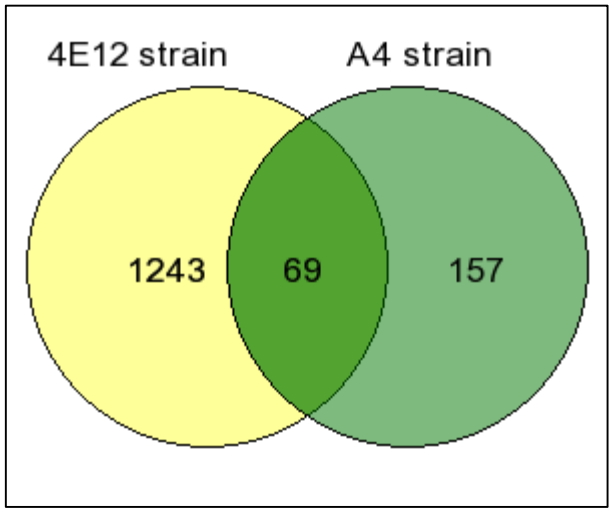


Figure 4.6: Comparison between number of significant genes in HBMEC/TNF co-cultured with IT4var14 and IT4var37 iRBC at 20 hours.
The Venn diagram showing 69 genes were found expressed in both IT4var37 and IT4var14 parasites incubated with TNF-stimulated HBMEC at 20 hours.

In this discussion, we tried to compare our previous chapter results with this chapter results regarding the functional and pathway analysis. Analysis of the functions and pathways of up-regulated genes at 6 hours of incubation IT4var14 parasite with HBMEC/TNF from the previous chapter revealed that among this group of genes, there are several genes involved in immune system (C3, TXNIP, EBI3, and CTSK) and arachidonic acid pathway (PLA2G4A). These gene expressions will be compared with the gene expressions from incubation IT4var37 with HBMEC/TNF at 6 hours in the next section.

Exposure of the TNF-stimulated HBMEC to IT4var37 parasite at 6 hours leads to reduce the expression levels of several important genes that may play role in immune system comparing to the previous chapter results (Figure 4.7). This reduction in C3, EBI3 and CTSK genes expressions suggest different parasite isolates with different *PfEMP-1* cause variations in modulating the host cells gene expression on the brain endothelial cells. However, the expression of TXNIP gene might have a common role in immune response as it was nearly equal in both different parasite strains

exposed to HBMEC/TNF at 6 hours. On the other hand, the level of PLA2G4A expression was around 1.8-fold change in this study compared to its expression from the prior chapter with 2.45-fold change. The expression of this gene from different parasite variants may suggest an importance of this gene in CM pathogenesis.

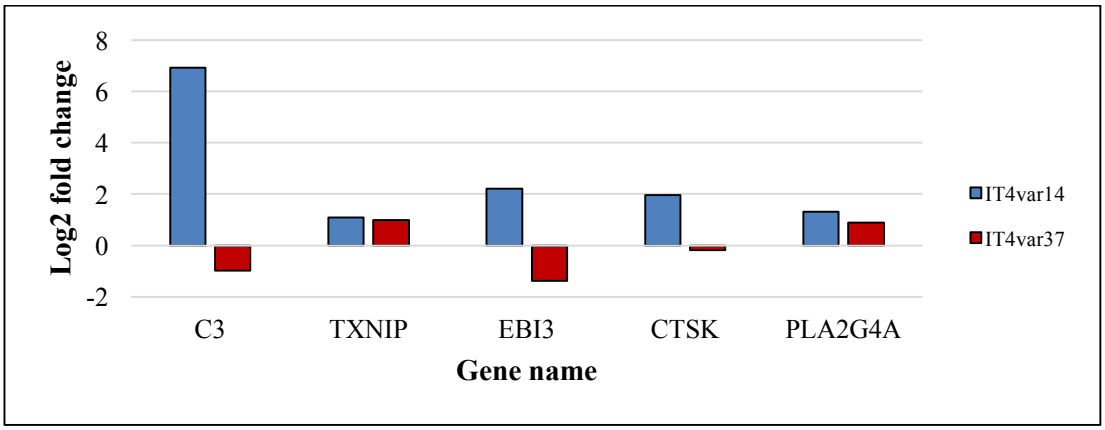


Figure 4.7: Comparison of the expression of selected genes representing immune system and arachidonic acid pathways in TNF-stimulated HBMEC incubated with IT4var14 and IT4var37 parasites at 6 hours.
The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with IT4var14 and IT4var37 parasite strains at 6 hours.

We compared in this study also the down-regulated genes that were involved in the immune system, positive and negative regulation of apoptotic process from the previous chapter with the findings in this chapter at 6 hours of incubation (Figure 4.8). We observed that the mRNA levels of genes related to the immune response such as FOS, CCL2 and RELB were decreased more with IT4var37 parasite than that with IT4var14 isolate, whereas the expression level of EGR1 with IT4var14 strain was more reduced. Response of the brain endothelial cells to the IT4var37 parasite at 6 hours of incubation in terms of the immune system was varied from one parasite strain to the other according to their *PfEMP-1*, and these observations may emphasize the role of parasite *var* gene switching to protect itself from destruction by the immune system.

With respect to genes involved in positive regulation of apoptotic process pathways from the previous chapter and comparing them with this study at 6 hours of incubation, we can find that there is a different response of HBMEC co-cultured with IT4var37 parasite strain than that with IT4var14 parasite in terms of GADD45B and ID1 genes, IT4var37 strain leads to increased expression of these two genes and may reduce the pro-apoptotic process. However, the transcriptional level of NFKBIA decreased more in this work compared with the previous chapter (Figure 4.8).

The expression levels of all genes correlated to negative regulation of apoptosis process pathway in this chapter were completely different from the previous results chapter. The co-culture model of IT4var14 parasite demonstrated that DUSP1, AQP1 and ID3 gene expressions were clearly reduced at 6 hours of incubation, while IT4var37 showed an opposite effect on these genes in HBMEC and this may lead to increased EC apoptosis and cell death (Figure 4.8). These observations also show more information about the response of HBMEC to different parasites isolates with different ups groups in the presence of TNF activation.

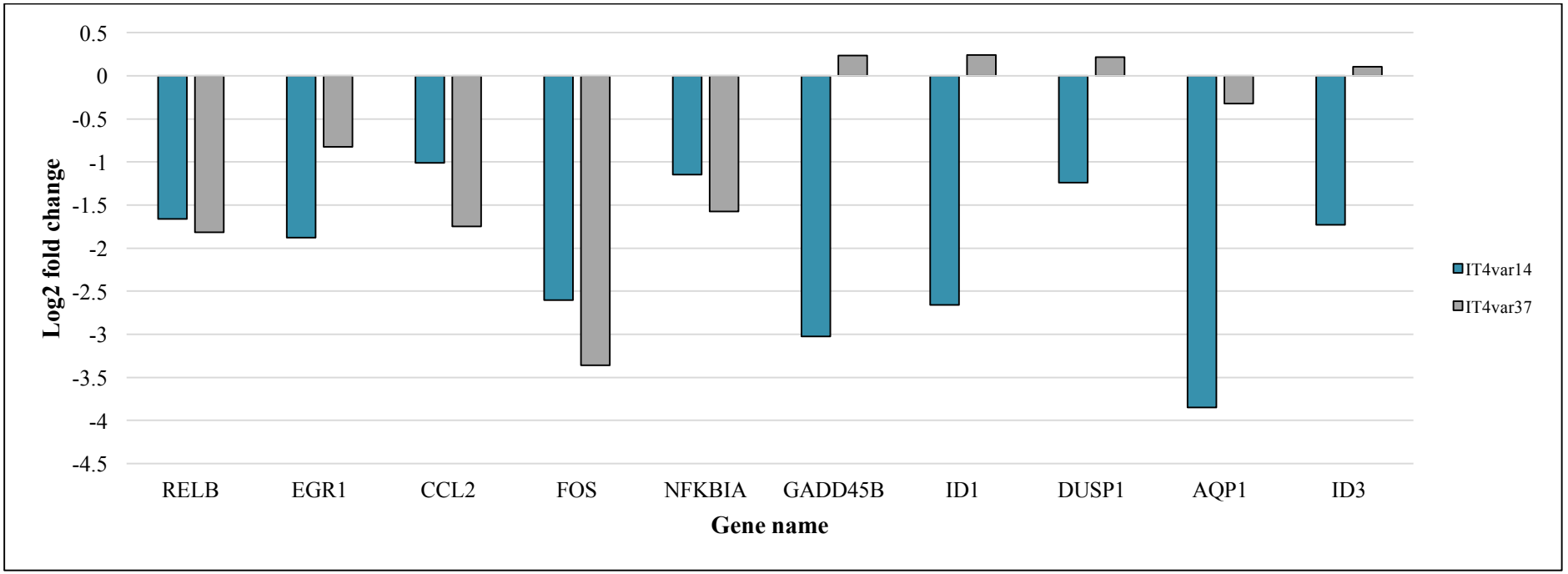


Figure 4.8: Comparison of the expression of selected genes representing immune system and positive and negative regulation of apoptotic process pathways in TNF-stimulated HBMEC incubated with IT4var14 and IT4var37 parasites at 6 hours.
The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with IT4var14 and IT4var37 parasite strains at 6 hours.

We identified in our study that co-culture of the brain endothelial cells to different *P. falciparum* iRBC (IT4var14 and IT4var37) in the presence of TNF stimulation at 20 hours can modulate the expression of several genes involved in the immune system. From the previous chapter, the IT4var14 experiments led to induced expression of CCL2, CCL20 and CXCL2 genes. While, the IT4var37 incubation with HBMEC/TNF works in other way and leads to reduce the expression of these three genes (Figure 4.9). The RNA-seq findings in this study illustrate a potential technique used by the malaria parasite based on *var* gene switching to protect itself from the host immune response to the infection.

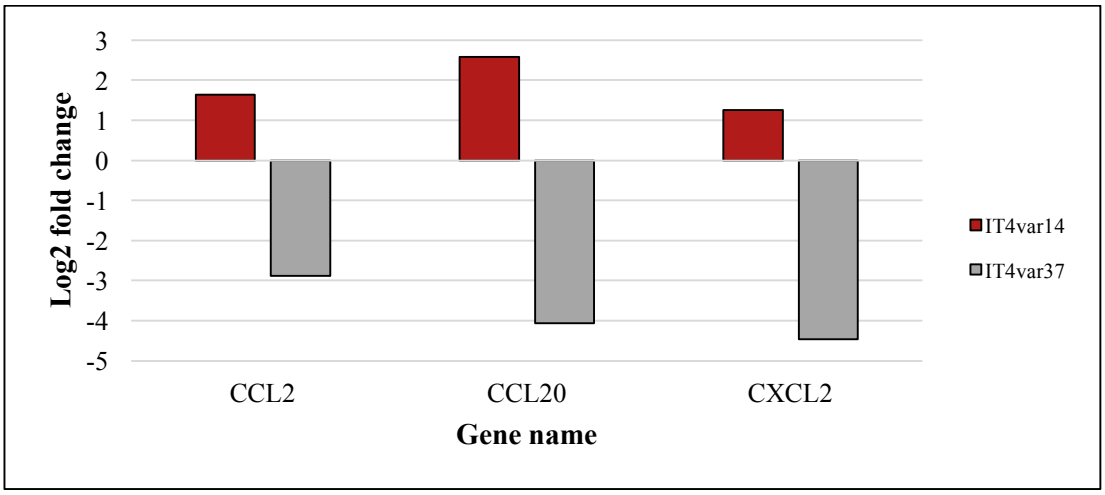


Figure 4.9: Comparison of the expression of selected genes representing immune system in TNF-stimulated HBMEC incubated with IT4var14 and IT4var37 parasites at 20 hours.
The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with IT4var14 and IT4var37 parasite strains at 20 hours.

One of the most interesting findings among the down-regulated genes in both co-culture models at 20 hours is the contribution of both *P. falciparum* parasite variants to the cell cycle pathway. It is possible that molecules released from rupturing the mature iRBC rather than *PfEMP-1* type may have a role in inducing pathway, and the mechanisms underpinning this need further investigation in the future.

In summary, the incubation of IT4var37 parasite isolate with human brain endothelial cells and TNF at 6 and 20 hours can modulate genes with differential expression, but these genes often expressed in opposite levels compared to the IT4var14 strain under the same conditions. These results confirm the ability of various parasites from the same family to modulate different gene expressions in host cells and emphasize also the capability of the parasite to protect itself and avoid the host defensive system to survive for long time using *PfEMP-1 var* gene switching technique. More information about the effect of different parasite isolates on the human brain endothelial cells may provide a useful link for CM therapeutic interventions.

CHAPTER 5

TRANSCRIPTIONAL CHANGES OF DERMAL ENDOTHELIAL CELL FOLLOWING INCUBATION WITH IT4VAR14

5.1 Introduction:

The adhesion of iRBC with human malaria parasite, *P. falciparum* to small blood vessels is a major virulence factor (Gray et al., 2003). Adhesion takes place due to specific interactions between the parasite protein termed *PfEMP-1,* encoded by *var* gene family, with several host cell adhesion molecules including ICAM-1 (Berendt et al., 1989) and EPCR (Turner et al., 2013) CD36 (Barnwell et al., 1989, Ockenhouse et al., 1989, Oquendo et al., 1989). Interestingly, evidence showing that parasites strains from children with severe malaria bind multiple endothelial receptors (Heddini et al., 2001), suggests that synergistic impacts between endothelial adhesion molecules might contribute to cytopathology.

One of the adhesion molecules which is up-regulated and binds iRBC in severe malaria is ICAM-1 (Berendt et al., 1989, Turner et al., 1994). CD36 is another target for malaria parasite interaction, however its role in the pathogenesis is still controversial (Cabrera et al., 2014). Study by Cooke *et al* using malaria patient isolates has found that ICAM-1 modulated rolling adhesion whereas CD36 mediated stationary adhesion (Cooke et al., 1994), which suggests that ICAM-1 capture and CD36 stationary adhesion might co-operate to enhance efficient sequestration. Indeed, it has been shown that the synergy between ICAM-1 and CD36 occurs under static binding assay conditions on HDMEC (McCormick et al., 1997). Interaction with several protein receptors, including ICAM-1 and CD36 have also been shown under flow conditions on HDMEC (Yipp et al., 2000). Dermal EC expresses both ICAM-1 and CD36 (McCormick et al., 1997), whereas brain EC does not express CD36 (Wassmer et al., 2011).

The IT4var14 parasite isolate has been identified as an ups Group B and classified as a long *PfEMP-1*varient with 7 domains (Buckee and Recker, 2012). This strain of

parasite has been shown to modulate the interaction with ICAM-1 receptor via DBLβ domain (Howell et al., 2008, Smith et al., 2000) and with CD36 via CIDRα domain (Baruch et al., 1997). The efficacy of binding IT4var14 parasite to ICAM-1 and CD36 proteins has been observed before in several studies under static conditions (McCormick et al., 1997, Madkhali et al., 2014) and under flow conditions (Gray et al., 2003, Madkhali et al., 2014) and they found that IT4var14 parasite can mediate the binding to ICAM-1 protein but its role in mediating the binding to CD36 was bigger.

We discussed in the previous chapters the ability of different parasite strains to modulate the gene expression of HBEMC/TNF at several time points of co-culture. It is interesting to assess the role of the IT4var14 as a good binder for ICAM-1 and CD36 proteins to mediate the gene expression levels of different microvascular endothelial cells such as HDMEC, so we aim in this part of the thesis to evaluate the gene expression of TNF-stimulated HDMEC in response to IT4var14 incubation at 2, 6 and 20 hours using RNA-seq technique and then compare the results to chapter 3 outcomes regarding the functional and pathway analysis.

5.2 Methods:

5.2.1 Parasite culture:

IT4var14 parasite lab isolate was cultured and used in this study as described before in the general methods chapter.

5.2.2 EC culture:

HDMEC was used in this chapter as showed previously in the general methods chapter.

5.2.3 Detection of mycoplasma infection in IT4var14 parasite isolate and HDMEC:

The potential existence of mycoplasma infection in the IT4var14 parasite and HDMEC was examined as explained before in the general methods chapter.

5.2.4 Characterization of the IT4var14 parasite and HDMEC:

In this chapter, FACS was used to confirm the expression of CD36, ICAM-1 and CD31 proteins in the presence and absence of TNF stimulation on HDMEC. Static adhesion assays were carried out to evaluate the binding of iRBC to CD36 and ICAM-1 proteins. The complete procedures were described previously in the general methods chapter.

5.2.5 Co-culture IT4var14 with HDMEC:

The method of co-culturing the IT4var14 parasite with TNF-stimulated HDMEC was described before in the general methods chapter.

5.2.6 RNA extraction, quality control and analysis of the RNA-seq data:

RNA from each incubated flask was extracted and examined its quality using an Aglient Bioanalyzer 2100 as shown before in the general methods chapter. The workflow for the analysis of the RNA-seq data was described in Figure 3.2.

5.3 Results:

5.3.1 Identification of mycoplasma infection in IT4var14 parasite and HDMEC:

The mycoplasma infection in cells can change the host gene expression (Hopfe et al., 2013), so in this study we have tried to avoid this kind of infection in both IT4var14 parasite and HDMEC cultures to ensure results with good quality. HDMEC and parasite cultures were showed no contamination with mycoplasma (Appendix 12).

5.3.2 Characterization of the IT4var14 parasite and HDMEC:

5.3.2.1 Static adhesion assays of IT4var14 parasite on ICAM-1 and CD36 proteins:

Regarding the binding levels to CD36 and ICAM-1, IT4var14 bound to both proteins at varying levels, in agreement with the results from Madkhali *et al* outcomes (Madkhali et al., 2014) (Appendix 2).

5.3.2.2 Detection the expression of HDMEC proteins receptors:

The endothelial cell markers CD36, ICAM-1 and CD31 expression, with and without TNF activation, were examined using FACS (Figure 5.1). Expression of CD36 and CD31 were slightly increased with TNF stimulation compared to non-TNF activation. However, ICAM-1 was hugely up-regulated with TNF treatment.

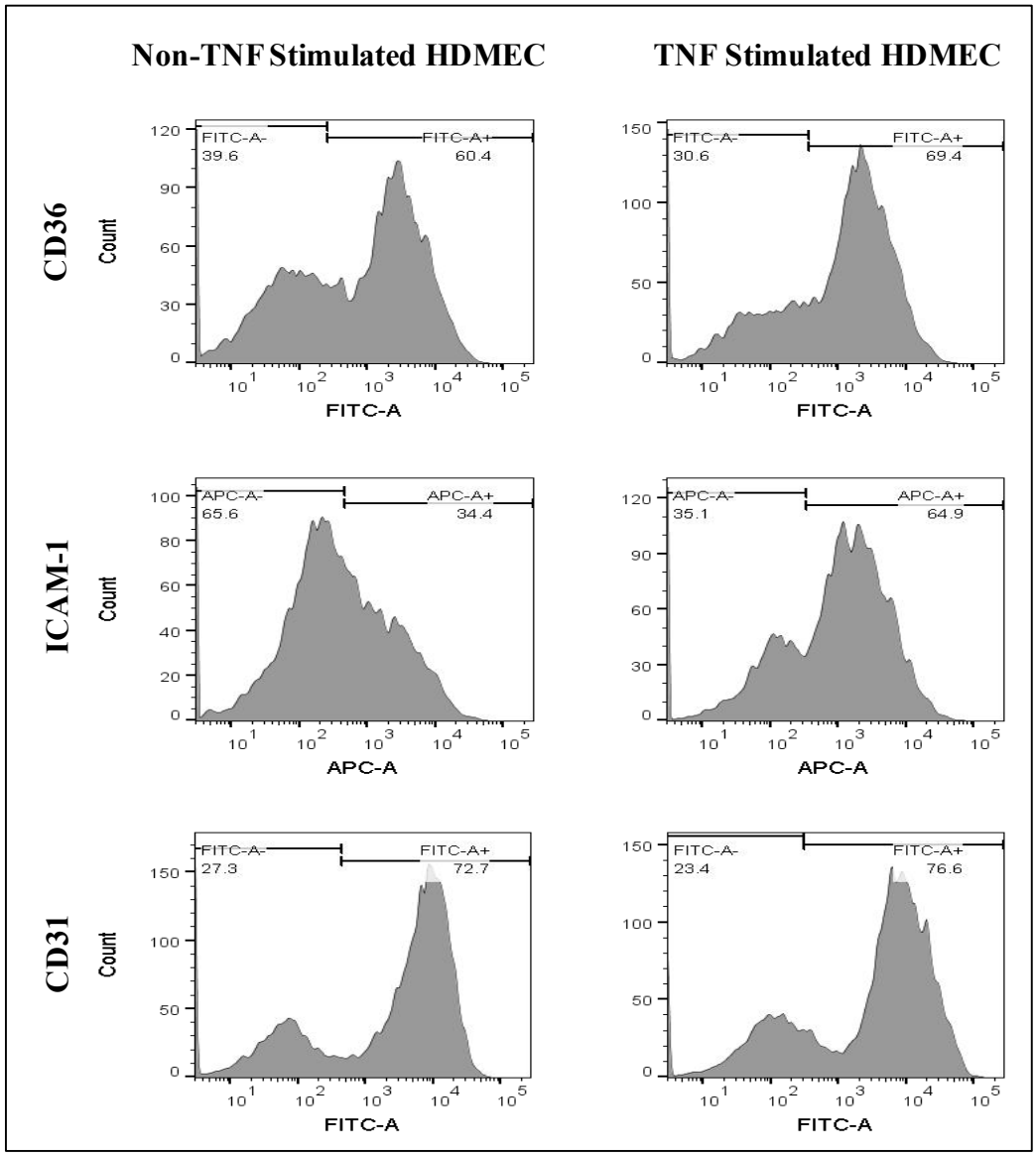


Figure 5.1: Detection of HDMEC receptor expression.
FACS analysis of CD36, ICAM-1 and CD31 expression on HDMEC without (left panel) and with (right panel) TNF stimulation. Stimulation was for 24 hrs using 10 ng/ml TNF. CD36, ICAM-1and an endothelial marker (CD31) are shown at the upper, the middle and the bottom panels respectively.

5.3.3 Quality control of RNA extraction samples:

In this chapter, all RNA samples were extracted from three independent co-culture experiments of IT4var14 with TNF-stimulated HDMEC at 0, 2, 6 and 20 hours. An Agilent 2100 Bioanalyzer machine was used to identify the quality of the RNA samples. All samples were with a RIN of more than 9 (Table 5.1).

Table 5.1: RIN calculated by Agilent 2100 Bioanalyzer.

First Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
1	Parasite co-cultured with HDMEC at 0 hour (TNF +ve)	9.4	41
2	Parasite co-cultured with HDMEC at 2 hours (TNF +ve)	10	89
3	Parasite co-cultured with HDMEC at 6 hours (TNF +ve)	10	58
4	Parasite co-cultured with HDMEC at 20 hours (TNF +ve)	10	89
Second Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
5	Parasite co-cultured with HDMEC at 0 hour (TNF +ve)	10	96
6	Parasite co-cultured with HDMEC at 2 hours (TNF +ve)	10	115
7	Parasite co-cultured with HDMEC at 6 hours (TNF +ve)	9.7	89
8	Parasite co-cultured with HDMEC at 20 hours (TNF +ve)	9.7	85
Third Experiment			
Sample number	**Description of sample**	**RIN**	**RNA Conc (ng/µl)**
9	Parasite co-cultured with HDMEC at 0 hour (TNF +ve)	9.9	82
10	Parasite co-cultured with HDMEC at 2 hours (TNF +ve)	9.8	91
11	Parasite co-cultured with HDMEC at 6 hours (TNF +ve)	9.9	279
12	Parasite co-cultured with HDMEC at 20 hours (TNF +ve)	9.3	78

The table presents the RIN and the RNA concentration of each sample. The high RIN calculated by the 2100 Bioanalyzer indicated the very high quality of the isolated RNA.

5.3.4 Identification of differentially expressed genes in HDMEC at 2 hours incubation:

Cuffdiff in FPKM was used in this thesis to measure the gene expression changes of TNF-activated HDMEC exposed to IT4var14 iRBC as described in chapter 3 section 3.3.6. At 2 hours of co-culture, 75 genes were identified as being significantly differentially expressed genes with 1Log2-fold change and adjusted FDR < 0.05. Of these 47 genes were found as over-expressed genes and 28 genes were under-expressed genes. The top nine over and eleven under-expressed genes are described in Figure 5.2. The most over-expressed gene at this time point was cytochrome P450

family 1 subfamily B member 1 (CYP1B1) with about 84 FC. On the other hand, the PARP1 binding protein (PARPBP) was the most under-expressed gene with around 16-fold reduction in expression. A table with a full description of the top nine over and eleven under-expressed genes is shown in appendix 13.

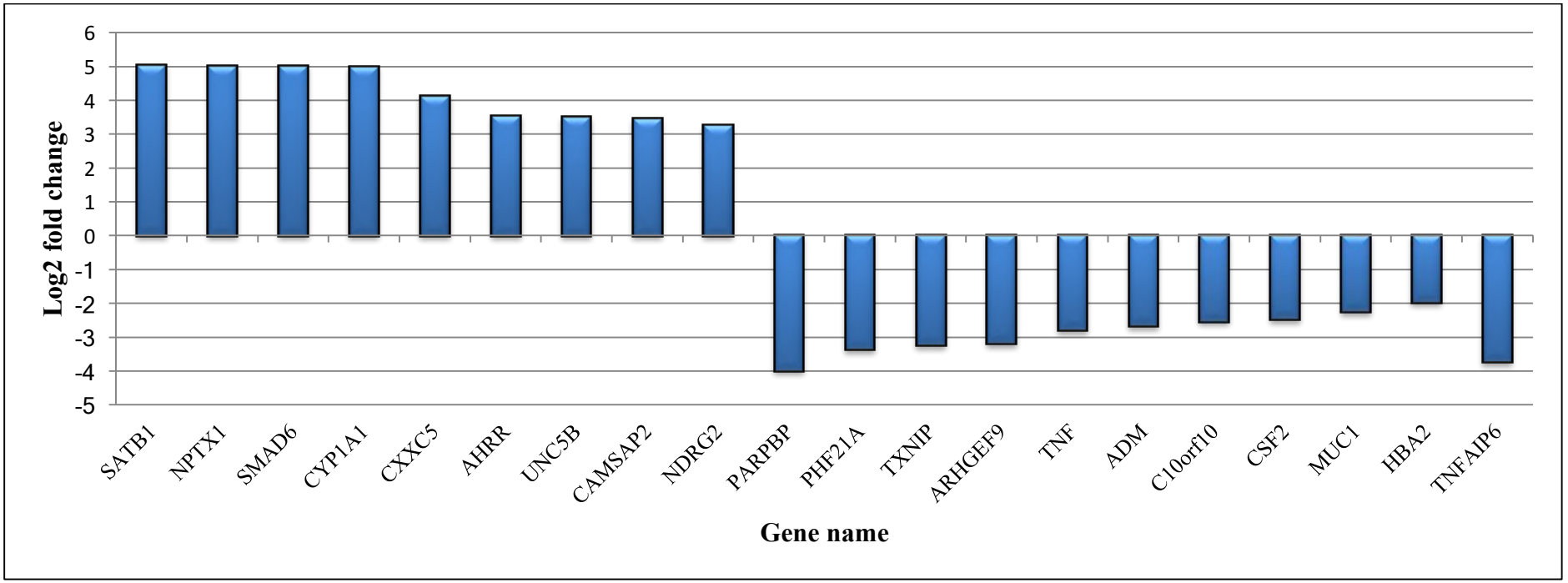


Figure 5.2: Top 9 up/11 down regulated genes in TNF-stimulated HDMEC incubated with IT4var14 parasite at 2 hours co-culture.
The differentially expressed genes in TNF-stimulated HDMEC co-cultured with IT4var14 parasite isolate at 2 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of the genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

5.3.5 Identification of differentially expressed genes in HDMEC at 6 hours incubation:

With respect to the gene expression levels of TNF-stimulated HDMEC co-cultured with IT4var14 iRBC at 6 hours, 77 significantly differentially expressed genes were identified in this study. Among these genes about 23% were shown as up-regulated genes and 77% were down-regulated genes. The top ten up and down regulated genes are shown in Figure 5.3, the highest FC was observed with carboxypeptidase M (CPM) followed by CYP1A1in up-regulated genes. MORN repeat containing 1 (MORN1) and zinc finger protein 345 were the top two down regulated genes (Figure 5.3). Further information about the top ten up and down-regulated genes can be seen in appendix 14.

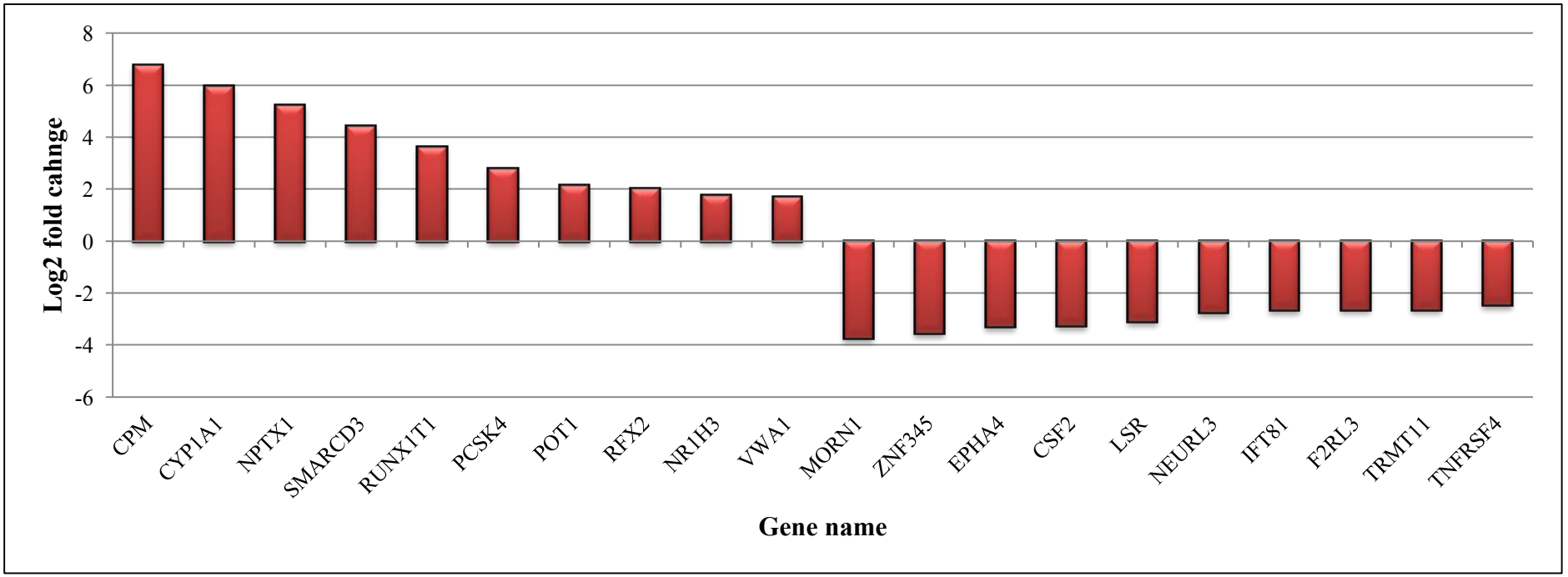


Figure 5.3: Top 10 up/down regulated genes in TNF-stimulated HDMEC incubated with IT4var14 parasite at 6 hours co-culture.
The differentially expressed genes in TNF-stimulated HDMEC co-cultured with IT4var14 parasite isolate at 6 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of the genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

5.3.6 Identification of differentially expressed genes in HDMEC at 20 hours incubation:

A total of 163 significantly differentially expressed genes were shown in TNF-stimulated HDMEC co-cultured with IT4var14 iRBC at 20 hours. 58 genes were found belonging to up-regulated genes, while 105 genes were identified belonging to down-regulated genes. The gene expression FC analysis among the top ten over-expressed genes revealed that the FCs varied from 6 to 89, and the most robust highly expressed gene was the CYP1A1 (Figure 5.4). In contrast, FCs of the top ten under-expressed genes varied from -9 to -20, and the lowest expressed gene was CSF2 (Figure 5.4). More information about the top ten up and down-regulated genes can be found in appendix 15.

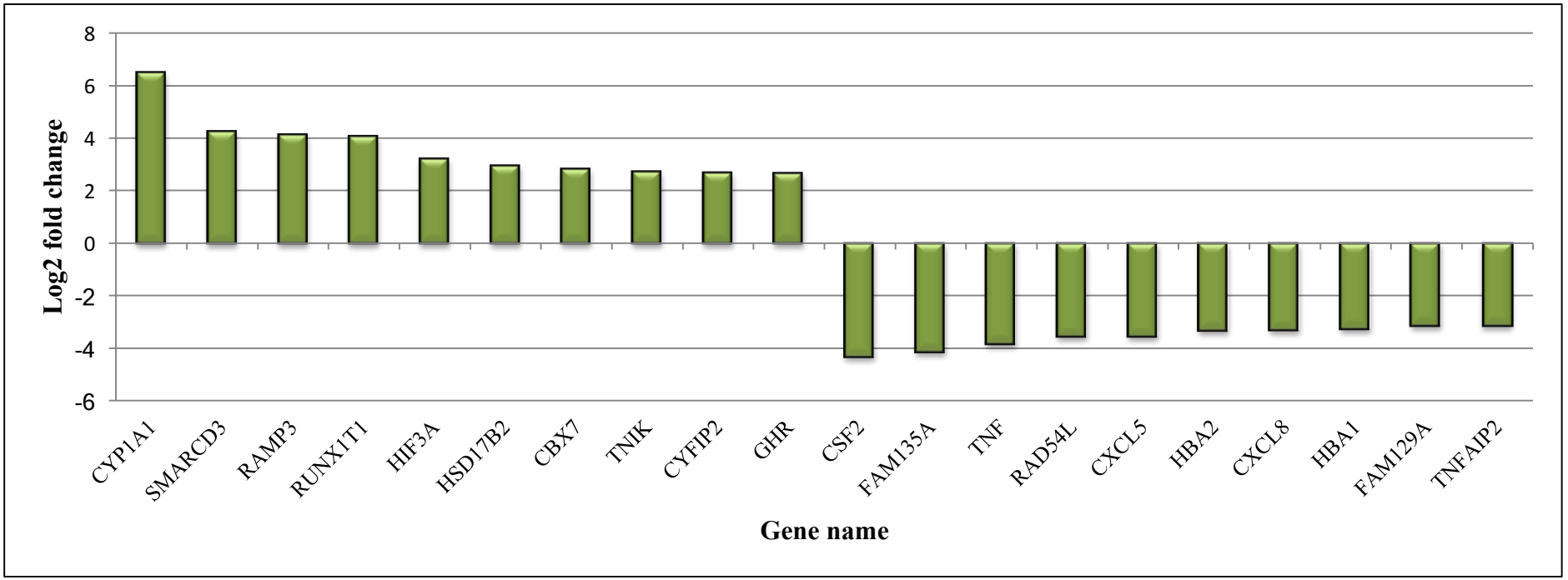


Figure 5.4: Top 10 up/down regulated genes in TNF-stimulated HDMEC incubated with IT4var14 parasite at 20 hours co-culture.
The differentially expressed genes in TNF-stimulated HDMEC co-cultured with IT4var14 parasite isolate at 20 hours vs those in control group at 0 hour were determined by Cuffdiff. The fold change is the ratio of *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) of the examined group compared to FPKM of the genes in the control group. The differentially expressed genes were ranked on their log2 fold change and the 10 genes with highest or lowest fold changes are shown here.

5.3.7 Functional and pathway analysis of differentially expressed genes:

In this chapter, analysis of the GO was performed on genes that were significantly differentially expressed as a result of incubation IT4var14 iRBC with TNF-stimulated HDMEC at 2, 6 and 20 hours according to the three GO categorizations; biological process, molecular function and cellular component. GO analysis data of co-culture models at 2, 6 and 20 hours can be seen in appendices 16, 17 and 18 respectively.

