CHAPTER III
LITERATURES REVIEW

1. Malaria

1.1 Life cycle of malaria

There are four different species of human malaria parasites namely, *Plasmodium falciparum, P. vivax, P. malaria,* and *P. ovale* *P. falciparum* is the most dangerous malaria species as it often leads to the death and can be fatal within few hours of the first symptom (Hyde *et al.*, 2002). The life cycle of *P. falciparum* is complex. In humans, *P. falciparum* infection begins when an infected *Anopheles* mosquito injected infected sporozoites into the peripheral circulation. Then sporozoites migrate and invade the liver within hepatocyte and undergo asexual multiplication producing merozoite form of the parasite. The infected hepatocytes rupture and release merozoites into the peripheral circulation before the invasion of the merozoites to red blood cells (RBCs). The complete round of multiplication is within 48-72 hours (Bannister *et al*., 2000). In additional multiplication results in the destruction of RBCs haemoglobin and the released merozoites invade more RBCs to carry on the cycle. The release of merozoites is thought to be responsible for the periodic fevers associated with malaria. Some merozoites do not divide but form male (microgametocytes) and female (macrogametocytes) sexual forms. These sexual forms are taken from the blood by a feeding *Anopheles* mosquito and fertilize in the mosquito midgut forming zygotes, which differentiate into motile forms (ookinetes). The ookinetes migrate through the mosquito gut and divide into oocytes on the external gut wall to form sporozoites. Thereafter, the sporozoites are released into the mosquito haemocoele and move to the salivary gland, where they await injection into another human host, thus completing the cycle (Fujioka and Aikawa, 2002). The life cycle of *P. falciparum* is shown in **Figure 1**.

The human malarial parasite *P. falciparum* interacted with wide temperature variation during its life cycle, ranging from 25°C or 26°C in the mosquito vector and 37°C in humans, to 41°C during febrile episodes in the patient (Anderson *et al.,* 1989). The repeated occurrence of fever at regular intervals is a characteristic of human

malaria. The period nature of the fever is ascribed by three factors. First, malaria parasite undergo repeated cycles of replication in host erythrocytes and the schizont rupture occurs at the end of each cycle is associated with fever. Second, the duration of each cycle is essentially constant, being 48 hours in *P. falciparum*, *P. vivax* and *P. ovale,* and 72 hours in *P. malariae* **(Figure 2)**. Finally, the parasites have an intriguing tendency to replicate in synchrony with each other *in vivo*, and therefore each generation of schizonts ruptures more or less simultaneously causing periodic paroxysms of fever (Golgi *et al.*, 1979).

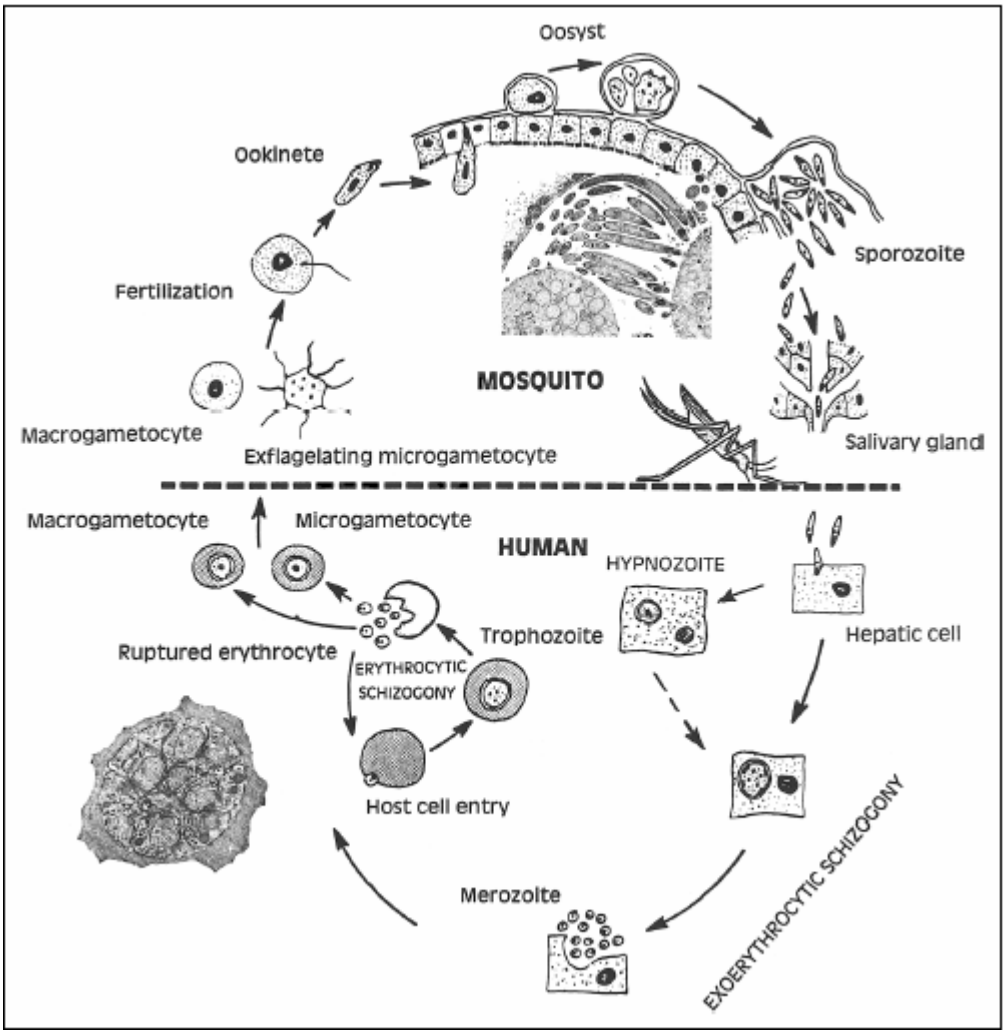

Figure 1 The life cycle of malaria parasites in the human host and Anopheline mosquito vector (Fujioka and Aikawa, 2002)

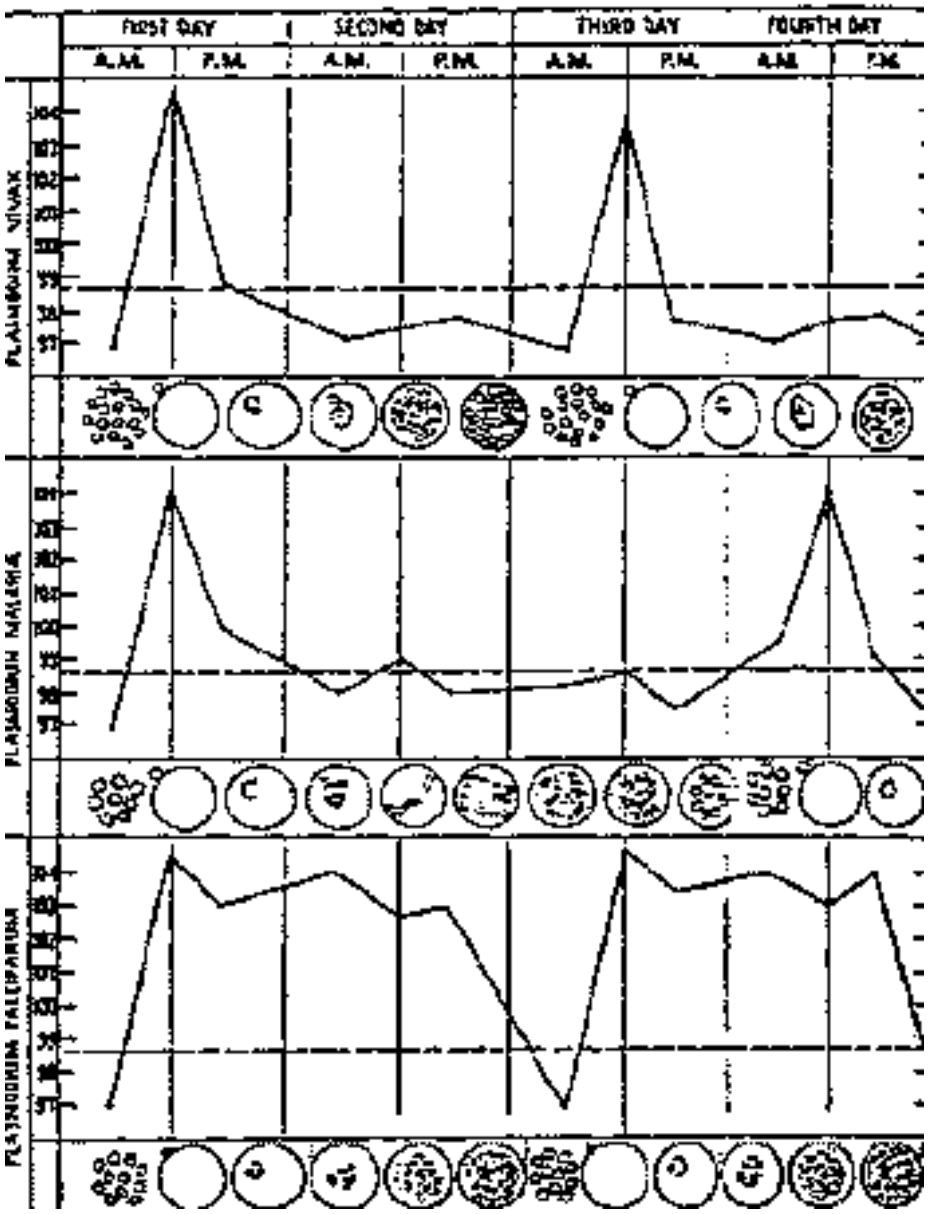

Figure 2 The periodic of malaria fever. The duration of each cycle is essentially constant, being 48 hours in *P. falciparum*, *P. vivax* and *P. ovale,* and 72 hours in *P. malariae* (Anderson *et al.,* 1989).

1.2 Clinical symptoms of malaria

In nonimmune individuals infected with *P. falciparum*, the median of pre-patent period from sporozoite inoculation to detectable parasitaemia is 10 days (range 5–10 days), and the median incubation period (time from sporozoite inoculation to development of symptoms) is 11 days (range 6–14 days). The incubation period may be significantly prolonged by the level of immunity acquired through previous exposures, as well as by antimalarial prophylaxis, or by prior partial treatment, which may mitigate, but not prevent the disease (Taylor *et al.,* 2000). Most nonimmune travelers develop symptoms of falciparum malaria within 1 month after departing from a malaria-endemic area (median 10 days). There have been reports of falciparum malaria presenting up to 4 years later (White *et al.,* 2003). The incubation period of other plasmodia species is usually longer (median 15–16 days), and both *P. vivax* and *P. ovale* malaria may relapse months or years after exposure due to the presence of hypnozoites in the liver. The longest reported incubation period for *P. vivax* is 30 years (White *et al.,* 2003).

