UNIVERSITÉ DE LA MÉDITERRANÉE – AIX-MARSEILLE II

École doctorale des Sciences de l'Environnement

THÈSE DE DOCTORAT

En vue d'obtenir le grade de

Docteur de l'Université de la Méditerranée

Spécialité «Environnement et Santé»

Influence des conditions environnementales sur le métabolisme de *Plasmodium falciparum*

Présentée et publiquement soutenue par

Marylin TORRENTINO-MADAMET

Le 1er décembre 2010

Membres du jury :

Professeur Yves JAMMES	Président et Directeur de thèse
Docteur Isabelle FLORENT	Rapporteur
Docteur Éric MARÉCHAL	Rapporteur
Professeur Daniel PARZY	Co-directeur de thèse

Institut de Médecine Tropicale du Service de Santé des Armées – IRBA Antenne de Marseille
Unité de Recherche en Pharmacologie et Physiopathologie Parasitaires
UMR-MD3 - Relations Hôtes-Parasites, Pharmacologie et Thérapeutique
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Sommaire

Préambule

Ayant débuté ma carrière scientifique au sein d'une institution de recherche du service de santé des armées, je n'aurai pu commencer ce rapport de thèse sans rappeler le souvenir du médecin officier de l'armée française, Charles Louis Alphonse Laveran. En 1907, Alphonse Laveran a reçu le Prix Nobel de Physiologie et de Médecine pour sa découverte de l'hématozoaire du paludisme.

1. Bref historique du paludisme

Le paludisme est une parasitose qui sévissait déjà avant l'apparition de l'homme. Les gorilles seraient à l'origine de la transmission du parasite à l'espèce humaine (Liu et *al.*, 2010). Ces parasites d'origine africaine auraient co-évolué depuis plusieurs milliers d'années avec les anthropoïdes africains, puis avec les hommes. Ainsi, l'histoire du paludisme se confond avec celle de l'humanité et peut se résumer en quatre grandes périodes :

- une période clinique et épidémiologique, qui commence des décennies avant Jésus-Christ, au cours de laquelle le paludisme fut longuement décrit et cité dans les écrits ;

- une période thérapeutique avec les premiers usages du Quinquina par les indiens du Pérou en 1630 ;

- une période parasitologique avec la découverte de l'agent infectieux responsable du paludisme par Alphonse Laveran qui cite : «*En 1880, à l'hôpital militaire de Constantine, je découvris sur les bords des corps sphériques pigmentés, dans le sang d'un malade atteint de fièvre palustre, des éléments filiformes ressemblant à des flagelles qui s'agitaient avec une grande vivacité en déplaçant les hématies voisines ; dès lors je n'eus plus de doutes sur la nature parasitaire des éléments que j'avais trouvés (...). En 1889, mon hématozoaire avait été retrouvé dans la plupart des régions palustres ; on ne pouvait plus mettre en doute ni son existence, ni son rôle pathogène (...). Après avoir tenté vainement de déceler le parasite dans l'air, dans l'eau ou dans le sol des localités palustres, et de le cultiver dans les milieux les plus variés, je suis arrivé à la conviction que le microbe se trouvait déjà en dehors du corps de l'homme, à l'état parasitaire, et très probablement à l'état de parasite des moustiques. J'ai émis cette opinion dès 1884 dans mon "Traité des fièvres palustres" et j'y suis revenu à plusieurs reprises.»*. L'hématozoaire identifié, il restait à découvrir quel vecteur était responsable de sa transmission à l'homme. C'est à Ronald Ross (Prix Nobel de Médecine de 1902) que revient le mérite d'avoir démontré que les hématozoaires du paludisme accomplissaient, chez leurs hôtes, plusieurs phases de leur développement et étaient transmis

par des moustiques. En parallèle, Grassi, Bastianelli et Bignami (1899) décrivaient le cycle de développement de *Plasmodium falciparum, Plasmodium vivax* et *Plasmodium malariae* chez *Anopheles claviger*. Cette période parasitologique s'achève avec la mise au point de la culture *in vitro* de *P. falciparum* sur des érythrocytes humains par Trager et Jensen en 1976 (Trager et Jensen, 1976).

- enfin, une période de lutte anti-vectorielle (drainage des marais, destruction des larves aquatiques, usage de moustiquaires…) et de lutte anti-paludique avec la découverte en 1930 des antipaludiques de synthèse. Les recherches sur la mise au point d'un vaccin débuteront en 1969 à partir de sporozoïtes irradiés de *P. berghei* (Nussenzweig et *al.*, 1969).

Dans les années 50, l'efficacité des antipaludiques a permis de diminuer fortement l'incidence du paludisme. Mais, dés le début du siècle dernier, les premiers cas de souches de *P. falciparum* résistantes à la quinine sont découverts (Tableau 1).

Antipaludiques	Première utilisation	Première résistance	Délai en années $1^{ère}$ Utilisation/$1^{ère}$ Résistance
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxine-pyriméthamine	1967	1967	0
Méfloquine	1977	1982	5
Atovaquone	1996	1996	0

Tableau 1 : Dates de première utilisation et du premier cas rapporté de résistance aux antipaludiques (Wongsrichanalai et al., 2002).

L'émergence et l'expansion de la résistance du parasite à la plupart des antipaludiques, même les plus récents, rendent indispensables la recherche de nouvelles cibles thérapeutiques ainsi que le développement d'associations de molécules. Cette résistance grandissante a amené l'OMS à préconiser l'utilisation des associations d'antipaludiques, notamment celles à base d'artémisinine et de ses dérivés (Artemisinin-based Combination Therapy, ACT) (OMS, 2010). L'action synergique de ces associations permet d'augmenter l'efficacité du traitement et de retarder l'apparition de la résistance de *P. falciparum* aux antipaludiques. Cependant, l'apparition de souches de sensibilité diminuée aux dérivés de l'artémisinine laisse supposer l'émergence de résistances dans un futur proche (Noedl et *al.*, 2010).

En parallèle, de nouvelles approches telles que les technologies à grande échelle sont apparues et marquent l'entrée dans une nouvelle période post-génomique.