Analysis of the enrichment pathways amongst the up and down regulated genes of TNF-activated HDMEC exposed to *P. falciparum* IT4var14 at 2, 6 and 20 hours was carried out using the Reactome. The analysis identified only 2 host response pathways involved in genes with over-expression (P<0.05 and at least 3 input genes) from incubation IT4var14 iRBC with HDMEC/TNF at 2 hours. Signalling by NOTCH and arachidonic acid metabolism were the only pathways identified in this time point of co-culture (Table 5.2).

Table 5.2: pathways of up regulated genes of HDMEC responded to *P. falciparum* and TNF exposure at 2 hours.

Rank	Pathway name	No. of genes	*P* value
1	Signalling by NOTCH	3	5.62E-3
2	Arachidonic acid metabolism	3	4.33E-2

The pathways were selected based on number of genes and *P* value <0.05.

At 6 hours of incubation, the pathway analysis identified 15 pathways in up-regulated genes. Peptide ligand-binding receptors, class A/1 (Rhodopsin-like receptors, GPCR ligand binding and hemostasis were all found as the top four pathways with 7 up-regulated genes but with different *P-values* (Figure 5.5).

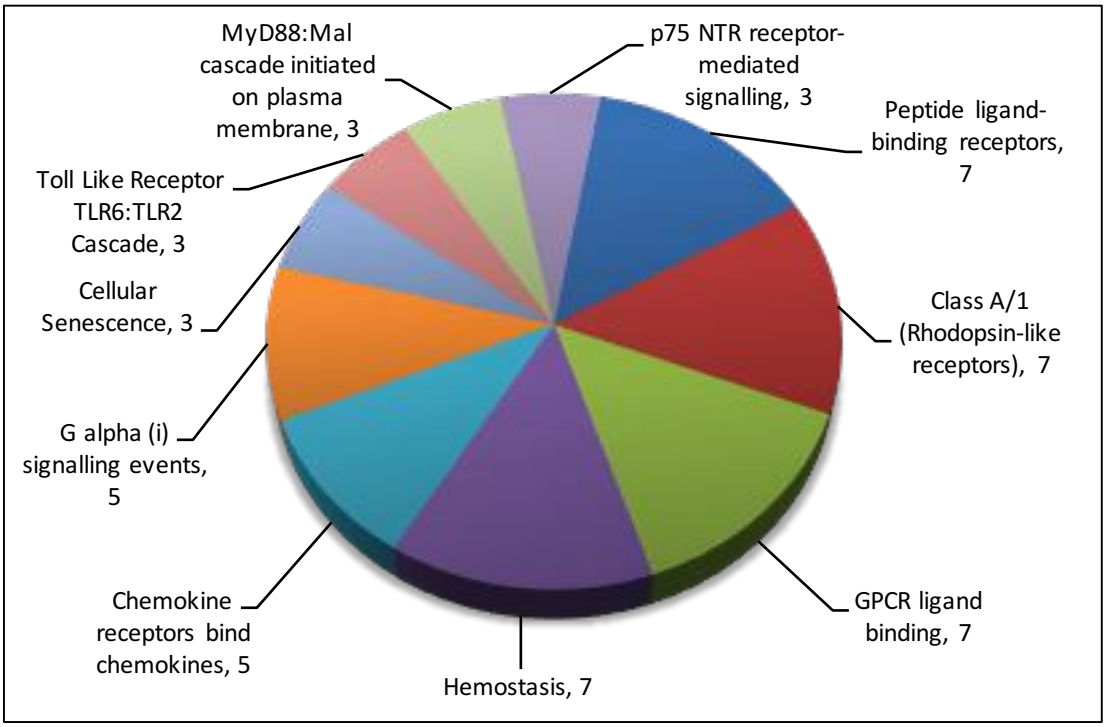


Figure 5.5: Top ten pathways of up regulated genes of HDMEC/TNF responding to iRBC at 6 hours.
The pathways were selected according to numbers of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

The analysis of the enriched pathways at 20 hours of exposure TNF-activated HDMEC to IT4var14 iRBC found 6 and 39 host response pathways in over and under-expressed genes respectively. The most significant pathway of the over-expressed genes based on the number of genes and the *P-value* was PPARA activates gene expression pathway. However, with respect to the gene numbers involved in each pathway, the immune system pathway was shown as the top pathway of under-expressed genes (Figure 5.6).

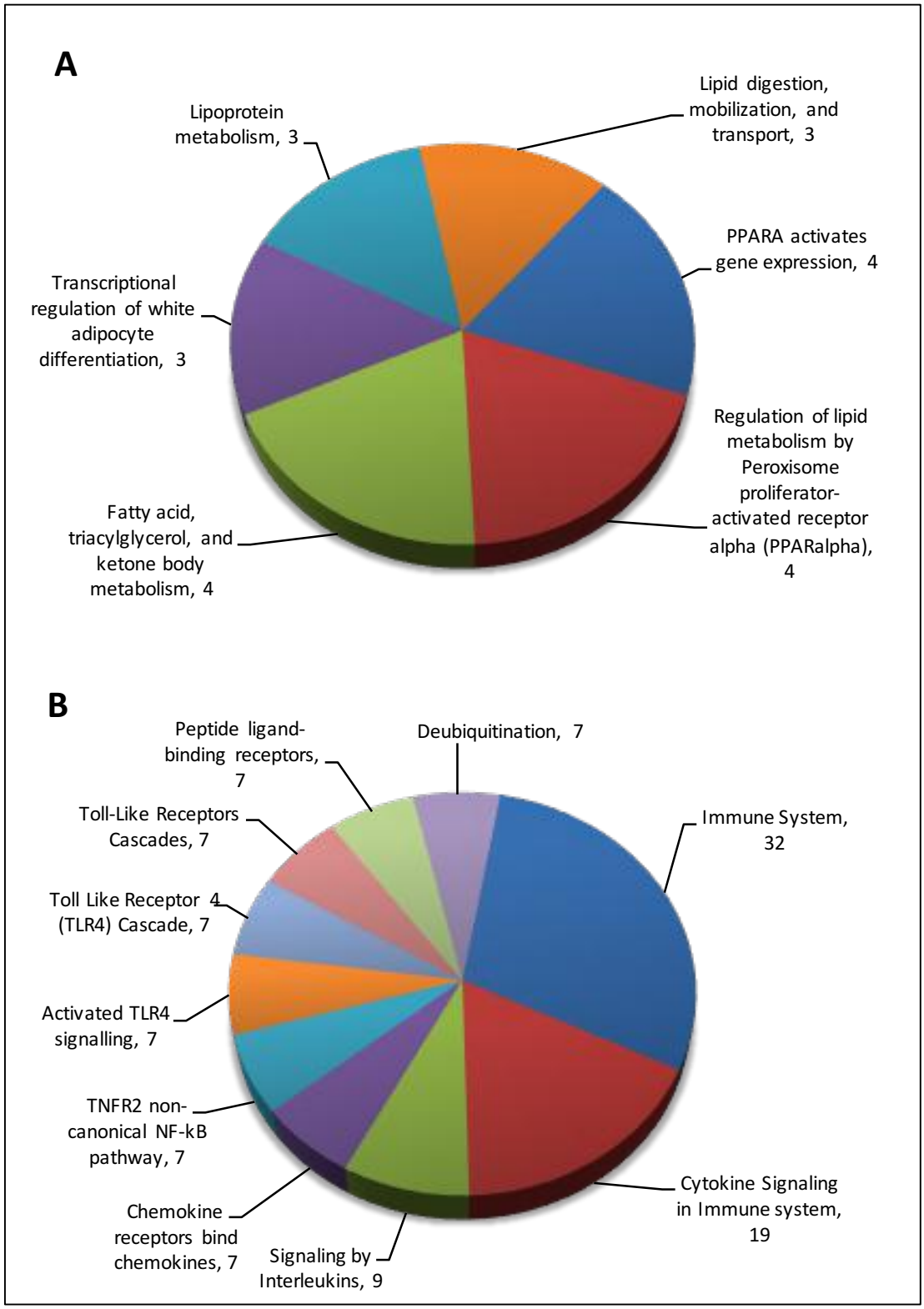


Figure 5.6: Top ten pathways of up and down regulated genes of HDMEC/TNF responding to iRBC at 20 hours.
A) pathways of up regulated genes and **B)** pathways of down regulated genes. The pathways were selected according to numbers of genes and *P* value <0.05. Values associated with each pathway represent the number of genes.

5.4 Discussion:

Multiple receptor interactions are essential for several adhesion processes, delivering signals, and increasing the adhesion strength (McCormick et al., 1997). Several studies demonstrated the role of receptors acting synergistically in modulating the adhesion of different cells (Languino et al., 1993, Duperray et al., 1997, Savill et al., 1992). McCormick *et al* found that ICAM-1 and CD36 work in a synergetic way to mediate the adhesion of a number of different parasite lines, such as IT4var14, under static conditions, with the majority of iRBCs binding in this system mediated by CD36 protein (McCormick et al., 1997). Other observations under flow conditions have also found co-operation between several receptors (Yipp et al., 2000, Ho et al., 2000).

This synergy is a property of malaria parasites expressing specific *var* genes, and the levels of synergy with IT4var14 strain were greater than those observed with other isolates (McCormick et al., 1997). Chapter 3 and 4 in this thesis have discussed the influence of different parasite isolates (IT4var14 and IT4var37) to modulate the transcriptional levels in TNF-stimulated HBMEC. In this chapter, we purposed to assess the effect of IT4var14 iRBC to change the mRNA expression levels in HDMEC using RNA-seq technique, then comparing the results with chapter 3 outcomes (using HBMEC).

IT4var14 parasite isolate had been identified as a good binder for ICAM-1 and CD36 proteins (Madkhali et al., 2014, Gray et al., 2003). HDMEC express both ICAM-1 (Swerlick et al., 1991) and CD36 (Swerlick et al., 1992) protein receptors and the expression of ICAM-1 can be modulated by cytokines such as TNF (McCormick et al., 1997). In this current study, the RNA-seq outcomes of TNF-activated HDMEC to IT4var14 iRBC at 2 hours demonstrated that 75 genes were identified as significantly

differentially expressed, while the incubation of IT4var14 iRBC with HBMEC/TNF from chapter 3 at the same time point showed 109 significant genes. There are differences in the results of the expressed genes in both types of endothelial cells (HDMEC and HBMEC) exposed to the same *P. falciparum* iRBC at 2 hours. Only 11 genes from both RNA-seq results overlapped as shown in the Venn diagram (Figure 5.7).

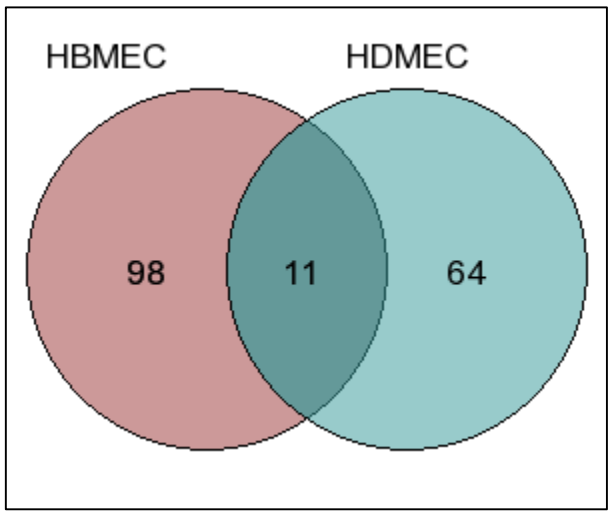


Figure 5.7: Comparison between number of significant genes in HBMEC/TNF and HDMEC/TNF co-cultured with IT4var14 parasite at 2 hours.
Venn diagram showing 11 genes were found expressed in the co-culture model of IT4var14 iRBC incubated with TNF-stimulated HBMEC and HDMEC at 2 hours.

At 6 hours of incubation IT4var14 iRBC with TNF-stimulated HDMEC, 77 genes were identified as differentially expressed. On the other hand, at the same period of incubation IT4var14 iRBC with HBMEC/TNF there were 88 genes observed with differential expression. Although, there was no big difference between number of significant expressed genes of HDMEC and HBMEC co-culture models, variation was observed in the genes themselves, with only 5 genes overlapping between the co-culture models (Figure 5.8).

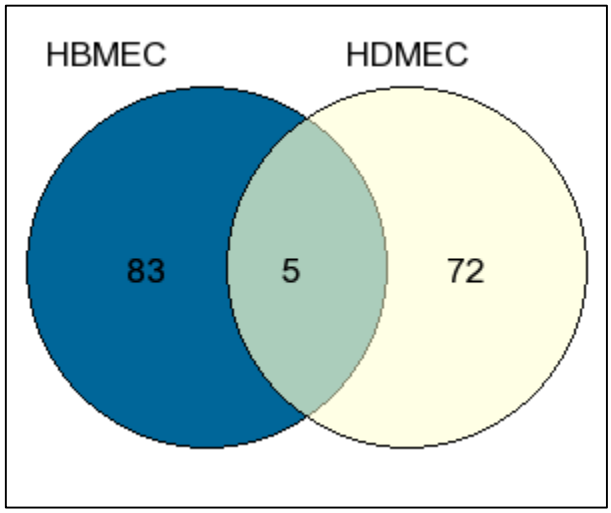


Figure 5.8: Comparison between number of significant genes in HBMEC/TNF and HDMEC/TNF co-cultured with IT4var14 parasite at 6 hours.
Venn diagram showing 5 genes were found expressed in the co-culture model of IT4var14 iRBC incubated with TNF-stimulated HBMEC and HDMEC at 6 hours.

The number of genes showing similarities and variances in differentially expressed genes in TNF-activated HDMEC and HBMEC co-cultured with IT4var14 at 20 hours are identified in Figure 5.9. 13 genes only were similar in both RNA-seq findings.

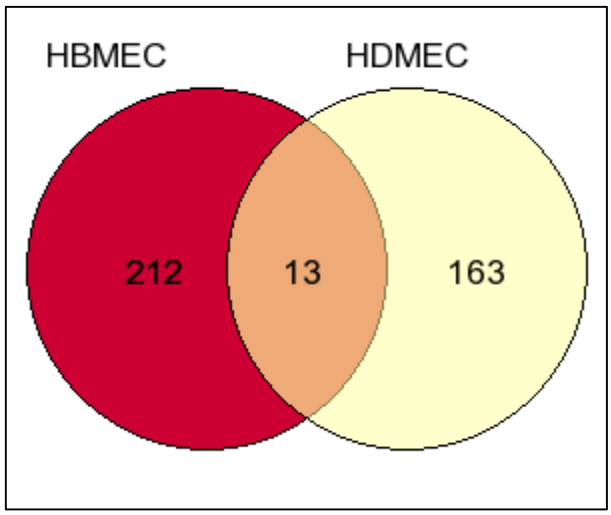


Figure 5.9: Comparison between number of significant genes in HBMEC/TNF and HDMEC/TNF co-cultured with IT4var14 parasite at 20 hours.
Venn diagram showing 13 genes were found expressed in the co-culture model of IT4var14 iRBC incubated with TNF-stimulated HBMEC and HDMEC at 20 hours.

According to the differences and similarities in the number of genes that were described in the previous paragraphs, we may observe that when ICAM-1 and CD36 proteins work in a synergetic manner during iRBC binding, they might mediate expression of a largely different set of genes in HDMEC than those seen with HBMEC co-cultured in the same conditions, and this may suggest a role of multiple protein receptors on the surface of endothelial cells to modulate the iRBC adhesion and then express different genes. Moreover, we described in chapter 3 the possibility of the gene expression changes as a results of binding IT4var14 parasite line to ICAM-1, however in this chapter it is perhaps the CD36 playing a major role to mediate the iRBC binding based on findings of several studies (McCormick et al., 1997, Yipp et al., 2000, Ho et al., 2000) and thereby give different expression patterns.

Our previous results from chapter 3 have been compared to this chapter findings in terms of the functional and pathway analysis. High-throughput screening from exposure of the TNF-activated HDMEC to IT4var14 iRBC at 2 hours could identify differential expressions in important genes involved in immune response pathway. The expression of IL32, EBI3 and CTSK genes were reduced in this study in comparison to chapter 3 results (Figure 5.10). In contrast, other genes also involved in immune system pathway such as FOS, CCL2 and CXCL1 were at the same levels of expression in both co-culture models from this study and chapter 3 as shown in Figure 5.10. These variations and similarities give more explanations about the effect of the malaria parasite that displays a long *PfEMP-1* variant on endothelial cells expressing both ICAM-1 and CD36 proteins.

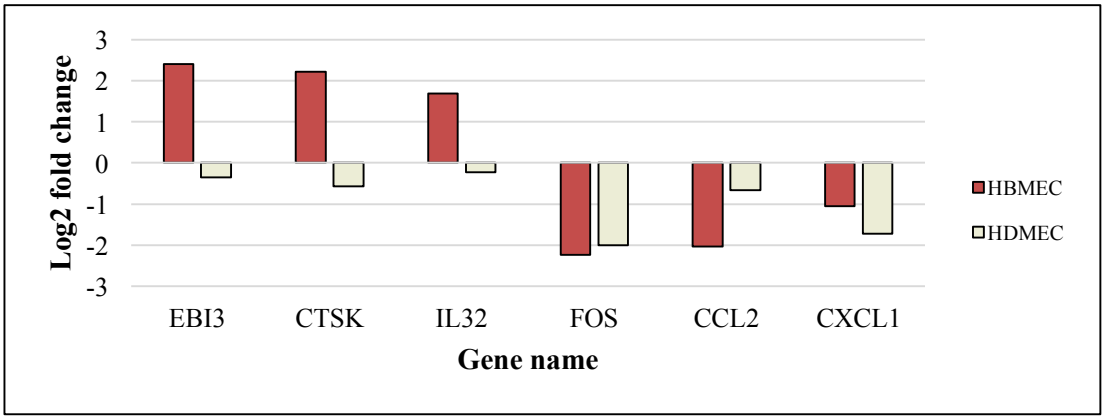


Figure 5.10: Comparison of the expression of selected genes representing immune system pathway in TNF-stimulated HDMEC and HBMEC incubated with IT4var14 iRBC at 2 hours.
The results show log2 fold change of differentially expressed genes in HDMEC/TNF and HBMEC/TNF co-cultured with the IT4var14 parasite strain at 2 hours.

Incubation of the IT4var14 iRBC with TNF-stimulated HDMEC at 6 hours leads to gene expression that might have a role in the immune system. C3, TXNIP and CTSK genes expressed in this current study had different up-regulation levels compared to chapter 3 outcomes, but the most fold change expressed gene is still C3, with nearly 121-fold change in HBMEC co-culture model from chapter 3. This investigation may suggest the role ICAM-1 as a major protein on HBMEC that modulates the adhesion to iRBC (Gray et al., 2003), however its role in HDMEC is changed because there is possibility of working in synergism with CD36. The expression level of PLA2G4A gene in this study is still over-expressed, especially when compared to the previous results from chapter 3 (Figure 5.11), which may indicate a link between expression of this gene and ICAM-1 expression on endothelium.

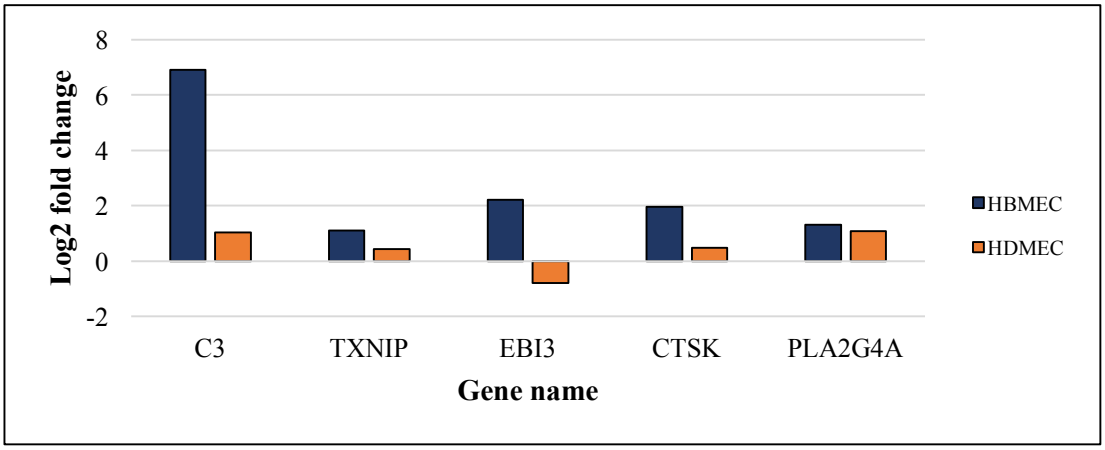


Figure 5.11: Comparison of the expression of selected genes representing immune system and arachidonic acid pathways in TNF-stimulated HDMEC and HBMEC incubated with IT4var14 parasite at 6 hours.
The results show log2 fold change of differentially expressed genes in HDMEC/TNF and HBMEC/TNF co-cultured with IT4var14 parasite strain at 6 hours.

The under-expressed genes that were involved in the immune system, as well as 'positive and negative regulation of apoptotic process' in this study also have been compared to chapter 3 findings at 6 hours of incubation (Figure 5.12). RELB, CCL2 and NFKBIA gene expressions were roughly the same, on the other hand the expression level of EGR1 gene was double in HDMEC/TNF exposed to IT4var14 iRBC compared to HBMEC/TNF. These observations may emphasize the role of ICAM-1 expression on endothelial cells to mediate the iRBC adhesion and develop the malaria pathogenesis even though it works in a synergism with CD36.

Variations in gene expression were also observed in several genes related to 'positive and negative regulation of apoptotic process' pathways in this study compared to chapter 3 results (Figure 5.12). Incubation of TNF-stimulated HDMEC with IT4var14 iRBC at 6 hours gave a little reduction in GADD45B gene expression in comparison to a high reduction observed in the same gene with HBMEC co-culture model. However, the expression pattern for ID1 is very different between HDMEC and HBMEC. Furthermore, a very little reduction in gene expression was found in

ID3 gene in this chapter, whereas the DUSP1 gene expression in HDMEC was nearly the same as HBMEC under the same conditions. Response of the ECs from different organs to IT4var14 iRBC *in vitro* is varied, and the reason may be due to the different array of proteins expressed on the surface of the microvascular endothelial cells and the nature of the endothelial cell organs (Ye et al., 2014).

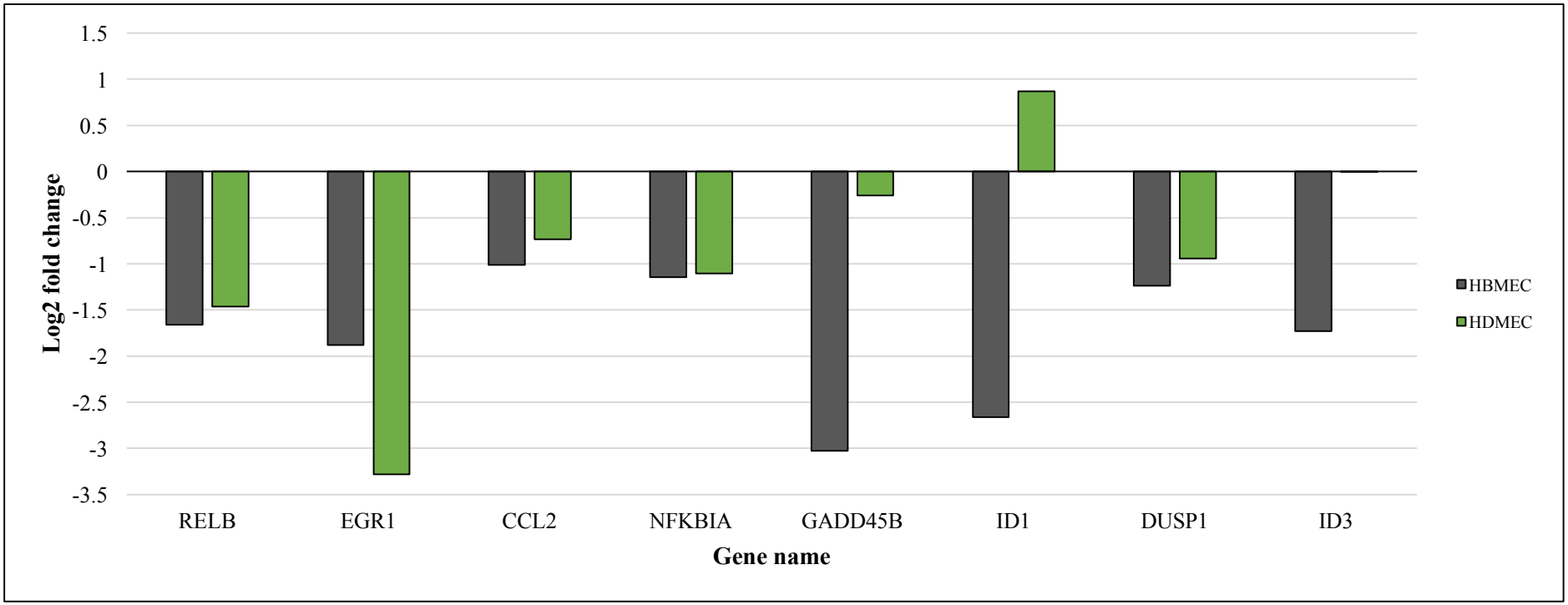


Figure 5.12: Comparison of the expression of selected genes representing immune system, positive and negative regulation of apoptotic process pathways in TNF-stimulated HDMEC and HBMEC incubated with IT4var14 parasite at 6 hours.
The results show log2 fold change of differentially expressed genes in HDMEC/TNF and HBMEC/TNF co-cultured with IT4var14 parasite strain at 6 hours.

At 20 hours of exposure HDMEC/TNF to IT4var14 iRBC, it can be seen that the expression level of genes involved in immune response pathway were completely different from that with HBMEC in chapter 3 results (Figure 5.13). In chapter 3, it has been shown that CCL2, CCL20 and CXCL2 genes were expressed at high levels, while in this chapter all of these selected genes were at low levels of expression. It is likely that the expression level of ICAM-1 and CD36 on HDMEC at this time point of co-culture are still at significant levels enhancing sequestration (Tripathi et al., 2006) and rupturing the mature iRBC may lead to changes in the transcriptional level in genes involved in immune pathway due to the release of soluble mediators.

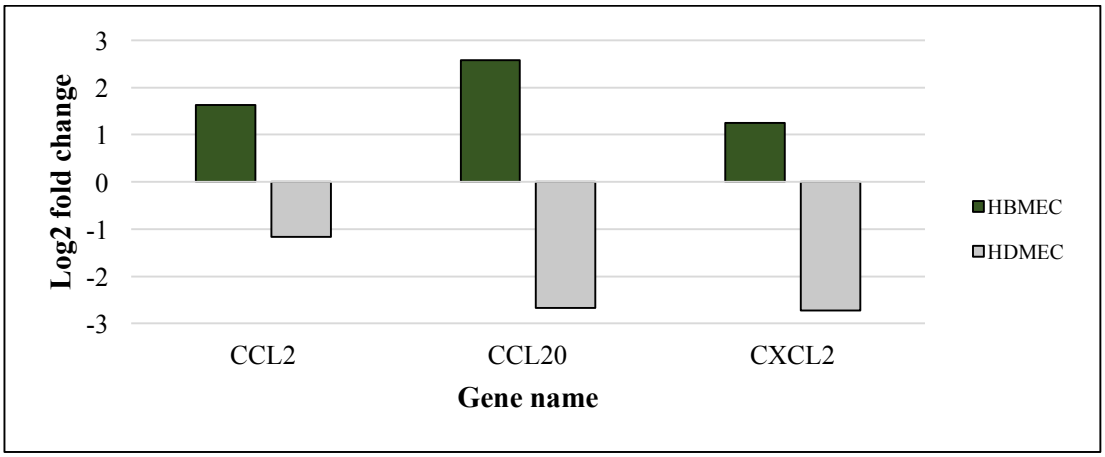


Figure 5.13: Comparison of the expression of selected genes representing immune system pathway in TNF-stimulated HDMEC and HBMEC incubated with IT4var14 parasite at 20 hours.
The results show log2 fold change of differentially expressed genes in HDMEC/TNF and HBMEC/TNF co-cultured with IT4var14 parasite strain at 20 hours.

Several similarities and differences were observed between HDMEC and HBMEC gene expression that were activated by TNF and incubated with IT4var14 iRBC at several time points. One possible explanation for the different patterns of expression is the lack of CD36 on HBMEC such that engagement with iRBC will lead to the transduction of different signals in HDMEC and HBMEC. Further work looking at the response to iRBC/ EC co-culture on the blocking of specific adhesion pathways

will be required to understand the contribution of key receptor/ ligand interactions on controlling the EC response to cytoadherence.

CHAPTER 6

EFFECTS OF TNF STIMULATION ON DIFFERNTIAL GENE EXPRESSION IN HBMEC CO-CULTURED WITH INFECTED AND UNINFECTED RBC

6.1 Introduction:

Infection by *P. falciparum* is a major cause of severe malaria disease associated with a range of clinical complications and syndromes including CM (Wu et al., 2011). The exact nature of the CM pathology is still not fully understood, but the cytoadherence and inflammation have been considered as two main mechanisms involved in this disease (Wu et al., 2011). There are several inflammatory and immune mediators likely to be implicated in this disease (Schofield and Grau, 2005, Marsh and Kinyanjui, 2006). During infection, factors such as high levels of circulating immune-complexes and plasma pro-inflammatory cytokines may play a crucial role in activating and damaging EC (Tripathi et al., 2009).

The cytoadherence mechanism of the asexual-stage iRBC is complex involving a family of parasite-encoded proteins (*PfEMP-1*) interacting with a range of host receptors such as ICAM-1, CD36, CD31, P-selectin (Cooke et al., 2000) and EPCR (Turner et al., 2013). This complication is elevated when these receptors cooperate to achieve efficient iRBC binding to EC (Gray et al., 2003, McCormick et al., 1997, Yipp et al., 2000).

Additionally, a feature of malaria infection is the production of high levels of TNF and pro-inflammatory mediators that may contribute to organ-related malaria disease (Brown et al., 1999b). These cytokines can activate the EC by over-expression of adhesion receptors to induce sequestration of iRBC within the microvessels, and contribute in some cases in chronic inflammation development by recruiting leukocytes into tissues (Pober and Cotran, 1990, Nitcheu et al., 2003). For instance, ICAM-1 is up-regulated in severe malaria and has been implicated in progression to cerebral malaria (Graninger et al., 1994, Berendt et al., 1989, Turner et al., 1994).

Usually, an *in vitro* model is used for studying the interaction between iRBC and EC *in vivo*, and this interaction can be promoted with TNF stimulation (Chakravorty et al., 2007). In the presence of the TNF, normal RBC also had an impact on the EC. The ability of uninfected RBC to bind to and modulate EC *in vitro* is not a novel result. Indeed, uninfected RBC can bind to EC, modulated by plasma factors such as fibronectin and fibrinogen (Wautier et al., 1983) and the binding can increase the expression of ICAM-1 in *in vitro* (Brown et al., 2001b).

A wide range of molecules can be expressed on the normal RBC surface which have been implicated in modulating adhesion to EC under several conditions. These include: (i) the blood group Lutheran molecule (LU), up-regulated on sickle cell RBC (sRBC), can bind to extracellular matrix protein laminin which contribute to the sickle cell pathogenesis related to vasoocclusive events (Eyler and Telen, 2006); (ii) VLA-4 (alpha4beta1) which can bind to VCAM-1 molecule on EC (Walmet et al., 2003); (iii) ICAM-4, a molecule related to Rhesus blood group, binds to integrins on platelets (alpha2beta4) which can be involved in thrombosis and on leukocytes (CD11-CD18) (Wautier and Wautier, 2004); and (iv) advanced glycation end products (AGEs) exist on normal RBC and bind to EC via their receptor (RAGE) (Wautier and Schmidt, 2004).

We propose in this chapter to compare the ability of IT4var14 iRBC to modulate the transcriptional expression levels of genes in HBMEC in the presence and absence of TNF, furthermore comparing the mRNA expression in TNF-stimulated HBMEC co-cultured with IT4var14 and uninfected RBC at 2, 6 and 20 hours using the high-throughput sequencing technique (RNA-seq).

6.2 Methods:

6.2.1 Parasite culture:

IT4var14 parasite line was cultured and used in this study as described previously in the general methods chapter.

6.2.2 EC culture:

HBMEC was cultured using the standard culturing techniques as shown before in the main methods chapter.

6.2.3 Detection of mycoplasma infection in IT4var14 parasite strain, RBC and HBMEC:

The possibility of mycoplasma infection in IT4var14, RBC and HBMEC were examined as described in the general methods chapter.

6.2.4 Co-culture ECs with malaria parasite:

IT4var14 iRBC was incubated with HBMEC in the presence and absence of TNF, at 0, 2, 6 and 20 hours as described in general methods chapter.

6.2.5 Co-culture ECs with normal RBC:

Uninfected RBC was co-cultured with TNF-stimulated HBMEC at 0, 2, 6 and 20 hours as parasite co-cultured methods that shown in the general methods chapter.

6.2.6 RNA extraction, quality control and RNA-seq data analysis:

RNA was isolated from each incubated flask and examined for its quality using Aglient Bioanalyzer 2100 as described previously in the main methods chapter. The RNA-seq data analysis workflow is described in Figure 3.2.

6.3 Results:

6.3.1 Comparing the differentially expressed genes in HBMEC stimulated and non-stimulated with TNF at 2 hours incubation:

Stimulating the brain EC cells by TNF in our co-culture models of IT4var14 iRBC at 2 hours can modulate differentially expressed genes in HBMEC, especially when compared to gene expressions of non-stimulated HBMEC at the same time point (Figure 6.1). Most of the top ten up-regulated genes seen in TNF-stimulated HBMEC had different expression levels compared to non-stimulated HBMEC. On the other hand, the pattern of expression levels of the top ten down-regulated genes in brain EC stimulated with TNF was largely similar to that without TNF, albeit with some exceptions (e.g. CMTM1 and AQP1) and a less marked effect (e.g. HES1 and KIT).

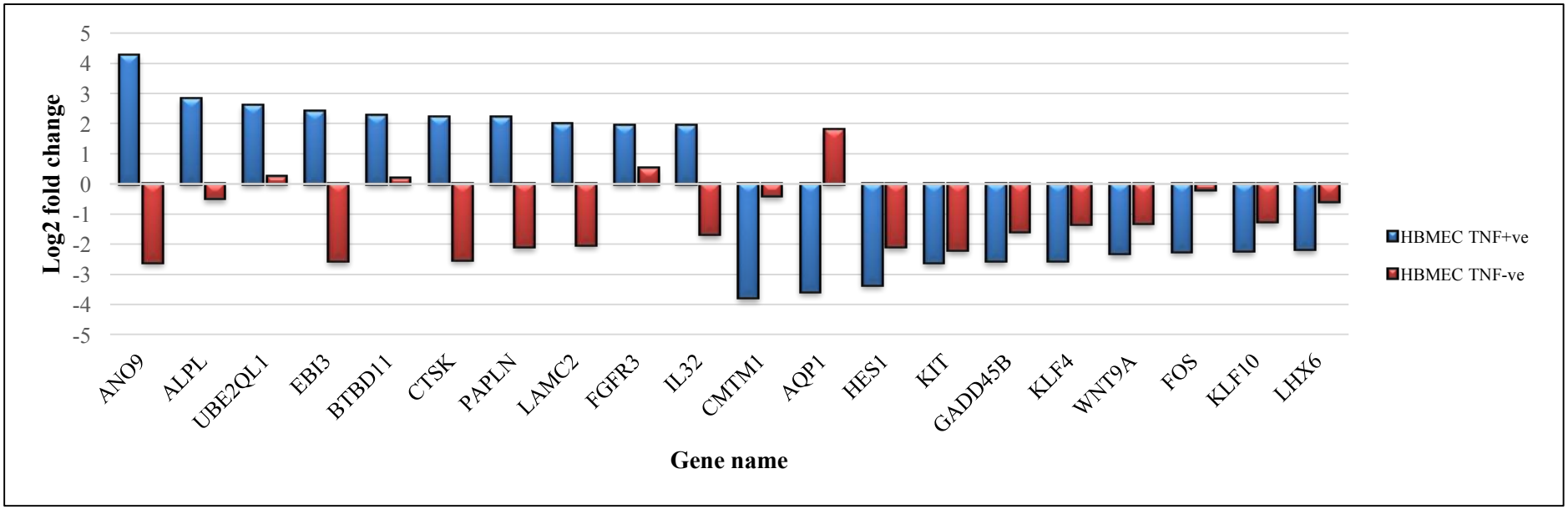


Figure 6.1: Comparison of the expression levels of the top ten up and down regulated genes of HBMEC stimulated and non-stimulated with TNF in co-culture model of IT4var14 iRBC at 2 hours.