The clinical symptoms of malaria are primarily due to schizont rupture and destruction of erythrocytes. Malaria can have a gradual or a fulminant course with nonspecific symptoms. The presentation of malaria often resembles those of common viral infections and may lead to a delay in diagnosis (Murphy *et al.,* 1996). The majority of patients experience fever (>92%), chill (79%), headache (70%), and diaphoresis (64%) (Genton *et al.,* 2001). Other symptoms including dizziness, malaise, myalgia, abdominal pain, nausea, vomiting, mild diarrhoea, dry cough, as well as physical signs such as fever, tachycardia, jaundice, pallor, orthostatic hypotension, hepatomegaly, and splenomegaly are also found. Clinical examination in nonimmune persons may be completely unremarkable even without fever (Andrej *et al.,* 2003). The characteristic of periodic fever in patient infected with *P. falciparum* and *P. vivax* are tertian, whereas the subtertain fever is commonly seen in patients' infected *P. falciparum* (Bruce-Chwatt *et al.,* 1986).

1.3 Periodic of fever in malaria

The patient infected with malaria shows highly periodic of fever and it relates to the parasite growth in both sexual and asexual erythrocytic stage of infection as

demonstrated by an exponential growth phase, which is terminated shortly after onset of periodic fever **(Figure 3)**. In addition, parasitaemia stabilizes and subsequently tend to decline slowly after the initial exponential growth phase. The pattern of decline of parasitaemia may relate to the acquisition of specific antiparasitic immunity and other growth limit factors such as erythrocyte availability. *P. falciparum* infects all ages of erythrocytes, whereas *P. vivax* and *P. ovale* infect young erythrocytes or reticulocytes and *P. malariae* infects old erythrocytes. As shown in **Figure 3b**, the steep drop in parasitaemia following an episode of extremely high fever is found (Kitchen *et al.,* 1949; Anderson *et at.,* 1989).

Data of periodic fever demonstrated that the simple form of host defense could promote synchronization and periodic phenomenal while tend to equilibrate parasite population density. However, the mechanism of host protection may reinforce this effect. The parasite rupturing schizonts cause fever by stimulating macrophage and other immune cell releasing pyrogenic cytokines including TNF. These could induce cell-mediated parasite killing by free oxygen radicals associated with other circulating antiparasitic factors that are maximal at the peak of the fever (Kwiatkowski *et al.,* 1989; Clark *et al.,* 1987; Mendis *et al.,* 1990). The high multiplication rate of *P. falciparum* in nonimmune individual could lead to large chaotic bursts of schizonts rupture that might contribute, such as through massive TNF release, to fatal outcome (Kwiatkowski *et al.,* 1991).

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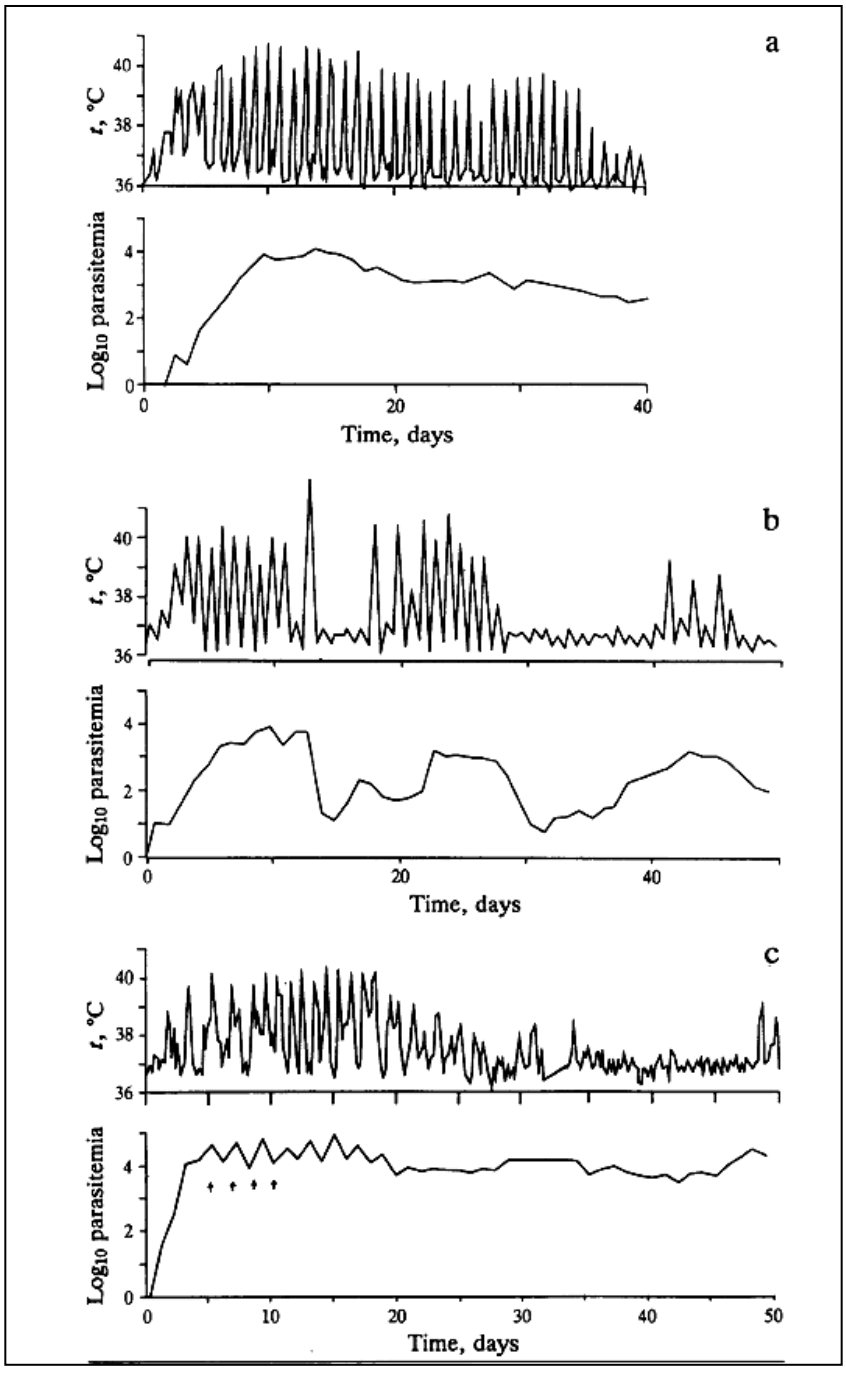

Figure 3 Exponential growth phases of malaria fever showing highly periodic fever which is related to the parasite growth cycle in both sexual and asexual erythrocytic stage of infection (Kwiatkowski *et al.,* 1987).

1.4 Tumor necrosis factor (TNF) in the pathogenesis of malaria fever.

When human is infected by pathogen, host protection by their immune is induced. In the early stage of an inflammatory response, the first cell that body produces to protect is neuthrophil, while vascular endothelial cells increase expression of E- and P-selectin in responses to acute inflammation (Kuby, 2000). Thrombin and histamine induce the increasing of P–selectin and cytokine such as IL-1 or TNF-α, which further induces E-selectin level resulting in neuthrophil adhesion and subsequent transendothelail migration to level up a gradient of chemoattactant. The chemotactic for neuthrophil between the inflammatory mediators makes the acute inflammatory response (Kuby, 2000). This response induces fever and increases the synthesis of hormones and production of white blood cell and acute-phase proteins in the liver. Many systemic acute phases affect toward the combined actions of IL-1, IL-6 and TNF-α. Each of this cytokine acts on the hypothalamus inducing a fever response (Kuby *et al.,* 2000).

Nadira and his colleagues explained the dynamics of fever and serum level of tumor necrosis factor in *P. vivax* in 9 patients infected with *P. vivax* (Nadira *et al.,* 1992). They found out that the levels of TNF were associated with temperature. They were lower or higher closely parallel but slightly preceded the up and down of temperature during a paroxysm of these patients (Nadira *et al.,* 1992). These data were in agreement with that shown in *P. falciparum*. There has been a clearly correlation among severities of disease, mild disease, nonfatal cerebral malaria, fatal cerebral malaria and TNF levels as shown in **Table 1** (Mendis et al., 1990; Grau *et al.*, 1989; Kwiatkowski *et al.,* 1990).

It is possible that cytokines including TNF do not directly involved in the temperature changes during malarial paroxysm, but is merely produced in parallel with other factors and mediates malarial fever. In addition, it has been postulated that TNF in *P. falciparum* may be involved in the mechanism of cerebral malaria (Grau *et al.*, 1989; Kwiatkowski *et al.,* 1990)

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Table 1 Plasma TNF levels in patients infected with *P. vivax* and *P. falciparum*

Disease and severity (no. of patients)	Time of plasma collection	Plasma TNF,* (pg/ml)
P. vivax (8)	Peak paroxysm	746.14 (264.6-2104.1)
(25)	Random	54.98 (17.38-173.8)
P. falciparum		
Mild (178)	Random	24 (20-29)
Severe		
(cerebral) (82)		51 (36-72)
Fatal (28)		269 (170-431)

*Geometric mean (and 95% confidence intervals) of TNF level measured in both studies by immunoassays.

2. Effect of febrile temperature of *Plasmodium falciparum* parasites

P. falciparum faces with different environments during its life cycle. The parasites is exposed to fluctuation of temperature ranging from 25°C in mosquito, to 37°C in human and 41°C in febrile episode. This reason forces an adaptive response in the malaria parasite. Thermoregulation of malaria parasite is important and influences not only the rate of development in human and mosquito, but also pathological effect such as cytoadherence.

2.1 Effect of febrile temperature on survival of *P. falciparum* parasites

Long and his colleagues cultured parasite in varying temperature ranging from 37 to 40°C, for four days. High temperature was found to decrease the parasite number and parasite growth was found to be inhibited by febrile temperature (Long *et al.,* 2001). Furthermore, Dominic Kwiatkowski found that the febrile temperature can synchronize parasites. The growth of *P. falciparum* with increased numbers was observed following 7 days culture at 37°C but not at 40°C (Kwiatkowski, 1988). Following the incubation of parasite culture for 48 days in conditions that mimick the cycle of tertian malaria at changing temperature from 37 to 40°C, or from 40 to 37°C on alternate days, the parasites became synchronized and acquired fixed relationship to the temperature cycle (**Figure 5**). Moreover, the febrile temperatures were capable of inhibiting parasite growth, although the ring stage resisted to the high temperature as shown in **Table 2** (Kwiatkowski, 1988).

However, temperature can induce parasite development and synchronization (Pavita *et al.,* 2004). The repeated exposure to varied temperature mimicking febrile episode promotes parasite development in human erythrocyte. Heat shock protein is mediated for cytoprotection and growth promotion (Pavita *et al.,* 2004).