2. Les technologies à grande échelle

Les projets de séquençage du génome de *P. falciparum* et d'*Anopheles gambiae* (principal moustique vecteur du paludisme) ont été initiés respectivement en 1996 et 1998, par un Consortium International des Centres de Génome réunissant trois grands groupes : The Sanger Center (Cambridge, Angleterre), The Institute of Genomic Research (Texas, Etats-Unis) et l'Université de Stanford (Californie, Etats-Unis). Les génomes de la souche 3D7 de *P. falciparum* et d'*A. gambiae* ont été publiés en 2002 (Gardner et *al*. 2002 ; Holt et *al*. 2002). L'annotation du génome de *P. falciparum*, fondée sur la combinaison de méthodes de détection d'ORFs (cadres ouverts de lecture) et sur l'étude d'homologie de séquence avec d'autres organismes (*Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Arabidopsis thaliana*), a permis de prédire environ 5300 gènes [http ://plasmodb.org/]. Sur les 5300 protéines prédites, 60% n'ont aucune fonction assignée (Brick et *al*., 2008 ; Paila et *al*., 2008) ; probablement en raison de l'extraordinaire richesse en adénines (A) et en thymines (T) du génome parasitaire (80,6% versus 60% chez l'homme) et de l'abondance de répétitions de faible complexité (Gardner et *al*., 2002 ; Winzeler et *al*., 2008). Le biais compositionnel au niveau des nucléotides engendre un biais protéique avec une préférence pour les acides aminés N, K, I, L, E et D, conséquence d'un échec de détection de similarité de séquences (Bastien et *al.*, 2004). L'ensemble de ces données a permis de reconstituer, au moins partiellement, de nombreuses voies métaboliques dont certaines spécifiques du parasite, permettent d'ouvrir de nouvelles perspectives thérapeutiques [http ://sites.huji.ac.il/malaria, 2010]. Actuellement, le plus grand défi est certainement d'élucider la fonction des protéines classées hypothétiques ainsi que de définir les interactions entre ces protéines (Wuchty et Ipsaro, 2007). Ce que peut permettre des approches globales, telles que les technologies de transcriptomique (Gardner et *al*., 2002 ; Le Roch et *al*., 2004 ; Bozdech et *al*., 2003 ; Oakley et *al*., 2007 ; Gunasekera et *al*., 2007 ; Daily et *al*., 2007 ; Hu et *al*., 2010) ou de protéomique (Florens et *al*., 2002 ; Lasonder et *al*., 2002 ; Tarun et *al*., 2008 ; Radfar et *al*., 2008 ; Prieto et *al*., 2008 ; Sturm et *al*., 2009).

Les premières études de transcriptomique ont montré qu'environ 60 % des gènes du parasite sont exprimés au cours du cycle érythrocytaire et l'existence d'une redondance dans l'utilisation des voies métaboliques tout au long du cycle de vie de *P. falciparum*. Ces études

révèlent un programme de co-expression des gènes pour certaines fonctions spécialisées sans organisation chromosomique particulière (Bozdech et *al*., 2003 ; Le Roch et *al*., 2003). Bozdech et *al*. ont représenté l'expression des gènes de *P. falciparum* avec un profil périodique au cours des 48 heures du cycle érythrocytaire (Figure 1). La comparaison des profils d'expression de différents clones (HB3, 3D7, et Dd2) par l'équipe de DeRisi (Llinas et *al*., 2006) montre que cette vague transcriptionnelle est très conservée [http ://www.plasmodb.org].

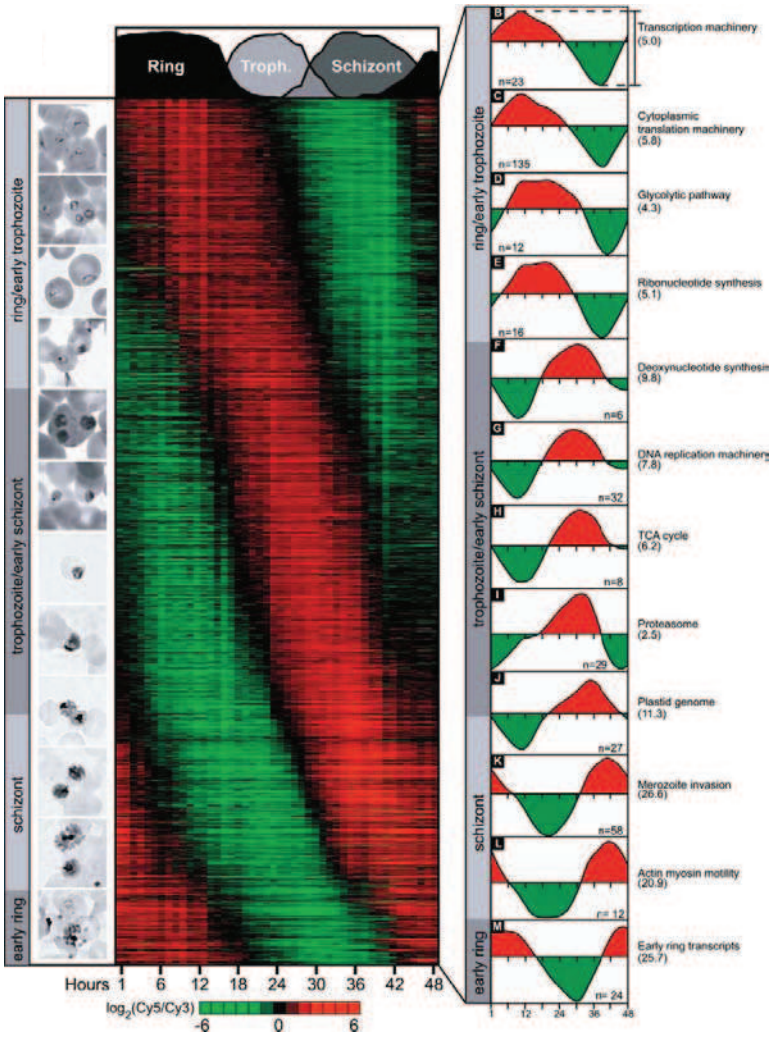

Figure 1. Profil transcriptionnel périodique des gènes de P. falciparum au cours des 48 heures du cycle érythrocytaire (Bozdech et al., 2003).

Ce profil périodique de l'expression des transcrits corrèle avec l'expression de la majorité des protéines parasitaires (Foth et *al*., 2008). Les données récentes sur le transcriptome de plasmodium montrent que l'expression des gènes peut varier en fonction

d'un stress associé à diverses antipaludiques (Gunasekera et *al*., 2007 ; Le Roch et *al*., 2008 ; Hu et *al.*, 2010).

3. Enjeu de ce projet de thèse

Dès le début du siècle dernier, la description du cycle biologique de *P. falciparum* laisse évoquer que le parasite est soumis à des conditions environnementales très hétérogènes pas toujours favorables à son développement qui lui imposent la mise en place de mécanismes de spécialisation et d'adaptation.