The results show log2 fold change of differentially expressed genes in HBMEC stimulated and non-stimulated with TNF and co-cultured with IT4var14 parasite strain at 2 hours.

6.3.2 Comparing the differentially expressed genes in HBMEC stimulated and non-stimulated with TNF at 6 hours incubation:

Gene expression of human brain EC incubated with IT4var14 iRBC in the presence and absence of TNF stimulation at 6 hours were different (Figure 6.2). Among the top ten over-expressed genes, C3 was observed as a highly significantly over-expressed gene in the presence of TNF, compared to a small reduction in expression of the same gene without TNF activation. As seen at 2 hours, the top ten under-expressed genes at this time point of co-culture demonstrated that most of the genes showed expression pattern in EC with both activated and non-activated TNF that were roughly on the same level of reduction, except AQP1 and FOS genes.

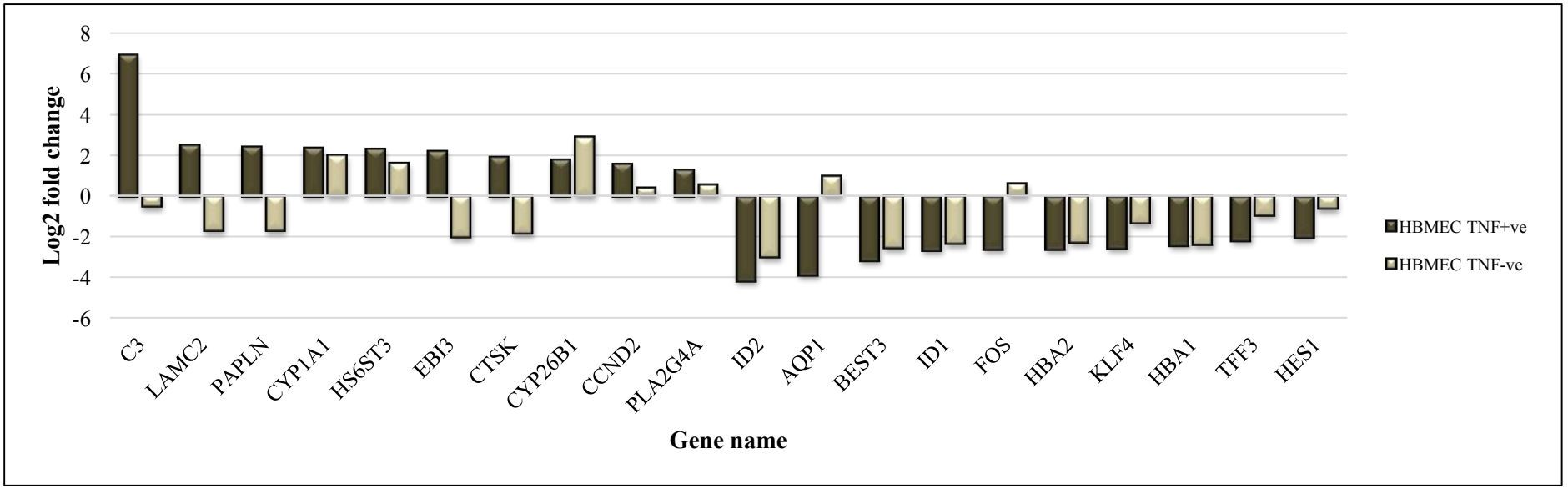


Figure 6.2: Comparison of the expression levels of the top ten up and down regulated genes of HBMEC stimulated and non-stimulated with TNF in co-culture model of IT4var14 iRBC at 6 hours.

The results show log2 fold change of differentially expressed genes in HBMEC stimulated and non-stimulated with TNF and co-cultured with IT4var14 parasite strain at 6 hours.

6.3.3 Comparing the differentially expressed genes in HBMEC stimulated and non-stimulated with TNF at 20 hours incubation:

At 20 hours of co-culture the IT4var14 iRBC with stimulated and non-stimulated TNF HBMEC, we observed that most of the gene expression levels of the top ten up-regulated genes in both conditions (presence and absence of TNF) were high, but genes in TNF stimulated EC were still at higher levels of expression compared to non-TNF stimulation, except for PRND and PAPLN genes, which had opposite expression levels under the two conditions (Figure 6.3). However, among the top ten down-regulated genes, the expression levels of the genes were mostly the same for with and without TNF, with some exceptions observed with FAM19A2 and GADD45B gene expression (Figure 6.3).

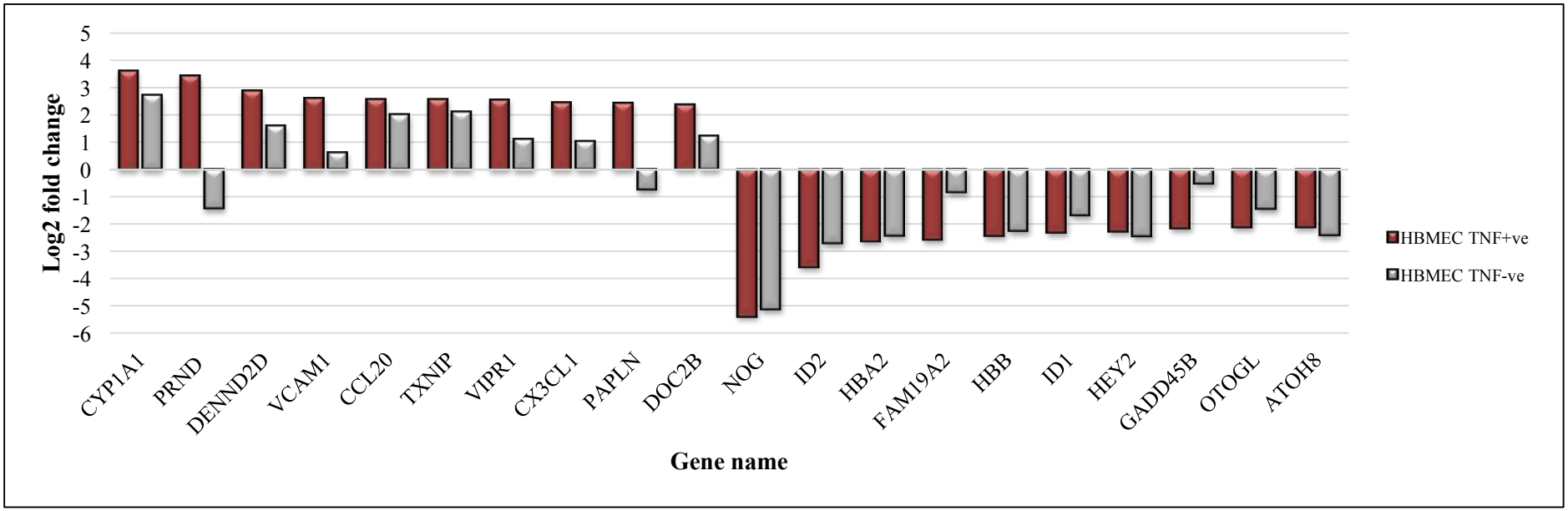


Figure 6.3: Comparison of the expression levels of the top ten up and down regulated genes of HBMEC stimulated and non-stimulated with TNF in co-culture model of IT4var14 iRBC at 20 hours.

The results show log2 fold change of differentially expressed genes in HBMEC stimulated and non-stimulated with TNF and co-cultured with IT4var14 parasite strain at 20 hours.

6.3.4 Comparing the differentially expressed genes in TNF-stimulated HBMEC Co-cultured with both IT4var14 iRBC and uninfected RBC at 2 hours:

In this study, co-culturing the TNF-activated HBMEC with both iRBC and normal RBC showed that the gene expression levels of EC in the co-culture model using uninfected RBC were slightly higher than that with iRBC in the top ten up-regulated genes (Figure 6.4). On the other hand, among the top ten down-regulated genes of the same co-culture models of both iRBC and normal RBC at 2 hours, the transcriptional levels of genes for iRBC co-culture had a slightly larger reduction compared to levels expressed with RBC co-culture (Figure 6.4), with the exception of CMTM1.

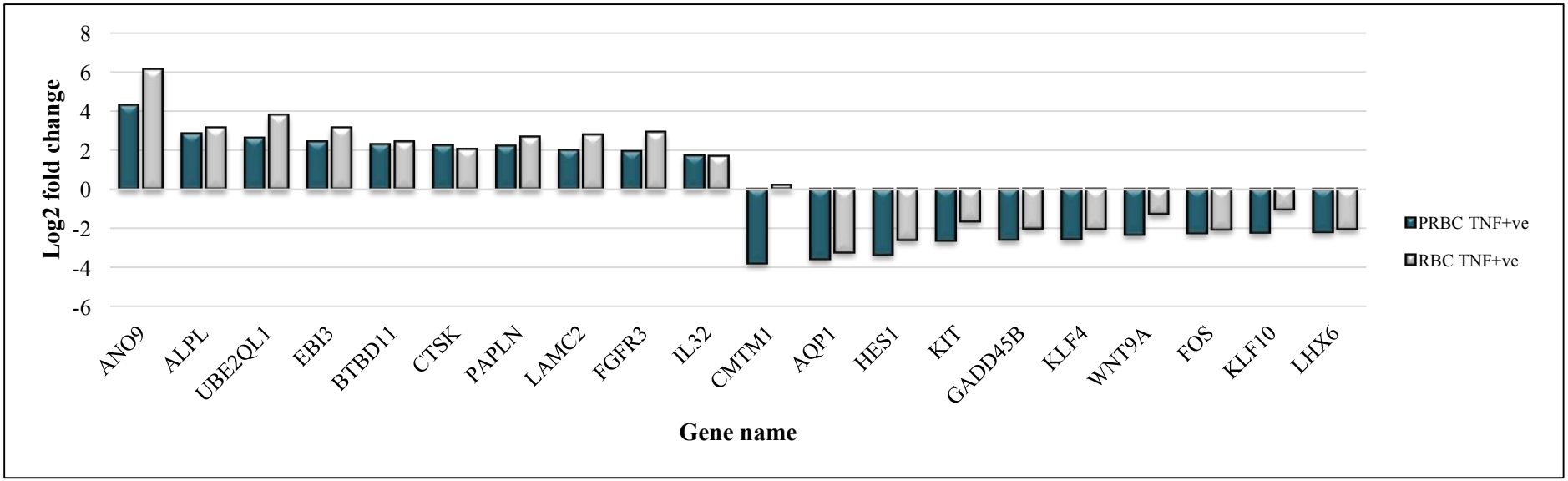


Figure 6.4: Comparison of the expression levels of the top ten up and down regulated genes of TNF- stimulated HBMEC co-cultured with both IT4var14 iRBC and uninfected RBC at 2 hours.

The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with both IT4var14 parasite strain and normal RBC at 2 hours.

6.3.5 Comparing the differentially expressed genes in TNF-stimulated HBMEC Co-cultured with both IT4var14 iRBC and uninfected RBC at 6 hours:

At 6 hours incubation with either iRBC or uninfected RBC on TNF-stimulated human brain EC, most of the top ten over-expressed gene expressions were nearly the same in both co-culture models (Figure 6.5). There was an exception in the expression of the C3 gene. Levels of mRNA expression among the top ten under-expressed genes of iRBC co-culture were roughly the same as those with normal RBC, with slightly higher reductions of the gene expression seen with IT4var14 iRBC incubation.

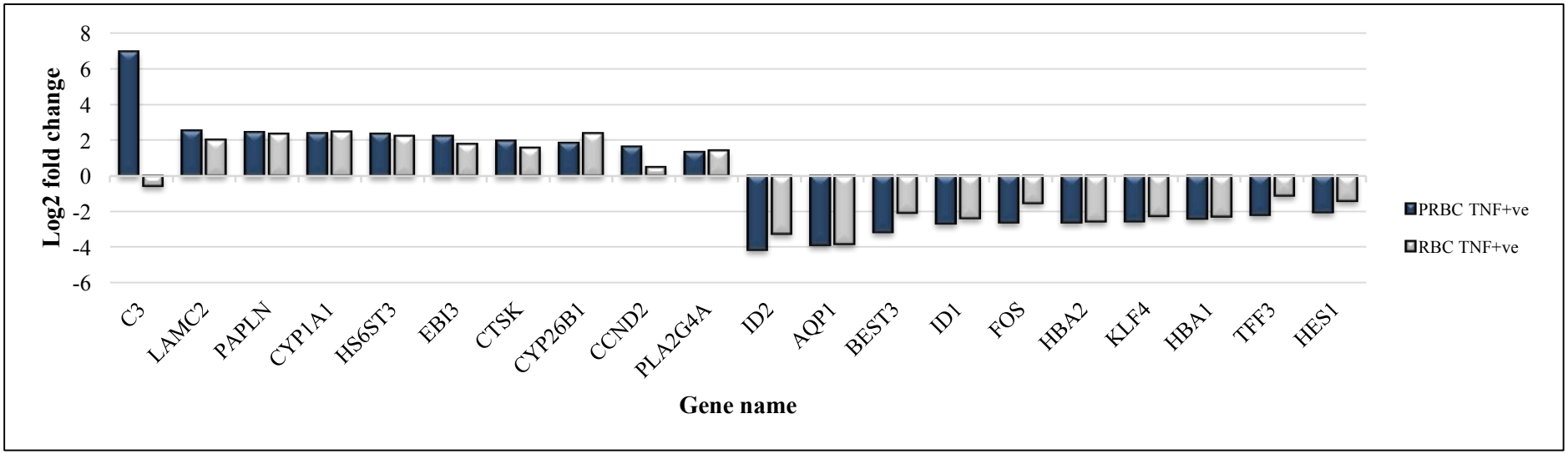


Figure 6.5: Comparison of the expression levels of the top ten up and down regulated genes of TNF- stimulated HBMEC co-cultured with both IT4var14 iRBC and uninfected RBC at 6 hours.

The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with both IT4var14 parasite strain and normal RBC at 6 hours.

6.3.6 Comparing the differentially expressed genes in TNF-stimulated HBMEC Co-cultured with both IT4var14 iRBC and uninfected RBC at 20 hours:

Incubation TNF-activated HBMEC with both IT4var14 iRBC and uninfected RBC at 20 hours again produced relatively similar patterns of differentially expressed genes in EC (Figure 6.6). At this time point of incubation with both co-culture models we observed that, among the top ten up-regulated genes, exposure of the EC to iRBC increased the expression of most of the genes compared to the levels seen with uninfected RBC. Vasoactive intestinal peptide receptor 1 (VIPR1) was the only gene showing slightly more induction with uninfected RBC than iRBC in the co-culture model. In contrast, transcriptional levels of the top ten down-regulated genes were observed with more reduction in EC exposed to IT4var14 iRBC than those with uninfected RBC. Haemoglobin subunit beta (HBB) was the only gene observed with a higher reduction in expression in uninfected RBC co-culture compared to iRBC co-culture.

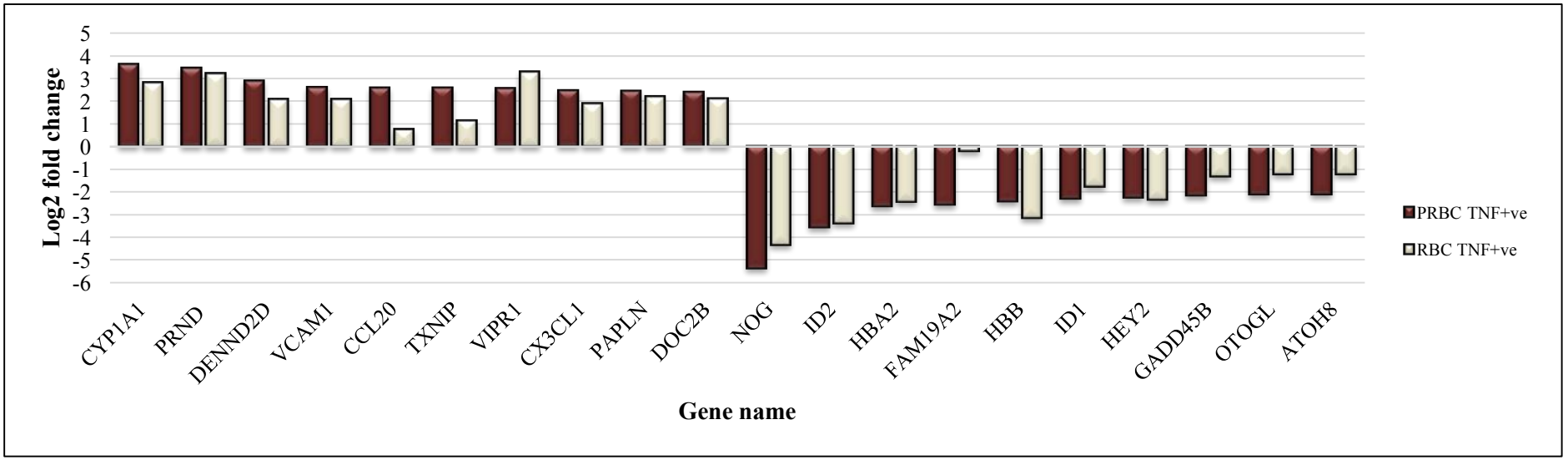


Figure 6.6: Comparison of the expression levels of the top ten up and down regulated genes of TNF- stimulated HBMEC co-cultured with both IT4var14 iRBC and uninfected RBC at 20 hours.

The results show log2 fold change of differentially expressed genes in HBMEC/TNF co-cultured with both IT4var14 parasite strain and normal RBC at 20 hours.

6.4 Discussion:

Pro-inflammatory cytokines have been implicated with CM disease (Schofield and Grau, 2005, Marsh and Kinyanjui, 2006), however a study from Mbale *et al* shows that TNF is not required for CM to occur (Mbale et al., 2016). High levels of these inflammatory mediators can activate the EC (Brown et al., 1999b) and the expression of several adhesion receptors such as ICAM-1 (Graninger et al., 1994, Turner et al., 1994, Berendt et al., 1989). Among these cytokines is TNF, which has a role in promoting the interaction between both iRBC and uninfected RBC to EC (Chakravorty et al., 2007).

With our work, we wanted to describe the detailed comparison of the mRNA expression levels of genes in HBMEC co-cultured with IT4var14 iRBC in the presence and absence of TNF at 2, 6 and 20 hours, as well as comparing the transcriptional expression levels of TNF-stimulated HBMEC incubated with both IT4var14 parasite isolate and uninfected RBC at 2, 6 and 20 hours using high-throughput RNA-seq technique.

Several studies have been investigated the role of TNF in mediating EC gene expression in an *in vitro* model of severe malaria using microarray technology (Barbier et al., 2011, Chakravorty et al., 2007). This study is the first to compare the mRNA expression levels of HBMEC co-cultured with IT4var14 iRBC in the presence and absence of TNF using the RNA-seq technology.

In the present study, it is interesting to know that comparing the gene expression profiles of the co-culture models before and after TNF stimulation, most of the top ten up or down-regulated genes were observed with high expression in the presence of TNF at the three different time points of co-culture (2, 6 and 20 hours) compared to non-TNF stimulation.

At 2 hours of incubation, the expression of EBI3, CTSK IL32 and FOS genes were completely different between with and without TNF categories; these genes were classified in chapter 3 as involved in the immune system pathway. On the other hand, co-culturing human brain EC with iRBC in the presence and absence of TNF at 6 hours demonstrated that the effect of the TNF stimulation on EC was obvious in both top ten over and under-expressed genes. C3 gene expression was the highest expressed gene observed in HBMEC after stimulation by TNF at this time point of co-culture model, but very little change was seen without TNF treatment; the role of C3 gene in immune response was discussed in chapter 3. The expression level of AQP1 in HBMEC, which was classified previously using the pathway analysis in chapter 3 as a negative regulator of apoptotic process, was totally different in the presence and absence of TNF stimulation. TNF has been identified as an inflammatory mediator that increase expression of ICAM-1, thus increasing the sequestration of the iRBC to EC (Chakravorty et al., 2007). This suggests a role of the TNF stimulation to modulate differentially expressed genes of EC co-cultured with iRBC at 6 hours.

It is clear from our work that exposure of the IT4var14 iRBC to brain EC before and after TNF stimulation can change the expression of the top ten up and down-regulated genes. However, the expression of genes at the 20 hours of co-culture was not totally different, despite genes with TNF-stimulated HBMEC were observed with higher expression than the pattern without TNF-stimulation. It is likely that at 20 hours of incubation iRBC with TNF-activated brain EC, the ICAM-1 expression may be decreased but is still with the significant levels (Tripathi et al., 2006), and this reduction in ICAM-1 might leads to reduce the expression of the significant genes.

Chakravorty and her colleague found that there was no significant modulation in transcriptional levels of EC exposed to iRBC or uninfected RBC without TNF stimulation, however significant changes of the EC transcriptome were observed in combination with TNF stimulation. Furthermore, in the same study, the author demonstrated that the expression level of ICAM-1 was significantly increased in EC when incubated with either iRBC or uninfected RBC but the level of ICAM-1 induction with iRBC was much higher than that observed with normal RBC (Chakravorty et al., 2007).

In our results, we demonstrate that at 2, 6 and 20 hours of exposure of TNF-stimulated HBMEC to both IT4var14 iRBC and uninfected RBC, the transcriptional levels of the top ten up and down-regulated genes were roughly the same. At 2 hours of incubation, it was the only exception that the expression levels of the top ten over-expressed genes were observed as being a little higher with uninfected RBC than iRBC co-culture. The C3 gene was the only gene expressed at different levels at 6 hours of co-culture between incubation with iRBC and RBC, with the increase in expression being associated with iRBC; the role of this gene in the immune system was discussed in chapter 3.

Our findings in this chapter have tried to explain the role of TNF stimulation in modulating the gene expression over the human brain EC in co-culture model of IT4var14 iRBC at different time points compare to non-TNF stimulation model of co-culture. On the other hand, the results also have compared the effect of the TNF activation on the HBMEC co-cultured with both iRBC and normal RBC at several time points of incubation to modulate genes with differential expression. Indeed, these outcomes may support the role of inflammatory cytokine especially TNF, in developing the CM disease. However, further investigations are required to study the

influence of other inflammatory mediators in interactions of iRBC to brain EC and thus understanding the pathogenesis of CM disease.

CHAPTER 7

GENERAL DISCUSSION, LIMITATIONS AND FUTURE PERSPECTIVES

7.1 General discussion:

Cerebral malaria is a complicated syndrome with no specific process that has been identified as being responsible for its pathogenesis. It has been proposed that CM pathogenesis can be explained by the integration of *P. falciparum* iRBC sequestration in the microvasculature of brain EC, which can be lethal through blood flow obstruction, inflammation mediation and endothelial activation and dysfunction. Cytoadhesion of iRBC to HBMEC can be enhanced by several host inflammatory cytokines such as TNF, which in turn results in an increased level of iRBC sequestration in the brain microvasculature (van der Heyde et al., 2006). It has been shown that the interaction between iRBC and EC lining brain microvessels can result in modulation several EC genes, which could be involved in activation of EC, intracellular signalling pathways, BBB disruption and EC apoptosis. The current study aims to understand how the *P. falciparum* parasite isolates can modulate the gene expression of ECs in order to better understand CM pathogenesis.

Several transcriptomic studies have been conducted to measure the level of gene expression in both *P. falciparum* parasites and ECs following the interaction between infected erythrocytes with endothelial cells using a microarray technique. Although the microarray is a cost effective, it is not a more sensitive manner to detect the aspects of RNA molecules (Wang et al., 2009, Casneuf et al., 2007). On the other hand, using the RNA-seq technique is more expensive than other NGS techniques, but it is more reliable and can detect 25% more genes (Sultan et al., 2008). Our study is the first RNA-seq study that investigates the response of the ECs to the iRBC co-adhesion interaction.

In chapter 3, we tried to address the question of how the IT4var14 parasite strain can modulate the gene expression of brain endothelial cells in the presence of TNF

stimulation at 2, 6 and 20 hours of co-culture. We demonstrated that iRBC adhesion induced expression of genes involved in immune system (IL32, EBI3 and CTSK) at 2 hours of co-culture. Stimulation of these genes in human brain EC might be resulting from the presence of the malaria parasite, with the host starting to defend itself against the infection. We also found that iRBC interaction reduced the transcriptional levels of genes involved in immune system (CCL2, CXCL1 and FOS) in HBMEC at 2 hours of incubation. Reduction in expression of CCL2 and CXCL1 genes may explain a potential role of HBMEC to protect itself from the malaria infection. On the other hand, down-regulation of FOS gene may lead to neuron dysfunction and hence damaging of the brain cells.

The ability of IT4var14 parasite to modulate gene expression and pathways in HBMEC differs from 2 to 6 hours of co-culture. At 6 hours of exposure the human brain EC to iRBC in the present of TNF, the transcriptional levels of C3, TXNIP, EBI3 and CTSK genes, that are involved in the immune system pathways, were high. When we compared the differential regulation of gene transcription of immune system pathway between 2 and 6 hours of co-culture, we observed that the expression of EBI3 and CTSK genes are downregulated at 6 hours, which may induce inflammation in the endothelium, resulting in higher cytoadherence. One of the most important observations in our study is the high expression of the C3 gene at 6 hours. We discussed in chapter 3 the possible role of C3 expression in CM. Moreover, interaction of iRBC to HBMEC at 6 hours led to increase expression of the PLA2G4A gene, which catalyses breakdown of phospholipids membrane to release arachidonic acid and promote prostanoid biosynthesis (Hua et al., 2015). Up-regulation of these genes in HBMEC at 6 hours of co-culture may result in the

increase of a pro-adhesive effect that potentially enhance the pathological sequestration in the CM.

On the other hand, we demonstrated that cytoadhesion of iRBC to brain endothelial cells at 6 hours of the co-incubation system can reduce expression of several genes involved in immune system (RELB, EGR1, CCL2 and FOS), positive regulation of apoptosis (NFKBIA, GADD45B and ID1) and negative regulation of apoptosis pathways (DUSP1, AQP1 and ID3). Increase in the time of co-culture from 2 to 6 hours might potentially induce the sequestration of iRBC on HBMEC that leads to increase the effect of iRBC modulation on the EC, this may be observed in the expression of CCL2 gene at 6 hours. CCL2 expression became less marked with time (-2.02 (6 hours) and -4.1 (2 hours) FC), thus it might increase the permeability of BBB and the likelihood of CM disease. However, at 6 hours of incubation, we observed that the transcriptional level of FOS gene was decreased with the time of co-culture and that may suggest the role of the IT4var14 parasite interaction to modulate genes in human brain EC which lead to induce CM pathology.

The differential expression of genes involved in pro and anti-apoptotic pathways resulting from interaction of the IT4var14 parasite with HBMEC in the presence of TNF opens questions such as, does the parasite want to kill the host cells or keep them alive? Or this process is a normal response from the host to protect itself? We suppose the needs of the parasite are to keep the host alive as long as it can because it depends on it to complete its life cycle and undergo transmission to the insect vector. Therefore, as well as inducing pathology in the host, the parasite may also activate protective mechanisms to prevent the interaction between itself and the host from causing too much damage that would prevent eventual gametocyte transmission.

One of the most critical time points that we used in our co-culture models was at 20 hours. It has been supposed that the ICAM-1 expression at this time point is still at a significant level enhancing sequestration (Tripathi et al., 2006), moreover most of the iRBC can be ruptured and released several molecules that modulate the gene expression in HBMEC such as hemozoin (Tyberghein et al., 2014). At 20 hours of co-culture, we observed that the mRNA level of CCL2 gene, which is involved in an immune response pathway, was totally different from that observed at 2 and 6 hours. Expression of CCL2 was induced at 20 hours of co-culture and this may explain the role of molecules that are released from rupturing iRBC to alter the gene expression levels in brain endothelial cells. In contrast, exposure of HBMEC to IT4var14 parasite and TNF at 20 hours reduced expression of genes mostly involved in cell cycle, and we do not have any understanding of why, at this time point, we should see decreased expression of these genes. The proposed effect of iRBC cytoadhesion to modulate gene expression pathways in the brain EC can be seen in Figure 7.1.

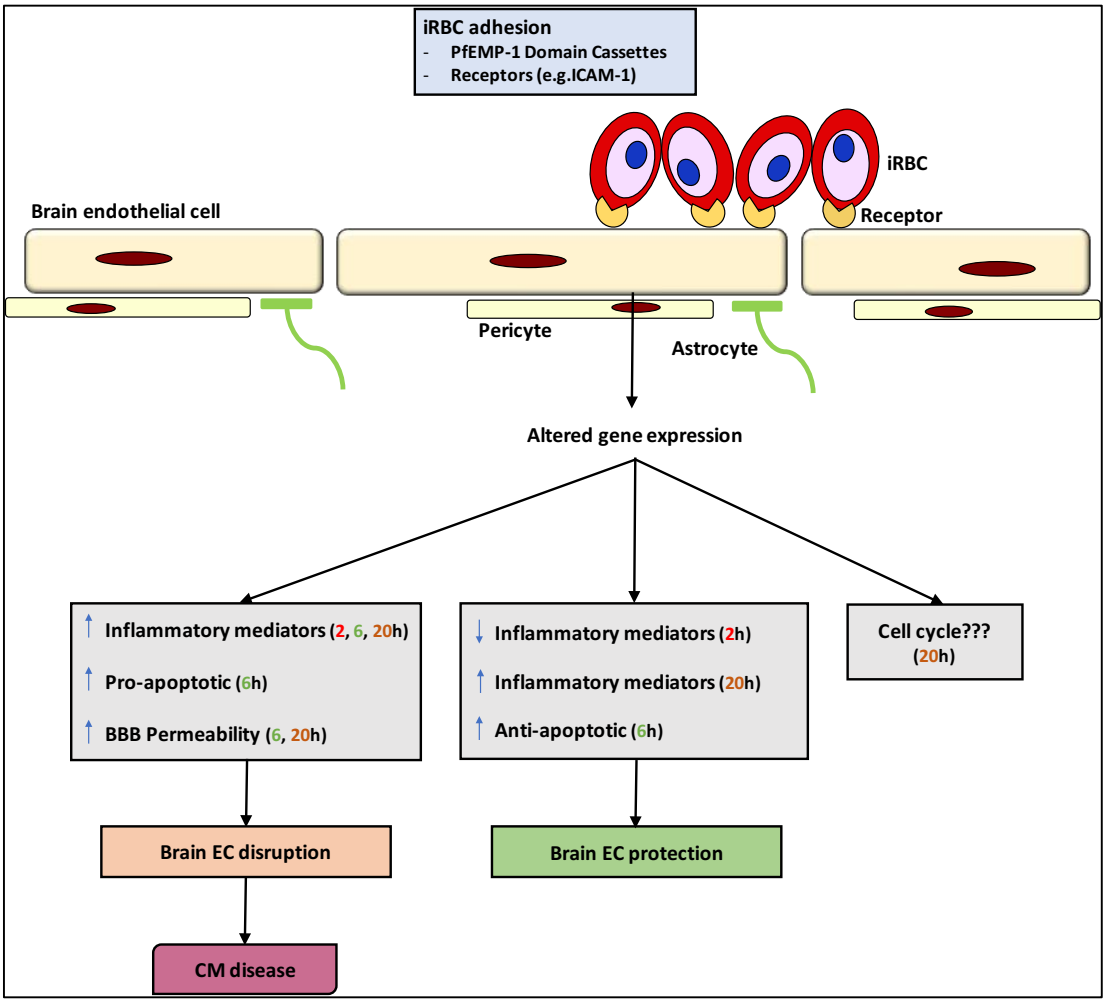


Figure 7.1: The proposed effect of iRBC cytoadhesion to modulate gene expression pathways in the brain EC.

In chapter 4, we tried to compare the transcriptional profile of HBMEC between two different *PfEMP-1* variants, IT4var14 (upsB) and IT4var37 (upsC). We found that exposure of brain endothelial cells to the IT4var37 parasite isolate in the presence of TNF can modulate different expression of genes in HBMEC, but these genes often expressed in opposite levels compared to IT4var14 interaction at 6 and 20 hours of co-culture. These findings may emphasize the ability of different parasite strains from the same family to cause different gene expressions in host cells and confirm also the ability of the parasite to protect itself from the host defensive system using *PfEMP-1 var* gene switching technique. Furthermore, these results may also show

the response of HBMEC to long and short *PfEMP-1* isolates (IT4var14 and IT4var37 isolates respectively).

In chapter 5, we used the RNA-seq technique to reveal the differences in the mRNA levels in HBMEC and HDMEC following the interaction with IT4var14 isolate in the presence of TNF stimulation. We demonstrated that the adhesion of iRBC led to modulate expression of several genes in the dermal endothelial cells, and there were variations in the expression of these genes when compared to HBMEC induced genes. These differences and similarities may be for several reasons; first: the nature of the endothelial cells such as, HDMEC express ICAM-1 and CD36 (McCormick et al., 1997) whereas HBMEC does not express CD36 (Wassmer et al., 2011). Second, the affinity of IT4var14 binding might play a role in these differences and similarities. The IT4var14 parasite can mediate binding to CD36 more than that with ICAM-1 (McCormick et al., 1997, Gray et al., 2003, Madkhali et al., 2014). Lastly, ICAM-1 and CD36 may work in a synergistic manner during iRBC binding (McCormick et al., 1997), thus they might mediate transcription of different sets of genes in HDMEC.

In the final chapter, we attempted to examine the ability of systemic TNF and uninfected erythrocytes to modulate gene expression in HBMEC during cytoadherence. To aid this work, first we co-cultured the brain endothelial cells to IT4var14 iRBC in the presence and absence of TNF and second incubated the HBMEC with uninfected RBC in the presence of TNF. We found that the inflammatory cytokine. It is possible that TNF activated HBMEC (Brown et al., 1999b) and then stimulated expression of the adhesion receptor ICAM-1 (Graninger et al., 1994, Turner et al., 1994, Berendt et al., 1989) to induce sequestration of iRBC on EC, thus leading to modulation of the downstream effects on mRNA expression

levels in brain EC. Exposure of HBMEC to IT4var14 parasite in the absence of TNF modulated different genes expression, especially when compared to transcriptional levels in TNF-stimulated HBMEC. These findings are in agreement with the published data of Chakravorty and colleagues (Chakravorty et al., 2007). On the other hand, we demonstrated that the transcriptional level of genes in brain EC incubated with both iRBC and uninfected erythrocyte in the present of TNF were nearly the same. We suggested that the main reason for these similarities in expression of genes with iRBC and uninfected erythrocytes co-culture models is probably that an infected erythrocyte contains many proteins that are found in an uninfected erythrocyte. Uninfected erythrocytes do not normally come into contact with EC, but with a malaria parasite inside them, they do.

7.2 Limitations and future perspectives:

Although this work generated data that contributed to the understanding of how the *P. falciparum* parasite can modulate the gene expressions of EC in response to the interaction in terms of understanding the mechanism of CM pathogenesis, there are limitations in the study which need to be considered in the future experiments. These include:

I. All parasite isolates that were used in this thesis were lab adapted. Although using patient strains in the lab may give more relevant results about the effect of the parasite on the EC in the co-culture model, it is sometimes difficult to culture and grow patient isolates in the lab, and it is not clear how long they retain their "patient" characteristics.

II. It has been suggested that ICAM-1 plays a key role in mediating sequestration of iRBC on EC, hence it can mediate the downstream effect on the human brain EC. To emphasize this role, it will be important to block the

ICAM-1 receptor on EC using ICAM-1 monoclonal antibody and examine the expression of genes following iRBC interaction.

III. Although we used the RNA-seq technique to assess the gene expression change in EC after co-culturing with iRBC at different time points, it was also interesting to understand how does the cytoadhesion alter the gene expressions in *P. falciparum* over time.

In future, we have several aims that should be achieved:

I. We depended in this thesis on understanding how the parasite can modulate the transcriptional levels in EC following cytoadhesion, but the need for experimental validation of these findings on the protein levels should be the next step to assure if these significant genes translate to proteins and perform their functions or not.