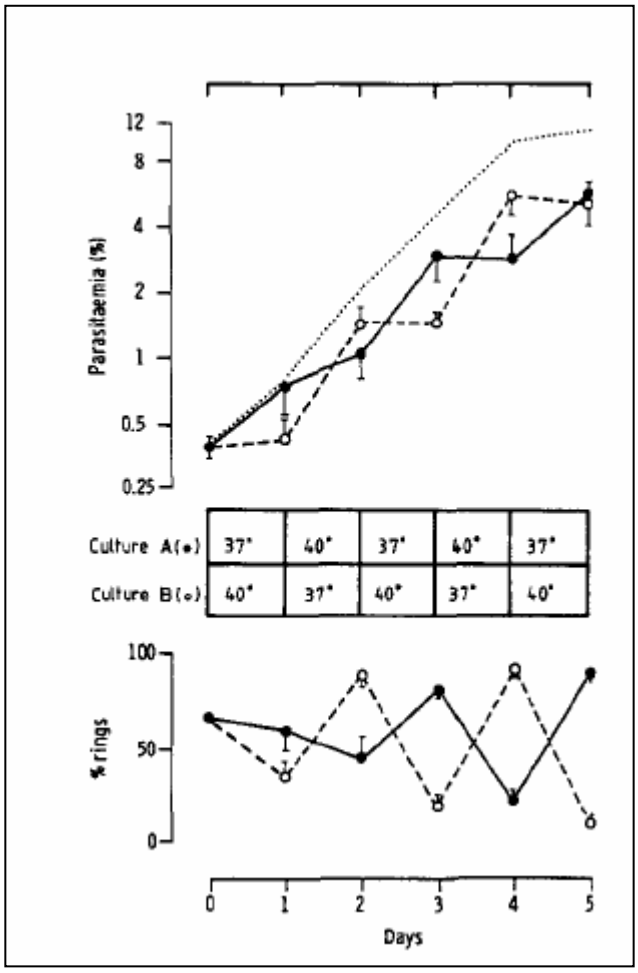

Figure 4 Erythrocytic cultures of *P. falciparum* incubated at 37°C and at 40°C on alternate days (seven experiments each). Starting cultures were asynchronous. Culture A (●) was at 40°C on days 2 and 4 ; and culture B (O) was at 40°C on days 1, 3, and 5 (Top panel). Growth rates expressed as geometric means with standard errors. Dotted line represents mean growth of control cultures maintained at 37°C (Bottom panel). Percentage of parasites at the ring stage of development (stages R and RT as defined in Materials and Methods) expressed as arithmetic means with standard errors. Cultures A and B differ significantly from 2 days onwards ($p < 0.01$ by paired t-test) (Kwiatkowski, 1988).

Table 2 Effect of incubation temperature at 40° C on parasitaemia of *P. falciparum*

Culture	Incubation temperature (° C)		Parasitaemia (between 0 and 48 h)
	0-24 h	24-48 h	
a	37	37	4.1 (2.9-5.6)
b	40	40	0.4(0.1-0.5)
c	40	37	3.6(3.0-4.3)
d	37	40	0.6 (0.5-0.7)

In addition, Miranda and her colleagues found the effect of febrile temperature on the survival of synchronous and asynchronous asexual erythrocytic-stage cultures of *P. falciparum* parasites by comparing the survival rates of parasites grown at 37°C and 41°C over the period of 48 h (Miranda *et al.,* 2007). They results show that elevated temperature had a deleterious effect on parasite survival in both synchronous and asynchronous cultures. The prolonged survival of synchronous cultures can be attributed to the fact that the starting cultures were solely comprised of ring-stage parasites that have been shown to be less susceptible to cultivation at elevated temperature than the mature forms (Miranda *et al.,* 2007; Kwiatkowski, 1989).

Moreover, the number and morphological appearance of "crisis forms" was significantly more evident following 4 h of treatment at 41°C. Previously, "crisis forms" of trophozoites and schizonts had been described in *P. falciparum* cultures undergoing death induced by treatment with antimalarial drugs and other experimentally induced forms of stress (Deponte and Becker, 2004; Nyakeriga *et al.,* 2006). This result is important to note that the presence of "crisis forms" has been ascribed as a marker of apoptotic cell death in malaria parasites. The results from previous researcher are in agreement with an earlier report showing an inhibitory effect of temperatures characteristic of the malaria paroxysm on *in vitro* parasite growth (Kwiatkowski, 1989; Long *et al.,* 2002) and suggest that the malaria paroxysm plays a significant role in limiting the exponential growth of parasites in a nonimmune host. To perceive the mechanism of febrile temperature-induced death in *P.falciparum* parasites, Miranda use TUNEL assay to discovered results to presence of "crisis forms" and TUNEL-positive parasites suggests that febrile temperature-induced parasite killing is mediated by the mechanism of apoptotic cell death (Miranda *et al.,* 2007). Howoever, further studies demonstrating the presence of additional markers of apoptotic cell death in heat-shocked parasites will be needed to firmly establish this conclusion (Miranda *et al.,* 2007).

2.2 Effect of febrile temperature on gene expression profile of *P. falciparum*

The knowledge of molecular factors and biologic pathways triggered in response to febrile illness during a malaria infection until now are unclear. Fang and his colleagues showed that temperature regulated the transcription of select RNAs, which

was up-regulated at cooler temperatures in mosquito to human and human with fever. The rate of transcription of the two genes designed from ribosomal RNA (rRNA) was investigated, i.e., A and S rRNA and three messenger RNA (mRNA). Those encoded the *P. falciparum* proteins HRPIII, MSP-1 and CSP. HRPIII and MSP-1 mRNA expression decreased when temperature was reduced, whereas the CSP mRNA expression was found to increase **(Figure 4)** (Fang *et al.,* 2002).

Miranda and her colleagues compared the global gene expression profiles in *P. falciparum* parasites cultivated at 37°C and after heat induction at 41°C for 2 h (Miranda *et al.,* 2007). They analyzed approximately 5,300 *P. falciparum* genes and found 336 protein-coding genes (6.3% of the genome) consistently show noticeably altered expression patterns in response to elevated temperature, with approximately equal numbers of genes being transcriptional up regulated (49%) and down regulated (51%) (**Table 3**). Of these 336 genes, 208 genes were annotated as "hypothetical proteins" in the *P. falciparum* genome database (Miranda *et al.,* 2007). For better understand the functional role of these genes during febrile illness and to improve the quality of annotations in the malaria genome database, they analyzed these genes by using sequence analysis techniques. They are able to detect conserved protein domains in 101 of these "hypothetical proteins," annotate them, and consequently make new functional predictions at differing levels of detail. From this remaining set, 76 showed conserved regions that were restricted to other *Plasmodium* species or other apicomplexans like *Theileria*. Another 28 proteins of the remaining set seemed to be entirely composed of low-complexity regions and seemed not to have any significant hits to other proteins in the non redundant protein database. A list of the newly annotated genes and their assigned functional and structural predictions is available (Miranda *et al.,* 2007).

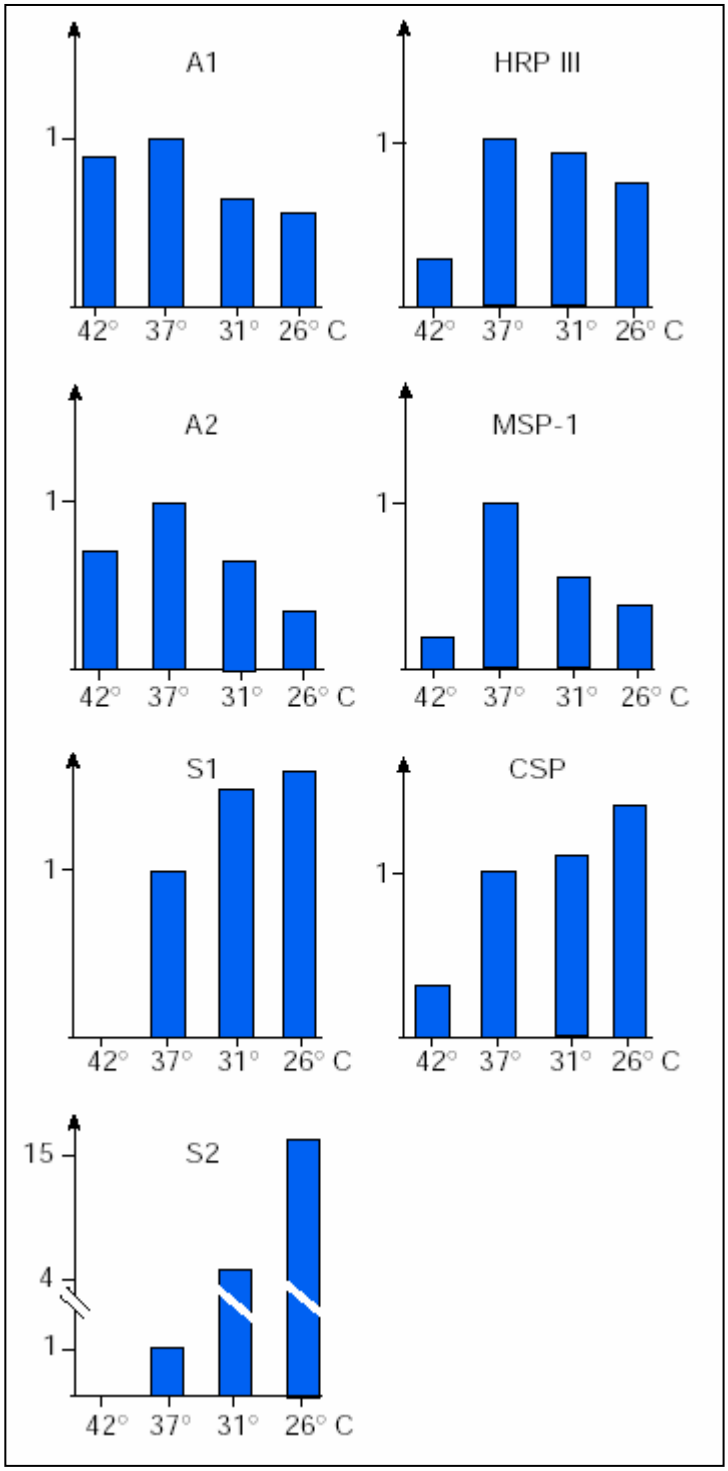

Figure 5 Effect of temperature on the selected genes transcription in the malaria parasite (Jun *et al.*, 2002)

Table 3 Febrile temperature-induced alterations in the *P. falciparum* gene expression regarding to their assigned biologic functions

Category	No. of genes		
	Total	Upregulated	Downregulated
Stress response/protein stability	22	15	7
DNA repair/replication	6	2	4
Chromatin/basal transcription	10	8	2
RNA processing	19	17	2
Translation	18	4	14
Ubiquitin-proteasome pathway	10	0	10
Secretion/protein-trafficking system	10	0	10
Glycosylation/GPI anchors	6	1	5
Signal transduction	10	8	2
Cytoskeleton	5	4	1
Lipid/fatty acid/isoprenid metabolism	14	6	8
Known transporters	7	0	7
Cell surface and adhesion	28	24	4
Basic metabolism	14	4	10
Miscellaneous	30	9	21
Apicomplexa specific	8	3	5
Plasmodium specific	72	26	46
P. falciparum specific	7	6	1
Low complexity	21	19	2
No hits	19	7	12
Total	336	163	173

2.3 Effect of febrile temperature on biological characteristics and their regulated genes

To obtain a specific representation of the cellular systems that might be altered in response to temperature stress, Miranda analyzed all the proteins encoded by the responding genes and classified them into specific biologic functional classes on the basis of the presence of conserved motifs and the pathways to which their orthologs according to her previous results. Several striking changes were seen across different functional classes (Miranda *et al.,* 2007).