Ainsi, du fait de son passage dans des environnements de concentrations variables en oxygène (glandes salivaires du moustique, circulation sanguine, circulation pulmonaire), l'oxygène nous est apparu comme un élément environnemental important. Dans cet objectif, notre intérêt s'est porté sur la réponse du métabolisme de *P. falciparum* en conditions hyperoxique et en présence de molécules inhibitrices du métabolisme respiratoire.

Cette thèse a débuté de façon concomitante avec le développement des plates-formes de transcriptomique et de protéomique au sein de notre unité de parasitologie. La mise en œuvre de ces technologies a demandé une grande mobilisation du personnel ; j'ai ainsi participé étroitement au développement des puces à ADN dédiées à *P. falciparum* et à la mise en place de la plate-forme transcritomique.

Le dernier volet abordé dans cette thèse est la mise en évidence d'une voie respiratoire alternative, évoquée mais jamais identifiée chez *P. falciparum*, qui pourrait être une cible thérapeutique potentielle en raison de son absence chez l'homme.

INTRODUCTION

1. Le paludisme à *Plasmodium falciparum*

Le paludisme est une érythrocytopathie parasitaire causée par un hématozoaire du genre *Plasmodium*, transmise à l'homme par la piqûre de moustique femelle du genre *Anopheles*.

Cinq espèces de plasmodies sont pathogènes pour l'homme : *P. falciparum, P. vivax*, *P. malariae, P. ovale* et *P. knolewsi*. Cependant, *P. falciparum* est l'espèce mortelle la plus répandue et responsable de la majorité des formes graves du paludisme, souvent mortelles.

1.1. Fléau mondial

Le paludisme demeure toujours un véritable fléau mondial. En Afrique, toutes les 30 secondes, un enfant meurt de cette pathologie. Environ 40% de la population mondiale vit dans des zones d'endémie palustre, soit 2,3 milliards d'individus. Le paludisme touche une centaine de pays dans le monde, particulièrement les zones tropicales défavorisées d'Afrique, d'Asie et d'Amérique Latine (Figure 2). L'Afrique est le continent le plus touché avec 90% des cas de paludisme recensés dans ses zones tropicales, dont 1 million d'enfants de moins de cinq ans.

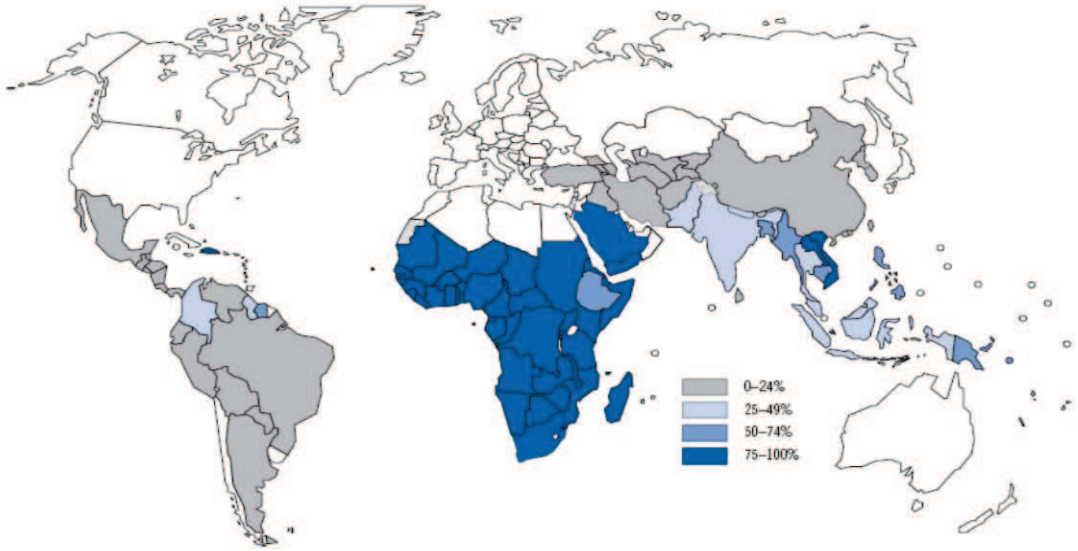

Figure 2. Pourcentage de paludisme à P. falciparum dans le monde en 2006 (source OMS : World Malaria Report 2008)

Les efforts déployés par les organismes internationaux ont permis de réduire la mortalité liée au paludisme à 1 million sur les 500 millions de cas observés chaque année (OMS, 2008). Cependant, le paludisme conserve un retentissement économique majeur sur les pays fragiles. Il existe une étroite corrélation entre le niveau de développement économique et l'incidence du paludisme (perte de croissance de 1,3% par an soit 12 milliards de dollars) (Breman et *al.*, 2004).

En Europe, l'accroissement des échanges professionnels et touristiques ainsi que les mouvements de population migrante sont responsables de l'augmentation du paludisme d'importation. La France est le premier pays européen touché avec 4000 cas recensés en 2009 (BEH, 2010). La majorité des cas survient chez des personnes n'ayant pas suivi de mesures préventives comme la protection contre les piqûres de moustiques et la chimioprophylaxie (Armengaud et *al.*, 2006 ; Doudier et *al.*, 2007).

1.2. Les signes cliniques du paludisme

L'accès palustre consiste généralement en des accès de fièvres accompagnés de multiples symptômes comme des malaises, nausées, maux de tête, douleurs musculaires et diarrhées modérées. La faible spécificité de ces symptômes, souvent attribués à des infections intestinales, est un des facteurs aggravant de l'endémie palustre.

Lors d'une primo-invasion, l'accès palustre simple comprend des épisodes fébriles, précédés de frissons et suivis de sueurs qui se répètent toutes les 48 heures. Les complications pouvant survenir sont dépendantes de plusieurs facteurs, dont notamment l'immunité éventuellement acquise par l'hôte, le fond génétique de l'hôte mais aussi du degré de chimiorésistance et de virulence de la population de *Plasmodium* impliquée dans l'infection.

Dans le cas de *P. falciparum*, il existe trois complications majeures qui peuvent être concomitantes et ont un pronostic mortel: l'anémie grave, le syndrome de détresse respiratoire et le neuropaludisme. L'anémie grave, cause principale de mortalité palustre, est définie par un hématocrite inférieur à 0,15 % associée à la présence de *P. falciparum*. Elle est causée par la corrélation de deux évènements: d'une part l'éclatement des érythrocytes causé par la libération du parasite et, d'autre part, la réduction de la production d'érythrocytes durant la maladie (Mackintosh et *al.*, 2004). La détresse respiratoire est souvent la cause de la survenue d'un oedème respiratoire chez l'adulte mais est dépendante d'une acidose métabolique chez l'enfant (Taylor et *al.*, 2002). Cette dernière est sans doute causée par le parasite lui-même et la réponse cytokines des cellules humaines à l'infection (Mackintosh et *al.*, 2004). Enfin, le neuropaludisme consiste en une encéphalopathie diffuse se traduisant par un syndrome

neurologique qui conduit généralement à un coma qui peut être suivi par la mort. La séquestration des globules rouges parasités, ainsi que la réponse de l'épithélium et du système immunitaire à cette obstruction des vaisseaux sanguins, semblent avoir un rôle important dans cette complication (Mackintosh et *al.*, 2004).