II. It is interesting to understand deeply how the IT4var37 parasite (CD36 binder) can modulate these huge number of significant changes in gene expression on HBMEC, despite this isolate not binding to ICAM-1. Furthermore, we should investigate more the functional pathways of these genes to distinguish between effect of the isolate that bind to ICAM-1 from other isolates that bind to other receptors.

III. It would be interesting to examine how the IT4var19 (EPCR binder) (Avril et al., 2012) can modulate the gene expression on HBMEC using co-culture system under different time points. This would be followed by pathway analysis to look at the gene functions and comparing the findings to our results in this thesis.

In brief, sequestration of the parasite may be not only the mediator of CM. There are many investigations have confirmed the role of inflammatory mediators in CM

infection. Nowadays, dysfunction of the vascular endothelium is thought to play an important role in CM pathogenesis. EC activation can be stimulated by the interaction of iRBC or the inflammatory response mediators. In December 2014, a comment was published in Nature that discussed the thought that microbiologists usually are paying their attention to the microbes' variables and often ignoring the host variables. It was stated that a microbe alone does not have the ability to cause the sickness without a host response. Many potential consequences result from contacts between the pathogen and the host to cause illness. It was thought that scientists should concentrate on the interactions between a pathogen and a host rather than the microbes alone (Casadevall and Pirofski, 2014).

REFERENCES

ABRAMS, E. T., BROWN, H., CHENSUE, S. W., TURNER, G. D., TADESSE, E., LEMA, V. M., MOLYNEUX, M. E., ROCHFORD, R., MESHNICK, S. R. & ROGERSON, S. J. 2003. Host response to malaria during pregnancy: placental monocyte recruitment is associated with elevated beta chemokine expression. *J Immunol,* 170**,** 2759-64.

ADAMS, S., BROWN, H. & TURNER, G. 2002. Breaking down the blood-brain barrier: signaling a path to cerebral malaria? *Trends Parasitol,* 18**,** 360-6.

AIRD, W. C., MOSNIER, L. O. & FAIRHURST, R. M. 2014. Plasmodium falciparum picks (on) EPCR. *Blood,* 123**,** 163-7.

ALEY, S. B., SHERWOOD, J. A., MARSH, K., EIDELMAN, O. & HOWARD, R. J. 1986. Identification of isolate-specific proteins on sorbitol-enriched Plasmodium falciparum infected erythrocytes from Gambian patients. *Parasitology,* 92 (Pt 3)**,** 511-25.

ALMELLI, T., NUEL, G., BISCHOFF, E., AUBOUY, A., ELATI, M., WANG, C. W., DILLIES, M. A., COPPEE, J. Y., AYISSI, G. N., BASCO, L. K., ROGIER, C., NDAM, N. T., DELORON, P. & TAHAR, R. 2014. Differences in gene transcriptomic pattern of Plasmodium falciparum in children with cerebral malaria and asymptomatic carriers. *PLoS One,* 9**,** e114401.

APARICIO-SIEGMUND, S. & GARBERS, C. 2015. The biology of interleukin-27 reveals unique pro- and anti-inflammatory functions in immunity. *Cytokine Growth Factor Rev,* 26**,** 579-86.

ARMAH, H., DODOO, A. K., WIREDU, E. K., STILES, J. K., ADJEI, A. A., GYASI, R. K. & TETTEY, Y. 2005a. High-level cerebellar expression of cytokines and adhesion molecules in fatal, paediatric, cerebral malaria. *Ann Trop Med Parasitol,* 99**,** 629-47.

ARMAH, H., WIRED, E. K., DODOO, A. K., ADJEI, A. A., TETTEY, Y. & GYASI, R. 2005b. Cytokines and adhesion molecules expression in the brain in human cerebral malaria. *Int J Environ Res Public Health,* 2**,** 123-31.

ASAGIRI, M., HIRAI, T., KUNIGAMI, T., KAMANO, S., GOBER, H. J., OKAMOTO, K., NISHIKAWA, K., LATZ, E., GOLENBOCK, D. T., AOKI, K., OHYA, K., IMAI, Y., MORISHITA, Y., MIYAZONO, K., KATO, S., SAFTIG, P. & TAKAYANAGI, H. 2008. Cathepsin K-dependent toll-like receptor 9 signaling revealed in experimental arthritis. *Science,* 319**,** 624-7.

AVRIL, M., GAMAIN, B., LEPOLARD, C., VIAUD, N., SCHERF, A. & GYSIN, J. 2006. Characterization of anti-var2CSA-PfEMP1 cytoadhesion inhibitory mouse monoclonal antibodies. *Microbes Infect,* 8**,** 2863-71.

AVRIL, M., TRIPATHI, A. K., BRAZIER, A. J., ANDISI, C., JANES, J. H., SOMA, V. L., SULLIVAN, D. J., JR., BULL, P. C., STINS, M. F. & SMITH, J. D. 2012. A restricted subset of var genes mediates adherence of Plasmodium falciparum-infected erythrocytes to brain endothelial cells. *Proc Natl Acad Sci U S A,* 109**,** E1782-90.

BAER, K., KLOTZ, C., KAPPE, S. H., SCHNIEDER, T. & FREVERT, U. 2007. Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. *PLoS Pathog,* 3**,** e171.

BAGGIOLINI, M., WALZ, A. & KUNKEL, S. L. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J Clin Invest,* 84**,** 1045-9.

BALDWIN, A. S. 2012. Regulation of cell death and autophagy by IKK and NF-kappaB: critical mechanisms in immune function and cancer. *Immunol Rev,* 246**,** 327-45.

BANG, Y. J., KWON, J. H., KANG, S. H., KIM, J. W. & YANG, Y. C. 1998. Increased MAPK activity and MKP-1 overexpression in human gastric adenocarcinoma. *Biochem Biophys Res Commun,* 250**,** 43-7.

BARBAZUK, W. B., EMRICH, S. J., CHEN, H. D., LI, L. & SCHNABLE, P. S. 2007. SNP discovery via 454 transcriptome sequencing. *Plant J,* 51**,** 910-8.

BARBIER, M., FAILLE, D., LORIOD, B., TEXTORIS, J., CAMUS, C., PUTHIER, D., FLORI, L., WASSMER, S. C., VICTORERO, G., ALESSI, M. C., FUSAI, T.,

NGUYEN, C., GRAU, G. E. & RIHET, P. 2011. Platelets alter gene expression profile in human brain endothelial cells in an in vitro model of cerebral malaria. *PLoS One,* 6**,** e19651.

BARNWELL, J. W., ASCH, A. S., NACHMAN, R. L., YAMAYA, M., AIKAWA, M. & INGRAVALLO, P. 1989. A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on Plasmodium falciparum-infected erythrocytes. *J Clin Invest,* 84**,** 765-72.

BARUCH, D. I., MA, X. C., SINGH, H. B., BI, X., PASLOSKE, B. L. & HOWARD, R. J. 1997. Identification of a region of PfEMP1 that mediates adherence of Plasmodium falciparum infected erythrocytes to CD36: conserved function with variant sequence. *Blood,* 90**,** 3766-75.

BAUD, V. & JACQUE, E. 2008. [The alternative NF-kB activation pathway and cancer: friend or foe?]. *Med Sci (Paris),* 24**,** 1083-8.

BEARE, N. A., SOUTHERN, C., CHALIRA, C., TAYLOR, T. E., MOLYNEUX, M. E. & HARDING, S. P. 2004. Prognostic significance and course of retinopathy in children with severe malaria. *Arch Ophthalmol,* 122**,** 1141-7.

BEESON, J. G., DREW, D. R., BOYLE, M. J., FENG, G., FOWKES, F. J. & RICHARDS, J. S. 2016. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS Microbiol Rev,* 40**,** 343-72.

BEKLEN, A., HUKKANEN, M., RICHARDSON, R. & KONTTINEN, Y. T. 2008. Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol Immunol,* 23**,** 425-31.

BERENDT, A. R., SIMMONS, D. L., TANSEY, J., NEWBOLD, C. I. & MARSH, K. 1989. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature,* 341**,** 57-9.

BERGER, S. S., TURNER, L., WANG, C. W., PETERSEN, J. E., KRAFT, M., LUSINGU, J. P., MMBANDO, B., MARQUARD, A. M., BENGTSSON, D. B., HVIID, L., NIELSEN, M. A., THEANDER, T. G. & LAVSTSEN, T. 2013. Plasmodium falciparum expressing domain cassette 5 type PfEMP1 (DC5-PfEMP1) bind PECAM1. *PLoS One,* 8**,** e69117.

BETTSCHEIDER, M., MURGATROYD, C. & SPENGLER, D. 2011. Simultaneous DNA and RNA isolation from brain punches for epigenetics. *BMC Res Notes,* 4**,** 314.

BHATT, S., WEISS, D. J., CAMERON, E., BISANZIO, D., MAPPIN, B., DALRYMPLE, U., BATTLE, K., MOYES, C. L., HENRY, A., ECKHOFF, P. A., WENGER, E. A., BRIET, O., PENNY, M. A., SMITH, T. A., BENNETT, A., YUKICH, J., EISELE, T. P., GRIFFIN, J. T., FERGUS, C. A., LYNCH, M., LINDGREN, F., COHEN, J. M., MURRAY, C. L. J., SMITH, D. L., HAY, S. I., CIBULSKIS, R. E. & GETHING, P. W. 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature,* 526**,** 207-211.

BICKFORD, J. S., BEACHY, D. E., NEWSOM, K. J., BARILOVITS, S. J., HERLIHY, J. D., QIU, X., WALTERS, J. N., LI, N. & NICK, H. S. 2013. A distal enhancer controls cytokine-dependent human cPLA2alpha gene expression. *J Lipid Res,* 54**,** 1915-26.

BIR, N., YAZDANI, S. S., AVRIL, M., LAYEZ, C., GYSIN, J. & CHITNIS, C. E. 2006. Immunogenicity of Duffy binding-like domains that bind chondroitin sulfate A and protection against pregnancy-associated malaria. *Infect Immun,* 74**,** 5955-63.

BOZON, B., DAVIS, S. & LAROCHE, S. 2002. Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus,* 12**,** 570-7.

BROWN, G. C. 2001. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta,* 1504**,** 46-57.

BROWN, H., HIEN, T. T., DAY, N., MAI, N. T., CHUONG, L. V., CHAU, T. T., LOC, P. P., PHU, N. H., BETHELL, D., FARRAR, J., GATTER, K., WHITE, N. & TURNER, G. 1999a. Evidence of blood-brain barrier dysfunction in human cerebral malaria. *Neuropathol Appl Neurobiol,* 25**,** 331-40.

BROWN, H., ROGERSON, S., TAYLOR, T., TEMBO, M., MWENECHANYA, J., MOLYNEUX, M. & TURNER, G. 2001a. Blood-brain barrier function in cerebral malaria in Malawian children. *Am J Trop Med Hyg,* 64**,** 207-13.

BROWN, H., TURNER, G., ROGERSON, S., TEMBO, M., MWENECHANYA, J., MOLYNEUX, M. & TAYLOR, T. 1999b. Cytokine expression in the brain in human cerebral malaria. *J Infect Dis,* 180**,** 1742-6.

BROWN, M. D., WICK, T. M. & ECKMAN, J. R. 2001b. Activation of vascular endothelial cell adhesion molecule expression by sickle blood cells. *Pediatr Pathol Mol Med,* 20**,** 47-72.

BRUCE-CHWATT, L. J. 1987. Malaria and its control: present situation and future prospects. *Annu Rev Public Health,* 8**,** 75-110.

BUCKEE, C. O. & RECKER, M. 2012. Evolution of the multi-domain structures of virulence genes in the human malaria parasite, Plasmodium falciparum. *PLoS Comput Biol,* 8**,** e1002451.

BUFFET, P. A., GAMAIN, B., SCHEIDIG, C., BARUCH, D., SMITH, J. D., HERNANDEZ-RIVAS, R., POUVELLE, B., OISHI, S., FUJII, N., FUSAI, T., PARZY, D., MILLER, L. H., GYSIN, J. & SCHERF, A. 1999. Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci U S A,* 96**,** 12743-8.

BUFFET, P. A., SAFEUKUI, I., DEPLAINE, G., BROUSSE, V., PRENDKI, V., THELLIER, M., TURNER, G. D. & MERCEREAU-PUIJALON, O. 2011. The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology. *Blood,* 117**,** 381-92.

BURGMANN, H., HOLLENSTEIN, U., WENISCH, C., THALHAMMER, F., LOOAREESUWAN, S. & GRANINGER, W. 1995. Serum concentrations of MIP-1 alpha and interleukin-8 in patients suffering from acute Plasmodium falciparum malaria. *Clin Immunol Immunopathol,* 76**,** 32-6.

CABRERA, A., NECULAI, D. & KAIN, K. C. 2014. CD36 and malaria: friends or foes? A decade of data provides some answers. *Trends Parasitol,* 30**,** 436-44.

CALIS, J. C., PHIRI, K. S., FARAGHER, E. B., BRABIN, B. J., BATES, I., CUEVAS, L. E., DE HAAN, R. J., PHIRI, A. I., MALANGE, P., KHOKA, M., HULSHOF, P. J., VAN LIESHOUT, L., BELD, M. G., TEO, Y. Y., ROCKETT, K. A., RICHARDSON, A., KWIATKOWSKI, D. P., MOLYNEUX, M. E. & VAN HENSBROEK, M. B. 2008. Severe anemia in Malawian children. *N Engl J Med,* 358**,** 888-99.

CANNON, B., SHABALINA, I. G., KRAMAROVA, T. V., PETROVIC, N. & NEDERGAARD, J. 2006. Uncoupling proteins: a role in protection against reactive oxygen species--or not? *Biochim Biophys Acta,* 1757**,** 449-58.

CASADEVALL, A. & PIROFSKI, L. A. 2014. Microbiology: Ditch the term pathogen. *Nature,* 516**,** 165-6.

CASNEUF, T., VAN DE PEER, Y. & HUBER, W. 2007. In situ analysis of cross-hybridisation on microarrays and the inference of expression correlation. *BMC Bioinformatics,* 8**,** 461.

CHAKRAVORTY, S. J., CARRET, C., NASH, G. B., IVENS, A., SZESTAK, T. & CRAIG, A. G. 2007. Altered phenotype and gene transcription in endothelial cells, induced by Plasmodium falciparum-infected red blood cells: pathogenic or protective? *Int J Parasitol,* 37**,** 975-87.

CHAKRAVORTY, S. J., HUGHES, K. R. & CRAIG, A. G. 2008. Host response to cytoadherence in Plasmodium falciparum. *Biochem Soc Trans,* 36**,** 221-8.

CHAMBERS, R. G. 2012. UN Envoy's response to estimates of global malaria mortality. *Lancet,* 379**,** 707-8.

CHEN, F., ZHAO, Q., WANG, S., WANG, H. & LI, X. 2016. Upregulation of Id3 inhibits cell proliferation and induces apoptosis in A549/DDP human lung cancer cells in vitro. *Mol Med Rep,* 14**,** 313-8.

CHEN, F. F., LIU, Y., WANG, F., PANG, X. J., ZHU, C. D., XU, M., YU, W. & LI, X. J. 2015. Effects of upregulation of Id3 in human lung adenocarcinoma cells on proliferation, apoptosis, mobility and tumorigenicity. *Cancer Gene Ther,* 22**,** 431-7.

CHEN, W., YANG, S., ABE, Y., LI, M., WANG, Y., SHAO, J., LI, E. & LI, Y. P. 2007. Novel pycnodysostosis mouse model uncovers cathepsin K function as a potential regulator of osteoclast apoptosis and senescence. *Hum Mol Genet,* 16**,** 410-23.

CHO, H. J., PARK, S. M., HWANG, E. M., BAEK, K. E., KIM, I. K., NAM, I. K., IM, M. J., PARK, S. H., BAE, S., PARK, J. Y. & YOO, J. 2010. Gadd45b mediates Fas-induced apoptosis by enhancing the interaction between p38 and retinoblastoma tumor suppressor. *J Biol Chem,* 285**,** 25500-5.

CLAESSENS, A., ADAMS, Y., GHUMRA, A., LINDERGARD, G., BUCHAN, C. C., ANDISI, C., BULL, P. C., MOK, S., GUPTA, A. P., WANG, C. W., TURNER, L., ARMAN, M., RAZA, A., BOZDECH, Z. & ROWE, J. A. 2012. A subset of group A-like var genes encodes the malaria parasite ligands for binding to human brain endothelial cells. *Proc Natl Acad Sci U S A,* 109**,** E1772-81.

CLARK, I. A., AWBURN, M. M., HARPER, C. G., LIOMBA, N. G. & MOLYNEUX, M. E. 2003. Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. *Malar J,* 2**,** 41.

CLARK, I. A., BUDD, A. C., ALLEVA, L. M. & COWDEN, W. B. 2006. Human malarial disease: a consequence of inflammatory cytokine release. *Malar J,* 5**,** 85.

CLAYTON, A., EVANS, R. A., PETTIT, E., HALLETT, M., WILLIAMS, J. D. & STEADMAN, R. 1998. Cellular activation through the ligation of intercellular adhesion molecule-1. *J Cell Sci,* 111 (Pt 4)**,** 443-53.

COLTEL, N., COMBES, V., WASSMER, S. C., CHIMINI, G. & GRAU, G. E. 2006. Cell vesiculation and immunopathology: implications in cerebral malaria. *Microbes Infect,* 8**,** 2305-16.

CONROY, A. L., PHIRI, H., HAWKES, M., GLOVER, S., MALLEWA, M., SEYDEL, K. B., TAYLOR, T. E., MOLYNEUX, M. E. & KAIN, K. C. 2010. Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. *PLoS One,* 5**,** e15291.

COOKE, B., COPPEL, R. & WAHLGREN, M. 2000. Falciparum malaria: sticking up, standing out and out-standing. *Parasitol Today,* 16**,** 416-20.

COOKE, B. M., BERENDT, A. R., CRAIG, A. G., MACGREGOR, J., NEWBOLD, C. I. & NASH, G. B. 1994. Rolling and stationary cytoadhesion of red blood cells parasitized by Plasmodium falciparum: separate roles for ICAM-1, CD36 and thrombospondin. *Br J Haematol,* 87**,** 162-70.

COWMAN, A. F., BERRY, D. & BAUM, J. 2012. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol,* 198**,** 961-71.

COX, F. E. 2010. History of the discovery of the malaria parasites and their vectors. *Parasit Vectors,* 3**,** 5.

CRAMER, J. P., NUSSLER, A. K., EHRHARDT, S., BURKHARDT, J., OTCHWEMAH, R. N., ZANGER, P., DIETZ, E., GELLERT, S., BIENZLE, U. & MOCKENHAUPT, F. P. 2005. Age-dependent effect of plasma nitric oxide on parasite density in Ghanaian children with severe malaria. *Trop Med Int Health,* 10**,** 672-80.

DALVI, S., NGUYEN, H. H., ON, N., MITCHELL, R. W., AUKEMA, H. M., MILLER, D. W. & HATCH, G. M. 2015. Exogenous arachidonic acid mediates permeability of human brain microvessel endothelial cells through prostaglandin E2 activation of EP3 and EP4 receptors. *J Neurochem,* 135**,** 867-79.

DE GROOT, C. J. & WOODROOFE, M. N. 2001. The role of chemokines and chemokine receptors in CNS inflammation. *Prog Brain Res,* 132**,** 533-44.

DEITSCH, K. W., LUKEHART, S. A. & STRINGER, J. R. 2009. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. *Nat Rev Microbiol,* 7**,** 493-503.

DELVES, M., PLOUFFE, D., SCHEURER, C., MEISTER, S., WITTLIN, S., WINZELER, E. A., SINDEN, R. E. & LEROY, D. 2012. The activities of current antimalarial drugs on the life cycle stages of Plasmodium: a comparative study with human and rodent parasites. *PLoS Med,* 9**,** e1001169.

DODDS, R. A., JAMES, I. E., RIEMAN, D., AHERN, R., HWANG, S. M., CONNOR, J. R., THOMPSON, S. D., VEBER, D. F., DRAKE, F. H., HOLMES, S., LARK, M. W. & GOWEN, M. 2001. Human osteoclast cathepsin K is processed intracellularly prior to attachment and bone resorption. *J Bone Miner Res,* 16**,** 478-86.

DONDORP, A., NOSTEN, F., STEPNIEWSKA, K., DAY, N., WHITE, N. & SOUTH EAST ASIAN QUININE ARTESUNATE MALARIA TRIAL, G. 2005. Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. *Lancet,* 366**,** 717-25.

DONDORP, A. M., FANELLO, C. I., HENDRIKSEN, I. C., GOMES, E., SENI, A., CHHAGANLAL, K. D., BOJANG, K., OLAOSEBIKAN, R., ANUNOBI, N., MAITLAND, K., KIVAYA, E., AGBENYEGA, T., NGUAH, S. B., EVANS, J., GESASE, S., KAHABUKA, C., MTOVE, G., NADJM, B., DEEN, J., MWANGA-AMUMPAIRE, J., NANSUMBA, M., KAREMA, C., UMULISA, N., UWIMANA, A., MOKUOLU, O. A., ADEDOYIN, O. T., JOHNSON, W. B., TSHEFU, A. K., ONYAMBOKO, M. A., SAKULTHAEW, T., NGUM, W. P., SILAMUT, K., STEPNIEWSKA, K., WOODROW, C. J., BETHELL, D., WILLS, B., ONEKO, M., PETO, T. E., VON SEIDLEIN, L., DAY, N. P., WHITE, N. J. & GROUP, A. 2010. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet,* 376**,** 1647-57.

DONDORP, A. M., SILAMUT, K., CHARUNWATTHANA, P., CHUASUWANCHAI, S., RUANGVEERAYUT, R., KRINTRATUN, S., WHITE, N. J., HO, M. & DAY, N. P. 2007. Levamisole inhibits sequestration of infected red blood cells in patients with falciparum malaria. *J Infect Dis,* 196**,** 460-6.

DONG, C., WANG, G., LI, B., XIAO, K., MA, Z., HUANG, H., WANG, X. & BAI, C. 2012. Anti-asthmatic agents alleviate pulmonary edema by upregulating AQP1 and AQP5 expression in the lungs of mice with OVA-induced asthma. *Respir Physiol Neurobiol,* 181**,** 21-8.

DOROVINI-ZIS, K., SCHMIDT, K., HUYNH, H., FU, W., WHITTEN, R. O., MILNER, D., KAMIZA, S., MOLYNEUX, M. & TAYLOR, T. E. 2011. The neuropathology of fatal cerebral malaria in malawian children. *Am J Pathol,* 178**,** 2146-58.

DREXLER, H. G. & UPHOFF, C. C. 2002. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. *Cytotechnology,* 39**,** 75-90.

DUFFY, P. E. & FRIED, M. 2003. Antibodies that inhibit Plasmodium falciparum adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. *Infect Immun,* 71**,** 6620-3.

DUPERRAY, A., LANGUINO, L. R., PLESCIA, J., MCDOWALL, A., HOGG, N., CRAIG, A. G., BERENDT, A. R. & ALTIERI, D. C. 1997. Molecular identification of a novel fibrinogen binding site on the first domain of ICAM-1 regulating leukocyte-endothelium bridging. *J Biol Chem,* 272**,** 435-41.

EDA, S. & SHERMAN, I. W. 2004. Plasmodium falciparum-infected erythrocytes bind to the RGD motif of fibronectin via the band 3-related adhesin. *Exp Parasitol,* 107**,** 157-62.

EISEN, M. B., SPELLMAN, P. T., BROWN, P. O. & BOTSTEIN, D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A,* 95**,** 14863-8.

EMERICH, D. F., DEAN, R. L., 3RD & BARTUS, R. T. 2002. The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct? *Exp Neurol,* 173**,** 168-81.

ENGELMANN, A., SPEIDEL, D., BORNKAMM, G. W., DEPPERT, W. & STOCKING, C. 2008. Gadd45 beta is a pro-survival factor associated with stress-resistant tumors. *Oncogene,* 27**,** 1429-38.

ENGLISH, M., SAUERWEIN, R., WARUIRU, C., MOSOBO, M., OBIERO, J., LOWE, B. & MARSH, K. 1997. Acidosis in severe childhood malaria. *QJM,* 90**,** 263-70.

ENGLISH, M., WALE, S., BINNS, G., MWANGI, I., SAUERWEIN, H. & MARSH, K. 1998. Hypoglycaemia on and after admission in Kenyan children with severe malaria. *QJM,* 91**,** 191-7.

ENGLISH, M. C., WARUIRU, C., LIGHTOWLER, C., MURPHY, S. A., KIRIGHA, G. & MARSH, K. 1996. Hyponatraemia and dehydration in severe malaria. *Arch Dis Child,* 74**,** 201-5.

ESSONE, J., N'DILIMABAKA, N., ONDZAGA, J., LEKANA-DOUKI, J. B., MBA, D. N., DELORON, P., MAZIER, D., GAY, F. & TOURE NDOUO, F. S. 2017. Comparison of apoptosis in human primary pulmonary endothelial cells and a brain microvascular endothelial cell line co-cultured with Plasmodium falciparum field isolates. *BMC Infect Dis,* 17**,** 454.

ESTEVA-FONT, C., JIN, B. J. & VERKMAN, A. S. 2014. Aquaporin-1 gene deletion reduces breast tumor growth and lung metastasis in tumor-producing MMTV-PyVT mice. *FASEB J,* 28**,** 1446-53.

ETIENNE, S., ADAMSON, P., GREENWOOD, J., STROSBERG, A. D., CAZAUBON, S. & COURAUD, P. O. 1998. ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells. *J Immunol,* 161**,** 5755-61.

EYLER, C. E. & TELEN, M. J. 2006. The Lutheran glycoprotein: a multifunctional adhesion receptor. *Transfusion,* 46**,** 668-77.

FABREGAT, A., SIDIROPOULOS, K., GARAPATI, P., GILLESPIE, M., HAUSMANN, K., HAW, R., JASSAL, B., JUPE, S., KORNINGER, F., MCKAY, S., MATTHEWS, L., MAY, B., MILACIC, M., ROTHFELS, K., SHAMOVSKY, V., WEBBER, M., WEISER, J., WILLIAMS, M., WU, G., STEIN, L., HERMJAKOB, H. & D'EUSTACHIO, P. 2016. The Reactome pathway Knowledgebase. *Nucleic Acids Res,* 44**,** D481-7.

FAIRHURST, R. M. 2015. Understanding artemisinin-resistant malaria: what a difference a year makes. *Curr Opin Infect Dis,* 28**,** 417-25.

FEBBRAIO, M., HAJJAR, D. P. & SILVERSTEIN, R. L. 2001. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest,* 108**,** 785-91.

FELTY, Q. & PORTHER, N. 2008. Estrogen-induced redox sensitive Id3 signaling controls the growth of vascular cells. *Atherosclerosis,* 198**,** 12-21.

FINK, K., BUSCH, H. J., BOURGEOIS, N., SCHWARZ, M., WOLF, D., ZIRLIK, A., PETER, K., BODE, C. & VON ZUR MUHLEN, C. 2013. Mac-1 directly binds to the endothelial protein C-receptor: a link between the protein C anticoagulant pathway and inflammation? *PLoS One,* 8**,** e53103.

FRANK, M. & DEITSCH, K. 2006. Activation, silencing and mutually exclusive expression within the var gene family of Plasmodium falciparum. *Int J Parasitol,* 36**,** 975-85.

GALLEGO-DELGADO, J., BASU-ROY, U., TY, M., ALIQUE, M., FERNANDEZ-ARIAS, C., MOVILA, A., GOMES, P., WEINSTOCK, A., XU, W., EDAGHA, I., WASSMER, S. C., WALTHER, T., RUIZ-ORTEGA, M. & RODRIGUEZ, A. 2016. Angiotensin receptors and beta-catenin regulate brain endothelial integrity in malaria. *J Clin Invest,* 126**,** 4016-4029.

GAO, H., GUI, J., WANG, L., XU, Y., JIANG, Y., XIONG, M. & CUI, Y. 2016. Aquaporin 1 contributes to chondrocyte apoptosis in a rat model of osteoarthritis. *Int J Mol Med,* 38**,** 1752-1758.

GARCIA, F., CEBRIAN, M., DGEDGE, M., CASADEMONT, J., BEDINI, J. L., NEVES, O., FILELLA, X., CINTA CID, M., CORACHAN, M. & GRAU, J. M. 1999. Endothelial cell activation in muscle biopsy samples is related to clinical severity in human cerebral malaria. *J Infect Dis,* 179**,** 475-83.

GARDNER, J. P., PINCHES, R. A., ROBERTS, D. J. & NEWBOLD, C. I. 1996. Variant antigens and endothelial receptor adhesion in Plasmodium falciparum. *Proc Natl Acad Sci U S A,* 93**,** 3503-8.

GARDNER, M. J., HALL, N., FUNG, E., WHITE, O., BERRIMAN, M., HYMAN, R. W., CARLTON, J. M., PAIN, A., NELSON, K. E., BOWMAN, S., PAULSEN, I. T., JAMES, K., EISEN, J. A., RUTHERFORD, K., SALZBERG, S. L., CRAIG, A., KYES, S., CHAN, M. S., NENE, V., SHALLOM, S. J., SUH, B., PETERSON, J., ANGIUOLI, S., PERTEA, M., ALLEN, J., SELENGUT, J., HAFT, D., MATHER, M. W., VAIDYA, A. B., MARTIN, D. M., FAIRLAMB, A. H., FRAUNHOLZ, M. J., ROOS, D. S., RALPH, S. A., MCFADDEN, G. I., CUMMINGS, L. M., SUBRAMANIAN, G. M., MUNGALL, C., VENTER, J. C., CARUCCI, D. J., HOFFMAN, S. L., NEWBOLD, C., DAVIS, R. W., FRASER, C. M. & BARRELL, B. 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature,* 419**,** 498-511.

GERONDAKIS, S. & SIEBENLIST, U. 2010. Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb Perspect Biol,* 2**,** a000182.

GERSTEN, M., ALIREZAEI, M., MARCONDES, M. C., FLYNN, C., RAVASI, T., IDEKER, T. & FOX, H. S. 2009. An integrated systems analysis implicates EGR1 downregulation in simian immunodeficiency virus encephalitis-induced neural dysfunction. *J Neurosci,* 29**,** 12467-76.

GIL-ARAUJO, B., TOLEDO LOBO, M. V., GUTIERREZ-SALMERON, M., GUTIERREZ-PITALUA, J., ROPERO, S., ANGULO, J. C., CHILOECHES, A. & LASA, M. 2014. Dual specificity phosphatase 1 expression inversely correlates with NF-kappaB activity and expression in prostate cancer and promotes apoptosis through a p38 MAPK dependent mechanism. *Mol Oncol,* 8**,** 27-38.

GILLRIE, M. R., KRISHNEGOWDA, G., LEE, K., BURET, A. G., ROBBINS, S. M., LOOAREESUWAN, S., GOWDA, D. C. & HO, M. 2007. Src-family kinase dependent disruption of endothelial barrier function by Plasmodium falciparum merozoite proteins. *Blood,* 110**,** 3426-35.

GLABINSKI, A. R., BALASINGAM, V., TANI, M., KUNKEL, S. L., STRIETER, R. M., YONG, V. W. & RANSOHOFF, R. M. 1996. Chemokine monocyte chemoattractant protein-1 is expressed by astrocytes after mechanical injury to the brain. *J Immunol,* 156**,** 4363-8.

GOEL, S., MUTHUSAMY, A., MIAO, J., CUI, L., SALANTI, A., WINZELER, E. A. & GOWDA, D. C. 2014. Targeted disruption of a ring-infected erythrocyte surface antigen (RESA)-like export protein gene in Plasmodium falciparum confers stable chondroitin 4-sulfate cytoadherence capacity. *J Biol Chem,* 289**,** 34408-21.

GRANINGER, W., PRADA, J., NEIFER, S., ZOTTER, G., THALHAMMER, F. & KREMSNER, P. G. 1994. Upregulation of ICAM-I by Plasmodium falciparum: in vitro and in vivo studies. *J Clin Pathol,* 47**,** 653-6.

GRAU, G. E., MACKENZIE, C. D., CARR, R. A., REDARD, M., PIZZOLATO, G., ALLASIA, C., CATALDO, C., TAYLOR, T. E. & MOLYNEUX, M. E. 2003. Platelet accumulation in brain microvessels in fatal pediatric cerebral malaria. *J Infect Dis,* 187**,** 461-6.

GRAU, G. E., TAYLOR, T. E., MOLYNEUX, M. E., WIRIMA, J. J., VASSALLI, P., HOMMEL, M. & LAMBERT, P. H. 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. *N Engl J Med,* 320**,** 1586-91.

GRAY, C., MCCORMICK, C., TURNER, G. & CRAIG, A. 2003. ICAM-1 can play a major role in mediating P. falciparum adhesion to endothelium under flow. *Mol Biochem Parasitol,* 128**,** 187-93.

GREENWALT, D. E., LIPSKY, R. H., OCKENHOUSE, C. F., IKEDA, H., TANDON, N. N. & JAMIESON, G. A. 1992. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood,* 80**,** 1105-15.

GREENWOOD, J., AMOS, C. L., WALTERS, C. E., COURAUD, P. O., LYCK, R., ENGELHARDT, B. & ADAMSON, P. 2003. Intracellular domain of brain

endothelial intercellular adhesion molecule-1 is essential for T lymphocyte-mediated signaling and migration. *J Immunol,* 171**,** 2099-108.

GREVE, J. M., DAVIS, G., MEYER, A. M., FORTE, C. P., YOST, S. C., MARLOR, C. W., KAMARCK, M. E. & MCCLELLAND, A. 1989. The major human rhinovirus receptor is ICAM-1. *Cell,* 56**,** 839-47.

GUERIN, P. J., OLLIARO, P., NOSTEN, F., DRUILHE, P., LAXMINARAYAN, R., BINKA, F., KILAMA, W. L., FORD, N. & WHITE, N. J. 2002. Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *Lancet Infect Dis,* 2**,** 564-73.

HANISCH, U. K. 2002. Microglia as a source and target of cytokines. *Glia,* 40**,** 140-55.

HANSEN, F. K., SUMANADASA, S. D., STENZEL, K., DUFFY, S., MEISTER, S., MAREK, L., SCHMETTER, R., KUNA, K., HAMACHER, A., MORDMULLER, B., KASSACK, M. U., WINZELER, E. A., AVERY, V. M., ANDREWS, K. T. & KURZ, T. 2014. Discovery of HDAC inhibitors with potent activity against multiple malaria parasite life cycle stages. *Eur J Med Chem,* 82**,** 204-13.

HANSEN, K. D., BRENNER, S. E. & DUDOIT, S. 2010. Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Res,* 38**,** e131.

HAO, L., LIAO, Q., TANG, Q., DENG, H. & CHEN, L. 2016. Id-1 promotes osteosarcoma cell growth and inhibits cell apoptosis via PI3K/AKT signaling pathway. *Biochem Biophys Res Commun,* 470**,** 643-9.

HARKNESS, K. A., SUSSMAN, J. D., DAVIES-JONES, G. A., GREENWOOD, J. & WOODROOFE, M. N. 2003. Cytokine regulation of MCP-1 expression in brain and retinal microvascular endothelial cells. *J Neuroimmunol,* 142**,** 1-9.

HEAP, G. A., YANG, J. H., DOWNES, K., HEALY, B. C., HUNT, K. A., BOCKETT, N., FRANKE, L., DUBOIS, P. C., MEIN, C. A., DOBSON, R. J., ALBERT, T. J., RODESCH, M. J., CLAYTON, D. G., TODD, J. A., VAN HEEL, D. A. & PLAGNOL, V. 2010. Genome-wide analysis of allelic expression imbalance in human primary cells by high-throughput transcriptome resequencing. *Hum Mol Genet,* 19**,** 122-34.

HEDDINI, A., PETTERSSON, F., KAI, O., SHAFI, J., OBIERO, J., CHEN, Q., BARRAGAN, A., WAHLGREN, M. & MARSH, K. 2001. Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. *Infect Immun,* 69**,** 5849-56.

HEINHUIS, B., NETEA, M. G., VAN DEN BERG, W. B., DINARELLO, C. A. & JOOSTEN, L. A. 2012. Interleukin-32: a predominantly intracellular proinflammatory mediator that controls cell activation and cell death. *Cytokine,* 60**,** 321-7.