2.3.1 Trafficking genes

From *Plasmodium* genome, about 47% of the genes showing altered transcription are predicted to be either transmembrane or secreted proteins. Moreover, a major component of the transcriptional response to temperature is directed at altering the cell surface and/or interactions with the host. About 22% (75 proteins) of the transcriptionally altered genes are predicted to contain the recently described Pexel motif or host target signal (consensus R/KXLXE/Q) (Hiller *et al.,* 2004; Marti *et al.,* 2004). In *P. falciparum*, 400 proteins (8% of the genome) are predicted to contain the putative Pexel motif. Of these, 225 proteins are identified as virulence proteins, and 160 are thought to be involved in the remodeling of the host erythrocyte (Marti, *et al.,* 2004). The Pexel motifs are fairly reliably detected, especially if constrained with the condition requiring them to be closely associated with a signal peptide, and show a more extended general amino acid compositional similarity around the motif. Furthermore, for several of the proteins with confidently identified Pexel motifs, for example, the rifins, Pfemp1, Psurf 4.2, some R45-like kinases and RESA-like DnaJ domain proteins, there is prior evidence for host targeting, supporting the predictive value of this motif (Hiller *et al.,* 2004; Marti *et al.,* 2004; Winter *et al.,* 2005). Nonetheless, further experimental evidence presented by additional molecules containing the Pexel motifs should fully authenticate the validity of the "Pexel motif rule." In Miranda studies, they found 72% of the proteins (54 of 75) with reliably predicted Pexel motifs encoded by the temperature-affected genes are upregulated, suggesting that there is a major extrusion of proteins into the host cytoplasm or membrane upon temperature elevation. The most prominent group of genes encoding

Pexel motif-containing proteins in our data set are the rifins. Several of the uncharacterized Pexel motif-containing proteins that show altered expression levels are *Plasmodium* specific predicted membrane proteins with large, low-complexity segments and might be involved in remodeling the erythrocyte and mediating interactions with the host, such as cytoadherence-mediated immune evasion. These results suggest that febrile illness conditions result in the en masse up regulation of proteins that might contribute toward parasite host interactions and cause necessary modifications in the host cell membrane to facilitate parasite sequestration (Miranda *et al.,* 2007).

2.3.2 Secreted and cell surface molecules

This class is basically defined as parasite-encoded polypeptides that are secreted outside the parasite cell or anchor themselves in the parasite membrane or host cell or membrane. The most striking molecules that showed a strong tendency for over expression are the Rif and Var proteins that are predicted to localize to the erythrocyte membrane (Ntumngia *et al.,* 2004). Proteins of the Rif and Var families are known to be involved in the binding of malaria parasites to receptors on the host cells, causing rosetting and sequestration, two phenomena that are associated with malaria pathogenesis (Ntumngia *et al.,* 2004). Two other surface molecules encoded by subtelomerically located genes are also up regulated, namely, PFA0135w, a homolog of the *P. falciparum* merozoite-associated tryptophan-rich antigen, and *P. yoelii* pAg-3 (Ntumngia *et al.,* 2004), a homolog of Psurf 4.2, a *P. falciparum* protein related to *P. vivax* Vir proteins (Winter *et al.,* 2005). Along with these proteins, other surface molecules that are over expressed include an ortholog of the ookinete-expressed protein SOAP of *P. berghei* (murine malaria), the so-called glycophorin-binding related antigen, a surface molecule with the anthrax-protective antigen domain (Templeton *et al.,* 2004), a protein with the membrane attack complex-perforin domain that has been implicated in invasion (Aravind *et al.,* 2003; Ishino *et al.,* 2005; Kadota *et al.,* 2004; Kaiser *et al.,* 2004), the merozoite surface protein 7, and the erythrocytebinding protein 3, a paralog of MAEBL. The elevated expression of the *P. falciparum* SOAP at febrile temperatures is of interest because this molecule is expressed in the micronemes of the ookinete in *P. berghei* malaria and

plays a role in adhesion to the mosquito basement layer (Dessens *et al.,* 2003). If this temperature induced increase in expression of *P. falciparum* SOAP also occurs at the level of translation, it could mean that *P. falciparum* SOAP may have acquired a different function or that this gene may have an additional function in the blood stages of the vertebrate host that was previously unknown. In a similar vein, the *P. falciparum* chitinase, a parasite enzyme shown to play an important role in the degradation of the insect peritrophic membrane, was also over expressed in their study (Vinetz *et al.,* 2000). These data again suggest a second function for this enzyme in modifying the deglycosylation of host molecules. However, direct biochemical studies will be needed to confirm the precise effects of altered expression of individual surface molecules in mediating different interactions with host cells. In contrast, other membrane proteins, such as at least six distinct small-molecule transporters predicted to be localized to the parasite membrane and two subunits of the vacuolar ATPase, are downregulated. The genome of *P. falciparum* possesses an intact pathway for the synthesis of glycosylphosphatidylinositol (GPI) anchors for membrane proteins, and this is consistent with the presence of several GPI-anchored proteins on the parasite membranes (Templeton *et al.,* 2004). In this study, five key enzymes in the GPI anchor biosynthesis pathway, including GPI transamidase and glycosyltransferase are consistently down regulated. This suggests that in response to elevated temperature, GPI-anchored proteins are likely to be depleted from the parasite membrane. This observation, taken together with the over expression of proteins released into the host, suggests that some type I membrane proteins on the parasite membrane might possibly be modulated to allow the aforesaid export. Interestingly, they also observed that a predicted secreted/cell surface glycosyltransferase (PF11_0487) is down regulated under febrile conditions. Sequence analysis showed that it contains a glycosyltransferase domain of the O-linked N-acetylglucosamine transferase family related to the plant Spindly-type proteins (Hartweck *et al.,* 2002). Miranda predicted that this protein might mediate as yet unnoticed glycosylation of serine and threonine residues in host or parasite proteins, which might be shut down or modulated in the febrile response (Miranda *et al.,* 2007).

2.3.3 Cytoplasmic systems and signal transduction

Miranda found that four of the five genes encoding conserved cytoskeletal proteins that were recovered in the study were up regulated, including tubulin and a homolog of the *Drosophila* actin-binding protein kelch (Anantharaman and Aravind, 2002). The only down regulated gene in this category was ADF3, an actin-depolymerizing factor related to gelsolin. A probable explanation for the observed expression pattern may be that the cytoskeleton is strengthened to compensate for the destabilizing effects of elevated temperature. All 10 genes related to cytoplasmic protein trafficking, vacuolar sorting, and secretion that were recovered in this study were found to be consistently down regulated. These included various small GTPases of the vesicular biogenesis and fusion pathway, a potential vesicular cargo-binding protein with the conserved GOLD domain (Anantharaman and Aravind, 2002), the microsomal signal peptidase, and one of the luminal disulfide bond isomerases. Similarly, 12 ribosomal protein genes and 2 genes for proteins with ribosome-associated functions were down regulated (Marques *et al.,* 2006). This apparent down regulation of several components of the protein synthesis and protein-trafficking apparatus as well as the ubiquitin-dependent protein degradation system might indicate a multilevel process to slow down the synthesis and turnover of proteins (Marques *et al.,* 2006). In terms of signal transduction, members of three distinct families of protein kinases are up regulated. Most interesting of these are the protein kinases of the Apicomplexa-specific R45 family. These predicted serine/threonine kinases are thus far found only in Apicomplexa and are characterized by several structural features that distinguish them from all other Ser/Thr kinases that have been characterized thus far. These unique structural features include the peculiar structure of the ATPbinding site in the N-terminal subdomain of the kinase and a conserved extension with a characteristic tryptophan N terminal to the kinase domain. These features suggest that these kinases target a unique set of substrates (Okley *et al.,* 2007). Furthermore, they possess a conserved Pexel motif, which has been shown to be required for their translocation to the host cytoplasm and are likely to phosphorylate targets in the host cytoplasm. The R45 family shows a lineage-specific expansion unique to the *P. falciparum* species (**Figure 6**), of which three members were found to be consistently up regulated. The fact that none of the other members of this large

lineage-specific expansion in Plasmodium are up regulated suggests that there is again a functional diversification of this recently diversified family, just as in the earlier-mentioned DnaJ proteins, with some members being recruited in the context of the febrile response (Okley *et al.,* 2007). In addition to the R45 family, two paralogous kinases of the GCN2 family of kinases, which are involved in regulating translation by phosphorylating components of the translation machinery (Wilson and Roach, 2002), are also up regulated. These kinases may also be exported to the host cytoplasm and may thereby interfere with the basic metabolism of the host cell. Two members of the calcium-dependent kinase family with EF-hand domains fused to the kinase domains are also strongly overexpressed. This family shows a lineage-specific expansion in various alveolates and might be widely used by organisms of this lineage in various signaling contexts (Marques *et al.,* 2006). In contrast, two genes for predicted calcium-binding proteins with EF-hand domains, and a mitogen-activated protein kinase are down regulated. Beyond this, no conserved signaling genes appear to be under any kind of regulation. This suggests that the transcriptional response to elevated temperature specifically affects only a small set of phosphorylation-dependent signaling pathways (Miranda *et al.,* 2007).