L'évolution du paludisme vers les formes graves peut être évitée par une prise en charge immédiate du patient et l'administration d'antipaludiques thérapeutiques comme la quinine ou une association quinine-dérivés d'artémisinine.

1.3. Éradiquer le paludisme

Les moyens de lutte contre le paludisme, recommandés par l'Organisation Mondiale de la Santé, impliquent simultanément la protection contre les vecteurs et la chimioprophylaxie contre le parasite. La lutte anti-vectorielle repose sur l'utilisation de moustiquaires imprégnées d'insecticides, l'éradication des gîtes larvaires aquatiques et l'utilisation d'insecticides. Cependant, les vecteurs *Anopheles* montrent une remarquable capacité à développer une résistance aux insecticides utilisés. Les molécules antipaludiques restent donc une mesure complémentaire nécessaire pour se protéger du paludisme en zone d'endémie.

1.3.1. Les molécules antipaludiques

Plusieurs molécules antipaludiques peuvent être utilisées en prophylaxie ou en thérapeutique. L'essentiel de l'arsenal antipaludique disponible actuellement agit sur les formes intraérythrocytaires de *P. falciparum* (Biot et *al*., 2008).

Les antipaludiques peuvent être classés selon différents critères :

- leur site d'action,
- leur mécanisme d'action,
- leur groupe chimique (Tableau 2),
- ainsi que leur cible parasitaire (Figure 3).

Forme parasitaire	Site d'action	Mécanisme d'action	Groupe chimique	Molécules
SCHIZONTICIDES	**érythrocytaire**	lysosomotropes	*amino-4-quinoléines*	chloroquine, amodiaquine
			aminoalcools	quinine, méfloquine, halofantrine, luméfantrine
			sesquiterpènes	artémisinine et ses dérivés (dihydroartémisinine, artéméther, artésunate, artémotil)
		antimétabolites	*antifoliques*	sulfadoxine, dapsone
			antifoliniques	proguanil, pyriméthamine
			antibiotiques	cyclines, clindamycine
			analogues de l'ubiquinone	atovaquone
		associations	*Artemisinin-based Combination Therapy (ACT)*	artéméther-luméfantrine, artésunate-méfloquine, artésunate-amodiaquine, artésunate-sulfadoxine-pyriméthamine
			non-ACT	sulfadoxine-pyriméthamine, méfloquine-sulfadoxine-pyriméthamine, atovaquone+proguanil, chlorproguanil+dapsone, choloroquine+proguanil
	tissulaires		*amino-8-quinoléines*	primaquine, tafénoquine
		antimétabolites	*antifoliniques*	proguanil
			antibiotiques	cyclines
GAMETOCYTOCIDES	**tissulaires**		*amino-8-quinoléines*	primaquine, tafénoquine

Tableau 2. Classification des molécules antipaludiques.

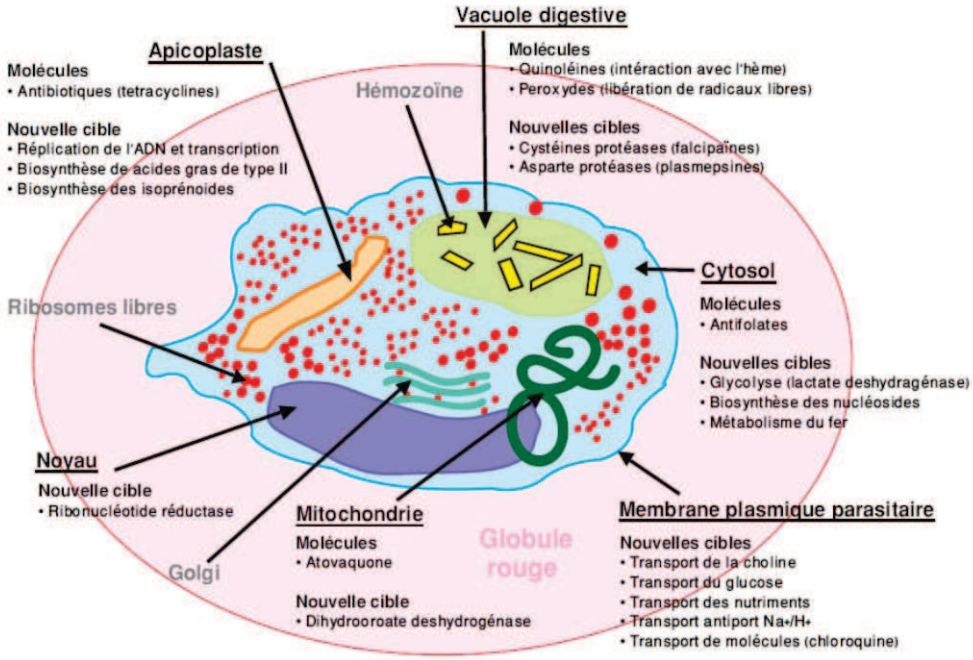

Figure 3. Cibles des antipaludiques. (Biot et al., 2008)

De par leur implication dans les voies métaboliques impliquées dans le stress oxydatif, la chloroquine et la malarone retiendront notre attention (Radfar et *al.*, 2008 ;. Srivastava et *al.*, 1997).

La chloroquine (CQ) s'est imposée de façon remarquable dans la chimioprophylaxie et la thérapie du paludisme, en raison de sa solubilité en milieu aqueux et de son faible coût de production. Cette amino-4-quinoléine, introduite dans les années 1940, présente le mécanisme d'action le mieux décrit. La CQ a la capacité de s'accumuler à des concentrations micromolaires dans la vacuole digestive du parasite, suivant le gradient de pH vacuolaire. Dans la vacuole digestive, elle se fixe à la ferriprotoporphyrine IX (FP IX) pour inhiber sa détoxification sous forme d'hémozoïne et empêcher les mécanismes de défense contre le stress oxydatif (Homewood et *al*., 1972 ; Pagola et *al*., 2000). Les isolats chloroquino-résistants présentent une altération de l'accumulation de la CQ dans la vacuole digestive (Wellems et *al*., 2001). L'extension de la chloroquino-résistance à la quasi-totalité des zones d'endémie palustre complique singulièrement cette prophylaxie (Cowman et *al*., 1990). La chimiorésistance est la conséquence de la pression de sélection induite par l'utilisation d'antipaludiques à grande échelle.