HEMINGWAY, J., SHRETTA, R., WELLS, T. N., BELL, D., DJIMDE, A. A., ACHEE, N. & QI, G. 2016. Tools and Strategies for Malaria Control and Elimination: What Do We Need to Achieve a Grand Convergence in Malaria? *PLoS Biol,* 14**,** e1002380.

HEMMER, C. J., LEHR, H. A., WESTPHAL, K., UNVERRICHT, M., KRATZIUS, M. & REISINGER, E. C. 2005. Plasmodium falciparum Malaria: reduction of endothelial cell apoptosis in vitro. *Infect Immun,* 73**,** 1764-70.

HO, M., HICKEY, M. J., MURRAY, A. G., ANDONEGUI, G. & KUBES, P. 2000. Visualization of Plasmodium falciparum-endothelium interactions in human microvasculature: mimicry of leukocyte recruitment. *J Exp Med,* 192**,** 1205-11.

HO, M., HOANG, H. L., LEE, K. M., LIU, N., MACRAE, T., MONTES, L., FLATT, C. L., YIPP, B. G., BERGER, B. J., LOOAREESUWAN, S. & ROBBINS, S. M. 2005. Ectophosphorylation of CD36 regulates cytoadherence of Plasmodium falciparum to microvascular endothelium under flow conditions. *Infect Immun,* 73**,** 8179-87.

HOPFE, M., DEENEN, R., DEGRANDI, D., KOHRER, K. & HENRICH, B. 2013. Host cell responses to persistent mycoplasmas--different stages in infection of HeLa cells with Mycoplasma hominis. *PLoS One,* 8**,** e54219.

HORA, R., KAPOOR, P., THIND, K. K. & MISHRA, P. C. 2016. Cerebral malaria--clinical manifestations and pathogenesis. *Metab Brain Dis,* 31**,** 225-37.

HORROCKS, P., PINCHES, R., KYES, S., KRIEK, N., LEE, S., CHRISTODOULOU, Z. & NEWBOLD, C. I. 2002. Effect of var gene disruption on switching in Plasmodium falciparum. *Mol Microbiol,* 45**,** 1131-41.

HORROCKS, P., PINCHES, R. A., CHAKRAVORTY, S. J., PAPAKRIVOS, J., CHRISTODOULOU, Z., KYES, S. A., URBAN, B. C., FERGUSON, D. J. & NEWBOLD, C. I. 2005. PfEMP1 expression is reduced on the surface of knobless Plasmodium falciparum infected erythrocytes. *J Cell Sci,* 118**,** 2507-18.

HOWELL, D. P., LEVIN, E. A., SPRINGER, A. L., KRAEMER, S. M., PHIPPARD, D. J., SCHIEF, W. R. & SMITH, J. D. 2008. Mapping a common interaction site used by Plasmodium falciparum Duffy binding-like domains to bind diverse host receptors. *Mol Microbiol,* 67**,** 78-87.

HUA, S., VIGNARAJAN, S., YAO, M., XIE, C., SVED, P. & DONG, Q. 2015. AKT and cytosolic phospholipase A2alpha form a positive loop in prostate cancer cells. *Curr Cancer Drug Targets,* 15**,** 781-91.

HUANG DA, W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res,* 37**,** 1-13.

HUNT, N. H. & GRAU, G. E. 2003. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. *Trends Immunol,* 24**,** 491-9.

IDRO, R., JENKINS, N. E. & NEWTON, C. R. 2005. Pathogenesis, clinical features, and neurological outcome of cerebral malaria. *Lancet Neurol,* 4**,** 827-40.

IDRO, R., KARAMAGI, C. & TUMWINE, J. 2004. Immediate outcome and prognostic factors for cerebral malaria among children admitted to Mulago Hospital, Uganda. *Ann Trop Paediatr,* 24**,** 17-24.

JANES, J. H., WANG, C. P., LEVIN-EDENS, E., VIGAN-WOMAS, I., GUILLOTTE, M., MELCHER, M., MERCEREAU-PUIJALON, O. & SMITH, J. D. 2011. Investigating the host binding signature on the Plasmodium falciparum PfEMP1 protein family. *PLoS Pathog,* 7**,** e1002032.

JENKINS, N., WU, Y., CHAKRAVORTY, S., KAI, O., MARSH, K. & CRAIG, A. 2007. Plasmodium falciparum intercellular adhesion molecule-1-based cytoadherence-related signaling in human endothelial cells. *J Infect Dis,* 196**,** 321-7.

JENSEN, A. T., MAGISTRADO, P., SHARP, S., JOERGENSEN, L., LAVSTSEN, T., CHIUCCHIUINI, A., SALANTI, A., VESTERGAARD, L. S., LUSINGU, J. P., HERMSEN, R., SAUERWEIN, R., CHRISTENSEN, J., NIELSEN, M. A., HVIID, L., SUTHERLAND, C., STAALSOE, T. & THEANDER, T. G. 2004. Plasmodium falciparum associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. *J Exp Med,* 199**,** 1179-90.

JENSEN, J. B. 1978. Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of Plasmodium falciparum. *Am J Trop Med Hyg,* 27**,** 1274-6.

JONES, M. W., ERRINGTON, M. L., FRENCH, P. J., FINE, A., BLISS, T. V., GAREL, S., CHARNAY, P., BOZON, B., LAROCHE, S. & DAVIS, S. 2001. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci,* 4**,** 289-96.

KERN, P., HEMMER, C. J., VAN DAMME, J., GRUSS, H. J. & DIETRICH, M. 1989. Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated Plasmodium falciparum malaria. *Am J Med,* 87**,** 139-43.

KEYSE, S. M. 2000. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Curr Opin Cell Biol,* 12**,** 186-92.

KHAN, J. A., MANDAL, T. K., DAS, T. K., SINGH, Y., PILLAI, B. & MAITI, S. 2011. Magnetite (Fe3O4) nanocrystals affect the expression of genes involved in the TGF-beta signalling pathway. *Mol Biosyst,* 7**,** 1481-6.

KHATRI, P., SIROTA, M. & BUTTE, A. J. 2012. Ten years of pathway analysis: current approaches and outstanding challenges. *PLoS Comput Biol,* 8**,** e1002375.

KIM, H., HIGGINS, S., LILES, W. C. & KAIN, K. C. 2011. Endothelial activation and dysregulation in malaria: a potential target for novel therapeutics. *Curr Opin Hematol,* 18**,** 177-85.

KIM, M. Y., SEO, E. J., LEE, D. H., KIM, E. J., KIM, H. S., CHO, H. Y., CHUNG, E. Y., LEE, S. H., BAIK, E. J., MOON, C. H. & JUNG, Y. S. 2010. Gadd45beta is a novel mediator of cardiomyocyte apoptosis induced by ischaemia/hypoxia. *Cardiovasc Res,* 87**,** 119-26.

KIRCHGATTER, K. & DEL PORTILLO, H. A. 2005. Clinical and molecular aspects of severe malaria. *An Acad Bras Cienc,* 77**,** 455-75.

KNAPSKA, E. & KACZMAREK, L. 2004. A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog Neurobiol,* 74**,** 183-211.

KOO, S. W., CASPER, K. A., OTTO, K. B., GIRA, A. K. & SWERLICK, R. A. 2003. Iron chelators inhibit VCAM-1 expression in human dermal microvascular endothelial cells. *J Invest Dermatol,* 120**,** 871-9.

KRAEMER, S. M., KYES, S. A., AGGARWAL, G., SPRINGER, A. L., NELSON, S. O., CHRISTODOULOU, Z., SMITH, L. M., WANG, W., LEVIN, E., NEWBOLD, C. I., MYLER, P. J. & SMITH, J. D. 2007. Patterns of gene recombination shape var gene repertoires in Plasmodium falciparum: comparisons of geographically diverse isolates. *BMC Genomics,* 8**,** 45.

KRAEMER, S. M. & SMITH, J. D. 2003. Evidence for the importance of genetic structuring to the structural and functional specialization of the Plasmodium falciparum var gene family. *Mol Microbiol,* 50**,** 1527-38.

KRAEMER, S. M. & SMITH, J. D. 2006. A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr Opin Microbiol,* 9**,** 374-80.

KWIATKOWSKI, D., HILL, A. V., SAMBOU, I., TWUMASI, P., CASTRACANE, J., MANOGUE, K. R., CERAMI, A., BREWSTER, D. R. & GREENWOOD, B. M. 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria. *Lancet,* 336**,** 1201-4.

KYRIACOU, H. M., STONE, G. N., CHALLIS, R. J., RAZA, A., LYKE, K. E., THERA, M. A., KONE, A. K., DOUMBO, O. K., PLOWE, C. V. & ROWE, J. A. 2006. Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia. *Mol Biochem Parasitol,* 150**,** 211-8.

LACKNER, P., HAMETNER, C., BEER, R., BURGER, C., BROESSNER, G., HELBOK, R., SPETH, C. & SCHMUTZHARD, E. 2008. Complement factors C1q, C3 and C5 in brain and serum of mice with cerebral malaria. *Malar J,* 7**,** 207.

LAMBROS, C. & VANDERBERG, J. P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol,* 65**,** 418-20.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol,* 10**,** R25.

LANGUINO, L. R., PLESCIA, J., DUPERRAY, A., BRIAN, A. A., PLOW, E. F., GELTOSKY, J. E. & ALTIERI, D. C. 1993. Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. *Cell,* 73**,** 1423-34.

LARSEN, C. M., DOSSING, M. G., PAPA, S., FRANZOSO, G., BILLESTRUP, N. & MANDRUP-POULSEN, T. 2006. Growth arrest- and DNA-damage-inducible 45beta gene inhibits c-Jun N-terminal kinase and extracellular signal-regulated kinase and decreases IL-1beta-induced apoptosis in insulin-producing INS-1E cells. *Diabetologia,* 49**,** 980-9.

LAVSTSEN, T., SALANTI, A., JENSEN, A. T., ARNOT, D. E. & THEANDER, T. G. 2003. Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J,* 2**,** 27.

LAVSTSEN, T., TURNER, L., SAGUTI, F., MAGISTRADO, P., RASK, T. S., JESPERSEN, J. S., WANG, C. W., BERGER, S. S., BARAKA, V., MARQUARD,

A. M., SEGUIN-ORLANDO, A., WILLERSLEV, E., GILBERT, M. T., LUSINGU, J. & THEANDER, T. G. 2012. Plasmodium falciparum erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. *Proc Natl Acad Sci U S A,* 109**,** E1791-800.

LAWSON, C., AINSWORTH, M., YACOUB, M. & ROSE, M. 1999. Ligation of ICAM-1 on endothelial cells leads to expression of VCAM-1 via a nuclear factor-kappaB-independent mechanism. *J Immunol,* 162**,** 2990-6.

LEE, J. I. & BURCKART, G. J. 1998. Nuclear factor kappa B: important transcription factor and therapeutic target. *J Clin Pharmacol,* 38**,** 981-93.

LI, H. & DURBIN, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics,* 25**,** 1754-60.

LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G., DURBIN, R. & GENOME PROJECT DATA PROCESSING, S. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25**,** 2078-9.

LI, H., RUAN, J. & DURBIN, R. 2008a. Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Res,* 18**,** 1851-8.

LI, L., WENG, Z., YAO, C., SONG, Y. & MA, T. 2015. Aquaporin-1 Deficiency Protects Against Myocardial Infarction by Reducing Both Edema and Apoptosis in Mice. *Sci Rep,* 5**,** 13807.

LI, M., WANG, I. X., LI, Y., BRUZEL, A., RICHARDS, A. L., TOUNG, J. M. & CHEUNG, V. G. 2011. Widespread RNA and DNA sequence differences in the human transcriptome. *Science,* 333**,** 53-8.

LI, W., LIU, Y., MUKHTAR, M. M., GONG, R., PAN, Y., RASOOL, S. T., GAO, Y., KANG, L., HAO, Q., PENG, G., CHEN, Y., CHEN, X., WU, J. & ZHU, Y. 2008b. Activation of interleukin-32 pro-inflammatory pathway in response to influenza A virus infection. *PLoS One,* 3**,** e1985.

LIN, C. Q., SINGH, J., MURATA, K., ITAHANA, Y., PARRINELLO, S., LIANG, S. H., GILLETT, C. E., CAMPISI, J. & DESPREZ, P. Y. 2000. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. *Cancer Res,* 60**,** 1332-40.

LIU, B., ZHANG, Y. H., JIANG, Y., LI, L. L., CHEN, Q., HE, G. Q., TAN, X. D. & LI, C. Q. 2015. Gadd45b is a novel mediator of neuronal apoptosis in ischemic stroke. *Int J Biol Sci,* 11**,** 353-60.

LIU, M., DICKINSON-COPELAND, C., HASSANA, S. & STILES, J. K. 2016. Plasmodium-infected erythrocytes (pRBC) induce endothelial cell apoptosis via a heme-mediated signaling pathway. *Drug Des Devel Ther,* 10**,** 1009-18.

LIU, N. 2015. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annu Rev Entomol,* 60**,** 537-59.

LIU, Z., MIAO, J. & CUI, L. 2011. Gametocytogenesis in malaria parasite: commitment, development and regulation. *Future Microbiol,* 6**,** 1351-69.

LODA, M., CAPODIECI, P., MISHRA, R., YAO, H., CORLESS, C., GRIGIONI, W., WANG, Y., MAGI-GALLUZZI, C. & STORK, P. J. 1996. Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. *Am J Pathol,* 149**,** 1553-64.

LU, Z., SERGHIDES, L., PATEL, S. N., DEGOUSEE, N., RUBIN, B. B., KRISHNEGOWDA, G., GOWDA, D. C., KARIN, M. & KAIN, K. C. 2006. Disruption of JNK2 decreases the cytokine response to Plasmodium falciparum glycosylphosphatidylinositol in vitro and confers protection in a cerebral malaria model. *J Immunol,* 177**,** 6344-52.

LUND, B. T., ASHIKIAN, N., TA, H. Q., CHAKRYAN, Y., MANOUKIAN, K., GROSHEN, S., GILMORE, W., CHEEMA, G. S., STOHL, W., BURNETT, M. E., KO, D., KACHUCK, N. J. & WEINER, L. P. 2004. Increased CXCL8 (IL-8) expression in Multiple Sclerosis. *J Neuroimmunol,* 155**,** 161-71.

LUO, J. L., KAMATA, H. & KARIN, M. 2005. The anti-death machinery in IKK/NF-kappaB signaling. *J Clin Immunol,* 25**,** 541-50.

MA, J., CHEN, W., ZHANG, L., TUCKER, B., ZHU, G., SASAKI, H., HAO, L., WANG, L., CI, H., JIANG, H., STASHENKO, P. & LI, Y. P. 2013. RNA interference-mediated silencing of Atp6i prevents both periapical bone erosion and inflammation in the mouse model of endodontic disease. *Infect Immun,* 81**,** 1021-30.

MA, N., HARDING, A. J., PAMPHLETT, R., CHAUDHRI, G. & HUNT, N. H. 1997. Increased c-fos expression in the brain during experimental murine cerebral malaria: possible association with neurologic complications. *J Infect Dis,* 175**,** 1480-9.

MADKHALI, A. M., ALKURBI, M. O., SZESTAK, T., BENGTSSON, A., PATIL, P. R., WU, Y., AL-HARTHI, S. A., JENSEN, A. T., PLEASS, R. & CRAIG, A. G. 2014. An analysis of the binding characteristics of a panel of recently selected ICAM-1 binding Plasmodium falciparum patient isolates. *PLoS One,* 9**,** e111518.

MAGUIRE, G. P., HANDOJO, T., PAIN, M. C., KENANGALEM, E., PRICE, R. N., TJITRA, E. & ANSTEY, N. M. 2005. Lung injury in uncomplicated and severe falciparum malaria: a longitudinal study in papua, Indonesia. *J Infect Dis,* 192**,** 1966-74.

MANNING, L., ROSANAS-URGELL, A., LAMAN, M., EDONI, H., MCLEAN, C., MUELLER, I., SIBA, P. & DAVIS, T. M. 2012. A histopathologic study of fatal paediatric cerebral malaria caused by mixed Plasmodium falciparum/Plasmodium vivax infections. *Malar J,* 11**,** 107.

MANZANO, R. G., MONTUENGA, L. M., DAYTON, M., DENT, P., KINOSHITA, I., VICENT, S., GARDNER, G. J., NGUYEN, P., CHOI, Y. H., TREPEL, J., AUERSPERG, N. & BIRRER, M. J. 2002. CL100 expression is down-regulated in advanced epithelial ovarian cancer and its re-expression decreases its malignant potential. *Oncogene,* 21**,** 4435-47.

MARSH, K. & KINYANJUI, S. 2006. Immune effector mechanisms in malaria. *Parasite Immunol,* 28**,** 51-60.

MARUYAMA, T., LI, J., VAQUE, J. P., KONKEL, J. E., WANG, W., ZHANG, B., ZHANG, P., ZAMARRON, B. F., YU, D., WU, Y., ZHUANG, Y., GUTKIND, J. S. & CHEN, W. 2011. Control of the differentiation of regulatory T cells and T(H)17 cells by the DNA-binding inhibitor Id3. *Nat Immunol,* 12**,** 86-95.

MBALE, E. W., MOXON, C. A., MUKAKA, M., CHAGOMERANA, M., GLOVER, S., CHISALA, N., OMAR, S., MOLYNEUX, M., SEYDEL, K., CRAIG, A. G., TAYLOR, T., HEYDERMAN, R. S. & MALLEWA, M. 2016. HIV coinfection influences the inflammatory response but not the outcome of cerebral malaria in Malawian children. *J Infect,* 73**,** 189-99.

MCCORMICK, C. J., CRAIG, A., ROBERTS, D., NEWBOLD, C. I. & BERENDT, A. R. 1997. Intercellular adhesion molecule-1 and CD36 synergize to mediate adherence of Plasmodium falciparum-infected erythrocytes to cultured human microvascular endothelial cells. *J Clin Invest,* 100**,** 2521-9.

MCGILVRAY, I. D., SERGHIDES, L., KAPUS, A., ROTSTEIN, O. D. & KAIN, K. C. 2000. Nonopsonic monocyte/macrophage phagocytosis of Plasmodium falciparum-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood,* 96**,** 3231-40.

MEDANA, I. M. & TURNER, G. D. 2006. Human cerebral malaria and the blood-brain barrier. *Int J Parasitol,* 36**,** 555-68.

MEDVEDEV, P., STANCIU, M. & BRUDNO, M. 2009. Computational methods for discovering structural variation with next-generation sequencing. *Nat Methods,* 6**,** S13-20.

MEISSNER, A., ZILLES, O., VARONA, R., JOZEFOWSKI, K., RITTER, U., MARQUEZ, G., HALLMANN, R. & KORNER, H. 2003. CC chemokine ligand 20 partially controls adhesion of naive B cells to activated endothelial cells under shear stress. *Blood,* 102**,** 2724-7.

MELIK-PARSADANIANTZ, S. & ROSTENE, W. 2008. Chemokines and neuromodulation. *J Neuroimmunol,* 198**,** 62-8.

MILLER, L. H., ACKERMAN, H. C., SU, X. Z. & WELLEMS, T. E. 2013. Malaria biology and disease pathogenesis: insights for new treatments. *Nat Med,* 19**,** 156-67.

MILLER, L. H., HUDSON-TAYLOR, D., GAMAIN, B. & SAUL, A. J. 2002. Definition of the minimal domain of CIDR1alpha of Plasmodium falciparum PfEMP1 for binding CD36. *Mol Biochem Parasitol,* 120**,** 321-3.

MILNER, D. A., JR., WHITTEN, R. O., KAMIZA, S., CARR, R., LIOMBA, G., DZAMALALA, C., SEYDEL, K. B., MOLYNEUX, M. E. & TAYLOR, T. E. 2014. The systemic pathology of cerebral malaria in African children. *Front Cell Infect Microbiol,* 4**,** 104.

MINSKY, N. & ROEDER, R. G. 2015. Direct link between metabolic regulation and the heat-shock response through the transcriptional regulator PGC-1alpha. *Proc Natl Acad Sci U S A,* 112**,** E5669-78.

MIRANDA, A. S., BRANT, F., ROCHA, N. P., CISALPINO, D., RODRIGUES, D. H., SOUZA, D. G., MACHADO, F. S., RACHID, M. A., TEIXEIRA, A. L., JR. & CAMPOS, A. C. 2013. Further evidence for an anti-inflammatory role of artesunate in experimental cerebral malaria. *Malar J,* 12**,** 388.

MISHRA, R. R., ADHIKARY, G., SIMONSON, M. S., CHERNIACK, N. S. & PRABHAKAR, N. R. 1998. Role of c-fos in hypoxia-induced AP-1 cis-element activity and tyrosine hydroxylase gene expression. *Brain Res Mol Brain Res,* 59**,** 74-83.

MOLYNEUX, M. E., TAYLOR, T. E., WIRIMA, J. J. & BORGSTEIN, A. 1989. Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. *Q J Med,* 71**,** 441-59.

MONTGOMERY, J., MPHANDE, F. A., BERRIMAN, M., PAIN, A., ROGERSON, S. J., TAYLOR, T. E., MOLYNEUX, M. E. & CRAIG, A. 2007. Differential var gene expression in the organs of patients dying of falciparum malaria. *Mol Microbiol,* 65**,** 959-67.

MONTGOMERY, S. B., SAMMETH, M., GUTIERREZ-ARCELUS, M., LACH, R. P., INGLE, C., NISBETT, J., GUIGO, R. & DERMITZAKIS, E. T. 2010. Transcriptome genetics using second generation sequencing in a Caucasian population. *Nature,* 464**,** 773-7.

MORTAZAVI, A., WILLIAMS, B. A., MCCUE, K., SCHAEFFER, L. & WOLD, B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods,* 5**,** 621-8.

MOTULSKY, E., SALIK, D., JANSSENS, X., PION, B., DUFRANE, R., CHAPUT, F., BOLAKY, N., GREGOIRE, F., CASPERS, L., PERRET, J., WILLERMAIN, F. & DELPORTE, C. 2014. Aquaporin-1 expression in proliferative vitreoretinopathy and in epiretinal membranes. *ScientificWorldJournal,* 2014**,** 876208.

MOXON, C. A., WASSMER, S. C., MILNER, D. A., JR., CHISALA, N. V., TAYLOR, T. E., SEYDEL, K. B., MOLYNEUX, M. E., FARAGHER, B., ESMON, C. T., DOWNEY, C., TOH, C. H., CRAIG, A. G. & HEYDERMAN, R. S. 2013. Loss of endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral malaria in African children. *Blood,* 122**,** 842-51.

MUGASA, J., QI, W., RUSCH, S., ROTTMANN, M. & BECK, H. P. 2012. Genetic diversity of expressed Plasmodium falciparum var genes from Tanzanian children with severe malaria. *Malar J,* 11**,** 230.

MURRAY, C. J., ROSENFELD, L. C., LIM, S. S., ANDREWS, K. G., FOREMAN, K. J., HARING, D., FULLMAN, N., NAGHAVI, M., LOZANO, R. & LOPEZ, A. D. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet,* 379**,** 413-31.

N'DILIMABAKA, N., TAOUFIQ, Z., ZOUGBEDE, S., BONNEFOY, S., LORTHIOIS, A., COURAUD, P. O., REBOLLO, A., SNOUNOU, G., MAZIER, D. & MORENO SABATER, A. 2014. P. falciparum isolate-specific distinct patterns of induced apoptosis in pulmonary and brain endothelial cells. *PLoS One,* 9**,** e90692.

NAG, S., MANIAS, J. L. & STEWART, D. J. 2009. Pathology and new players in the pathogenesis of brain edema. *Acta Neuropathol,* 118**,** 197-217.

NESS, J. M., HARVEY, C. R., WASHINGTON, J. D., ROTH, K. A., CARROLL, S. L. & ZHANG, J. 2008. Differential activation of c-fos and caspase-3 in hippocampal neuron subpopulations following neonatal hypoxia-ischemia. *J Neurosci Res,* 86**,** 1115-24.

NETEA, M. G., AZAM, T., LEWIS, E. C., JOOSTEN, L. A., WANG, M., LANGENBERG, D., MENG, X., CHAN, E. D., YOON, D. Y., OTTENHOFF, T., KIM, S. H. & DINARELLO, C. A. 2006. Mycobacterium tuberculosis induces interleukin-32 production through a caspase- 1/IL-18/interferon-gamma-dependent mechanism. *PLoS Med,* 3**,** e277.

NEWBOLD, C., CRAIG, A., KYES, S., ROWE, A., FERNANDEZ-REYES, D. & FAGAN, T. 1999. Cytoadherence, pathogenesis and the infected red cell surface in Plasmodium falciparum. *Int J Parasitol,* 29**,** 927-37.

NEWBOLD, C., WARN, P., BLACK, G., BERENDT, A., CRAIG, A., SNOW, B., MSOBO, M., PESHU, N. & MARSH, K. 1997. Receptor-specific adhesion and clinical disease in Plasmodium falciparum. *Am J Trop Med Hyg,* 57**,** 389-98.

NEWTON, C. R., HIEN, T. T. & WHITE, N. 2000. Cerebral malaria. *J Neurol Neurosurg Psychiatry,* 69**,** 433-41.

NEWTON, C. R., TAYLOR, T. E. & WHITTEN, R. O. 1998. Pathophysiology of fatal falciparum malaria in African children. *Am J Trop Med Hyg,* 58**,** 673-83.

NITCHEU, J., BONDUELLE, O., COMBADIERE, C., TEFIT, M., SEILHEAN, D., MAZIER, D. & COMBADIERE, B. 2003. Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J Immunol,* 170**,** 2221-8.

OCHIEL, D. O., AWANDARE, G. A., KELLER, C. C., HITTNER, J. B., KREMSNER, P. G., WEINBERG, J. B. & PERKINS, D. J. 2005. Differential regulation of beta-chemokines in children with Plasmodium falciparum malaria. *Infect Immun,* 73**,** 4190-7.

OCHOLA, L. B., SIDDONDO, B. R., OCHOLLA, H., NKYA, S., KIMANI, E. N., WILLIAMS, T. N., MAKALE, J. O., LILJANDER, A., URBAN, B. C., BULL, P. C., SZESTAK, T., MARSH, K. & CRAIG, A. G. 2011. Specific receptor usage in Plasmodium falciparum cytoadherence is associated with disease outcome. *PLoS One,* 6**,** e14741.

OCKENHOUSE, C. F., BETAGERI, R., SPRINGER, T. A. & STAUNTON, D. E. 1992a. Plasmodium falciparum-infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. *Cell,* 68**,** 63-9.

OCKENHOUSE, C. F., TANDON, N. N., MAGOWAN, C., JAMIESON, G. A. & CHULAY, J. D. 1989. Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor. *Science,* 243**,** 1469-71.

OCKENHOUSE, C. F., TEGOSHI, T., MAENO, Y., BENJAMIN, C., HO, M., KAN, K. E., THWAY, Y., WIN, K., AIKAWA, M. & LOBB, R. R. 1992b. Human vascular endothelial cell adhesion receptors for Plasmodium falciparum-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. *J Exp Med,* 176**,** 1183-9.

OLARERIN-GEORGE, A. O. & HOGENESCH, J. B. 2015. Assessing the prevalence of mycoplasma contamination in cell culture via a survey of NCBI's RNA-seq archive. *Nucleic Acids Res,* 43**,** 2535-42.

OLLIARO, P. 2008. Editorial commentary: mortality associated with severe Plasmodium falciparum malaria increases with age. *Clin Infect Dis,* 47**,** 158-60.

OLUMESE, P. E., ADEYEMO, A. A., GBADEGESIN, R. A. & WALKER, O. 1997. Retinal haemorrhage in cerebral malaria. *East Afr Med J,* 74**,** 285-7.

OQUENDO, P., HUNDT, E., LAWLER, J. & SEED, B. 1989. CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. *Cell,* 58**,** 95-101.

OWENS, D. M. & KEYSE, S. M. 2007. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene,* 26**,** 3203-13.

PACHER, P., BECKMAN, J. S. & LIAUDET, L. 2007. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev,* 87**,** 315-424.

PAN, Q., SHAI, O., LEE, L. J., FREY, B. J. & BLENCOWE, B. J. 2008. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet,* 40**,** 1413-5.

PAPPA, V., SEYDEL, K., GUPTA, S., FEINTUCH, C. M., POTCHEN, M. J., KAMPONDENI, S., GOLDMAN-YASSEN, A., VEENSTRA, M., LOPEZ, L., KIM, R. S., BERMAN, J. W., TAYLOR, T. & DAILY, J. P. 2015. Lipid metabolites of the phospholipase A2 pathway and inflammatory cytokines are associated with brain volume in paediatric cerebral malaria. *Malar J,* 14**,** 513.

PASTERNAK, N. D. & DZIKOWSKI, R. 2009. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum. *Int J Biochem Cell Biol,* 41**,** 1463-6.

PATIL, P. R., GEMMA, S., CAMPIANI, G. & CRAIG, A. G. 2011. Broad inhibition of plasmodium falciparum cytoadherence by (+)-epigallocatechin gallate. *Malar J,* 10**,** 348.

PENG, Z., CHENG, Y., TAN, B. C., KANG, L., TIAN, Z., ZHU, Y., ZHANG, W., LIANG, Y., HU, X., TAN, X., GUO, J., DONG, Z., LIANG, Y., BAO, L. & WANG, J. 2012. Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome. *Nat Biotechnol,* 30**,** 253-60.

PERCARIO, S., MOREIRA, D. R., GOMES, B. A., FERREIRA, M. E., GONCALVES, A. C., LAURINDO, P. S., VILHENA, T. C., DOLABELA, M. F. & GREEN, M. D. 2012. Oxidative stress in malaria. *Int J Mol Sci,* 13**,** 16346-72.

PERK, J., IAVARONE, A. & BENEZRA, R. 2005. Id family of helix-loop-helix proteins in cancer. *Nat Rev Cancer,* 5**,** 603-14.

PERSIDSKY, Y., RAMIREZ, S. H., HAORAH, J. & KANMOGNE, G. D. 2006. Blood-brain barrier: structural components and function under physiologic and pathologic conditions. *J Neuroimmune Pharmacol,* 1**,** 223-36.

PICARDI, E., HORNER, D. S., CHIARA, M., SCHIAVON, R., VALLE, G. & PESOLE, G. 2010. Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. *Nucleic Acids Res,* 38**,** 4755-67.

PINO, P., TAOUFIQ, Z., NITCHEU, J., VOULDOUKIS, I. & MAZIER, D. 2005. Blood-brain barrier breakdown during cerebral malaria: suicide or murder? *Thromb Haemost,* 94**,** 336-40.

PINO, P., VOULDOUKIS, I., DUGAS, N., CONTI, M., NITCHEU, J., TRAORE, B., DANIS, M., DUGAS, B. & MAZIER, D. 2004. Induction of the CD23/nitric oxide pathway in endothelial cells downregulates ICAM-1 expression and decreases cytoadherence of Plasmodium falciparum-infected erythrocytes. *Cell Microbiol,* 6**,** 839-48.

PINO, P., VOULDOUKIS, I., KOLB, J. P., MAHMOUDI, N., DESPORTES-LIVAGE, I., BRICAIRE, F., DANIS, M., DUGAS, B. & MAZIER, D. 2003. Plasmodium falciparum--infected erythrocyte adhesion induces caspase activation and apoptosis in human endothelial cells. *J Infect Dis,* 187**,** 1283-90.

POBER, J. S. & COTRAN, R. S. 1990. Cytokines and endothelial cell biology. *Physiol Rev,* 70**,** 427-51.

POUGET, J. G., GONCALVES, V. F., SCHIZOPHRENIA WORKING GROUP OF THE PSYCHIATRIC GENOMICS, C., SPAIN, S. L., FINUCANE, H. K., RAYCHAUDHURI, S., KENNEDY, J. L. & KNIGHT, J. 2016. Genome-Wide Association Studies Suggest Limited Immune Gene Enrichment in Schizophrenia Compared to 5 Autoimmune Diseases. *Schizophr Bull,* 42**,** 1176-84.

POUVELLE, B., MATARAZZO, V., JURZYNSKI, C., NEMETH, J., RAMHARTER, M., ROUGON, G. & GYSIN, J. 2007. Neural cell adhesion molecule, a new

cytoadhesion receptor for Plasmodium falciparum-infected erythrocytes capable of aggregation. *Infect Immun,* 75**,** 3516-22.

PUNSAWAD, C., MANEERAT, Y., CHAISRI, U., NANTAVISAI, K. & VIRIYAVEJAKUL, P. 2013. Nuclear factor kappa B modulates apoptosis in the brain endothelial cells and intravascular leukocytes of fatal cerebral malaria. *Malar J,* 12**,** 260.

QADAH, T., FINLAYSON, J., NORTH, E. & GHASSEMIFAR, R. 2015. Molecular characterization of Hb Hamilton Hill (HBA2: c.388delC), a novel HBA2 variant generating a premature termination codon and truncated HBA2 chain. *Hemoglobin,* 39**,** 88-94.

QIN, F., ZHANG, H., SHAO, Y., LIU, X., YANG, L., HUANG, Y., FU, L., GU, F. & MA, Y. 2016. Expression of aquaporin1, a water channel protein, in cytoplasm is negatively correlated with prognosis of breast cancer patients. *Oncotarget,* 7**,** 8143-54.

RANSOHOFF, R. M. 2002. The chemokine system in neuroinflammation: an update. *J Infect Dis,* 186 Suppl 2**,** S152-6.

RANSON, H. & LISSENDEN, N. 2016. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends Parasitol,* 32**,** 187-96.

RASOOL, S. T., TANG, H., WU, J., LI, W., MUKHTAR, M. M., ZHANG, J., MU, Y., XING, H. X., WU, J. & ZHU, Y. 2008. Increased level of IL-32 during human immunodeficiency virus infection suppresses HIV replication. *Immunol Lett,* 117**,** 161-7.

RAZIN, S. 2006. The Genus Mycoplasma and Related Genera (Class Mollicutes). *Prokaryotes: A Handbook on the Biology of Bacteria, Vol 4, Third Edition***,** 836-904.

RICKLIN, D., REIS, E. S., MASTELLOS, D. C., GROS, P. & LAMBRIS, J. D. 2016. Complement component C3 - The "Swiss Army Knife" of innate immunity and host defense. *Immunol Rev,* 274**,** 33-58.

RILEY, E. M., COUPER, K. N., HELMBY, H., HAFALLA, J. C., DE SOUZA, J. B., LANGHORNE, J., JARRA, W. B. & ZAVALA, F. 2010. Neuropathogenesis of human and murine malaria. *Trends Parasitol,* 26**,** 277-8.

ROBERTS, D. J., CRAIG, A. G., BERENDT, A. R., PINCHES, R., NASH, G., MARSH, K. & NEWBOLD, C. I. 1992. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature,* 357**,** 689-92.

ROBINSON, B. A., WELCH, T. L. & SMITH, J. D. 2003. Widespread functional specialization of Plasmodium falciparum erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. *Mol Microbiol,* 47**,** 1265-78.

ROGERSON, S. J., TEMBENU, R., DOBANO, C., PLITT, S., TAYLOR, T. E. & MOLYNEUX, M. E. 1999. Cytoadherence characteristics of Plasmodium falciparum-infected erythrocytes from Malawian children with severe and uncomplicated malaria. *Am J Trop Med Hyg,* 61**,** 467-72.

ROTTEM, S. 2003. Interaction of mycoplasmas with host cells. *Physiol Rev,* 83**,** 417-32.

ROTTMANN, M., LAVSTSEN, T., MUGASA, J. P., KAESTLI, M., JENSEN, A. T., MULLER, D., THEANDER, T. & BECK, H. P. 2006. Differential expression of var gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children. *Infect Immun,* 74**,** 3904-11.

ROWE, J. A., CLAESSENS, A., CORRIGAN, R. A. & ARMAN, M. 2009. Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med,* 11**,** e16.

ROWE, J. A., MOULDS, J. M., NEWBOLD, C. I. & MILLER, L. H. 1997. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature,* 388**,** 292-5.

RUG, M., PRESCOTT, S. W., FERNANDEZ, K. M., COOKE, B. M. & COWMAN, A. F. 2006. The role of KAHRP domains in knob formation and cytoadherence of P falciparum-infected human erythrocytes. *Blood,* 108**,** 370-8.

SAHU, A., SUNYER, J. O., MOORE, W. T., SARRIAS, M. R., SOULIKA, A. M. & LAMBRIS, J. D. 1998. Structure, functions, and evolution of the third complement component and viral molecular mimicry. *Immunol Res,* 17**,** 109-21.