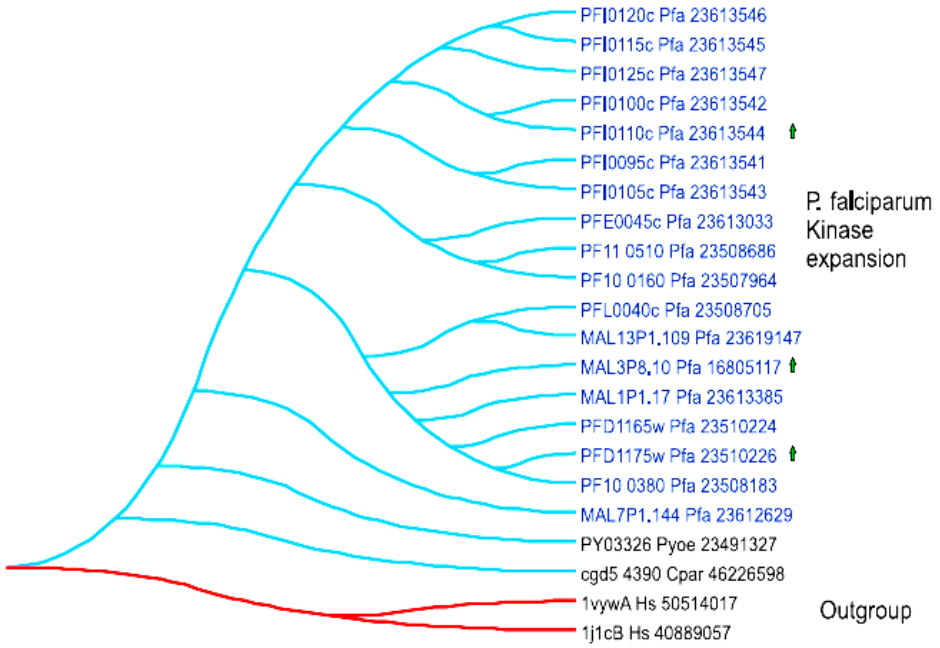

Figure 6 Tree of R45 protein kinase family showing lineage-specific expansion. A phylogenetic tree of the R45 protein kinase lineage-specific expansion in *P. falciparum*, with human kinases with structures as a outgroup, is shown. The *P. falciparum* proteins which are found to be up regulated in this study are indicated by small green arrows pointing up. The proteins are denoted by their gene name followed by the specie abbreviation and GenBank identifier (Miranda *et al.,* 2007).

2.3.4 RNA metabolism

Miranda found that 17 genes for proteins involved in different aspects of RNA metabolism, particularly splicing, mRNA maturation, and posttranscriptional gene regulation, are overexpressed when compared to those of three genes for RNA metabolism proteins that are down regulated (Miranda *et al.,* 2007). A striking, opposite regulation of two genes for Sm proteins was observed in their study. The classical Sm protein, Sm-G, which is a core component of the U1, U2, U4, and U5 spliceosomal particles, is strongly upregulated, whereas LSM6, which is a component of the U6 spliceosomal particle and decapping-dependent RNA degradation pathway, is downregulated. This pattern might indicate a change in stoichiometry of the spliceosomal components, which might affect the splicing or stability of specific mRNAs (Miranda *et al.,* 2007). The earlier work reported a family of predicted RNA-binding proteins with multiple Zn-chelating CCCH domains (typified by PFE1245w), which show a lineage-specific expansion in *Plasmodium* (Templeton, *et al.*, 2004). Two members of this expansion show a strong over expression in response to temperature stress and might participate in an apicomplexa-specific posttranscriptional regulatory mechanism. These observations point to a major potential regulatory input occurring at the level of mRNA stability and perhaps splicing (Miranda *et al.,* 2007).

2.3.5 Nuclear functions

Eight genes for chromatin components are up regulated, in comparison, only two genes are down regulated (Miranda *et al.,* 2007). The up regulated genes include the histones (H2B and H4) and the NAD-dependent histone deacetylase of the Sir2p family (*Pf*Sir2). Several genes of the DNA replication and repair systems, including the RP-A and RF-C are down regulated, whereas a Rad25-like helicase/ATPase and a DNA repair nuclease, Dem1p of the RecB family are up regulated (Miranda *et al.,* 2007). The exact implication of these changes in the expression patterns of the nuclear proteins is unclear, but it might indicate a tendency for condensation of chromatin and a possible slowdown in replication. A few DNA-binding proteins other than the histones that are associated with chromatin structure maintenance also show up regulation, namely, the BRIGHT domain protein (MAL6P1.39), which is likely to be a component of the SWI2/SNF2-dependent chromatin remodeling complexes, and

the histone-type nuclear factor Y homolog (PF14_0374). They observed that the mRNA levels of three predicted specific transcription factors show noticeable changes in response to elevated temperature. Two of these, PFL0455c with two Cterminal C2H2 zinc finger domains, and PFD0200c with the recently identified ApiAP2 DNA-binding domain, are up regulated (Miranda *et al.,* 2007). In contrast, the third transcription factor, PFE1025c, has a DNA-binding domain related to the plant p24/PBF-2 transcription factors and the ciliate TIF1 transcription factor and is down regulated. In ciliates, the orthologous transcription factor TIF1 is known to be required for the transcription of ribosomal DNA in ribosomal biogenesis (Saha *et al.,* 2001). It is likely that the *Plasmodium* protein plays a similar role, and its down regulation is consistent with the down regulation of several other ribosomal components. Another striking observation we made was that about 26% (90 genes) of the genes showing a change in transcription in response to febrile conditions map to the subtelomeric gene arrays that, in addition to members of the rif, var, and Dnaj families, also encode several other proteins. This observation indicates a strong bias in the preferential regulation of genes associated with chromosome ends (0.001 chance probability of obtaining the observed numbers by the chi test) and point to probable special chromatin-related changes in the subtelomeric regions. In particular, they noticed that at least 70% of subtelomeric genes found in our data set were overexpressed, suggesting there might be an increased accessibility of particular regions of subtelomeric chromatin to allow increased transcription of certain genes (Miranda *et al.,* 2007).

2.3.6 General metabolism

Miranda did not observe expression patterns suggesting systematic down or up regulation of entire metabolic pathways; however, expression of genes for specific components of a few metabolic pathways did seem to show alterations. The most striking alterations were seen in the case of lipid metabolism (Sciara *et al.,* 2003). *Plasmodium* possesses multiple paralogs of a fatty acyl coenzyme A synthetase, some of which have been shown to function on long-chain fatty acids. Recently, these proteins have been demonstrated to be exported in specific vesicular structures to the host cell (Matesanz *et al.,* 2003). They observed that three members of this family are

strongly or moderately over expressed under temperature stress. Furthermore, a serine Cpalmitoyltransferase (ortholog of yeast Lcb2p), which functions in sphingolipid biosynthesis, is also up regulated, and this protein is predicted on the basis of the Pexel motif to be exported into the host cell. Likewise, two paralogous genes encoding phospholipases that are predicted to convert fatty acid monoglycerides to free fatty acids are also over expressed (Matesanz *et al.,* 2003). Interestingly, the gene for an enzyme catalyzing the opposite step in the pathway, a membrane-associated lysophosphatidic acyltransferase, is strongly down regulated, implying a two-level modification of the pathway in the same general direction. These patterns suggest potential mechanisms for modification of the lipids of the host and the parasite that might be conducive for the localization of the parasite proteins and also allow the formation and maintenance of the parasitophorous membrane. PFB0590w encodes a predicted monooxygenase related to the bacteria antibiotic biosynthesis monooxygenases is down regulated under febrile conditions (Sciara *et al.,* 2003). It would be of interest to further investigate whether it might be involved in the modification of as-yet-unknown metabolites in the parasite. The gene for allantoicase, which is involved in purine degradation, is also quite strongly up regulated. This suggests that under heat shock conditions, there might be a shift to utilization of purine breakdown products as a secondary nitrogen source (Sciara *et al.,* 2003). A Cof-like phosphatase of the HAD superfamily of hydrolases, which belongs to a family of highly conserved hydrolases, is strongly over expressed in their study. However, the functional implications of this protein remain largely unclear. They believe that a combination of gene expression data, sequence analysis, and biologic experiments has helped us piece together the potential activities involved in the febrile temperature response in *P. falciparum* and is depicted in **Figure 7** (Miranda *et al.,* 2007). Miranda note, particularly, that a large number of polypeptides that are predicted or known to be exported into the host cell or expressed on the host cell surface are over expressed to various degrees under temperature stress (Miranda *et al.,* 2007). In particular, the PRESAN domain proteins, such as the DnaJ family, might form specific complexes in the host cytoplasm and modify its properties in response to the temperature elevation. In terms of a general intracellular response, the up regulation of several genes related to mRNA metabolism and splicing appears to suggest a major posttranscriptional

regulatory response. In terms of protein stability, trafficking, and protein synthesis itself, a general tendency to slow down synthesis of new proteins and degradation of existing proteins is suggested by our data. On a more pragmatic note, they observed that several *Plasmodium*- or apicomplexan- specific gene families and other enzymes with no close homologs in humans are over expressed. If this observation were to be reflected in comparable elevated protein levels, then they might serve as potential targets for therapeutic intervention or as vaccine candidates (Miranda *et al.,* 2007). In summary, Miranda data present for the first time a comprehensive view of the alterations in gene expression and predicted biochemical pathways in *P. falciparum* parasites exposed in vitro to temperatures characteristic of febrile illness, independent of confounding factors, such as host genetics and immune status (Miranda *et al.,* 2007).