L'introduction récente de la Malarone® (association atovaquone-proguanil) dans la chimioprophylaxie du paludisme est déjà victime de l'apparition de résistances même si elles sont limitées (Biagini et *al*., 2008). La cible de l'atovaquone est le complexe cytochrome *bc1* de la chaîne respiratoire mitochondriale, impliquée dans l'équilibre redox chez les eucaryotes. Une mutation ponctuelle (codon 268) du gène *pfcytb*, codant pour le cytochrome b, confère une très forte résistance à l'atovaquone, empêchant toute synergie d'action avec le proguanil sur le potentiel transmembranaire mitochondrial (Schwobel et *al*., 2003).

La nécessité d'identifier de nouvelles stratégies de lutte est évidente et le développement d'un vaccin efficace devient urgent. Mais la conception d'un vaccin antipaludique se heurte à de nombreuses complications liées à la complexité du cycle biologique du parasite et au fait que le parasite ait développé de nombreux mécanismes d'échappement au système immunitaire de l'hôte.

L'éradication du paludisme demande encore de nombreuses années d'efforts, la recherche de nouvelles cibles thérapeutiques s'impose et implique une meilleure connaissance de la biologie de *P. falciparum* et sur ses interactions avec l'hôte.

2. La biologie de *Plasmodium falciparum*

2.1. Classification phylogénétique, un protiste du phylum Apicomplexa

Les plasmodies appartiennent à l'embranchement des *Sporozoaires* (*Apicomplexa*), règne des *Protista* et à l'ordre des *Haemosporididae*. Le phylum des *Apicomplexa* est un embranchement du règne des protistes. La classification phylogénétique fine, des protistes en général et des Apicomplexes en particulier, a fait l'objet de nombreuses polémiques qui ont animé l'évolution des classifications. Ainsi, plusieurs classifications phylogénétiques du genre *Plasmodium* ont été proposées selon des critères taxonomiques. Les caractéristiques communes aux différents ordres composant ce phylum sont l'existence d'une phase de sporogonie lors du cycle cellulaire et la présence d'un complexe apical indispensable au mécanisme d'invasion. La classification la plus représentative de notre étude est celle récemment proposée par Cavalier-Smith en 2004 (Figure 4).

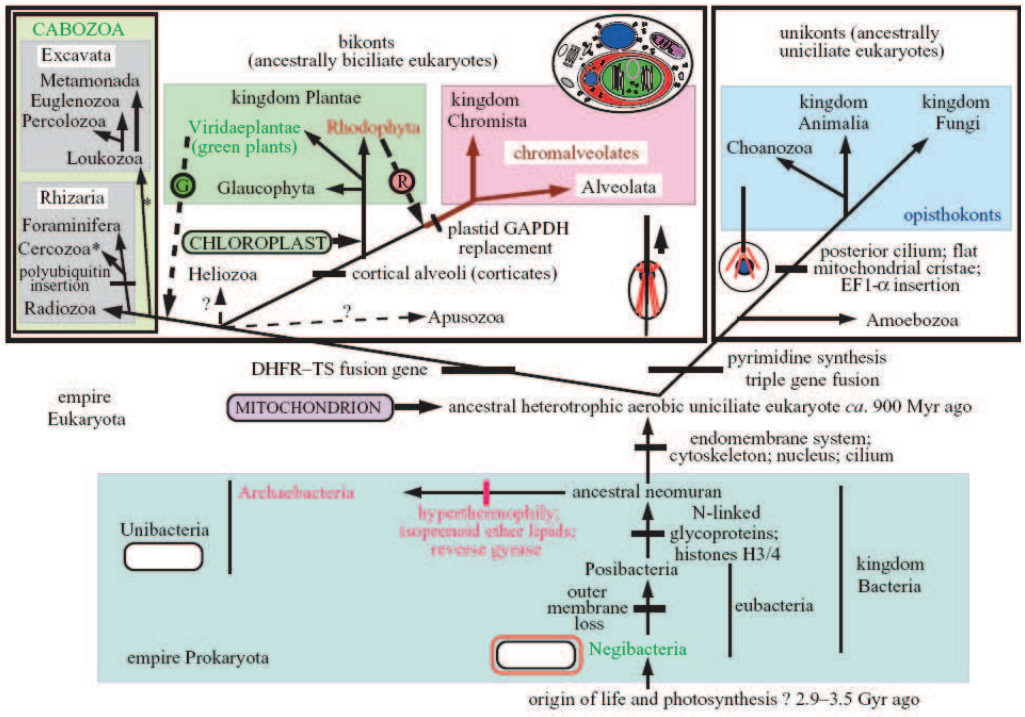

Figure 4. L'arbre de vie selon Cavalier-Smith (Cavalier-Smith, 2004).

Cet arbre de vie, propose la « racine » du vivant au niveau des Negibactéries et la « racine » des eucaryotes entre les Bichontes et les Unichontes. Ce règne comprend quatre groupes majeurs (Alveolata, Cabozoa, Amoebozoa et Choanozoa) et le phylum Apusozoa. Le règne des Protista n'est pas indiqué mais est classé dans le groupe des Alveolata.

Cette représentation synthétique propose un "eucaryote ancestral" qui serait un unicellulaire phagotrophe unicillié, issu d'un ancêtre bactérien par apparition simultanée du cytosquelette, du système endomembranaire, du noyau et des cils. Cet "eucaryote ancestral" aurait réalisé très tôt une endosymbiose avec une alpha-protéobactérie à l'origine des mitochondries. Deux schémas d'organisation des cellules eucaryotes se seraient séparés, d'une part les Unichontes (issus d'une cellule eucaryote ancestrale uniciliée), et d'autre part les Bichontes (issus d'une cellule eucaryote ancestrale biciliée). Dans cette représentation, la racine des protistes se situerait au niveau du lien ancestral entre Bichontes et Unichontes. Ce scénario repose sur des données moléculaires, ultra-structurales et paléontologiques. Il tient notamment compte des théories endosymbiotiques de l'origine de la mitochondrie (acquise lors de l'ingestion de Negibactéries distinctes par un eucaryote primitif) et de l'origine des Chromalveolés par ingestion d'une algue rouge unicellulaire par un eucaryote unicellulaire.