SALANTI, A., STAALSOE, T., LAVSTSEN, T., JENSEN, A. T., SOWA, M. P., ARNOT, D. E., HVIID, L. & THEANDER, T. G. 2003. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. *Mol Microbiol,* 49**,** 179-91.

SALVADO, M. D., ALFRANCA, A., HAEGGSTROM, J. Z. & REDONDO, J. M. 2012. Prostanoids in tumor angiogenesis: therapeutic intervention beyond COX-2. *Trends Mol Med,* 18**,** 233-43.

SAVILL, J., HOGG, N., REN, Y. & HASLETT, C. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest,* 90**,** 1513-22.

SAXENA, M. & MUSTELIN, T. 2000. Extracellular signals and scores of phosphatases: all roads lead to MAP kinase. *Semin Immunol,* 12**,** 387-96.

SCHINDL, M., OBERHUBER, G., OBERMAIR, A., SCHOPPMANN, S. F., KARNER, B. & BIRNER, P. 2001. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. *Cancer Res,* 61**,** 5703-6.

SCHINDLER, S. M., LITTLE, J. P. & KLEGERIS, A. 2014. Microparticles: a new perspective in central nervous system disorders. *Biomed Res Int,* 2014**,** 756327.

SCHOFIELD, L. & GRAU, G. E. 2005. Immunological processes in malaria pathogenesis. *Nat Rev Immunol,* 5**,** 722-35.

SCHOFIELD, L., NOVAKOVIC, S., GEROLD, P., SCHWARZ, R. T., MCCONVILLE, M. J. & TACHADO, S. D. 1996. Glycosylphosphatidylinositol toxin of Plasmodium up-regulates intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin expression in vascular endothelial cells and increases leukocyte and parasite cytoadherence via tyrosine kinase-dependent signal transduction. *J Immunol,* 156**,** 1886-96.

SCHROEDER, A., MUELLER, O., STOCKER, S., SALOWSKY, R., LEIBER, M., GASSMANN, M., LIGHTFOOT, S., MENZEL, W., GRANZOW, M. & RAGG, T. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *Bmc Molecular Biology,* 7.

SCHUTYSER, E., STRUYF, S. & VAN DAMME, J. 2003. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev,* 14**,** 409-26.

SEMPLE, B. D., KOSSMANN, T. & MORGANTI-KOSSMANN, M. C. 2010. Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. *J Cereb Blood Flow Metab,* 30**,** 459-73.

SEYDEL, K. B., KAMPONDENI, S. D., VALIM, C., POTCHEN, M. J., MILNER, D. A., MUWALO, F. W., BIRBECK, G. L., BRADLEY, W. G., FOX, L. L., GLOVER, S. J., HAMMOND, C. A., HEYDERMAN, R. S., CHILINGULO, C. A., MOLYNEUX, M. E. & TAYLOR, T. E. 2015. Brain swelling and death in children with cerebral malaria. *N Engl J Med,* 372**,** 1126-37.

SHEN, J., ZHANG, Y., YU, H., SHEN, B., LIANG, Y., JIN, R., LIU, X., SHI, L. & CAI, X. 2016. Role of DUSP1/MKP1 in tumorigenesis, tumor progression and therapy. *Cancer Med,* 5**,** 2061-8.

SIANO, J. P., GRADY, K. K., MILLET, P. & WICK, T. M. 1998. Short report: Plasmodium falciparum: cytoadherence to alpha(v)beta3 on human microvascular endothelial cells. *Am J Trop Med Hyg,* 59**,** 77-9.

SMITH, J. D. 2014. The role of PfEMP1 adhesion domain classification in Plasmodium falciparum pathogenesis research. *Mol Biochem Parasitol,* 195**,** 82-7.

SMITH, J. D., CHITNIS, C. E., CRAIG, A. G., ROBERTS, D. J., HUDSON-TAYLOR, D. E., PETERSON, D. S., PINCHES, R., NEWBOLD, C. I. & MILLER, L. H. 1995.

Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell,* 82**,** 101-10.

SMITH, J. D., CRAIG, A. G., KRIEK, N., HUDSON-TAYLOR, D., KYES, S., FAGAN, T., PINCHES, R., BARUCH, D. I., NEWBOLD, C. I. & MILLER, L. H. 2000. Identification of a Plasmodium falciparum intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. *Proc Natl Acad Sci U S A,* 97**,** 1766-71.

SMITH, J. D., GAMAIN, B., BARUCH, D. I. & KYES, S. 2001. Decoding the language of var genes and Plasmodium falciparum sequestration. *Trends Parasitol,* 17**,** 538-45.

SMITH, R. C., VEGA-RODRIGUEZ, J. & JACOBS-LORENA, M. 2014. The Plasmodium bottleneck: malaria parasite losses in the mosquito vector. *Mem Inst Oswaldo Cruz,* 109**,** 644-61.

SNOW, R. W., GUERRA, C. A., NOOR, A. M., MYINT, H. Y. & HAY, S. I. 2005. The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature,* 434**,** 214-7.

SOBOTA, R. S., DARA, A., MANNING, J. E., NIANGALY, A., BAILEY, J. A., KONE, A. K., THERA, M. A., DJIMDE, A. A., VERNET, G., LEISSNER, P., WILLIAMS, S. M., PLOWE, C. V. & DOUMBO, O. K. 2016. Expression of complement and toll-like receptor pathway genes is associated with malaria severity in Mali: a pilot case control study. *Malar J,* 15**,** 150.

SPRINGER, A. L., SMITH, L. M., MACKAY, D. Q., NELSON, S. O. & SMITH, J. D. 2004. Functional interdependence of the DBLbeta domain and c2 region for binding of the Plasmodium falciparum variant antigen to ICAM-1. *Mol Biochem Parasitol,* 137**,** 55-64.

STAALSOE, T., MEGNEKOU, R., FIEVET, N., RICKE, C. H., ZORNIG, H. D., LEKE, R., TAYLOR, D. W., DELORON, P. & HVIID, L. 2001. Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of Plasmodium falciparum-infected erythrocytes that protect against placental parasitemia. *J Infect Dis,* 184**,** 618-26.

STAMATOVIC, S. M., KEEP, R. F., KUNKEL, S. L. & ANDJELKOVIC, A. V. 2003. Potential role of MCP-1 in endothelial cell tight junction 'opening': signaling via Rho and Rho kinase. *J Cell Sci,* 116**,** 4615-28.

STAMATOVIC, S. M., SHAKUI, P., KEEP, R. F., MOORE, B. B., KUNKEL, S. L., VAN ROOIJEN, N. & ANDJELKOVIC, A. V. 2005. Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. *J Cereb Blood Flow Metab,* 25**,** 593-606.

STAUNTON, D. E., MERLUZZI, V. J., ROTHLEIN, R., BARTON, R., MARLIN, S. D. & SPRINGER, T. A. 1989. A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell,* 56**,** 849-53.

STORM, J. & CRAIG, A. G. 2014. Pathogenesis of cerebral malaria--inflammation and cytoadherence. *Front Cell Infect Microbiol,* 4**,** 100.

STURM, A., AMINO, R., VAN DE SAND, C., REGEN, T., RETZLAFF, S., RENNENBERG, A., KRUEGER, A., POLLOK, J. M., MENARD, R. & HEUSSLER, V. T. 2006. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science,* 313**,** 1287-90.

SUBRAMANIAN, A., TAMAYO, P., MOOTHA, V. K., MUKHERJEE, S., EBERT, B. L., GILLETTE, M. A., PAULOVICH, A., POMEROY, S. L., GOLUB, T. R., LANDER, E. S. & MESIROV, J. P. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A,* 102**,** 15545-50.

SUBUDHI, A. K., BOOPATHI, P. A., PANDEY, I., KAUR, R., MIDDHA, S., ACHARYA, J., KOCHAR, S. K., KOCHAR, D. K. & DAS, A. 2015a. Disease specific modules and hub genes for intervention strategies: A co-expression network based approach for Plasmodium falciparum clinical isolates. *Infect Genet Evol,* 35**,** 96-108.

SUBUDHI, A. K., BOOPATHI, P. A., PANDEY, I., KOHLI, R., KARWA, R., MIDDHA, S., ACHARYA, J., KOCHAR, S. K., KOCHAR, D. K. & DAS, A. 2015b. Plasmodium falciparum complicated malaria: Modulation and connectivity between exportome and variant surface antigen gene families. *Mol Biochem Parasitol,* 201**,** 31-46.

SULTAN, M., SCHULZ, M. H., RICHARD, H., MAGEN, A., KLINGENHOFF, A., SCHERF, M., SEIFERT, M., BORODINA, T., SOLDATOV, A., PARKHOMCHUK, D., SCHMIDT, D., O'KEEFFE, S., HAAS, S., VINGRON, M., LEHRACH, H. & YASPO, M. L. 2008. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science,* 321**,** 956-60.

SUSOMBOON, P., MANEERAT, Y., DEKUMYOY, P., KALAMBAHETI, T., IWAGAMI, M., KOMAKI-YASUDA, K., KAWAZU, S., TANGPUKDEE, N., LOOAREESUWAN, S. & KANO, S. 2006. Down-regulation of tight junction mRNAs in human endothelial cells co-cultured with Plasmodium falciparum-infected erythrocytes. *Parasitol Int,* 55**,** 107-12.

SUTHERLAND, C. J., TANOMSING, N., NOLDER, D., OGUIKE, M., JENNISON, C., PUKRITTAYAKAMEE, S., DOLECEK, C., HIEN, T. T., DO ROSARIO, V. E., AREZ, A. P., PINTO, J., MICHON, P., ESCALANTE, A. A., NOSTEN, F., BURKE, M., LEE, R., BLAZE, M., OTTO, T. D., BARNWELL, J. W., PAIN, A., WILLIAMS, J., WHITE, N. J., DAY, N. P., SNOUNOU, G., LOCKHART, P. J., CHIODINI, P. L., IMWONG, M. & POLLEY, S. D. 2010. Two nonrecombining sympatric forms of the human malaria parasite Plasmodium ovale occur globally. *J Infect Dis,* 201**,** 1544-50.

SWERLICK, R. A., GARCIA-GONZALEZ, E., KUBOTA, Y., XU, Y. L. & LAWLEY, T. J. 1991. Studies of the modulation of MHC antigen and cell adhesion molecule expression on human dermal microvascular endothelial cells. *J Invest Dermatol,* 97**,** 190-6.

SWERLICK, R. A., LEE, K. H., WICK, T. M. & LAWLEY, T. J. 1992. Human dermal microvascular endothelial but not human umbilical vein endothelial cells express CD36 in vivo and in vitro. *J Immunol,* 148**,** 78-83.

TAOUFIQ, Z., PINO, P., DUGAS, N., CONTI, M., TEFIT, M., MAZIER, D. & VOULDOUKIS, I. 2006. Transient supplementation of superoxide dismutase protects endothelial cells against Plasmodium falciparum-induced oxidative stress. *Mol Biochem Parasitol,* 150**,** 166-73.

TAYLOR, S. M., CERAMI, C. & FAIRHURST, R. M. 2013. Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis. *PLoS Pathog,* 9**,** e1003327.

TAYLOR, T. E., FU, W. J., CARR, R. A., WHITTEN, R. O., MUELLER, J. S., FOSIKO, N. G., LEWALLEN, S., LIOMBA, N. G. & MOLYNEUX, M. E. 2004. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat Med,* 10**,** 143-5.

THOMPSON, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science,* 267**,** 1456-62.

TOBIUME, K., MATSUZAWA, A., TAKAHASHI, T., NISHITOH, H., MORITA, K., TAKEDA, K., MINOWA, O., MIYAZONO, K., NODA, T. & ICHIJO, H. 2001. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO Rep,* 2**,** 222-8.

TRAGER, W. & JENSEN, J. B. 1976. Human malaria parasites in continuous culture. *Science,* 193**,** 673-5.

TRAPNELL, C., PACHTER, L. & SALZBERG, S. L. 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics,* 25**,** 1105-1111.

TRAPNELL, C., ROBERTS, A., GOFF, L., PERTEA, G., KIM, D., KELLEY, D. R., PIMENTEL, H., SALZBERG, S. L., RINN, J. L. & PACHTER, L. 2012.

Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc,* 7**,** 562-78.

TRAPNELL, C., WILLIAMS, B. A., PERTEA, G., MORTAZAVI, A., KWAN, G., VAN BAREN, M. J., SALZBERG, S. L., WOLD, B. J. & PACHTER, L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol,* 28**,** 511-5.

TREERATANAPIBOON, L., PSATHAKI, K., WEGENER, J., LOOAREESUWAN, S., GALLA, H. J. & UDOMSANGPETCH, R. 2005. In vitro study of malaria parasite induced disruption of blood-brain barrier. *Biochem Biophys Res Commun,* 335**,** 810-8.

TRIPATHI, A. K., SHA, W., SHULAEV, V., STINS, M. F. & SULLIVAN, D. J., JR. 2009. Plasmodium falciparum-infected erythrocytes induce NF-kappaB regulated inflammatory pathways in human cerebral endothelium. *Blood,* 114**,** 4243-52.

TRIPATHI, A. K., SULLIVAN, D. J. & STINS, M. F. 2006. Plasmodium falciparum-infected erythrocytes increase intercellular adhesion molecule 1 expression on brain endothelium through NF-kappaB. *Infect Immun,* 74**,** 3262-70.

TRIPATHI, A. K., SULLIVAN, D. J. & STINS, M. F. 2007. Plasmodium falciparum-infected erythrocytes decrease the integrity of human blood-brain barrier endothelial cell monolayers. *J Infect Dis,* 195**,** 942-50.

TURNER, G. D., LY, V. C., NGUYEN, T. H., TRAN, T. H., NGUYEN, H. P., BETHELL, D., WYLLIE, S., LOUWRIER, K., FOX, S. B., GATTER, K. C., DAY, N. P., TRAN, T. H., WHITE, N. J. & BERENDT, A. R. 1998. Systemic endothelial activation occurs in both mild and severe malaria. Correlating dermal microvascular endothelial cell phenotype and soluble cell adhesion molecules with disease severity. *Am J Pathol,* 152**,** 1477-87.

TURNER, G. D., MORRISON, H., JONES, M., DAVIS, T. M., LOOAREESUWAN, S., BULEY, I. D., GATTER, K. C., NEWBOLD, C. I., PUKRITAYAKAMEE, S., NAGACHINTA, B. & ET AL. 1994. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. *Am J Pathol,* 145**,** 1057-69.

TURNER, L., LAVSTSEN, T., BERGER, S. S., WANG, C. W., PETERSEN, J. E., AVRIL, M., BRAZIER, A. J., FREETH, J., JESPERSEN, J. S., NIELSEN, M. A., MAGISTRADO, P., LUSINGU, J., SMITH, J. D., HIGGINS, M. K. & THEANDER, T. G. 2013. Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature,* 498**,** 502-5.

TYBERGHEIN, A., DEROOST, K., SCHWARZER, E., ARESE, P. & VAN DEN STEEN, P. E. 2014. Immunopathological effects of malaria pigment or hemozoin and other crystals. *Biofactors,* 40**,** 59-78.

VAN DER HEYDE, H. C., NOLAN, J., COMBES, V., GRAMAGLIA, I. & GRAU, G. E. 2006. A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. *Trends Parasitol,* 22**,** 503-8.

VAN DER POUW KRAAN, T. C., BERNINK, F. J., YILDIRIM, C., KOOLWIJK, P., BAGGEN, J. M., TIMMERS, L., BEEK, A. M., DIAMANT, M., CHEN, W. J., VAN ROSSUM, A. C., VAN ROYEN, N., HORREVOETS, A. J. & APPELMAN, Y. E. 2014. Systemic toll-like receptor and interleukin-18 pathway activation in patients with acute ST elevation myocardial infarction. *J Mol Cell Cardiol,* 67**,** 94-102.

VERKMAN, A. S., RUIZ-EDERRA, J. & LEVIN, M. H. 2008. Functions of aquaporins in the eye. *Prog Retin Eye Res,* 27**,** 420-33.

VICENT, S., GARAYOA, M., LOPEZ-PICAZO, J. M., LOZANO, M. D., TOLEDO, G., THUNNISSEN, F. B., MANZANO, R. G. & MONTUENGA, L. M. 2004. Mitogen-activated protein kinase phosphatase-1 is overexpressed in non-small cell lung

cancer and is an independent predictor of outcome in patients. *Clin Cancer Res,* 10**,** 3639-49.

VIEBIG, N. K., GAMAIN, B., SCHEIDIG, C., LEPOLARD, C., PRZYBORSKI, J., LANZER, M., GYSIN, J. & SCHERF, A. 2005a. A single member of the Plasmodium falciparum var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep,* 6**,** 775-81.

VIEBIG, N. K., WULBRAND, U., FORSTER, R., ANDREWS, K. T., LANZER, M. & KNOLLE, P. A. 2005b. Direct activation of human endothelial cells by Plasmodium falciparum-infected erythrocytes. *Infect Immun,* 73**,** 3271-7.

VOGEL, C. F., WU, D., GOTH, S. R., BAEK, J., LOLLIES, A., DOMHARDT, R., GRINDEL, A. & PESSAH, I. N. 2013. Aryl hydrocarbon receptor signaling regulates NF-kappaB RelB activation during dendritic-cell differentiation. *Immunol Cell Biol,* 91**,** 568-75.

WALMET, P. S., ECKMAN, J. R. & WICK, T. M. 2003. Inflammatory mediators promote strong sickle cell adherence to endothelium under venular flow conditions. *Am J Hematol,* 73**,** 215-24.

WALTER, P. R., GARIN, Y. & BLOT, P. 1982. Placental pathologic changes in malaria. A histologic and ultrastructural study. *Am J Pathol,* 109**,** 330-42.

WANG, H. Y., CHENG, Z. & MALBON, C. C. 2003. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. *Cancer Lett,* 191**,** 229-37.

WANG, Q. & DOERSCHUK, C. M. 2000. Neutrophil-induced changes in the biomechanical properties of endothelial cells: roles of ICAM-1 and reactive oxygen species. *J Immunol,* 164**,** 6487-94.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WASSMER, S. C., CIANCIOLO, G. J., COMBES, V. & GRAU, G. E. 2006a. [LMP-420, a new therapeutic approach for cerebral malaria?]. *Med Sci (Paris),* 22**,** 343-5.

WASSMER, S. C., COMBES, V., CANDAL, F. J., JUHAN-VAGUE, I. & GRAU, G. E. 2006b. Platelets potentiate brain endothelial alterations induced by Plasmodium falciparum. *Infect Immun,* 74**,** 645-53.

WASSMER, S. C., DE SOUZA, J. B., FRERE, C., CANDAL, F. J., JUHAN-VAGUE, I. & GRAU, G. E. 2006c. TGF-beta1 released from activated platelets can induce TNF-stimulated human brain endothelium apoptosis: a new mechanism for microvascular lesion during cerebral malaria. *J Immunol,* 176**,** 1180-4.

WASSMER, S. C., LEPOLARD, C., TRAORE, B., POUVELLE, B., GYSIN, J. & GRAU, G. E. 2004. Platelets reorient Plasmodium falciparum-infected erythrocyte cytoadhesion to activated endothelial cells. *J Infect Dis,* 189**,** 180-9.

WASSMER, S. C., MOXON, C. A., TAYLOR, T., GRAU, G. E., MOLYNEUX, M. E. & CRAIG, A. G. 2011. Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. *Cell Microbiol,* 13**,** 198-209.

WAUTIER, J. L., PINTIGNY, D., WAUTIER, M. P., PATON, R. C., GALACTEROS, F., PASSA, P. & CAEN, J. P. 1983. Fibrinogen, a modulator of erythrocyte adhesion to vascular endothelium. *J Lab Clin Med,* 101**,** 911-20.

WAUTIER, J. L. & SCHMIDT, A. M. 2004. Protein glycation: a firm link to endothelial cell dysfunction. *Circ Res,* 95**,** 233-8.

WAUTIER, J. L. & WAUTIER, M. P. 2004. Erythrocytes and platelet adhesion to endothelium are mediated by specialized molecules. *Clin Hemorheol Microcirc,* 30**,** 181-4.

WHITE, N. J., PUKRITTAYAKAMEE, S., HIEN, T. T., FAIZ, M. A., MOKUOLU, O. A. & DONDORP, A. M. 2014. Malaria. *Lancet,* 383**,** 723-35.

WILLIAM, T., RAHMAN, H. A., JELIP, J., IBRAHIM, M. Y., MENON, J., GRIGG, M. J., YEO, T. W., ANSTEY, N. M. & BARBER, B. E. 2013. Increasing incidence of

Plasmodium knowlesi malaria following control of P. falciparum and P. vivax Malaria in Sabah, Malaysia. *PLoS Negl Trop Dis,* 7**,** e2026.

WU, Y., SZESTAK, T., STINS, M. & CRAIG, A. G. 2011. Amplification of P. falciparum Cytoadherence through induction of a pro-adhesive state in host endothelium. *PLoS One,* 6**,** e24784.

XIA, H., YE, J., BAI, H. & WANG, C. 2013. [Effects of cetuximab combined with celecoxib on apoptosis and KDR and AQP1 expression in lung cancer]. *Zhongguo Fei Ai Za Zhi,* 16**,** 625-31.

XIE, Y. F., SHU, R., JIANG, S. Y., LIU, D. L. & ZHANG, X. L. 2011. Comparison of microRNA profiles of human periodontal diseased and healthy gingival tissues. *Int J Oral Sci,* 3**,** 125-34.

YE, M., SANCHEZ, H. M., HULTZ, M., YANG, Z., BOGORAD, M., WONG, A. D. & SEARSON, P. C. 2014. Brain microvascular endothelial cells resist elongation due to curvature and shear stress. *Sci Rep,* 4**,** 4681.

YIPP, B. G., ANAND, S., SCHOLLAARDT, T., PATEL, K. D., LOOAREESUWAN, S. & HO, M. 2000. Synergism of multiple adhesion molecules in mediating cytoadherence of Plasmodium falciparum-infected erythrocytes to microvascular endothelial cells under flow. *Blood,* 96**,** 2292-8.

YIPP, B. G., HICKEY, M. J., ANDONEGUI, G., MURRAY, A. G., LOOAREESUWAN, S., KUBES, P. & HO, M. 2007. Differential roles of CD36, ICAM-1, and P-selectin in Plasmodium falciparum cytoadherence in vivo. *Microcirculation,* 14**,** 593-602.

YIPP, B. G., ROBBINS, S. M., RESEK, M. E., BARUCH, D. I., LOOAREESUWAN, S. & HO, M. 2003. Src-family kinase signaling modulates the adhesion of Plasmodium falciparum on human microvascular endothelium under flow. *Blood,* 101**,** 2850-7.

YOUNG, L., SUNG, J., STACEY, G. & MASTERS, J. R. 2010. Detection of Mycoplasma in cell cultures. *Nat Protoc,* 5**,** 929-34.

YOZA, B. K., HU, J. Y., COUSART, S. L., FORREST, L. M. & MCCALL, C. E. 2006. Induction of RelB participates in endotoxin tolerance. *J Immunol,* 177**,** 4080-5.

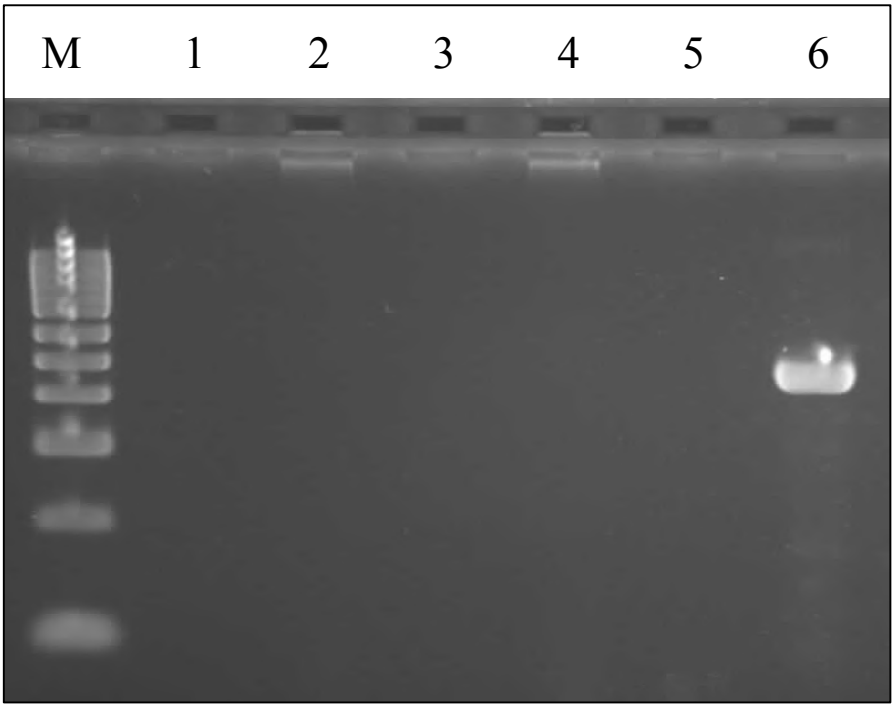
ZHANG, J. G., LI, X. Y., WANG, Y. Z., ZHANG, Q. D., GU, S. Y., WU, X., ZHU, G. H., LI, Q. & LIU, G. L. 2014. ROCK is involved in vasculogenic mimicry formation in hepatocellular carcinoma cell line. *PLoS One,* 9**,** e107661.

ZHAO, S., GUO, Y., SHENG, Q. & SHYR, Y. 2014. Advanced heat map and clustering analysis using heatmap3. *Biomed Res Int,* 2014**,** 986048.

ZHOU, J. & CHNG, W. J. 2013. Roles of thioredoxin binding protein (TXNIP) in oxidative stress, apoptosis and cancer. *Mitochondrion,* 13**,** 163-9.

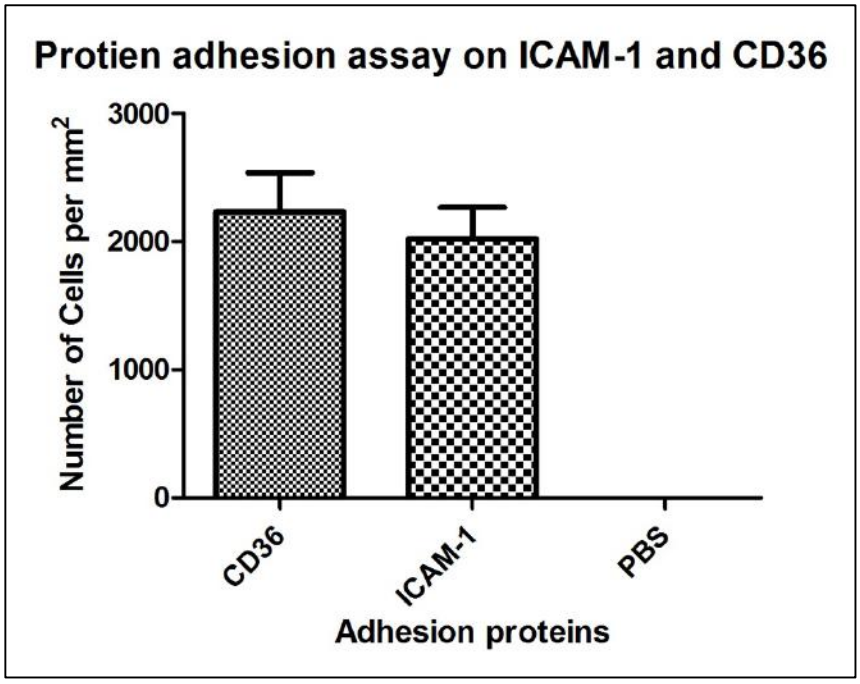
APPENDICES

Appendix1:



Screening for mycoplasma infection in IT4var14 parasite and HBMEC.
Lane M, Promega pGEM® DNA Markers (Cat.# G1741); lane 1, IT4var14 parasite; lane 2, HBMEC; lane 3, HBMEC growth media; lane 4, Parasite growth media; lane 5, -ve control; lane 6, +ve control.

Appendix 2:



Static Adhesion of IT4var14 parasite to ICAM-1 and CD36.
2 µl spots of 50 µg/ml ICAM-1, CD36 and PBS were placed onto 60 mm dishes and standard protein static binding assays carried out with iRBC suspended in binding buffer at a haematocrit of 1% and a parasitemia of 3%. The outcomes show the mean of binding and the bars represent standard error (SE).

Appendix 3:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 2h	Log_2 Fold Change	P-value	FDR
ENSG00000185101	ANO9	loanoctamin 9	chr11:417932-442011	0.0403979	0.782521	4.27578	0.0002	0.0322867
ENSG00000162551	ALPL	alkaline phosphatase, liver/bone/kidney	chr1:21509371-21578412	0.149733	1.06215	7	0.00035	0.0466528
ENSG00000215218	UBE2QL1	ubiquitin conjugating enzyme E2 Q family like 1	chr5:6448622-6494909	0.146029	0.895465	2.61638	0.00035	0.0466528
ENSG00000105246	EBI3	Epstein-Barr virus induced 3	chr19:4229497-4237531	0.662777	3.51066	2.40515	0.0002	0.0322867
ENSG00000151136	BTBD11	BTB domain containing 11	chr12:107318412-107659642	0.96968	4.68185	2.2715	5e-05	0.0111762
ENSG00000143387	CTSK	cathepsin K	chr1:150796207-150808323	3.85458	17.9079	2.21595	5e-05	0.0111762
ENSG00000100767	PAPLN	papilin, proteoglycan-like sulfated glycoprotein	chr14:73237496-73274640	1.78988	8.2048	2.19661	5e-05	0.0111762
ENSG00000058085	LAMC2	laminin subunit gamma 2	chr1:183186237-183244900	2.53443	10.0178	1.98284	5e-05	0.0111762
ENSG00000068078	FGFR3	fibroblast growth factor receptor 3	chr4:1793306-1808872	0.617387	2.35091	1.92897	0.00035	0.0466528
ENSG00000008517	IL32	interleukin 32	chr16:3065296-3087100	67.7863	219.525	1.69532	5e-05	0.0111762
ENSG00000089505	CMTM1	CKLF like MARVEL transmembrane domain containing 1	chr16:66552562-66579137	0.782157	0.057398	-3.76839	5e-05	0.0111762
ENSG00000240583	AQP1	aquaporin 1	chr7:30651942-30925516	42.8405	3.62057	-.	5e-05	0.0111762
ENSG00000114315	HES1	hes family bHLH transcription factor 1	chr3:194136144-194138732	85.1669	8.44216	-3.33461	5e-05	0.0111762
ENSG00000157404	KIT	KIT proto-oncogene receptor tyrosine kinase	chr4:54657917-54740715	2.80319	0.454706	-2.62406	5e-05	0.0111762

ENSG00000099860	GADD45 B	growth arrest and DNA damage inducible beta	chr19:2476121-2478259	91.6759	15.5255	-2.5619	5e-05	0.0111762
ENSG00000136826	KLF4	Kruppel-like factor 4	chr9:107484851-107490482	6.1146	1.05327	-2.53738	5e-05	0.0111762
ENSG00000143816	WNT9A	Wnt family member 9A	chr1:227918655-227947898	2.07033	0.4154	-2.31729	5e-05	0.0111762
ENSG00000170345	FOS	FBJ murine osteosarcoma viral oncogene homolog	chr14:75278773-75282230	12.2643	2.60224	-2.23664	5e-05	0.0111762
ENSG00000155090	KLF10	Kruppel-like factor 10	chr8:102648778-102655902	22.8943	4.90231	-2.22345	5e-05	0.0111762
ENSG00000106852	LHX6	LIM homeobox 6	chr9:122202576-122229626	11.7219	2.58449	-2.18126	5e-05	0.0111762

A full description of top ten up and down regulated genes in HBMEC/TNF interacted with IT4var14 parasite at 2 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 2 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix 4:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 6h	Log_2 Fold Change	P-value	FDR
ENSG00000125730	C3	complement component 3	chr19:6677703-6737603	0.00336973	0.408815	6.92267	5e-05	0.013328
ENSG00000058085	LAMC2	laminin subunit gamma 2	chr1:183186237-183244900	2.6679	15.2133	2.51156	5e-05	0.013328
ENSG00000100767	PAPLN	papilin, proteoglycan-like sulfated glycoprotein	chr14:73237496-73274640	1.88296	10.2331	2.44217	5e-05	0.013328
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	3.93862	20.5203	2.38129	5e-05	0.013328
ENSG00000185352	HS6ST3	heparan sulfate 6-O-sulfotransferase 3	chr13:96090838-96839562	0.0938523	0.476322	2.34347	5e-05	0.013328
ENSG00000105246	EBI3	Epstein-Barr virus induced 3	chr19:4229497-4237531	0.697018	3.2518	2.22197	5e-05	0.013328
ENSG00000143387	CTSK	cathepsin K	chr1:150796207-150808323	4.06572	15.743	1.95312	5e-05	0.013328
ENSG00000003137	CYP26B1	cytochrome P450 family 26 subfamily B member 1	chr2:72129237-72148038	0.505962	1.78734	1.82071	0.0002	0.036154
ENSG00000118971	CCND2	cyclin D2	chr12:4248764-4305350	0.694087	2.11321	1.60624	5e-05	0.013328
ENSG00000116711	PLA2G4A	phospholipase A2 group IVA	chr1:186828952-186988981	8.92585	22.208	1.31502	5e-05	0.013328
ENSG00000115738	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	chr2:8666635-8684453	60.8568	3.42801	-4.14998	5e-05	0.013328
ENSG00000240583	AQP1	aquaporin 1 (Colton blood group)	chr7:30651942-30925516	45.1979	3.12909	-3.85244	5e-05	0.013328
ENSG00000127325	BEST3	bestrophin 3	chr12:69643359-69699476	0.410644	0.0464148	-3.14523	0.0001	0.0209693
ENSG00000125968	ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	chr20:31605282-31606515	559.984	88.4692	-2.66214	5e-05	0.013328

ENSG00000170345	FOS	FBJ murine osteosarcoma viral oncogene homolog	chr14:75278773-75282230	12.9083	2.12447	-2.60312	5e-05	0.013328
ENSG00000188536	HBA2	hemoglobin subunit alpha 2	chr16:172846-173710	30.8493	5.11327	-2.59292	5e-05	0.013328
ENSG00000136826	KLF4	Kruppel-like factor 4 (gut)	chr9:107484851-107490482	6.45825	1.11226	-2.53765	5e-05	0.013328
ENSG00000206172	HBA1	hemoglobin subunit alpha 1	chr16:176679-177522	9.21904	1.73907	-2.4063	0.0001	0.0209693
ENSG00000160180	TFF3	trefoil factor 3	chr21:42311666-42315651	2.87366	0.629691	-2.19017	0.0002	0.036154
ENSG00000114315	HES1	hes family bHLH transcription factor 1	chr3:194136144-194138732	89.9483	22.1294	-2.02313	5e-05	0.013328

A full description of top ten up and down regulated genes in HBMEC/TNF interacted with IT4var14parasite at 6 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 6 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix 5:

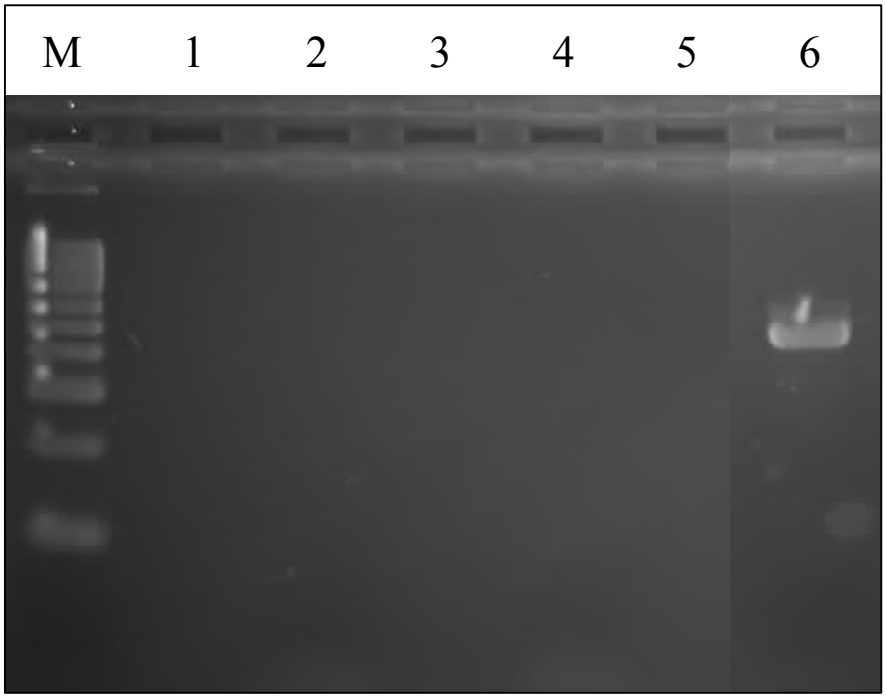
Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 20h	Log_2 Fold Change	P-value	FDR
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	3.76103	46.2772	3.6211	5e-05	0.00818138
ENSG00000171864	PRND	prion protein 2	chr20:4721909-4728460	0.0571954	0.622117	3.44322	0.00015	0.0176118
ENSG00000162777	DENND2D	DENN domain containing 2D	chr1:111187173-111243440	0.0805743	0.595492	2.88569	0.0002	0.021817
ENSG00000162692	VCAM1	vascular cell adhesion molecule 1	chr1:100719741-100739045	0.533853	3.25258	2.60707	5e-05	0.00818138
ENSG00000115009	CCL20	C-C motif chemokine ligand 20	chr2:227813841-227817564	0.234889	1.40141	2.57683	0.0004	0.0339912
ENSG00000265972	TXNIP	thioredoxin interacting protein	chr1:145992434-145996600	11.1227	66.2085	2.57351	5e-05	0.00818138
ENSG00000114812	VIPR1	vasoactive intestinal peptide receptor 1	chr3:42489298-42537573	0.0766432	0.450533	2.5554	5e-05	0.00818138
ENSG00000006210	CX3CL1	C-X3-C motif chemokine ligand 1	chr16:57372457-57385048	0.206721	1.14051	2.46392	5e-05	0.00818138
ENSG00000100767	PAPLN	papilin, proteoglycan-like sulfated glycoprotein	chr14:73237496-73274640	1.79858	9.7613	2.44021	5e-05	0.00818138
ENSG00000272636	DOC2B	double C2 domain beta	chr17:142788-181636	0.173891	0.906035	2.38138	5e-05	0.00818138
ENSG00000183691	NOG	noggin	chr17:56593698-56595590	5.49245	0.134192	-5.35508	5e-05	0.00818138
ENSG00000115738	ID2	inhibitor of DNA binding 2	chr2:8666635-8684453	58.1397	4.92853	-3.56029	5e-05	0.00818138
ENSG00000188536	HBA2	hemoglobin subunit alpha 2	chr16:172846-173710	29.3289	4.74045	-2.62923	5e-05	0.00818138
ENSG00000198673	FAM19A2	family with sequence similarity 19 (chemokine (C-C motif)-like), member A2	chr12:61708258-62417431	0.576024	0.0976469	-2.56048	0.0006	0.0433268

ENSG00000244734	HBB	hemoglobin subunit beta	chr11:5225463-5229395	51.6099	9.63441	-2.42138	5e-05	0.00818138
ENSG00000125968	ID1	inhibitor of DNA binding 1	chr20:31605282-31606515	534.501	107.681	-2.31143	5e-05	0.00818138
ENSG00000135547	HEY2	hes related family bHLH transcription factor with YRPW motif 2	chr6:125578557-125761269	4.14179	0.864693	-2.25999	5e-05	0.00818138
ENSG00000099860	GADD45 B	growth arrest and DNA damage inducible beta	chr19:2476121-2478259	91.8943	20.5668	-2.15966	5e-05	0.00818138
ENSG00000165899	OTOGL	otogelin like	chr12:80209452-80379090	0.63461	0.14639	-2.11605	0.00035	0.0311176
ENSG00000168874	ATOH8	atonal bHLH transcription factor 8	chr2:85751343-85788066	29.1582	6.75063	-2.11081	5e-05	0.00818138

A full description of top ten up and down regulated genes in HBMEC/TNF interacted with IT4var14 parasite at 20 hours.