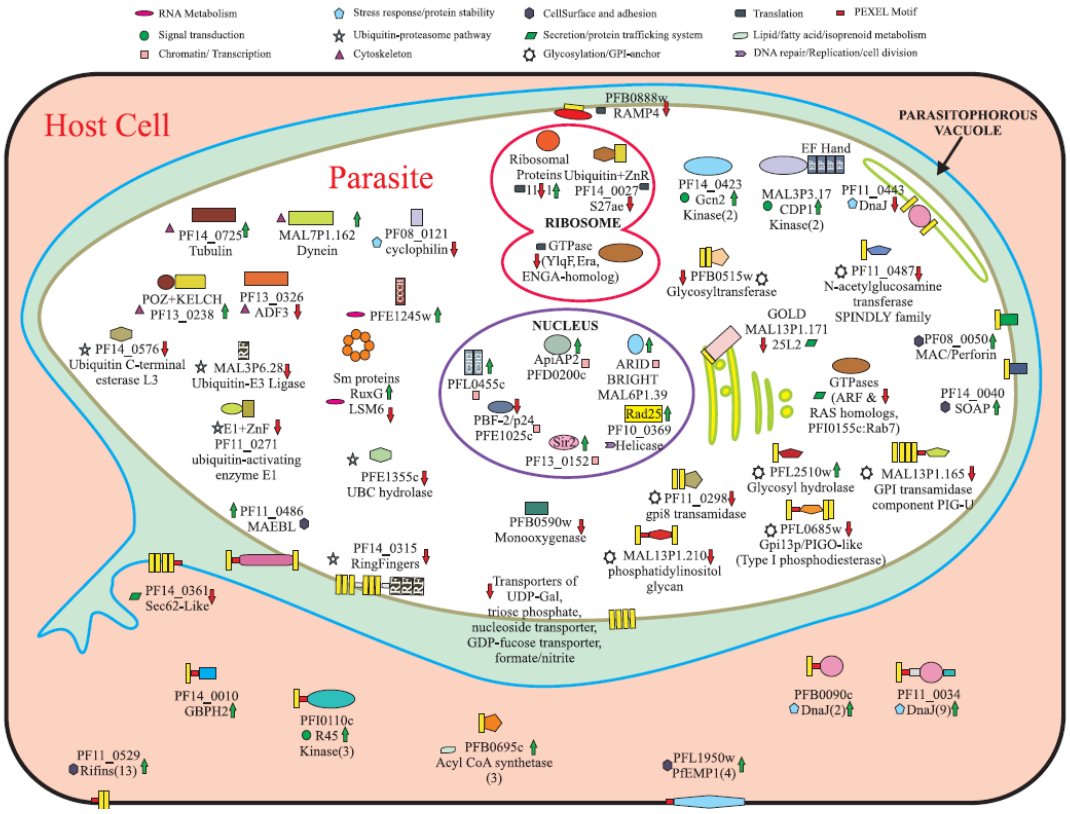

Figure 7 Cell cartoon with subcellular localization of various proteins showing altered mRNA levels under febrile temperatures. The cells and organelles are schematically shown and not drawn to scale. The membranous system depicted on the right side of the parasite cell is the Golgi and vesicular transport system. The alveolar membrane or endoplasmic reticulum system is shown to the extreme right. The domain architectures of proteins that show altered temperature response and their localizations are indicated. The representative protein name is given and the number of proteins, if any, is given in parentheses. The function legend is given on the top of the figure, and each protein is marked with a function symbol when its function is known. Green arrows pointing up indicate up regulation, while red arrows pointing down indicate down regulation. Abbreviations: RF, ring finger; GBPH2, glycophorin-binding protein homolog 2; MAC, membrane attack complex; CoA, coenzyme A. ZnF- is a zinc finger domain. All the GPI anchor biosynthesis-related proteins are shown inside the parasite cell and are likely to colocalize with the Golgi and vesicular transport system involved in secretion and maturation of proteins (Miranda *et al.,* 2007).

2.4 Effect of febrile temperature on *P. falciparum* pathology

Febrile temperature is commonly found in symptomatic human malaria and this phenomenon induces the parasites pathological evolvement in human (Udomsangpetch *et al.,* 2002). Cerebral malaria in *P. falciparum* infected patient is caused by the increase of TNF-α, resulting in the over-expression for adhesion molecules leading to fever by host immune response (Gimenez *et al.,* 2003). There are a few reports on the effect of febrile temperature on inducing cytoadherence of the ring stage *P. falciparum.* A study conducted in 10 patients with acute malaria symptoms showed that the parasite did not adhere to CD36 and ICAM-1 at 37°C. However, after briefly heated these parasites at 40° C, all of ring stage adhered to CD36 and some isolates adhered to ICAM-1. Furthermore, the experiment using A4 *P. falciparum* clone revealed the same finding, but after briefly heating, A4 was able to bind to both vascular receptors. The cytoadherence of the trophozoite stage was also increased after briefly heating. These are caused by the increase in trafficking of Pf EMP-1 (Udomsangpetch *et al.,* 2002). Febrile not only induces the cytoadherence, but they also influence the parasite clearance by antipyretics. Studies in 50 children infected with *P. falciparum* and were treated with quinine alone or quinine with an antipyretic paracetamol found that the fever clearance time for the group treated with paracetamol was shorter than that treated with the antipyretic, although the difference was not statistically significant. Parasites clearance time was found to be significantly prolonged in the group who received paracetamol, possibly through decreasing the production of TNF and oxygen radical (Christian *et al*., 1997)

The effect of febrile illness on malaria pathogenesis is now unclear. A generalized up regulation in the expression of genes that are identified as virulence factors and potential erythrocyte remodeling proteins strongly suggest that febrile illness directly affects malaria pathogenesis (Chen *et al.,* 1998). In previous observation, Miranda point to special attention to EMP-1, the most-studied virulence protein of *P. falciparum.* They find that in four of five microarray experiments, there was a consistent up regulation in the expression of five var genes. Among these, four var transcripts encode full-length Var proteins, and one of the transcripts is a truncated transcript and could have a regulatory role. How elevated temperature upregulated the expression of multiple var genes is not known (Chen *et al.,* 1998). While

simultaneous transcription of multiple var genes in a parasite isolate culture has been described earlier (Chen *et al.,* 1998), of the 60 var genes present in the *P. falciparum* genome, in a single parasite at a given time, only one var gene is expressed. The regulation of the expression of var family genes is thought to be controlled by several factors. One recently identified factor is a transcriptional regulatory protein, *P. falciparum* Sir2 (PfSir2), a molecule that has been shown to maintain the subtelomeric var genes in a silent state by deacetylating the histones that are bound to their promoter (Freitas *et al.,* 2005; Ralph and Scherf, 2005). Interestingly, they found that following heat shock, there is an average 2.4-fold increase in the level of PfSir2 expression. Some malaria researchers believe that a permutation of events, such as frequent recombinations, deletions, and gene conversions, give rise to a limitless var repertoire for antigenic variation and thus make it impossible to attain sterilizing immunity against blood-form parasites (Dzikowski *et al.,* 2006). It is understanding to assume that in an area where malaria is endemic, clinically immune adults possess immunity against a multitude of var alleles. How febrile illness influences var-mediated malaria pathogenesis is not known (Dzikowski *et al.,* 2006). In sub-Saharan Africa, the regulation of var gene expression in young children, who are the most susceptible to cerebral malaria, has not been studied. Therefore, on the basis of their results, it is tempting to hypothesize that malaria-induced fever causes enhanced expression of multiple Var proteins leading to enhanced cytoadherence in vivo, thereby modulating the pathogenesis of disease in a susceptible (Miranda *et al.,* 2007).

2.5 Effect of febrile temperature on drug transporter

Channels and transporter are integral membrane proteins that can translocate molecules and ions across biological membranes (Cecilia *et al.,* 1997). There are two classes of membrane transporter proteins which differ in the functional characteristics and mechanisms of action. A channel provides a diffusion pathway through the membrane allowing the passage of solutes by size and charge down their electrochemical gradient. It may be regulated by range of physiological signals. Unlike channels, transporters do not provide an open diffusion pathway but only move substances into and out of the cells (Cecilia *et al*., 1997).

2.6 Effect of febrile temperature on *P. falciparum* heat shock protein

In all organisms, there is an intricate network of stress responses, which ensures that cells and tissues are protected from acute and often toxic changes in the environment. Stress has broad connotations with biological systems that change the survival of the cell (Morimoto *et al*., 1997*)*. The expression of diverse stress-responsive gene and proteins which function to protect the cell and to re-establish homeostasis are induced by exposing to the stress (Morimoto *et al*., 1997*)*. These include response to extreme temperatures, UV light, oxidants, toxic chemicals, pharmacological active molecules and mutagens. Severe stress often results in a generalized arrest of DNA and RNA synthesis, if prolonged, it can lead to withdrawal of the stressed cells from the cell cycle and tissue abnormalities in multi-cellular organisms due to developmental delays (Morimoto *et al*., 1997*)*. Heat-shock response is a highly ordered pattern of genetically defined responses, which are initiated by stress. Although the exposure of cells and organisms to elevated temperatures represents the prototypical stress. Heat-shock response is induced by conditions as diverse as infection with viral and bacterial agents or exposure to transition heavy metals, amino acid analogues and oxidants (Morimoto *et al*., 1997*)*. The complexity that underlies the transcriptional regulation of heat-shock genes is exemplified by a diverse array of stress conditions, which can be separated into three major categories including: (i) environmental stress such as heat shock, amino acid analogues, drugs, toxic chemicals and heavy metals; (ii) non-stress conditions, including the cell cycle, growth factors, serum stimulation, development, differentiation and activation by certain oncogenes and (iii) pathophysiological and disease states, including oxidative stress, fever, inflammation, infection, myocardial stress and ischaemia, neural degenerative diseases and cancer (**Figure 8**) (Morimoto *et al*., 1997*)*. Although the acute response to stress may be critical for the recovery and long-term survival of the affected tissues, the chronic expression of heat shock proteins (Hsps) and molecular chaperones may also be deleterious for protein biogenesis and cell growth (Morimoto *et al*., 1997*)*. Investigations of the cellular response to heat shock and other types of physiological stresses have allowed the identification of families of heat shock proteins (Hsps) (Arrigo *et al*., 1998). Heat shock proteins range in molecular size from 8 to 150 kDa (**Table 1**) (Whitley *et al*., 1998). Among these are Hsp60, Hsp70,

and Hsp90 families containing proteins that display chaperone function(s) (Arrigo *et al*., 1998). Heat shock proteins are usually named according to their molecular size *i.e.;* 70 kDa proteins are referred to as heat shock protein 70 (Hsp70) and the gene coding for the protein would be *hsp* 70 (Whitley *et al*., 1998). Stress proteins including the Hsps have been proposed as general markers of cellular stress and their use for environmental monitoring is often suggested (Ait-Aisse *et al*., 2000). Hsps have generated renewed interest because these proteins may not only be involved in cellular protection against stress, but also in essential physiological processes in unstressed cells (Arrigo *et al*., 1998). Hsps are highly conserved abundant proteins that are found in different cellular compartments including the cytosol, mitochondria, nucleus, nucleolus, endoplasmic reticulum (ER), lysosomes and the plasma membrane (Multhoff *et al*., 1998). Many Hsps are always present in the cell while the expressions of other Hsps are increased by stress. They are rapidly induced through transcription (Whitley *et al*., 1998).

Gene transcription is controlled by heat shock transcription factors. Inactive heat shock factors exist as monomers. Once activated, they trimerize into an active form, which is capable of binding to the promoter site of the Hsp gene, which then initiates transcription (Whitley *et al*., 1998). Abnormal levels of Hsps have been found in a number of disorders including atherosclerosis, congestive heart failure, fever, infection and aging (Whitley *et al*., 1998). Several physical and chemical conditions favor the appropriate folding of proteins and are thus hazardous to cells (Smith *et al*., 1998). It has been recognized that mild temperature elevation can induce heat shock response in cells. This response is characterized by a rapid shutdown of the synthesis of most proteins by Hsp. A similar response occurs after other proteotoxic insults (Smith *et al*., 1998).