La majorité des parasites apicomplexes (*Plasmodium*, *Toxoplasma*, *Babesia*...) présente deux organites contenant des génomes extra-chromosomaux, la mitochondrie et l'apicoplaste (un chloroplaste vestigial). Sur le plan phylogénétique, cette structure plastidiale a pour origine une endosymbiose secondaire entre deux eucaryotes, avec ingestion d'une algue rouge unicellulaire par un protiste, ancêtre de l'ensemble des Apicomplexes. Le plaste de l'algue rouge résulte lui-même d'une endosymbiose primaire d'une cyanobactérie et un eucaryote primitif. L'endosymbiose secondaire a été suivie d'une disparition de la plupart des structures sub-cellulaires de l'algue et s'est accompagnée d'un transfert génétique vers le noyau de la cellule hôte. L'hypothèse d'une origine végétale de l'apicoplaste est confortée par le mécanisme d'adressage des protéines codées par le génome nucléaire. L'analyse des séquences peptidiques a en effet révélé la présence d'une séquence signal bipartite N-terminal dans les protéines de l'apicoplaste. Ce domaine bipartite comprend un peptide signal qui permet l'entrée dans le système endomembranaire et un peptide d'adressage impliqué au niveau de l'import dans l'apicoplaste (Foth et *al*., 2003), médiés par un peptide de transit de type chloroplastique. L'apicoplaste est un organite homologue non photosynthétique du choloroplaste des plantes et des algues (McFadden et *al*., 1996 ; Kohler et *al*., 1997). Le génome de l'apicoplaste d'une taille de 35 kb ne code que pour environ 30 protéines (60 % de fonctions putatives) ; son maintien est donc dépendant de protéines codées par le génome nucléaire (Waller et *al*., 2000). La séquence de son génome est homologue à celle des chloroplastes, de plantes supérieures et d'algues, excepté que l'ADN ne porte pas de gènes impliqués dans la photosynthèse (Gardner et *al*., 1991a ; Gardner et *al*., 1991b, Wilson et *al*.,

1991 ; Wilson et *al*., 1996 ; Wilson et Williamson, 1997). Bien qu'essentiel à la survie du parasite, son rôle reste peu clair ; il serait impliqué dans les voies métaboliques de synthèse des acides gras (Surolia et *al*., 2001), des isoprènoïdes (Jomaa et *al*., 1999), et des formes intermédiaires de l'hème (Dhanasekaran et *al*., 2004). Ces voies métaboliques, distinctes de celles de l'hôte, sont donc des cibles thérapeutiques de choix et reflètent l'origine végétale de l'apicoplaste (Ralph et *al*., 2001). Cette origine végétale a aussi ouvert la voie à une recherche de molécules thérapeutiques innovantes correspondant à de nouvelles familles de médicaments que l'on peut qualifier de médicaments-herbicides.

2.2. Cycle biologique de *Plasmodium falciparum*

Le cycle évolutif de *P. falciparum* est complexe et s'effectue en deux phases, une phase exogène sexuée et une phase endogène asexuée, impliquant respectivement deux hôtes : un arthropode hématophage (l'anophèle femelle) et un hôte intermédiaire vertébré (l'homme) (Figure 5).

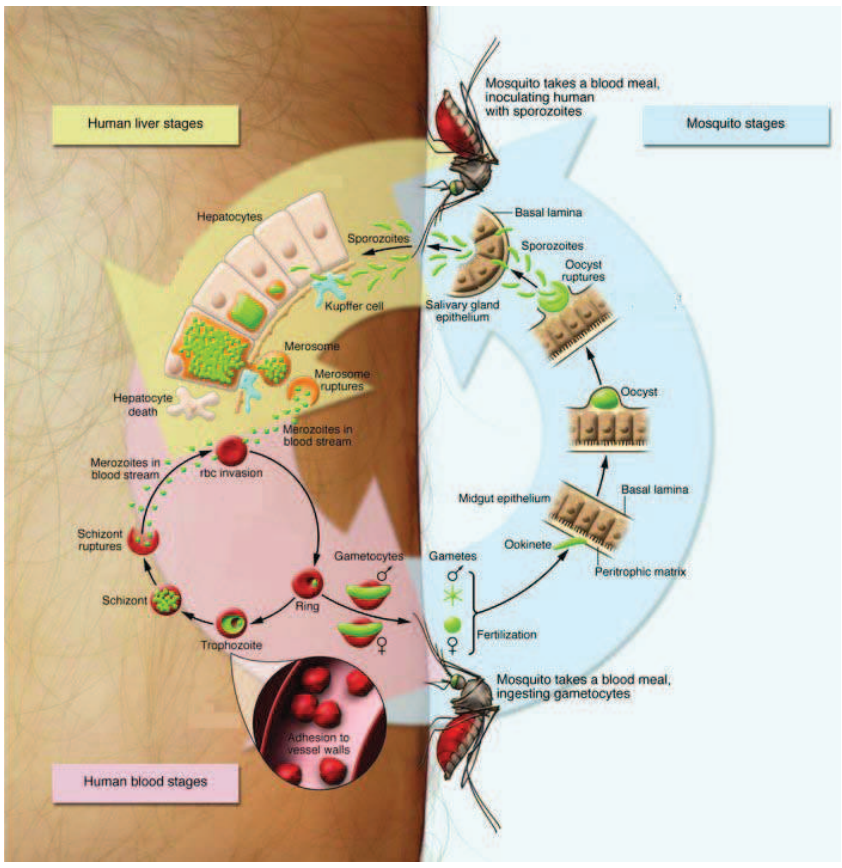

Figure 5. Cycle biologique de Plasmodium falciparum (Greenwood et al., 2008).

2.2.1. La phase exogène sexuée chez l'anophèle

Lors d'un repas sanguin sur un individu infecté, l'anophèle femelle ingère des gamétocytes, stade sanguin sexué des plasmodies. Au cours d'un processus d'exflagellation, un gamétocyte mâle donne 8 gamètes mâles haploïdes qui peuvent fusionner avec un macrogamète femelle haploïde. Cette fécondation aboutit à la formation d'un ookinète diploïde qui traverse activement la paroi stomacale du moustique et forme, à la surface externe de cette paroi, un oocyste, moins de 24 heures après le repas sanguin. A l'intérieur de cet oocyste, les cellules parasitaires se multiplient pour libérer au bout de quelques jours près d'un millier de sporozoïtes haploïdes. Les sporozoïtes gagnent ensuite les glandes salivaires et s'y accumulent. Au cours d'un nouveau repas sanguin, l'anophèle régurgite quelques dizaines de sporozoïtes dans la circulation sanguine de l'hôte vertébré, lesquels gagnent rapidement le foie de l'homme.