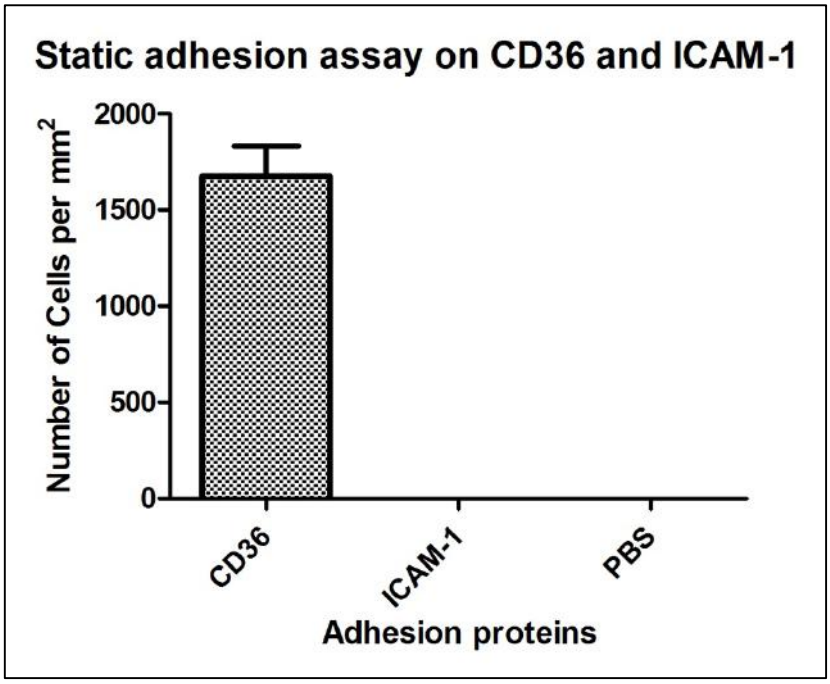
The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 20 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix 6:



Screening for mycoplasma infection in IT4var37 parasite and HBMEC.
Lane M, Promega pGEM® DNA Markers (Cat.# G1741); lane 1, IT4var37 parasite; lane 2, HBMEC; lane 3, HBMEC growth media; lane 4, Parasite growth media; lane 5, -ve control; lane 6, +ve control.

Appendix 7:



Static Adhesion of IT4var37 parasite to CD36 and ICAM-1.
2 µl spots of 50 µg/ml CD36, ICAM-1 and PBS were placed onto 60 mm dishes and standard protein static binding assays carried out with iRBC suspended in binding buffer at a haematocrit of 1% and a parasitemia of 3%. The outcomes show the mean of binding and the bars represent standard error (SE).

Appendix 8:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 6h	Log_2 Fold Change	P-value	FDR
ENSG00000171246	NPTX1	neuronal pentraxin 1	chr17:80467147-80477843	0.321097	5.1817	4.01234	5e-05	0.00137067
ENSG00000109846	CRYAB	crystallin alpha B	chr11:111908564-111926872	0.0713443	1.01792	3.83469	5e-05	0.00137067
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	4.39485	43.3712	3.30285	5e-05	0.00137067
ENSG00000055955	ITIH4	inter-alpha-trypsin inhibitor heavy chain family member 4	chr3:52812974-52897596	0.211062	1.51545	2.84401	0.00105	0.0188944
ENSG00000171227	TMEM37	transmembrane protein 37	chr2:119429900-119438520	0.315049	2.025	2.68428	0.0011	0.0195588
ENSG00000010319	SEMA3G	semaphorin 3G	chr3:52433052-52445085	0.552651	3.04747	2.46317	5e-05	0.00137067
ENSG00000221866	PLXNA4	plexin A4	chr7:132123331-132648688	0.600831	3.27809	2.44782	5e-05	0.00137067
ENSG00000099338	CATSPERG	cation channel sperm associated auxiliary subunit gamma	chr19:38335774-38370943	0.22432	1.0911	2.28216	0.0011	0.0195588
ENSG00000139187	KLRG1	killer cell lectin like receptor G1	chr12:8950043-9010760	0.829622	4.02734	2.2793	5e-05	0.00137067
ENSG00000092068	SLC7A8	solute carrier family 7 member 8	chr14:23125294-23183674	0.929502	4.31291	2.21413	5e-05	0.00137067
ENSG00000159166	LAD1	ladinin 1	chr1:201359007-201401190	2.54089	0.0713469	-5.15434	5e-05	0.00137067
ENSG00000164400	CSF2	colony stimulating factor 2	chr5:132073789-132076170	346.736	9.82972	-5.14055	5e-05	0.00137067
ENSG00000189283	FHIT	fragile histidine triad	chr3:59749309-61251459	7.18337	0.222274	-5.01425	5e-05	0.00137067
ENSG00000163734	CXCL3	C-X-C motif chemokine ligand 3	chr4:74036588-74038807	516.207	21.0543	-4.61576	5e-05	0.00137067

ENSG00000163121	NEURL3	neuralized E3 ubiquitin protein ligase 3	chr2:96497642-96508109	26.9812	1.32155	-4.35165	5e-05	0.00137067
ENSG00000163082	SGPP2	sphingosine-1-phosphate phosphatase 2	chr2:222424516-222560948	1.66013	0.0883764	-4.23149	5e-05	0.00137067
ENSG00000198535	C2CD4A	C2 calcium dependent domain containing 4A	chr15:62066976-62070917	5.71198	0.360262	-3.98688	5e-05	0.00137067
ENSG00000108342	CSF3	colony stimulating factor 3	chr17:40015360-40017813	411.958	30.1414	-3.77268	5e-05	0.00137067
ENSG00000006210	CX3CL1	C-X3-C motif chemokine ligand 1	chr16:57372457-57385048	504.14	37.4382	-3.75124	5e-05	0.00137067
ENSG00000123610	TNFAIP6	TNF alpha induced protein 6	chr2:151357591-151380048	23.0279	1.73143	-3.73335	5e-05	0.00137067

A full description of top ten up and down regulated genes in HBMEC/TNF interacted with IT4var37 parasite at 6 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 6 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix 9:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 20h	Log_2 Fold Change	P-value	FDR
ENSG00000129214	SHBG	sex hormone binding globulin	chr17:7561874-7633383	0.208592	13.4055	6.00599	0.00515	0.0347625
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	4.4154	119.122	4.75375	5e-05	0.000799012
ENSG00000132470	ITGB4	integrin subunit beta 4	chr17:75721327-75765711	0.428689	9.7628	4.50929	5e-05	0.000799012
ENSG00000122432	SPATA1	spermatogenesis associated 1	chr1:84498324-84574480	0.0721602	1.57334	4.44648	0.0006	0.00655469
ENSG00000240583	AQP1	aquaporin 1 (Colton blood group)	chr7:30651942-30925516	0.591723	10.1785	4.10446	5e-05	0.000799012
ENSG00000167244	IGF2	insulin like growth factor 2	chr11:2129111-2161341	0.0499515	0.767225	3.94105	5e-05	0.000799012
ENSG00000101115	SALL4	spalt like transcription factor 4	chr20:51782330-51802520	0.195634	2.50621	3.67928	0.00015	0.00206973
ENSG00000167748	KLK1	kallikrein 1	chr19:50819147-50823787	0.18301	2.23133	3.60791	0.0013	0.0119027
ENSG00000171227	TMEM37	transmembrane protein 37	chr2:119429900-119438520	0.316035	3.56295	3.49492	5e-05	0.000799012
ENSG00000068831	RASGRP2	RAS guanyl releasing protein 2	chr11:64726910-64745456	0.430621	4.09626	3.24982	5e-05	0.000799012
ENSG00000164400	CSF2	colony stimulating factor 2	chr5:132073789-132076170	348.492	1.57203	-7.79235	5e-05	0.000799012
ENSG00000007908	SELE	selectin E	chr1:169662006-169894267	1043.6	8.67812	-6.90996	5e-05	0.000799012
ENSG00000232070	TMEM253	transmembrane protein 253	chr14:21016762-21104722	3.0488	0.0268986	-6.82457	0.0018	0.0154996
ENSG00000163734	CXCL3	C-X-C motif chemokine ligand 3	chr4:74036588-74038807	518.849	4.89534	-6.72776	5e-05	0.000799012
ENSG00000108342	CSF3	colony stimulating factor 3	chr17:40015360-40017813	414.151	4.5349	-6.51294	5e-05	0.000799012

ENSG00000198535	C2CD4A	C2 calcium dependent domain containing 4A	chr15:62066976-62070917	5.74157	0.0749541	-6.25929	0.00265	0.0210758
ENSG00000227507	LTB	lymphotoxin beta	chr6:31580524-31582522	143.925	2.12652	-6.08068	5e-05	0.000799012
ENSG00000163121	NEURL3	neuralized E3 ubiquitin protein ligase 3	chr2:96497642-96508109	27.1193	0.640732	-5.40346	5e-05	0.000799012
ENSG00000123610	TNFAIP6	TNF alpha induced protein 6	chr2:151357591-151380048	23.1399	0.547297	-5.40192	5e-05	0.000799012
ENSG00000171236	LRG1	leucine rich alpha-2-glycoprotein 1	chr19:4522530-4540474	5.9073	0.154951	-5.25261	5e-05	0.000799012

A full description of top ten up and down regulated genes in HBMEC/ TNF interacted with IT4var37 parasite at 20 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 20 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix 10:

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Aging	6	3.5E-3	5.1E0
Negative regulation of cell proliferation	8	1.1E-2	1.5E1
Response to drug	6	3.9E-2	4.5E1
Ovulation from ovarian follicle	2	4.2E-2	4.7E1
Regulation of insulin-like growth factor receptor signaling pathway	2	4.2E-2	4.7E1
Negative regulation of platelet activation	2	4.8E-2	5.2E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Inflammatory response	44	2.3E-27	3.9E-24
Chemokine-mediated signaling pathway	16	3.1E-14	5.2E-11
Immune response	30	7.1E-13	1.2E-9
Chemotaxis	17	9.7E-12	1.6E-8
Response to lipopolysaccharide	19	1.0E-11	1.7E-8
Cellular response to lipopolysaccharide	16	3.7E-11	6.2E-8
Signal transduction	42	1.6E-8	2.6E-5
Cell chemotaxis	11	1.7E-8	2.9E-5
Positive regulation of NF-kappab transcription factor activity	14	3.4E-8	5.7E-5
Cellular response to interleukin-1	10	5.0E-7	8.4E-4
Positive regulation of inflammatory response	10	6.3E-7	1.1E-3
Positive regulation of leukocyte chemotaxis	6	3.3E-6	5.6E-3
Defense response	9	3.5E-6	5.8E-3
Lipopolysaccharide-mediated signaling pathway	7	4.1E-6	6.8E-3
Cell-cell signaling	15	1.1E-5	1.8E-2
Aging	12	1.7E-5	2.8E-2
Cell adhesion	20	1.8E-5	3.1E-2
Cellular response to tumor necrosis factor	10	2.0E-5	3.3E-2
Neutrophil chemotaxis	8	3.1E-5	5.3E-2
Apoptotic process	22	3.4E-5	5.7E-2

Positive regulation of gene expression	14	6.6E-5	1.1E-1
Defense response to virus	11	9.0E-5	1.5E-1
I-kappab kinase/NF-kappab signaling	7	1.6E-4	2.7E-1
Positive regulation of transcription from RNA polymerase II promoter	29	1.7E-4	2.9E-1
Response to molecule of bacterial origin	4	2.0E-4	3.3E-1
Nucleotide-binding oligomerization domain containing signaling pathway	5	3.4E-4	5.7E-1
Leukocyte migration involved in inflammatory response	4	3.8E-4	6.4E-1
Single organismal cell-cell adhesion	8	4.6E-4	7.8E-1
Lymphocyte chemotaxis	5	5.3E-4	8.9E-1
Positive regulation of nitric-oxide synthase biosynthetic process	4	6.5E-4	1.1E0
Positive regulation of ERK1 and ERK2 cascade	10	6.8E-4	1.1E0
Negative regulation of inflammatory response	7	7.3E-4	1.2E0
Response to virus	8	7.8E-4	1.3E0
Regulation of cell proliferation	10	1.0E-3	1.7E0
Protein kinase B signaling	5	1.0E-3	1.7E0
Humoral immune response	6	1.1E-3	1.8E0
Negative regulation of cell proliferation	15	1.1E-3	1.9E0
Tumor necrosis factor-mediated signaling pathway	8	1.2E-3	2.0E0
Response to muscle stretch	4	1.2E-3	2.0E0
Positive regulation of I-kappab kinase/NF-kappab signaling	9	1.7E-3	2.8E0
Regulation of inflammatory response	6	1.7E-3	2.8E0
Type I interferon signaling pathway	6	1.8E-3	3.0E0
Nucleotide-binding oligomerization domain containing 2 signaling pathway	3	1.8E-3	3.0E0
Myeloid dendritic cell differentiation	4	2.1E-3	3.4E0
Monocyte chemotaxis	5	2.5E-3	4.1E0
Positive regulation of nitric oxide biosynthetic process	5	2.7E-3	4.5E0
G-protein coupled receptor signaling pathway	24	2.8E-3	4.6E0
Interferon-gamma-mediated signaling pathway	6	2.8E-3	4.7E0
Positive regulation of neutrophil chemotaxis	4	3.2E-3	5.2E0

Positive regulation of interleukin-6 production	5	3.2E-3	5.3E0
Positive regulation of interleukin-10 production	4	3.6E-3	5.9E0
Positive regulation of tumor necrosis factor production	5	3.8E-3	6.2E0
Leukocyte cell-cell adhesion	4	4.6E-3	7.5E0
Positive regulation of cell proliferation	15	4.9E-3	7.9E0
Positive regulation of angiogenesis	7	4.9E-3	8.0E0
Positive regulation of peptidyl-tyrosine phosphorylation	6	5.3E-3	8.5E0
Positive regulation of interferon-beta production	4	5.8E-3	9.2E0
Innate immune response	14	6.2E-3	1.0E1
Cellular response to lipoteichoic acid	3	6.3E-3	1.0E1
Hyperosmotic response	3	6.3E-3	1.0E1
Negative regulation of smooth muscle cell proliferation	4	7.0E-3	1.1E1
Face morphogenesis	4	7.7E-3	1.2E1
Regulation of tumor necrosis factor-mediated signaling pathway	4	7.7E-3	1.2E1
Negative regulation of blood pressure	4	7.7E-3	1.2E1
Negative regulation of ERK1 and ERK2 cascade	5	8.0E-3	1.3E1
Cellular response to organic cyclic compound	5	8.5E-3	1.3E1
Positive regulation of T cell proliferation	5	9.0E-3	1.4E1
Cytokine-mediated signaling pathway	7	9.2E-3	1.4E1
Response to antibiotic	4	9.3E-3	1.5E1
Response to hypoxia	8	9.4E-3	1.5E1
Response to muramyl dipeptide	3	9.4E-3	1.5E1
Positive regulation of macrophage chemotaxis	3	9.4E-3	1.5E1
Neutrophil mediated immunity	3	9.4E-3	1.5E1
Negative regulation of apoptotic process	14	9.8E-3	1.5E1
Response to interleukin-1	4	1.0E-2	1.6E1
Positive regulation of gtpase activity	16	1.1E-2	1.7E1
Extrinsic apoptotic signaling pathway in absence of ligand	4	1.1E-2	1.7E1
Positive regulation of leukocyte migration	3	1.1E-2	1.7E1
Negative regulation of gene expression	7	1.1E-2	1.7E1

Positive regulation of protein secretion	4	1.3E-2	2.0E1
Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	4	1.4E-2	2.1E1
Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	5	1.5E-2	2.2E1
Positive regulation of interleukin-2 production	3	1.5E-2	2.3E1
Activation of MAPK activity	6	1.6E-2	2.3E1
Response to progesterone	4	1.6E-2	2.4E1
Negative regulation of NF-kappab transcription factor activity	5	1.6E-2	2.4E1
Negative regulation of viral genome replication	4	1.7E-2	2.5E1
Regulation of I-kappab kinase/NF-kappab signaling	3	1.7E-2	2.5E1
Positive regulation of cellular protein metabolic process	3	1.7E-2	2.5E1
Extracellular matrix organization	8	1.8E-2	2.6E1
Positive regulation of DNA replication	4	1.9E-2	2.8E1
Positive regulation of monocyte chemotaxis	3	2.0E-2	2.8E1
Positive regulation of macrophage derived foam cell differentiation	3	2.0E-2	2.8E1
Establishment of endothelial barrier	3	2.0E-2	2.8E1
Positive regulation of chemokine production	3	2.2E-2	3.1E1
Toll-like receptor 4 signaling pathway	3	2.5E-2	3.4E1
Positive regulation of interferon-gamma production	4	2.5E-2	3.4E1
Divalent metal ion transport	2	2.7E-2	3.7E1
Nucleotide-binding oligomerization domain containing 1 signaling pathway	2	2.7E-2	3.7E1
Activation of MAPK activity involved in innate immune response	2	2.7E-2	3.7E1
T cell proliferation	3	2.7E-2	3.7E1
Defense response to protozoan	3	2.7E-2	3.7E1
Muscle cell cellular homeostasis	3	2.7E-2	3.7E1
Positive regulation of protein kinase B signaling	5	2.8E-2	3.8E1
Calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules	3	3.3E-2	4.3E1
Positive regulation of NF-kappab import into nucleus	3	3.3E-2	4.3E1
Angiogenesis	8	3.3E-2	4.3E1

Muscle organ development	5	3.3E-2	4.4E1
Positive regulation of translation	4	3.6E-2	4.6E1
Positive regulation of cell-matrix adhesion	3	3.6E-2	4.6E1
Cellular response to cytokine stimulus	3	3.6E-2	4.6E1
Negative regulation of peptidyl-serine phosphorylation	3	3.9E-2	4.9E1
Response to bacterium	3	3.9E-2	4.9E1
Negative regulation of toll-like receptor 3 signaling pathway	2	4.0E-2	5.0E1
Cytokine secretion involved in immune response	2	4.0E-2	5.0E1
Positive regulation of mononuclear cell proliferation	2	4.0E-2	5.0E1
Positive regulation of cellular extravasation	2	4.0E-2	5.0E1
T cell antigen processing and presentation	2	4.0E-2	5.0E1
Positive regulation of cell migration	7	4.1E-2	5.0E1
Response to interferon-gamma	3	4.2E-2	5.1E1
Cellular response to hydrogen peroxide	4	4.3E-2	5.2E1
Cellular response to interferon-gamma	4	4.3E-2	5.2E1
Response to tumor necrosis factor	3	4.5E-2	5.4E1
Positive regulation of interleukin-12 production	3	4.5E-2	5.4E1
Positive regulation of interleukin-8 production	3	4.9E-2	5.7E1
Positive regulation of smooth muscle cell proliferation	4	4.9E-2	5.7E1
Transcription from RNA polymerase II promoter	13	4.9E-2	5.7E1
Positive regulation of transcription, DNA-templated	13	5.0E-2	5.8E1

Cellular Component – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Plasma membrane	37	1.7E-2	1.8E1
Integral component of membrane	44	1.9E-2	2.0E1
Cell junction	8	2.5E-2	2.6E1

Cellular Component – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**

Extracellular space	44	3.0E-8	3.7E-5
Integral component of plasma membrane	42	8.6E-7	1.1E-3
Cell surface	22	8.3E-6	1.0E-2
Plasma membrane	83	1.3E-5	1.7E-2
External side of plasma membrane	11	4.8E-4	6.0E-1
I-kappab/NF-kappab complex	3	1.6E-3	2.0E0
Extracellular region	36	1.7E-3	2.1E0
Perinuclear region of cytoplasm	18	3.1E-3	3.9E0
Membrane raft	9	5.5E-3	6.8E0
Alpha9-beta1 integrin-vascular cell adhesion molecule-1 complex	2	2.6E-2	2.8E1
Mitochondrial outer membrane	6	4.5E-2	4.4E1

Molecular Function – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Transmembrane receptor protein tyrosine phosphatase activity	3	4.8E-3	6.0E0
Carbohydrate binding	5	3.3E-2	3.5E1
Amino acid transmembrane transporter activity	3	3.4E-2	3.6E1
Microtubule binding	5	4.0E-2	4.0E1
Insulin-like growth factor II binding	2	4.8E-2	4.7E1

Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Chemokine activity	12	4.4E-11	6.1E-8
Cytokine activity	15	1.3E-7	1.9E-4
CXCR chemokine receptor binding	5	4.0E-6	5.6E-3
Growth factor activity	12	1.4E-5	1.9E-2
Receptor binding	15	3.6E-4	5.0E-1
Receptor activity	10	3.0E-3	4.1E0
Protein self-association	5	3.5E-3	4.7E0
CCR chemokine receptor binding	4	3.6E-3	4.9E0
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	4	3.6E-3	4.9E0

Heparin binding	8	6.4E-3	8.5E0
Tumor necrosis factor receptor binding	4	7.0E-3	9.3E0
Identical protein binding	19	1.4E-2	1.8E1
Integrin binding	6	1.4E-2	1.8E1
Tumor necrosis factor-activated receptor activity	3	4.2E-2	4.5E1

Functions analysis enrichment result at 6 hours incubation of IT4var37 with HBMEC/TNF.

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

Appendix 11:

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Cholesterol biosynthetic process	6	1.2E-3	1.9E0
Positive regulation of insulin-like growth factor receptor signaling pathway	4	2.3E-3	3.8E0
Regulation of cell growth	7	7.0E-3	1.1E1
Drug transmembrane transport	4	7.1E-3	1.1E1
Regulation of insulin-like growth factor receptor signaling pathway	3	8.8E-3	1.4E1
Regulation of glucose metabolic process	4	1.1E-2	1.7E1
Phospholipid translocation	4	1.1E-2	1.7E1
Response to estradiol	7	1.3E-2	1.9E1
Cellular protein metabolic process	8	1.3E-2	1.9E1
Type B pancreatic cell proliferation	3	1.5E-2	2.2E1
Oxidation-reduction process	22	1.5E-2	2.3E1
Transmembrane transport	12	1.5E-2	2.3E1
G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	5	1.6E-2	2.4E1
Negative regulation of smooth muscle cell proliferation	4	2.3E-2	3.2E1
Positive regulation of MAPK cascade	6	2.9E-2	3.9E1
Negative regulation of camp biosynthetic process	3	3.0E-2	4.0E1
Isoprenoid biosynthetic process	3	3.4E-2	4.4E1
Smooth muscle hyperplasia	2	4.2E-2	5.1E1
Angiogenesis	10	4.9E-2	5.7E1
Regulation of blood pressure	5	4.9E-2	5.7E1
Intracellular signal transduction	15	4.9E-2	5.7E1
Response to fatty acid	3	4.9E-2	5.7E1
Monocyte differentiation	3	4.9E-2	5.7E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Inflammatory response	57	7.8E-18	1.4E-14
Defense response to virus	31	2.1E-12	3.8E-9

DNA replication	28	7.6E-11	1.4E-7
Type I interferon signaling pathway	18	2.8E-10	5.0E-7
Interferon-gamma-mediated signaling pathway	18	1.6E-9	2.9E-6
Immune response	44	1.2E-8	2.2E-5
Negative regulation of viral genome replication	13	2.7E-8	4.8E-5
Negative regulation of type I interferon production	11	1.3E-7	2.3E-4
Cell chemotaxis	15	1.9E-7	3.4E-4
Apoptotic process	50	2.4E-7	4.3E-4
Positive regulation of NF-kappab transcription factor activity	21	2.7E-7	4.9E-4
Response to virus	19	2.9E-7	5.3E-4
Chemotaxis	20	3.1E-7	5.5E-4
Chemokine-mediated signaling pathway	15	5.9E-7	1.1E-3
Positive regulation of neutrophil chemotaxis	9	1.1E-6	2.0E-3
Response to lipopolysaccharide	22	2.1E-6	3.7E-3
Cellular response to lipopolysaccharide	18	2.1E-6	3.8E-3
DNA replication initiation	10	2.8E-6	5.0E-3
Positive regulation of inflammatory response	14	5.0E-6	8.9E-3
Positive regulation of interleukin-6 production	11	7.9E-6	1.4E-2
Innate immune response	38	8.1E-6	1.5E-2
G1/S transition of mitotic cell cycle	16	1.1E-5	2.0E-2
Positive regulation of I-kappab kinase/NF-kappab signaling	20	2.1E-5	3.7E-2
Lipopolysaccharide-mediated signaling pathway	9	2.6E-5	4.7E-2
Defense response	12	5.8E-5	1.0E-1
Positive regulation of interferon-beta production	8	6.5E-5	1.2E-1
Cellular response to interferon-gamma	11	7.0E-5	1.3E-1
Positive regulation of tumor necrosis factor production	10	7.9E-5	1.4E-1
I-kappab kinase/NF-kappab signaling	11	1.1E-4	2.0E-1
Positive regulation of gene expression	25	1.2E-4	2.2E-1
Signal transduction	72	1.6E-4	2.8E-1
Regulation of inflammatory response	11	1.7E-4	3.0E-1

Positive regulation of ERK1 and ERK2 cascade	19	2.0E-4	3.6E-1
Regulation of transcription involved in G1/S transition of mitotic cell cycle	7	2.1E-4	3.8E-1
Regulation of cell proliferation	19	4.0E-4	7.2E-1
Cellular response to interleukin-1	11	4.6E-4	8.2E-1
Cellular response to organic cyclic compound	10	4.8E-4	8.6E-1
Positive regulation of leukocyte chemotaxis	6	5.3E-4	9.5E-1
Toll-like receptor signaling pathway	7	5.4E-4	9.7E-1
TRIF-dependent toll-like receptor signaling pathway	7	6.7E-4	1.2E0
Negative regulation of bone resorption	5	9.3E-4	1.7E0
Negative regulation of cell proliferation	30	1.1E-3	2.0E0
Toll-like receptor 2 signaling pathway	4	1.1E-3	2.0E0
Positive regulation of NF-kappab import into nucleus	6	1.1E-3	2.0E0
Positive regulation of fibroblast proliferation	9	1.2E-3	2.1E0
Cell-cell signaling	22	1.2E-3	2.1E0
Positive regulation of transcription from RNA polymerase II promoter	59	1.4E-3	2.5E0
Angiogenesis	20	1.4E-3	2.5E0
DNA duplex unwinding	8	1.6E-3	2.8E0
Negative regulation of NF-kappab transcription factor activity	10	1.9E-3	3.3E0
Positive regulation of DNA-directed DNA polymerase activity	4	1.9E-3	3.4E0
Response to type I interferon	4	1.9E-3	3.4E0
DNA synthesis involved in DNA repair	7	2.3E-3	4.0E0
Negative regulation of apoptotic process	32	2.4E-3	4.2E0
Positive regulation of T cell proliferation	9	2.4E-3	4.2E0
Positive regulation of smooth muscle cell proliferation	9	2.4E-3	4.2E0
Tumor necrosis factor-mediated signaling pathway	13	2.5E-3	4.3E0
Nucleotide-binding oligomerization domain containing signaling pathway	6	2.6E-3	4.6E0
Leukocyte cell-cell adhesion	6	2.6E-3	4.6E0
Cell proliferation	27	3.0E-3	5.2E0
DNA replication checkpoint	4	3.0E-3	5.2E0

Positive regulation of interleukin-8 production	6	3.1E-3	5.5E0
Positive regulation of type I interferon production	8	3.7E-3	6.5E0
Somitogenesis	7	4.0E-3	6.9E0
Cellular response to tumor necrosis factor	12	4.2E-3	7.2E0
Viral entry into host cell	10	4.2E-3	7.4E0
Response to molecule of bacterial origin	4	4.3E-3	7.5E0
Hyperosmotic response	4	4.3E-3	7.5E0
Negative regulation of protein ubiquitination	7	5.2E-3	8.9E0
Monocyte chemotaxis	7	5.8E-3	1.0E1
Positive regulation of DNA replication	7	5.8E-3	1.0E1
DNA damage checkpoint	6	6.0E-3	1.0E1
Fibroblast migration	4	6.0E-3	1.0E1
Positive regulation of chemokine biosynthetic process	4	6.0E-3	1.0E1
Positive regulation of T cell migration	4	6.0E-3	1.0E1
Positive regulation of cell migration	16	6.5E-3	1.1E1
Single organismal cell-cell adhesion	11	6.6E-3	1.1E1
Humoral immune response	8	7.0E-3	1.2E1
JAK-STAT cascade	6	7.9E-3	1.3E1
Leukocyte migration involved in inflammatory response	4	8.0E-3	1.3E1
Response to muramyl dipeptide	4	8.0E-3	1.3E1
Positive regulation of macrophage chemotaxis	4	8.0E-3	1.3E1
Immunoglobulin mediated immune response	4	8.0E-3	1.3E1
Positive regulation of apoptotic process	22	8.4E-3	1.4E1
Regulation of cell migration	9	8.7E-3	1.4E1
Positive regulation of cytokine secretion involved in immune response	3	8.9E-3	1.5E1
Negative regulation of macrophage apoptotic process	3	8.9E-3	1.5E1
Positive regulation of hyaluronan biosynthetic process	3	8.9E-3	1.5E1
Response to interleukin-1	6	9.0E-3	1.5E1
Myd88-dependent toll-like receptor signaling pathway	6	9.0E-3	1.5E1
Positive regulation of defense response to virus by	5	1.0E-2	1.7E1

host			
Positive regulation of interleukin-1 beta secretion	5	1.0E-2	1.7E1
Response to exogenous dsrna	6	1.0E-2	1.7E1
Positive regulation of interferon-alpha production	4	1.0E-2	1.7E1
Cell morphogenesis	8	1.1E-2	1.8E1
Cellular senescence	5	1.2E-2	1.9E1
Cell division	24	1.2E-2	2.0E1
DNA damage response, detection of DNA damage	6	1.3E-2	2.1E1
Translesion synthesis	6	1.3E-2	2.1E1
Myd88-independent toll-like receptor signaling pathway	4	1.3E-2	2.1E1
Regulation of cytokine production	4	1.3E-2	2.1E1
Positive regulation of nitric-oxide synthase biosynthetic process	4	1.3E-2	2.1E1
Positive regulation of interleukin-17 production	4	1.3E-2	2.1E1
Regulation of type I interferon production	4	1.3E-2	2.1E1
Regulation of innate immune response	4	1.3E-2	2.1E1
Response to interferon-gamma	5	1.4E-2	2.2E1
Nucleotide-excision repair, DNA gap filling	5	1.4E-2	2.2E1
Aging	14	1.4E-2	2.2E1
Detection of virus	3	1.4E-2	2.3E1
Intracellular transport of viral protein in host cell	3	1.4E-2	2.3E1
Nucleotide-binding oligomerization domain containing 2 signaling pathway	3	1.4E-2	2.3E1
Cellular response to interleukin-3	3	1.4E-2	2.3E1
Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	6	1.5E-2	2.3E1
NIK/NF-kappab signaling	8	1.5E-2	2.4E1
Cell adhesion	29	1.6E-2	2.5E1
Positive regulation of peptidyl-tyrosine phosphorylation	9	1.6E-2	2.5E1
Positive regulation of interleukin-12 production	5	1.6E-2	2.5E1
Response to tumor necrosis factor	5	1.6E-2	2.5E1
Positive regulation of protein tyrosine kinase activity	5	1.8E-2	2.8E1
Strand displacement	5	1.8E-2	2.8E1