Miranda found two chaperones, the HSP70 and HSP90 orthologs, which have been implicated in the heat shock response across the phylogenetic spectrum of life, show an increased expression (Aravind *et al.,* 2003). *P. falciparum*, in contrast to other *Plasmodium* species and other apicomplexa, shows a dramatic lineage-specific expansion of a particular family of DnaJ domain proteins (Aravind, *et al.,* 2003). Outside of apicomplexa, orthologs of these proteins are currently encountered only in plants, further suggesting an ultimate origin from the plastid progenitor. Nine

members of this DnaJ expansion show elevated expression in Miranda study. In *P. falciparum*, these proteins are characterized by an additional C-terminal domain that is predicted to form a multihelical bundle enriched in charged amino acids that may serve as a surface for mediating interactions with specific protein targets. These DnaJ domain proteins also contain an N-terminal hydrophobic signal and a Pexel motif, suggesting that they are secreted into the host cell wherein they might stabilize certain complexes by acting in conjunction with their usual functional partner, HSP70. In addition to the nine members of this expansion that are expressed under elevated temperature conditions Miranda observed that there are several other members of the expansion that are not expressed. This observation suggests that after the recent lineage-specific expansion in *P. falciparum*, some were adapted for specific roles in the febrile response, whereas other members of the expansion may be deployed under as-yet-unknown conditions. This suggests that the expansion of this family might have a role in terms of multiple specific adaptations of *P. falciparum* (Marques *et al.,* 2006). Most of these *P. falciparum*-specific RESA-type DnaJ domain proteins were found to contain an additional conserved N-terminal domain. They accordingly named this conserved domain the PRESAN domain for *Plasmodium* RESA N-terminal domain (Marques *et al.,* 2006). Overall, they detected at least 67 proteins in *P. falciparum* with complete copies of the PRESAN domain and several additional fragmentary versions (5 to 10) of the domain which might represent mispredicted genes or pseudogenes (Marques *et al.,* 2006). In the publicly available draft of protein sequences of *P. yoelii, P. berghei,* and *P. vivax* in the GenBank database, we detected at least one protein each with a copy of the PRESAN domain. No versions of this domain were detected in other apicomplexan genera, suggesting that the domain was "invented" after the divergence of the lineage leading to genus *Plasmodium* but underwent a dramatic proliferation only in *P. falciparum* (Marques *et al.,* 2006). A secondary structure prediction based on the amino acid frequency, a hidden Markov model, and a position-specific score matrix derived from the multiple alignment of the PRESAN family revealed that it is composed of an all-helical fold. The core domain is predicted to contain six conserved helical segments, which are likely to form a compact bundle. Most of the highly conserved positions seen throughout the family are hydrophobic residues that are likely to form the buried core of the helical bundle.

Less conserved regions are enriched in both positively and negatively charged polar residues and likely comprise the exposed surface, which suggests a role for the PRESAN domain in protein-protein interactions (Marques *et al.,* 2006). Further iterative searches with the PRESAN domain led to the identification of the conserved extracellular domains within the Vir superfamily of proteins, including the *P. falciparum* protein PfSURFIN4.2. Both of these domains are helical and share a similar pattern of secondary structural elements, however, the Vir superfamily contains conserved cysteines that are absent in the PRESAN domains. This suggests that the two domains are likely to have emerged from a common ancestor, with the Vir superfamily specializing in extracellular interactions, whereas the PRESAN superfamily specialized in cytoplasmic interactions (Marques *et al.,* 2006). Paradoxically, 10 different genes for proteins of the ubiquitin metabolism system were observed to be consistently down regulated in this study. These include proteasomal enzymes, different E1 and E3 enzymes, as well as some ubiquitin C-terminal hydrolases (Marques *et al.,* 2006). As a validation of our microarray results, Miranda noted that polyubiquitin (PFL0585w), the only ubiquitin pathway gene found to be up regulated in their data set (1.53-fold change), was also up regulated in response to elevated temperature in an earlier published report (Horrocks and Newbold, 2000). To establish a relationship between their microarray data and its biologic relevance, they measured total ubiquitination of proteins isolated from parasites incubated at 37°C and 41°C, using a rabbit polyclonal bovine antiubiquitin antibody. Comparison of expression levels obtained by ECL-based semiquantitative Western blot analysis of *P. falciparum* parasite extracts collected after incubation at 37°C or 41°C for 2 h demonstrated that temperature elevation causes a generalized down regulation in the ubiquitination process (Marques *et al.,* 2006). On the basis of its immunoreactivity with antiubiquitin antibody, there is a significant depression in ubiquitination of both high-molecular-mass and low-molecular-mass protein adducts following treatment at 41°C. A quantitative analysis based on intensities of bands measured between the areas marked by asterisks that includes the high- and low-molecular-weight proteins from parasites incubated at 37°C and 41°C gave IOD units of 19,047 and 1,291, respectively, demonstrating a 14.8-fold down regulation in the ubiquitination process (Marques *et al.,* 2006). The significance of this biologic assay is twofold. First, it

confirms that changes in expression are occurring at the protein level as well as the mRNA level. Second, while their microarray data capture changes in expression of individual enzymes in the ubiquitin pathway, this assay quantifies total ubiquitination of all parasite proteins. It may seem rather counterintuitive that the ubiquitin pathway is downregulated in response to elevated temperature, which undoubtedly results in the accumulation of misfolded proteins that may become toxic to the cell if not removed. However, depression of the ubiquitin pathway may be a mechanism to increase the halflives of certain proteins under stressful conditions (Marques *et al.,* 2006). A recent study suggested that protein degradation by ubiquitination and HSP-assisted refolding do, in fact, act in concert with one another and may even at times compete for the same substrates (misfolded proteins) (Marques *et al.,* 2006). Another plausible explanation for a generalized depression in the ubiquitin pathway could be a parasite strategy to conserve energy at times of duress. It is estimated that approximately 30% of nascent proteins are degraded by the proteasome in unstressed cells (Schubert *et al.,* 2000); therefore, even a slight decrease in the ubiquitin pathway will result in a considerable increase in energy available for other cellular processes (Miranda *et al.,* 2007).

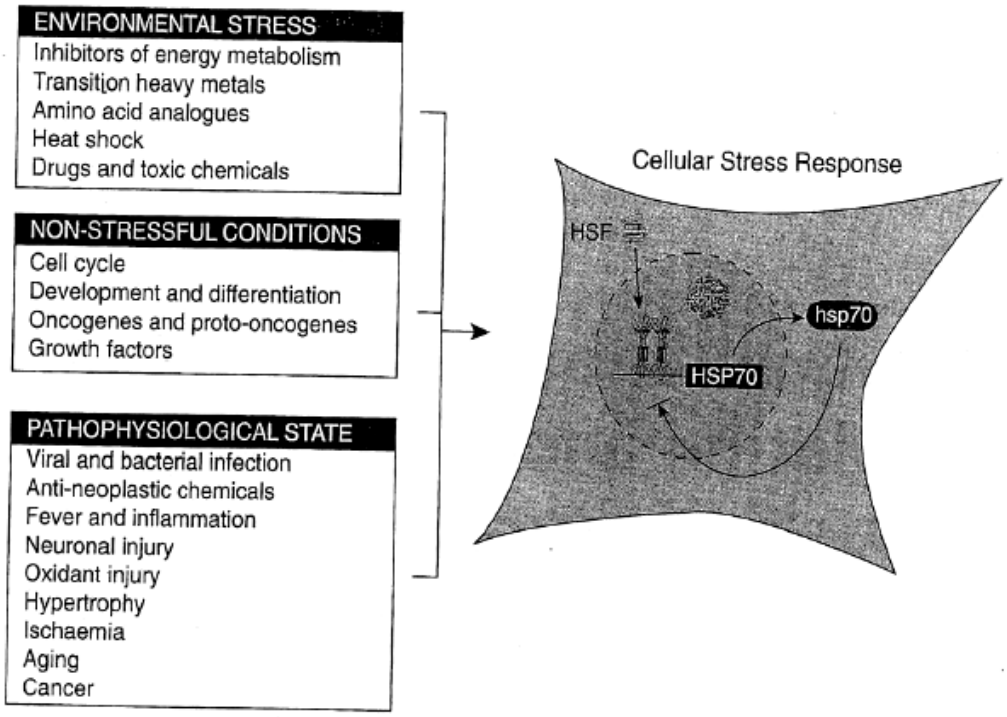

Figure 8 Cell stress response. Conditions that induce heat-shock gene expression. The diagram depicts the stress-dependent activity of heat shock transcription factor (HSF), which occurs in response to environmental, non-stressful, and pathophysiology stress resulting in elevated transcription of the heat shock protein 70 (HSP70) gene and expression of heat shockprotein 70 (hsp70) (Morimoto *et al.,*1997)

Many of the major heat shock proteins, *i.e.,* Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 assist in the folding of newly synthesized proteins, refolding of denatured proteins or promoting their degradation after stress or injury. They are therefore referred to as "molecular chaperones" because of these functions **(Figure 9)** (Whitley *et al*., 1998). Molecular chaperones are critically important because they appear to be necessary in the critical steps of the three dimensional folding of some newly formed proteins within the cell (Whitley *et al*., 1998). Although the amino acid sequence alone is sufficient to dictate the native conformation of small proteins *in vitro,* most polypeptides would fail to fold efficiently in the highly concentrated and complex cellular environment without the assistance of molecular chaperones (Smith *et al*., 1998). Chaperones ensure that newly formed polypeptides proceed correctly through folding and unfolding to eventually achieve a functional shape (Whitley *et al*., 1998). Many molecular chaperones and their associated co-chaperones are constitutively expressed in all cells (Smith *et al*., 1998). They are found in all components of the cell, but the endoplasmic reticulum (ER) provides a highly specialized environment for chaperone activity as it has its own complex chaperone machinery. The two major chaperones in the ER are the Bip/Grp78 and Grp94/gp96 members of the Hsp70 and Hsp90 families, respectively. Compared to the cytoplasm, the lumen of the ER is a distinct folding environment in which the redox potential is oxidizing and there is a relatively high concentration of calcium (Ca^{2+}) (Smith *et al*., 1998). After chain elongation is complete, the incompletely folded nascent chain may undergo sequential rounds of chaperone binding. Only fully folded chains and properly assembled oligomeric complexes exit efficiently from the ER and progress to the Golgi apparatus. Mis-folded or unassembled proteins are retained in the ER by continued chaperone interactions but combinations of chaperone interactions vary with different substrates. The ER chaperone machinery has been characterized as a quality control station. Apart from their role in protein folding, several components of the chaperone machinery appear to function as regulatory factors for a variety of signaling proteins. Hsp90 has been found to interact with multiple regulatory proteins including the steroid hormone receptors and several transcription factors unrelated to steroid receptors (Smith *et al*., 1998). Molecular chaperones differ in their ability to stabilize non-native polypeptides and to mediate protein folding defining "holding" and

"folding" systems (Luders *et al*., 1998). Mammalian cytosolic and nuclear chaperone Hsc70 can act as both a "holding" and a "folding" system depending on the chaperone cofactors which associate with it (Luders *et al*., 1998).