2.2.2. La phase hépatique (schizogonie pré- ou exo-érythrocytaire) chez l'homme

Les sporozoïtes envahissent les hépatocytes où ils se multiplient (schizonte pré-érythrocytaire). L'éclatement des hépatocytes libère dans la circulation sanguine les mérozoïtes qui se multiplient au cours de la schizogonie érythrocytaire. Cette phase hépatique est totalement asymptomatique sur le plan clinique.

2.2.3. La phase intra-érythrocytaire (schizogonie érythrocytaire) chez l'homme

Les mérozoïtes envahissent les érythrocytes par invagination de la membrane cellulaire en une vacuole parasitophore reliée au milieu plasmatique par un pertuis. A l'intérieur de l'érythrocyte, les mérozoïtes se différencient en forme en anneau. Après une période de 15 heures, le trophozoïte entre dans un processus de synthèses nucléique (ADN et ARN) et protéique. Le métabolisme de digestion de l'hémoglobine érythrocytaire est également activé. Simultanément, commencent l'induction d'une nouvelle voie de perméabilité (New Permeation Pathways, NPP) à la surface de l'érythrocyte (Tanneur et *al.*, 2006 ; Lew et *al*., 2003) et l'export de protéines parasitaires vers la membrane érythrocytaire modifiant ainsi sa perméabilité pour les échanges nutritionnels (Das et *al.*, 1994). Les NPP sont des canaux ioniques qui favorisent les échanges de métabolites. Physiologiquement, ces NPP contribuent à réguler le volume de l'érythrocyte parasité de façon ATP dépendante et sont induits par le stress oxydatif (Tanneur et *al.*, 2006). Lors de cette synthèse protéique

accrue, le parasite met en place un réseau de membranes assurant les connexions entre la vacuole parasitophore et la membrane érythrocytaire. Ces invaginations appelées «tâches de Maurer» sont le siège de la maturation des molécules néo-synthétisées et exportées vers le globule rouge. L'agrégation de protéines parasitaires à la surface de l'érythrocyte produit de petites protubérances appelées «knobs» (Leech et *al*., 1984). Ces complexes présentent une protéine, PfEMP1, qui confère aux parasites des propriétés d'adhésion observées *in vitro* et déterminantes dans la physiopathologie du neuropaludisme (Pouvelle et *al.*, 2000). Cette propriété à cytoadhérer entraîne une adhésion des érythrocytes infectés à l'endothélium des micro-vaisseaux périphériques et une séquestration dans certains organes comme la rate, le poumon et le cerveau (MacPherson et *al*., 1985). Ceci explique l'absence de parasite aux stades matures dans la circulation sanguine lors d'une infection à *P.falciparum*. Vers la 36ème heure du cycle, la schizogonie érythrocytaire commence avec la division nucléaire et la morphogenèse des mérozoïtes. Le schizonte croît jusqu'à épuiser quasiment la réserve d'hémoglobine, accumulant le pigment malarique (hémozoïne) dans la vacuole digestive. Il synthétise et assemble les éléments constitutionnels des 16 à 32 futurs mérozoïtes en commençant par les organites apicaux avec prolifération du réticulum endoplasmique et des ribosomes et division de la mitochondrie et de l'apicoplaste. L'éclatement des hématies contenant les formes schizontes matures (ou rosettes) libère les mérozoïtes qui vont envahir des hématies saines et entreprendre un nouveau cycle érythrocytaire ou évoluer en gamétocytes. Brièvement extracellulaire, les mérozoïtes sont exposés directement à l'environnement de l'hôte soit au système immunitaire et aux antipaludiques dans la circulation sanguine. La schizogonie érythrocytaire dure, *in vivo*, approximativement 48 heures pour *P. falciparum*. Les gamétocytes ne sont habituellement détectables dans la circulation sanguine qu'après plusieurs cycles de schizogonie érythrocytaire.

Les formes parasitaires du stade intra-érythrocytaire sont caractérisées par des formes phénotypiques aux caractéristiques morphologiques différentes en fonction de leur âge (Figure 6).

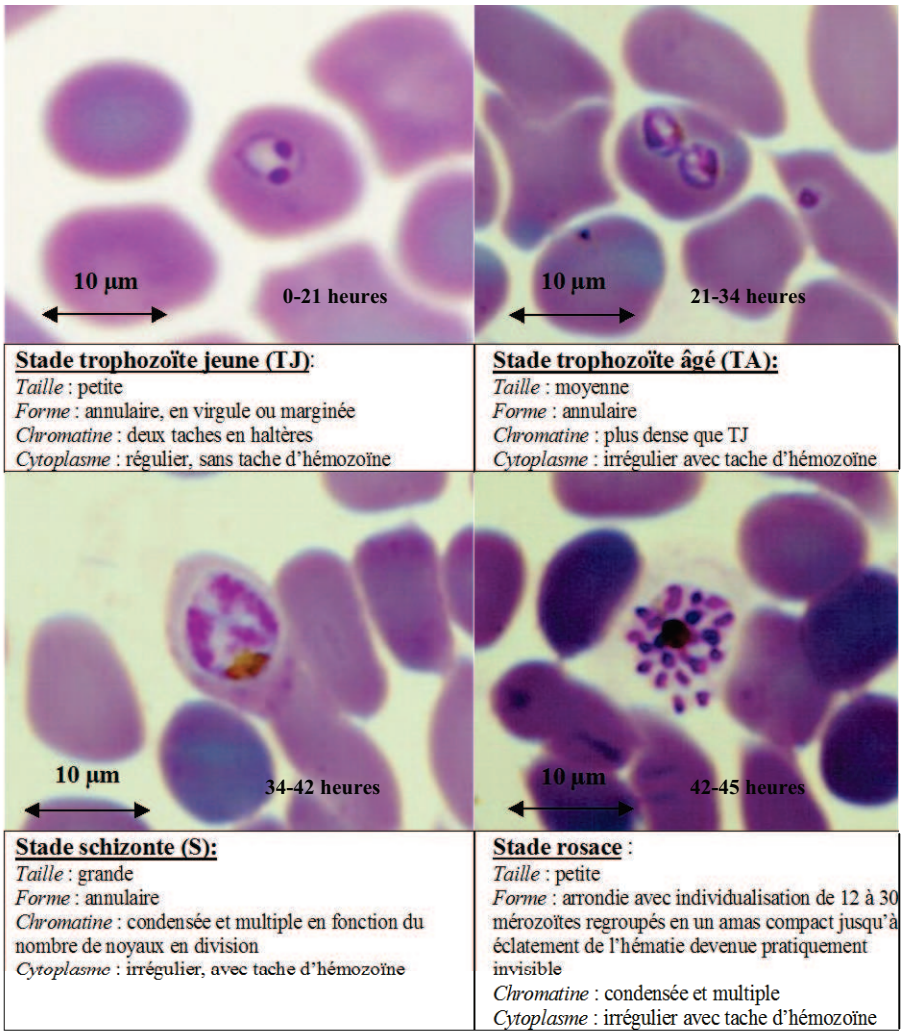

Figure 6. Morphologie des stades intra-érythrocytaires de P. falciparum en microscopie optique.