Mitotic chromosome condensation	4	2.0E-2	3.0E1
DNA strand elongation involved in DNA replication	4	2.0E-2	3.0E1
Positive regulation of type 2 immune response	3	2.1E-2	3.2E1
Positive regulation of dendritic cell differentiation	3	2.1E-2	3.2E1
Regulation of apoptotic process	16	2.2E-2	3.3E1
Lymphocyte chemotaxis	5	2.3E-2	3.5E1
Response to muscle stretch	4	2.3E-2	3.5E1
Positive regulation of monocyte chemotaxis	4	2.3E-2	3.5E1
Positive regulation of chemokine production	4	2.8E-2	4.0E1
Positive regulation of cytokine-mediated signaling pathway	3	2.9E-2	4.1E1
Positive regulation of macrophage cytokine production	3	2.9E-2	4.1E1
Positive regulation of viral entry into host cell	3	2.9E-2	4.1E1
Antigen processing and presentation of endogenous peptide antigen via MHC class I	3	2.9E-2	4.1E1
Viral process	20	2.9E-2	4.1E1
Regulation of tumor necrosis factor-mediated signaling pathway	5	2.9E-2	4.1E1
Necroptotic process	4	3.2E-2	4.4E1
Toll-like receptor 4 signaling pathway	4	3.2E-2	4.4E1
Positive regulation of interferon-gamma production	6	3.4E-2	4.7E1
Cytokine-mediated signaling pathway	11	3.5E-2	4.7E1
Positive regulation of transcription, DNA-templated	30	3.6E-2	4.8E1
Defense response to protozoan	4	3.7E-2	4.9E1
Error-free translesion synthesis	4	3.7E-2	4.9E1
Myeloid dendritic cell differentiation	4	3.7E-2	4.9E1
Cellular response to zinc ion	4	3.7E-2	4.9E1
Error-prone translesion synthesis	4	3.7E-2	4.9E1
T cell proliferation	4	3.7E-2	4.9E1
Negative regulation of platelet activation	3	3.7E-2	4.9E1
Toll-like receptor 3 signaling pathway	3	3.7E-2	4.9E1
Neutrophil activation	3	3.7E-2	4.9E1

Positive regulation of protein ubiquitination	7	4.0E-2	5.2E1
Protein complex assembly	10	4.0E-2	5.2E1
Cell migration	13	4.1E-2	5.3E1
Positive regulation of MAPK cascade	8	4.1E-2	5.3E1
Positive regulation of smooth muscle cell migration	4	4.3E-2	5.4E1
Positive regulation of JNK cascade	7	4.3E-2	5.5E1
Protein stabilization	11	4.4E-2	5.5E1
Double-strand break repair	7	4.6E-2	5.7E1
Neutrophil chemotaxis	7	4.6E-2	5.7E1
Negative regulation of activated T cell proliferation	3	4.7E-2	5.8E1
Positive regulation of type I interferon-mediated signaling pathway	3	4.7E-2	5.8E1
Response to interferon-beta	3	4.7E-2	5.8E1
Positive regulation of T cell apoptotic process	3	4.7E-2	5.8E1
Cellular response to lipoteichoic acid	3	4.7E-2	5.8E1
Regulation of autophagy	6	4.7E-2	5.8E1
Stress-activated MAPK cascade	4	4.8E-2	5.9E1
Protein heterooligomerization	7	4.9E-2	5.9E1
Cell cycle	15	5.0E-2	6.0E1

Cellular Component – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Plasma membrane	109	6.8E-3	8.6E0
Vacuolar membrane	3	3.4E-2	3.7E1
Golgi membrane	20	4.8E-2	4.7E1

Cellular Component – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Cytoplasm	267	8.2E-8	1.2E-4
Membrane	127	3.4E-6	4.9E-3
MCM complex	6	9.5E-6	1.4E-2
Nucleoplasm	151	1.0E-5	1.4E-2
Cytosol	169	8.2E-5	1.2E-1

Extracellular space	79	2.2E-4	3.2E-1
Nucleus	252	3.8E-4	5.4E-1
Perinuclear region of cytoplasm	41	1.2E-3	1.7E0
Early endosome	20	1.5E-3	2.2E0
Cell surface	36	2.3E-3	3.2E0
Ctf18 RFC-like complex	4	2.8E-3	3.9E0
Replication fork	5	3.6E-3	4.9E0
Intracellular membrane-bounded organelle	36	3.6E-3	5.0E0
TAP complex	3	4.4E-3	6.1E0

Molecular Function – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Insulin-like growth factor I binding	5	8.1E-5	1.2E-1
Insulin-like growth factor II binding	4	4.7E-4	6.9E-1
Symporter activity	6	4.2E-3	6.0E0
Transmembrane transporter activity	5	1.9E-2	2.5E1
Enzyme inhibitor activity	4	2.4E-2	3.0E1
Collagen binding	5	3.7E-2	4.2E1

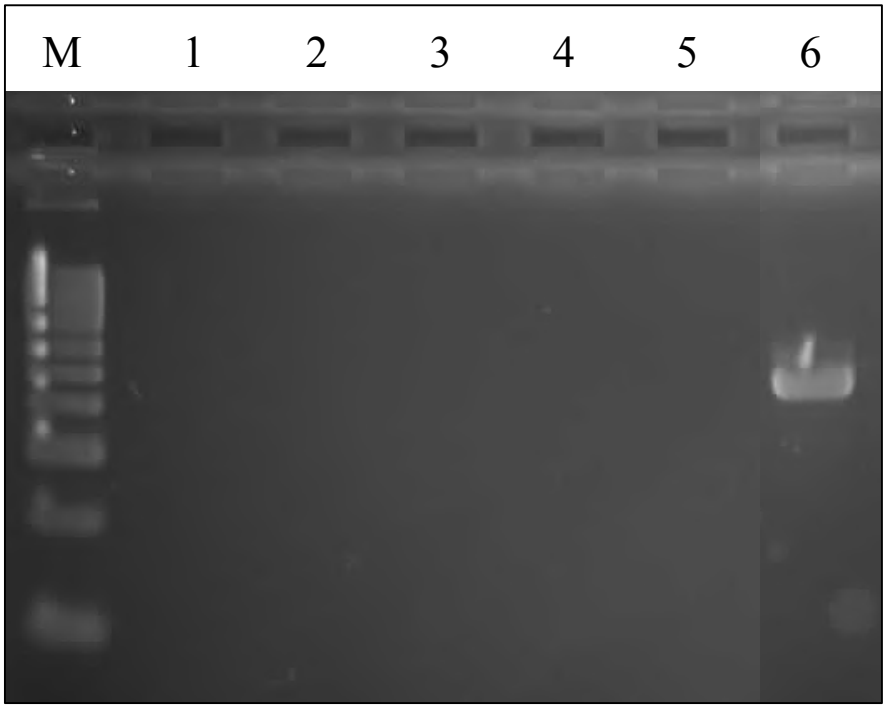
Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Protein binding	436	5.3E-11	8.1E-8
Chemokine activity	13	3.8E-7	5.8E-4
Cytokine activity	20	8.7E-5	1.3E-1
Double-stranded RNA binding	11	1.4E-4	2.2E-1
TAP1 binding	4	2.5E-4	3.8E-1
2'-5'-oligoadenylate synthetase activity	4	2.5E-4	3.8E-1
CXCR chemokine receptor binding	5	2.8E-4	4.2E-1
Cytokine receptor activity	8	4.9E-4	7.5E-1
Identical protein binding	50	5.6E-4	8.6E-1
DNA replication origin binding	5	6.8E-4	1.0E0
Growth factor activity	17	8.3E-4	1.3E0

DNA clamp loader activity	4	3.1E-3	4.7E0
TAP2 binding	3	4.7E-3	7.0E0
Peptide antigen-transporting atpase activity	3	4.7E-3	7.0E0
Tumor necrosis factor receptor binding	6	5.5E-3	8.1E0
Single-stranded DNA-dependent atpase activity	4	6.3E-3	9.2E0
Signal transducer activity	17	8.3E-3	1.2E1
Protein self-association	7	9.8E-3	1.4E1
Ubiquitin protein ligase binding	21	1.2E-2	1.7E1
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	5	1.2E-2	1.7E1
DNA helicase activity	5	1.4E-2	2.0E1
C3HC4-type RING finger domain binding	3	2.2E-2	2.9E1
Virus receptor activity	8	2.2E-2	2.9E1
4 iron, 4 sulfur cluster binding	6	2.6E-2	3.3E1
Single-stranded RNA binding	6	2.8E-2	3.5E1
Receptor activity	16	2.9E-2	3.7E1
Zinc ion binding	61	3.0E-2	3.7E1
ATP-dependent DNA helicase activity	5	3.8E-2	4.5E1
SUMO binding	3	3.8E-2	4.5E1
K63-linked polyubiquitin binding	4	3.9E-2	4.5E1
Actin filament binding	11	4.0E-2	4.7E1
Ligase activity	18	4.5E-2	5.1E1
Enzyme binding	21	4.8E-2	5.3E1

Functions analysis enrichment result at 20 hours incubation of IT4var37 with HBMEC/TNF.

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

Appendix12:



Screening for mycoplasma infection in IT4var14 parasite and HDMEC.
Lane M, Promega pGEM® DNA Markers (Cat.# G1741); lane 1, IT4var14 parasite; lane 2, HDMEC; lane 3, HDMEC growth media; lane 4, Parasite growth media; lane 5, -ve control; lane 6, +ve control.

Appendix13:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 2h	Log_2 Fold Change	P-value	FDR
ENSG00000138061	CYP1B1	cytochrome P450 family 1 subfamily B member 1	chr2:37923186-38239590	0.0559435	4.70835	6.39511	0.00035	0.0450679
ENSG00000182568	SATB1	SATB homeobox	chr3:17157161-18920401	0.190542	6.24003	5.03337	5e-05	0.01043
ENSG00000171246	NPTX1	neuronal pentraxin 1	chr17:80467147-80477843	0.645323	20.8499	5.01388	5e-05	0.01043
ENSG00000137834	SMAD6	SMAD family member 6	chr15:66702227-66782848	1.75016	56.0449	5.00102	5e-05	0.01043
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	6.51256	206.834	4.98911	5e-05	0.01043
ENSG00000171604	CXXC5	CXXC finger protein 5	chr5:139647298-139683882	3.15975	55.2379	4.12778	5e-05	0.01043
ENSG00000063438	AHRR	aryl-hydrocarbon receptor repressor	chr5:271620-438291	0.411271	4.77446	3.53718	5e-05	0.01043
ENSG00000107731	UNC5B	unc-5 netrin receptor B	chr10:71212569-71302864	1.68203	18.9589	3.49461	5e-05	0.01043
ENSG00000118200	CAMSAP 2	calmodulin regulated spectrin associated protein family member 2	chr1:200739557-200860704	2.56607	28.216	3.45888	0.0001	0.0182982
ENSG00000165795	NDRG2	NDRG family member 2	chr14:21016762-21104722	0.609393	5.81043	3.2532	5e-05	0.01043
ENSG00000185480	PARPBP	PARP1 binding protein	chr12:102120184-102480645	7.47861	0.471243	-3.98823	5e-05	0.01043
ENSG00000135365	PHF21A	PHD finger protein 21A	chr11:45929322-46121178	24.5969	2.40407	-3.35493	5e-05	0.01043
ENSG00000265972	TXNIP	thioredoxin interacting protein	chr1:145992434-145996600	70.4704	7.51412	-3.22934	5e-05	0.01043
ENSG00000131089	ARHGEF9	Cdc42 guanine nucleotide exchange factor 9p	chrX:63634966-63809274	12.0347	1.33333	-3.17409	0.0004	0.0485116

ENSG00000232810	TNF	tumor necrosis factor	chr6:31575566-31578336	25.7731	3.72734	-2.78965	0.0002	0.0311343
ENSG00000148926	ADM	adrenomedullin	chr11:10304679-10307397	285.044	44.673	-2.67371	5e-05	0.01043
ENSG00000165507	C10orf10	chromosome 10 open reading frame 10	chr10:44811023-44995891	108.482	18.8563	-2.52434	5e-05	0.01043
ENSG00000164400	CSF2	colony stimulating factor 2	chr5:132073789-132076170	133.908	24.209	-2.46762	5e-05	0.01043
ENSG00000185499	MUC1	mucin 1, cell surface associated	chr1:155169407-155192916	6.13405	1.29673	-2.24196	0.0001	0.0182982
ENSG00000188536	HBA2	hemoglobin subunit alpha 2	chr16:172846-173710	465.773	119.592	-1.9615	5e-05	0.01043

A full description of top ten up and down regulated genes in HDMEC/TNF interacted with IT4var14 parasite at 2 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 2 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix14:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 6h	Log_2 Fold Change	P-value	FDR
ENSG00000135678	CPM	carboxypeptidase M	chr12:68808171-68971570	0.0537127	5.77092	6.74739	5e-05	0.011
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	6.69851	412.925	5.94589	5e-05	0.011
ENSG00000171246	NPTX1	neuronal pentraxin 1	chr17:80467147-80477843	0.664586	24.5275	5.2058	5e-05	0.011
ENSG00000082014	SMARCD 3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	chr7:151232488-151277896	0.232953	4.98085	4.41828	5e-05	0.011
ENSG00000079102	RUNX1T1	RUNX1 translocation partner 1	chr8:91954966-92103286	0.449452	5.45003	3.60003	5e-05	0.011
ENSG00000115257	PCSK4	proprotein convertase subtilisin/kexin type 4	chr19:1481427-1497927	1.47031	10.1969	2.79393	0.00035	0.0463974
ENSG00000128513	POT1	protection of telomeres 1	chr7:124822385-125379321	4.04539	17.6908	2.12864	5e-05	0.011
ENSG00000087903	RFX2	regulatory factor X2	chr19:5978402-6199572	1.89238	7.75019	2.03403	5e-05	0.011
ENSG00000025434	NR1H3	nuclear receptor subfamily 1 group H member 3	chr11:47239301-47269032	4.47314	15.1365	1.75868	0.0003	0.04136
ENSG00000179403	VWA1	von Willebrand factor A domain containing 1	chr1:1434860-1442882	26.1843	84.5726	1.69149	0.0001	0.0181404
ENSG00000116151	MORN1	MORN repeat containing 1	chr1:2321252-2391707	10.4087	0.773552	-3.75014	0.0003	0.04136
ENSG00000251247	ZNF345	zinc finger protein 345	chr19:36797517-36916291	5.86974	0.494505	-3.56924	5e-05	0.011
ENSG00000116106	EPHA4	EPH receptor A4	chr2:221418026-221574454	6.88857	0.698906	-3.30103	5e-05	0.011

ENSG00000164400	CSF2	colony stimulating factor 2	chr5:132073789-132076170	138.834	14.6357	-3.2458	5e-05	0.011
ENSG00000105699	LSR	lipolysis stimulated lipoprotein receptor	chr19:35248329-35267964	6.28833	0.730187	-3.10634	0.0002	0.0318154
ENSG00000163121	NEURL3	neuralized E3 ubiquitin protein ligase 3	chr2:96497642-96508109	44.5928	6.63587	-2.74845	5e-05	0.011
ENSG00000122970	IFT81	intraflagellar transport 81	chr12:110124334-110218797	14.913	2.37758	-2.64901	0.0002	0.0318154
ENSG00000127533	F2RL3	F2R like thrombin/trypsin receptor 3	chr19:16888859-16892606	8.84846	1.414	-2.64565	0.00035	0.0463974
ENSG00000066651	TRMT11	tRNA methyltransferase 11 homolog	chr6:125986429-126039276	14.0548	2.24743	-2.64471	0.0002	0.0318154
ENSG00000186827	TNFRSF4	TNF receptor superfamily member 4	chr1:1211325-1214138	256.968	46.2719	-2.47338	5e-05	0.011

A full description of top ten up and down regulated genes in HDMEC/TNF interacted with IT4var14 parasite at 6 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 6 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix15:

Ensemble Gene ID	Gene Name	Description	Coordinates	FPKM PRBC 0h	FPKM PRBC 20h	Log_2 Fold Change	P-value	FDR
ENSG00000140465	CYP1A1	cytochrome P450 family 1 subfamily A member 1	chr15:74719541-74725610	3.76103	46.2772	3.6211	5e-05	0.00818138
ENSG00000171864	PRND	prion protein 2	chr20:4721909-4728460	0.0571954	0.622117	3.44322	0.00015	0.0176118
ENSG00000162777	DENND2D	DENN domain containing 2D	chr1:111187173-111243440	0.0805743	0.595492	2.88569	0.0002	0.021817
ENSG00000162692	VCAM1	vascular cell adhesion molecule 1	chr1:100719741-100739045	0.533853	3.25258	2.60707	5e-05	0.00818138
ENSG00000115009	CCL20	C-C motif chemokine ligand 20	chr2:227813841-227817564	0.234889	1.40141	2.57683	0.0004	0.0339912
ENSG00000265972	TXNIP	thioredoxin interacting protein	chr1:145992434-145996600	11.1227	66.2085	2.57351	5e-05	0.00818138
ENSG00000114812	VIPR1	vasoactive intestinal peptide receptor 1	chr3:42489298-42537573	0.0766432	0.450533	2.5554	5e-05	0.00818138
ENSG00000006210	CX3CL1	C-X3-C motif chemokine ligand 1	chr16:57372457-57385048	0.206721	1.14051	2.46392	5e-05	0.00818138
ENSG00000100767	PAPLN	papilin, proteoglycan-like sulfated glycoprotein	chr14:73237496-73274640	1.79858	9.7613	2.44021	5e-05	0.00818138
ENSG00000272636	DOC2B	double C2 domain beta	chr17:142788-181636	0.173891	0.906035	2.38138	5e-05	0.00818138
ENSG00000183691	NOG	noggin	chr17:56593698-56595590	5.49245	0.134192	-5.35508	5e-05	0.00818138
ENSG00000115738	ID2	inhibitor of DNA binding 2	chr2:8666635-8684453	58.1397	4.92853	-3.56029	5e-05	0.00818138
ENSG00000188536	HBA2	hemoglobin subunit alpha 2	chr16:172846-173710	29.3289	4.74045	-2.62923	5e-05	0.00818138
ENSG00000198673	FAM19A2	family with sequence similarity 19 (chemokine (C-C motif)-like), member A2	chr12:61708258-62417431	0.576024	0.0976469	-2.56048	0.0006	0.0433268

ENSG00000244734	HBB	hemoglobin subunit beta	chr11:5225463-5229395	51.6099	9.63441	-2.42138	5e-05	0.00818138
ENSG00000125968	ID1	inhibitor of DNA binding 1	chr20:31605282-31606515	534.501	107.681	-2.31143	5e-05	0.00818138
ENSG00000135547	HEY2	hes related family bHLH transcription factor with YRPW motif 2	chr6:125578557-125761269	4.14179	0.864693	-2.25999	5e-05	0.00818138
ENSG00000099860	GADD45 B	growth arrest and DNA damage inducible beta	chr19:2476121-2478259	91.8943	20.5668	-2.15966	5e-05	0.00818138
ENSG00000165899	OTOGL	otogelin like	chr12:80209452-80379090	0.63461	0.14639	-2.11605	0.00035	0.0311176
ENSG00000168874	ATOH8	atonal bHLH transcription factor 8	chr2:85751343-85788066	29.1582	6.75063	-2.11081	5e-05	0.00818138

A full description of top ten up and down regulated genes in HDMEC/TNF interacted with IT4var14 parasite at 20 hours.

The table presents the ensemble gene ID of the top ten up and down regulated genes, gene name, location of the gene in the genome, *Fragments Per Kilobase of exon per Million fragments mapped* (FPKM) at 0 and 20 hours, log2 fold change, P-value and FDR value of each gene involve in this study. PRBC indicates the *P. falciparum* infected erythrocyte.

Appendix16:

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Response to hydrogen peroxide	4	3.4E	4.8E-2
Oxygen transport	3	1.5E-4	2.2E-1
Positive regulation of apoptotic process	5	4.7E-4	6.6E-1
Regulation of cell proliferation	4	1.5E-3	2.1E0
Positive regulation of smooth muscle cell proliferation	3	2.5E-3	3.5E0
Response to wounding	3	2.8E-3	3.8E0
Positive regulation of gene expression	4	4.1E-3	5.6E0
Positive regulation of transcription from RNA polymerase II promoter	6	6.3E-3	8.5E0
Negative regulation of cell division	2	8.7E-3	1.2E1
Cellular response to phorbol 13-acetate 12-myristate	2	8.7E-3	1.2E1
Positive regulation of podosome assembly	2	1.2E-2	1.6E1
Negative regulation of cell proliferation	4	1.3E-2	1.6E1
Negative regulation of glucose import	2	1.4E-2	1.8E1
Response to lipopolysaccharide	3	1.8E-2	2.2E1
Negative regulation of apoptotic process	4	1.8E-2	2.3E1
Protein phosphorylation	4	1.8E-2	2.3E1
Monocyte differentiation	2	2.1E-2	2.6E1
Blood coagulation	3	2.2E-2	2.7E1
Negative regulation of transcription, DNA-templated	4	2.3E-2	2.8E1
Hydrogen peroxide catabolic process	2	2.5E-2	3.0E1
Transcription from RNA polymerase II promoter	4	2.5E-2	3.0E1
Positive regulation of transcription, DNA-templated	4	2.5E-2	3.0E1
Positive regulation of gtpase activity	4	3.2E-2	3.7E1
Apoptotic process	4	3.2E-2	3.7E1
Positive regulation of cell death	2	3.6E-2	4.0E1
Cellular response to fibroblast growth factor stimulus	2	3.7E-2	4.1E1
Cellular response to epidermal growth factor stimulus	2	4.0E-2	4.4E1

MAPK cascade	3	4.2E-2	4.5E1
Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	2	4.5E-2	4.8E1
Positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	2	4.9E-2	5.1E1
Regulation of mitotic cell cycle	2	4.9E-2	5.1E1

Biological Process – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Negative regulation of transcription from RNA polymerase II promoter	11	5.2E-6	7.6E-3
Angiogenesis	6	1.8E-4	2.6E-1
Transcription, DNA-templated	14	4.0E-4	5.9E-1
Positive regulation of transcription, DNA-templated	7	1.3E-3	1.9E0
Transforming growth factor beta receptor signaling pathway	4	1.4E-3	2.0E0
Negative regulation of Notch signaling pathway involved in somitogenesis	2	4.8E-3	6.8E0
Negative regulation of transcription, DNA-templated	6	6.3E-3	8.9E0
Regulation of transcription, DNA-templated	10	7.7E-3	1.1E1
Positive regulation of transcription from RNA polymerase II promoter	8	7.8E-3	1.1E1
Negative regulation of sequence-specific DNA binding transcription factor activity	3	9.0E-3	1.2E1
Positive regulation of smooth muscle cell proliferation	3	9.0E-3	1.2E1
Enucleate erythrocyte differentiation	2	1.2E-2	1.6E1
Negative regulation of cell proliferation	5	1.4E-2	1.9E1
Tube formation	2	1.9E-2	2.4E1
Omega-hydroxylase P450 pathway	2	2.1E-2	2.7E1
Notch signaling involved in heart development	2	2.1E-2	2.7E1
Pulmonary valve morphogenesis	2	2.4E-2	3.0E1
Epithelial to mesenchymal transition involved in endocardial cushion formation	2	3.1E-2	3.7E1
Positive regulation of apoptotic process	4	3.4E-2	4.0E1
Blood vessel morphogenesis	2	4.0E-2	4.5E1
Epoxygenase P450 pathway	2	4.2E-2	4.7E1
Negative regulation of gene expression	3	4.2E-2	4.7E1
Positive regulation of pri-mirna transcription from RNA polymerase II promoter	2	4.7E-2	5.0E1

Cellular Component – Up Regulated Genes: Go term	Size	P-value	FDR
Cytosol	12	1.1E-3	1.1E0
Haptoglobin-hemoglobin complex	2	5.0E-3	4.8E0
Extracellular region	7	1.3E-2	1.2E1
Hemoglobin complex	2	1.5E-2	1.4E1
Endocytic vesicle lumen	2	2.0E-2	1.8E1
Cytoplasm	12	4.0E-2	3.3E1
Cytosol	12	1.1E-3	1.1E0
Haptoglobin-hemoglobin complex	2	5.0E-3	4.8E0

Cellular Component – Down Regulated Genes: Go term	Size	P-value	FDR
Cytoplasm	22	1.3E-3	1.4E0
Nucleus	21	5.5E-3	5.8E0
Organelle membrane	3	1.6E-2	1.6E1

Molecular Function – Up Regulated Genes: Go term	Size	P-value	FDR
Protein binding	21	4.8E-4	5.1E-1
RNA polymerase II core promoter proximal region sequence-specific DNA binding	5	1.2E-3	1.3E0
Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	4	3.9E-3	4.0E0
Haptoglobin binding	2	4.1E-3	4.2E0
Oxygen transporter activity	2	1.9E-2	1.8E1
Peroxidase activity	2	3.0E-2	2.7E1
Protein complex binding	3	3.2E-2	2.9E1
Transcription factor activity, sequence-specific DNA binding	5	3.9E-2	3.4E1
Protein binding	21	4.8E-4	5.1E-1

Molecular Function – Up Regulated Genes: Go term	Size	P-value	FDR
Protein binding	32	1.5E-4	1.8E-1
Protein dimerization activity	5	3.5E-4	4.1E-1

Transcription factor activity, sequence-specific DNA binding	9	1.1E-3	1.3E0
RNA polymerase II regulatory region sequence-specific DNA binding	5	1.2E-3	1.4E0
Protein homodimerization activity	7	5.4E-3	6.2E0
Identical protein binding	7	6.1E-3	6.9E0
DNA binding	10	1.0E-2	1.2E1
Transcription factor binding	4	2.6E-2	2.6E1
Sequence-specific DNA binding	5	2.8E-2	2.9E1
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	2	3.3E-2	3.3E1
Heme binding	3	3.8E-2	3.6E1
RNA polymerase II core promoter proximal region sequence-specific DNA binding	4	4.5E-2	4.2E1
Activating transcription factor binding	2	4.8E-2	4.4E1

Functions analysis enrichment result at 2 hours incubation of IT4var14 with HDMEC/TNF.

Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

Appendix17:

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Cellular lipid metabolic process	2	3.1E-2	3.1E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Signal transduction	15	1.2E-5	1.8E-2
Inflammatory response	9	2.6E-5	3.8E-2
Chemotaxis	6	4.4E-5	6.4E-2
Immune response	9	5.5E-5	8.0E-2
Chemokine-mediated signaling pathway	5	7.9E-5	1.1E-1
Blood coagulation	5	2.9E-3	4.1E0
Positive regulation of interferon-beta production	3	3.4E-3	4.8E0
Positive regulation of protein kinase activity	3	1.0E-2	1.3E1
Peptidyl-tyrosine phosphorylation	4	1.3E-2	1.7E1
Negative regulation of ERK1 and ERK2 cascade	3	1.5E-2	1.9E1
Response to lipopolysaccharide	4	1.6E-2	2.0E1
Cell chemotaxis	3	1.9E-2	2.4E1
Positive regulation of interleukin-12 biosynthetic process	2	1.9E-2	2.4E1
Toll-like receptor 2 signaling pathway	2	1.9E-2	2.4E1
Protein heterooligomerization	3	2.0E-2	2.5E1
Positive regulation of inflammatory response	3	2.3E-2	2.9E1
Response to molecule of bacterial origin	2	2.9E-2	3.4E1
Cellular response to lipoteichoic acid	2	2.9E-2	3.4E1
Cell proliferation	5	3.0E-2	3.5E1
Oxygen transport	2	4.7E-2	5.0E1
Positive regulation of macrophage derived foam cell differentiation	2	5.0E-2	5.2E1

Cellular Component – Down Regulated Genes: Go term	Size	P-value	FDR
Plasma membrane	24	4.4E-4	5.1E-1

Cell surface	7	4.3E-3	4.9E0
Membrane	14	7.6E-3	8.4E0
Perinuclear region of cytoplasm	7	8.2E-3	9.1E0
Haptoglobin-hemoglobin complex	2	1.1E-2	1.2E1
Extracellular region	11	1.4E-2	1.5E1
Integral component of plasma membrane	10	1.8E-2	1.9E1
Hemoglobin complex	2	3.4E-2	3.3E1
Extracellular space	9	3.6E-2	3.4E1
Endocytic vesicle lumen	2	4.5E-2	4.1E1

Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Chemokine activity	4	5.1E-4	6.1E-1
Identical protein binding	9	2.5E-3	2.9E0
Haptoglobin binding	2	9.6E-3	1.1E1
Receptor binding	5	2.6E-2	2.7E1
CXCR chemokine receptor binding	2	2.8E-2	2.9E1
Oxygen transporter activity	2	4.4E-2	4.2E1

Functions analysis enrichment result at 6 hours incubation of IT4var14 with HDMEC/TNF.
Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.

Appendix18:

Biological Process – Up Regulated Genes: Go term	Size	P-value	FDR
Receptor internalization	3	6.7E-3	9.4E0
Negative regulation of branching involved in ureteric bud morphogenesis	2	1.1E-2	1.5E1
Steroid hormone mediated signaling pathway	3	1.2E-2	1.6E1
Negative regulation of interferon-gamma-mediated signaling pathway	2	1.4E-2	1.9E1
Positive regulation of endothelial cell proliferation	3	1.7E-2	2.2E1
Negative regulation of cholesterol storage	2	1.7E-2	2.2E1
Cellular response to insulin stimulus	3	2.0E-2	2.6E1
Positive regulation of phagocytosis, engulfment	2	2.3E-2	2.9E1
Response to lipid	2	2.3E-2	2.9E1
Secondary heart field specification	2	2.3E-2	2.9E1
Positive regulation of lipoprotein lipase activity	2	2.8E-2	3.4E1
Negative regulation of macrophage derived foam cell differentiation	2	3.7E-2	4.2E1
Negative regulation of substrate adhesion-dependent cell spreading	2	3.7E-2	4.2E1
Cell adhesion	5	4.2E-2	4.7E1
Apoptotic cell clearance	2	4.2E-2	4.7E1
Lipoprotein transport	2	4.2E-2	4.7E1
Cell surface receptor signaling pathway	4	4.3E-2	4.8E1
Anterior/posterior axis specification	2	4.8E-2	5.1E1
Monocyte differentiation	2	4.8E-2	5.1E1
Response to iron ion	2	5.0E-2	5.3E1
Response to vitamin A	2	5.0E-2	5.3E1

Biological Process – Down Regulated Genes: Go term	Size	P-value	FDR
Inflammatory response	16	5.9E-9	9.2E-6
Immune response	16	2.4E-8	3.7E-5
Lipopolysaccharide-mediated signaling pathway	6	1.1E-6	1.8E-3
Positive regulation of NF-kappab transcription factor activity	9	1.2E-6	1.8E-3

Chemokine-mediated signaling pathway	7	3.8E-6	5.9E-3
Chemotaxis	8	7.7E-6	1.2E-2
Response to molecule of bacterial origin	4	1.6E-5	2.5E-2
Cell chemotaxis	6	4.0E-5	6.3E-2
Protein heterooligomerization	6	4.7E-5	7.3E-2
Response to lipopolysaccharide	8	5.2E-5	8.2E-2
Positive regulation of inflammatory response	6	7.0E-5	1.1E-1
Apoptotic process	13	1.2E-4	1.9E-1
Positive regulation of I-kappab kinase/NF-kappab signaling	7	3.8E-4	5.9E-1
I-kappab kinase/NF-kappab signaling	5	4.3E-4	6.7E-1
Cellular response to lipopolysaccharide	6	5.5E-4	8.5E-1
Regulation of tumor necrosis factor-mediated signaling pathway	4	7.2E-4	1.1E0
Interferon-gamma-mediated signaling pathway	5	8.2E-4	1.3E0
Positive regulation of gene expression	8	9.1E-4	1.4E0
Negative regulation of viral genome replication	4	1.7E-3	2.6E0
Positive regulation of macrophage chemotaxis	3	1.8E-3	2.8E0
Oxygen transport	3	3.4E-3	5.2E0
Cellular response to tumor necrosis factor	5	4.1E-3	6.2E0
Positive regulation of chemokine production	3	4.4E-3	6.7E0
Tumor necrosis factor-mediated signaling pathway	5	5.2E-3	7.9E0
T cell proliferation	3	5.5E-3	8.3E0
Hydrogen peroxide catabolic process	3	6.1E-3	9.1E0
Regulation of inflammatory response	4	6.1E-3	9.2E0
Positive regulation of neutrophil chemotaxis	3	7.4E-3	1.1E1
Leukocyte cell-cell adhesion	3	9.5E-3	1.4E1
Nucleotide-binding oligomerization domain containing signaling pathway	3	9.5E-3	1.4E1
Positive regulation of interferon-beta production	3	1.1E-2	1.6E1
Nucleotide-binding oligomerization domain containing 1 signaling pathway	2	1.2E-2	1.7E1
Positive regulation of cell death	3	1.3E-2	1.8E1
Myd88-dependent toll-like receptor signaling pathway	3	1.6E-2	2.2E1

Defense response to virus	5	1.6E-2	2.3E1
Aging	5	1.6E-2	2.3E1
Response to exogenous dsrna	3	1.7E-2	2.4E1
Signal transduction	14	1.9E-2	2.6E1
Cell adhesion	8	1.9E-2	2.6E1
Positive regulation of ERK1 and ERK2 cascade	5	2.0E-2	2.7E1
Negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	3	2.0E-2	2.7E1
Protein homooligomerization	5	2.1E-2	2.8E1
Regulation of cell proliferation	5	2.4E-2	3.2E1
Positive regulation of cell adhesion	3	2.7E-2	3.4E1
Positive regulation of nitric oxide biosynthetic process	3	2.7E-2	3.4E1
Response to virus	4	2.7E-2	3.5E1
Bicarbonate transport	3	2.8E-2	3.6E1
Extracellular matrix organization	5	2.9E-2	3.7E1
Positive regulation of interleukin-6 production	3	2.9E-2	3.7E1
Nucleotide-binding oligomerization domain containing 2 signaling pathway	2	2.9E-2	3.7E1
JNK cascade	3	3.4E-2	4.2E1
Toll-like receptor 2 signaling pathway	2	3.5E-2	4.3E1
Positive regulation of interleukin-12 biosynthetic process	2	3.5E-2	4.3E1
Response to hydrogen peroxide	3	3.6E-2	4.4E1
Cellular response to interferon-gamma	3	4.5E-2	5.1E1
Negative regulation of ERK1 and ERK2 cascade	3	4.6E-2	5.2E1
Negative regulation of lipid storage	2	4.6E-2	5.2E1
Neutrophil activation	2	4.6E-2	5.2E1
Cellular response to organic cyclic compound	3	4.7E-2	5.3E1
Positive regulation of T cell proliferation	3	4.9E-2	5.4E1

Cellular Component – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Receptor complex	4	4.3E-3	4.6E0
Perinuclear region of cytoplasm	7	5.0E-3	5.4E0

Nuclear chromatin	4	1.3E-2	1.4E1
Dendritic spine	3	2.7E-2	2.6E1
Cell junction	5	3.0E-2	2.9E1
Apical plasma membrane	4	3.9E-2	3.5E1

Cellular Component – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Extracellular space	21	4.2E-5	5.0E-2
Haptoglobin-hemoglobin complex	3	1.8E-4	2.2E-1
Cytosol	33	7.3E-4	8.7E-1
Plasma membrane	38	9.7E-4	1.1E0
Hemoglobin complex	3	1.9E-3	2.3E0
Cell surface	10	3.2E-3	3.7E0
Endocytic vesicle lumen	3	3.5E-3	4.0E0
Extracellular region	18	7.1E-3	8.1E0
Membrane	22	8.5E-3	9.7E0
Perinuclear region of cytoplasm	9	2.2E-2	2.3E1
Protein complex	7	2.7E-2	2.8E1
Early endosome	5	3.9E-2	3.7E1
Golgi apparatus	10	5.0E-2	4.6E1
Cytoplasm	38	5.0E-2	4.6E1

Molecular Function – Up Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Chromatin binding	6	4.5E-3	5.3E0
Lipoprotein particle binding	2	1.4E-2	1.5E1
High-density lipoprotein particle binding	2	2.2E-2	2.4E1
Low-density lipoprotein receptor activity	2	3.6E-2	3.5E1

Molecular Function – Down Regulated Genes: Go term	**Size**	**P-value**	**FDR**
Chemokine activity	6	8.8E-6	1.1E-2
Haptoglobin binding	3	9.8E-5	1.3E-1

Cytokine activity	7	5.3E-4	6.9E-1
CXCR chemokine receptor binding	3	1.1E-3	1.5E0
Identical protein binding	13	1.2E-3	1.5E0
Oxygen transporter activity	3	2.8E-3	3.6E0
Peroxidase activity	3	7.0E-3	8.7E0
Protein binding	63	1.2E-2	1.4E1
Tumor necrosis factor receptor binding	3	1.2E-2	1.4E1
Oxygen binding	3	3.0E-2	3.2E1

Functions analysis enrichment result at 20 hours incubation of IT4var14 with HDMEC/TNF.
Enriched functions of up and down regulated genes are listed in the table and separated into 3 GO term categories (biological process, molecular function and cellular component). Size, number of expressed genes associated with the term. FDR, False discovery rate.