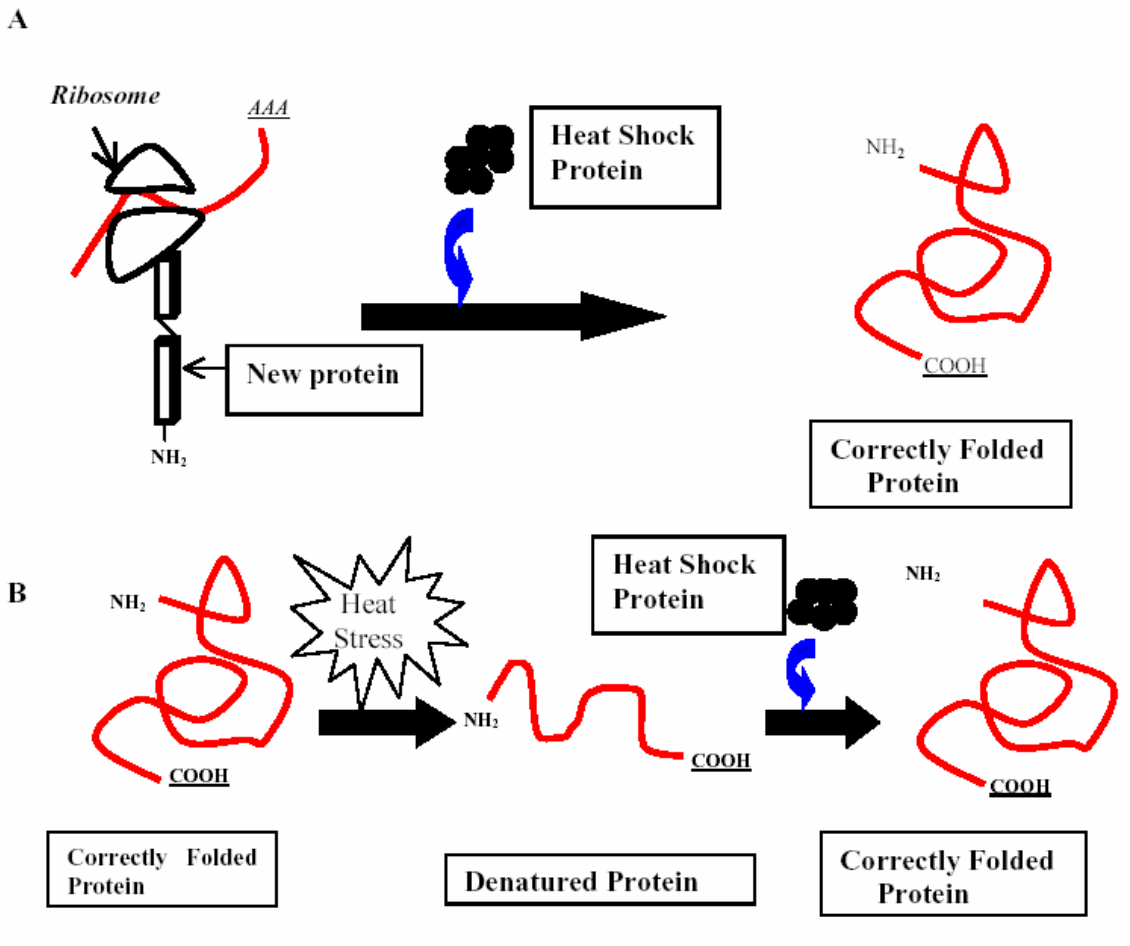

Figure 9 Functions of heat shock proteins. Two of functions of heat shock proteins. A: As new proteins are being produced; heat shock protein assists in the folding of protein into functional proteins. B: After stress, heat shock proteins also assist in refolding or degradation of damaged or denatured proteins (modified from Whitley *et al.*, 1998).

3. Heat shock proteins in *P. falciparum*

Like many prokaryotes and eukaryotes, the malaria parasite also synthesizes several stress proteins (Sharma, 1992). Techniques like recombinant DNA technology have been applied for investigation of malarial Hsp. Five Hsp-encoding genes from *P. falciparum* which are located on different chromosomes have been identified. The genes encoding three Hsp70 and one Hsp90 of the *P. falciparum* have been cloned. These genes are classified as *Pf*Hsp70-I, *Pf*Hsp70-II, and *Pf*Hsp90. The biological roles of these proteins in malaria are not fully understood but it is possible that they provide protection to the parasite from various stress encountered in the host (Sharma, 1992). In this process, Hsps probably bind to the toxic molecules as well as damaged proteins, so as to remove them out of the parasite. Their involvement in the stage–specific parasite transformation to increase the infectivity and virulence as observed in other parasites remains to be determined. Malaria Hsps are antigenic in humans. This antigenicity could be attributed to the non-homologous sequences in the *C*-terminal region (Sharma *et al.*, 1992). The upstream sequence of the Hsp-encoding gene contains several conserved regions. These regions are known as heat shock elements (HSE), where the transcriptional activator, heat shock factor (HSF) binds to initiate transcription. At high temperatures however, this factor (HSF) binds more efficiently to HSE, thus enhancing the transcription of the Hsp-encoding genes (Sharma *et al.,* 1992).

3.1 *Plasmodium falicparum* Hsp70 (*Pf*Hsp 70)

P. falciparum Hsp70 (*Pf*Hsp70) has an apparent molecular mass of 70 kDa. PfHsp70 is synthesized by all asexual blood stages of the parasite, and is reported to be a soluble cytoplasmic protein (Sharma, 1992). *Pf*Hs70 are barely detected in the salivary gland of sporozoites (Kumar and Zheng, 1992). They are expressed at elevated levels in the parasites undergoing development in liver cells (exo-erythrocytic stages). Temperature shift studies in blood-stage parasites of the *P. falciparum* have also shown that the Hsp70 like protein is heat inducible. *Pf*Hsp70 is located in the nuclear and cytoplasmic compartments of the parasite (Kumar and Zheng, 1992).

3.2 Effect of heat on PfHsp70

The effect of various body temperatures encountered during malarial fever on the synthesis of *P. falciparum* heat shock protein *Pf*Hsp70 and parasite growth rates among 5 different isolates were carried out (Biswas and Sharma, 1994). The results showed that after the exposure of the parasite to 39°C for 30 minutes, the amount of *Pf*Hsp70 in all 5 isolates increased markedly and significantly, whereas parasite growth rates and the amount of total blood stage antigens remained almost unaffected. This indicated that the *Pf*Hsp70-encoding gene responded to heat shock by producing higher amounts of *Pf*Hsp70 proteins presumably to protect the parasite from being killed during malaria fever (Biswas and Sharma, 1994). In various experiments, *P. falciparum* was subjected to heat shock for varying times and temperatures in order to test for their viability, growth, and expression (Joshi *et al*., 1992). Results showed that the majority of parasites remained viable after heat shock but their growth was affected. Those growing at 37°C grew faster compared to those exposed to 35°C, 39°C or 41°C for certain periods of time before returning them to 37°C for 48 hours. At 41°C, the maximum numbers of parasites were found dead after heat-shock; the death rate was increased if they were exposed for 30 minutes or longer (Joshi *et al*., 1992). PfHsp70 gene are expressed and enhanced after heat shock, and these results in the malaria parasites being able to survive *in vivo* during fever. This is probably due to their over expression (Joshi *et al*., 1992).

3.3 Structure of *Pf*Hsp70

Cloned *Pf*Hsp70 family from *P. falciparum* of 75 kDa and 72 kDa were shown to share sequence similarities with eukaryote Hsp70 (Kumar and Zheng, 1992). Like eukaryotic Hsp70, they contain three domains, an *N*-terminal ATPase domain of 45kDa, a central substrate binding domain of 15 kDa and a *C*-terminal domain of approximately 10 kDa. At the *C*-terminal, there is a highly conserved GMP motif that was shown to cause an immune response in mice (Kumar and Zhenga, 1998). *Pf*hsp70 contains a conserved EEVD motif also found in Hsp70 of eukaryote, which binds to TPR motif of HOP.

3.4 Heat shock protein DnaJ homologues from *P. falciparum* (Pfj 1)

The complete sequence of a DnaJ homologue, Pfj1 from *P.falciparum* was determined by Watanabe (Watanabe, 1997). Comparison of Pfj1, DnaJ and Mdj1 (mitochondrial DnaJ homologue of Saccharomyces cerevisiae) revealed five characteristic regions. Four repeats of Cys-x-x-Cys-x-Gly-x-Gly (204-254 aa) were found which resembled a zinc-finger motif. It was found that this region actually binds two zinc ions and functions in substrate-binding. Down stream of the repeats is a region with 140 amino acids (270-403 aa), which also contain conserved amino acids. The determined sequence contained 1881 nucleotides encoding 627 amino acids. Pfj1 contained an additional 200 amino acid (424-627 aa) with a stretch rich in lysine and proline. Pfj1 belongs to the classical DnaJ group which functions in prokaryotes or mitochondria. According to the classification in Cheetham (Cheetham *et al.,* 1998) Pfj1 belongs to Type I. Type I have full domain conservation with DnaJ. Pfj1 may play important roles in the adaptation to homeothermal vertebrate hosts (Watanabe, 1997).

3.5 Immunology of *Pf*Hsp70

Frequently antibodies against heat shock proteins are generated as a result of parasite infection and the best characterized is the *Plasmodium* chaperone member of the Hsp70 family (Tsuji *et al.*, 1994). Different *P. falciparum* Hsp70 genes have been identified. Three monoclonal antibodies generated by immunization of mice with *P. berghei* infected red blood cells were found to react with the Hsp70 (Tsuji *et al.*, 1994). These monoclonal antibodies react not only with sporozoites of *P. berghei* but also with sporozoites of several other rodent and human *Plasmodium* species (Tsuji *et al.*, 1994). Specific antibodies against *Pf*Hsp70 were detected in the sera of individuals exposed to the parasite (Kumar *et al.*, 1990). *P. falciparum* from different geographical locations showed conserved genes for Hsps, thus they are likely to be immune targets in various epidemic areas (Kumar *et al.*, 1990). Lymphocytes from two tested immune donors responded in proliferation assays to purified *Pf*Hsp70 and recombinant protein (*Pf*Hsp70). Similar response was seen in lymphocytes from non-immune individuals and raised questions pertaining to a generalized responsiveness of

lymphocytes to some common determinants present in heat shock-related proteins in various pathogens (Kumar *et al.,* 1990).

A gene was cloned that had encoded a novel protein with sequence domains, some similar to the Gly – Gly – Met – Pro (GGMP) repeat region found near the carboxyl terminus of the Hsp70 of *P. falciparum*. The other exhibited highly significant resemblance to analogous acidic and basic domains found in numerous eukaryotic transcription factors (Uparanukraw *et al.*, 1993). Early Hsps of a wide variety of organisms have been found to be targets of cellular and humoral immune response (Kumar and Zheng, 1998). Hsp70 of the malaria parasite *P. falciparum* have been shown to be targets of the natural immune response. Antibodies recognize specific epitopes in *Pf*Hsp70 and T –cells which are targets of antibody–dependent cell-mediated cytotoxicity. It was found that the GGMP repeat region in the *Pf*Hsp70 immunogen was a dominant epitope recognized by large proportion of mice. Further evidence to show a dominant antibody response against the GGMP epitope came from the studies in which the peptide was conjugated to the carrier molecule bovine serum albumin (BSA).