2.2.4. La gamétocytogénèse

La gamétocytogénèse est un processus qui conduit une partie des parasites à un arrêt du cycle cellulaire et à initier une phase de différenciation cellulaire en formes sexuées. Cette transformation conduit à des changements morphologiques, de l'expression protéique et du métabolisme. Les mécanismes permettant la différenciation du mérozoïte en gamétocyte sont mal connus.

En culture *in vitro*, l'induction de la gamétocytogénèse dépend de facteurs environnementaux tels que la parasitémie, le stress oxydatif et l'action de certains

antipaludiques comme la CQ (Graves et *al*., 1984). Cinq stades sont décrits dans la maturation des gamétocytes de *P. falciparum*. Les gamétocytes immatures (stade I à IV) sont séquestrés dans les tissus profonds, en particulier dans la moelle osseuse (Smalley et *al*., 1981). Cette séquestration favoriserait la maturation des gamétocytes durant 9 à 12 jours et éviterait leur élimination lors de leur passage dans la rate (Garnham et *al*., 1966). Pour *P. falciparum*, les premiers stades de différenciation en gamétocytes sont morphologiquement identiques à ceux des trophozoïtes jeunes (Talman et *al*., 2004). Le marqueur principal de l'engagement dans la voie gamétocytaire est le développement d'une vacuole et de microtubules, responsable de l'élongation et de la symétrie du microorganisme. Au stade V, rencontré dans la circulation périphérique, l'hématie est déformée par le gamétocyte mature allongé, le gamétocyte mâle est reconnaissable par son plus grand noyau, la rareté des ribosomes et des mitochondries. Cette maturation permet la préparation du cycle sexué chez le moustique où les phénomènes de division cellulaire se réinitient.

L'une des conséquences du métabolisme actif, de la rapidité de division et de différenciation du parasite au cours de son cycle biologique chez l'hôte vertébré est la production d'espèces réactives de l'oxygène (Reactive Oxygen Species, ROS). Ces ROS dérivent de l'oxygène par des réductions à un électron, tels que l'anion superoxyde $O_2°$-, le radical hydroxyle OH°, le monoxyde d'azote NO et le peroxyde d'hydrogène H_2O_2. Le stress oxydatif se définit comme un déséquilibre entre les prooxydants et les antioxydants. Il est actuellement admis que le stress oxydatif génère une production excessive de ROS susceptible d'engendrer une oxydation des composants biologiques comme les lésions ADN (Barzilai et Yamamoto, 2004), la peroxydation des lipides (Meagher et Fitzgerald, 2000) et l'oxydation des protéines (Pantke et *al*., 1999). Pour contrer leurs effets néfastes, le parasite possède plusieurs systèmes de défense antioxydants permettant de maintenir un faible taux de ROS intracellulaire tels que la glutathione S-transferase, les superoxydes dismutases et les systèmes glutarédoxine et thiorédoxine (Bozdech et *al.*, 2004).

Au stress oxydatif endogène lié au propre métabolisme du parasite (digestion de l'hémoglobine dans la vacuole digestive et respiration mitochondriale), se surajoute un stress oxydatif exogène imposé au parasite au sein de l'érythrocyte par les différentes pressions partielles d'O_2 dans la circulation sanguine. Le paradoxe des ROS en biologie est qu'ils constituent des espèces extrêmement toxiques, tout en étant indispensables à la vie.

3. Stress oxydatif chez *Plasmodium falciparum*

3.1. Source endogène de ROS, métabolisme parasitaire

Chez *P. falciparum*, deux compartiments cellulaires sont susceptibles d'être source de ROS de par leur activité métabolique : la vacuole digestive et la mitochondrie (Figure 7 ; Oliveira et *al*., 2002).

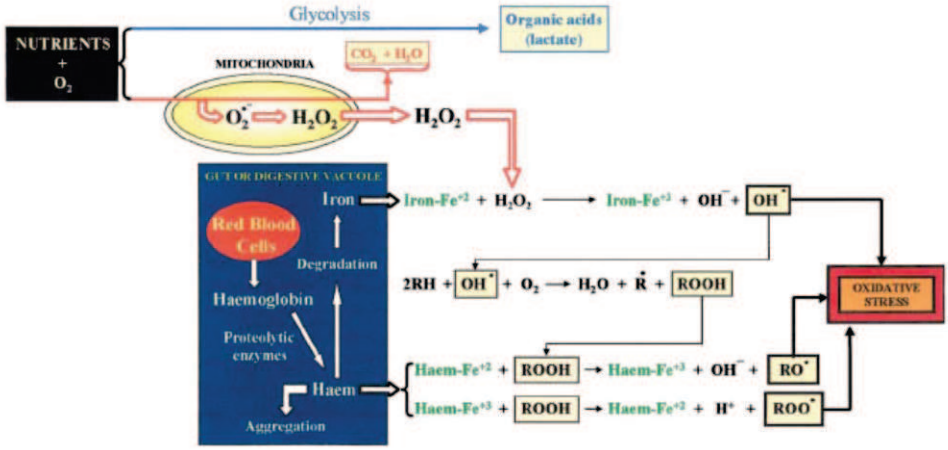

Figure 7. Sources de stress oxydatif chez P. falciparum.
(Oliveira et al., 2002)

3.1.1. La vacuole digestive

La vacuole digestive est un compartiment acide de type lysosomal adapté pour le métabolisme de digestion de l'hémoglobine. Cette vacuole est un site d'acidification (pH 5-5,4), de protéolyse de l'hémoglobine, de transport peptidique, de détoxification de l'hème et des radicaux libres. *P. falciparum* dispose d'une voie métabolique de synthèse *de novo* des acides aminés très limitée. La digestion de l'hémoglobine est pour le parasite une source complémentaire d'acides aminés, les antipaludiques spécifiques de cette voie inhibant la croissance parasitaire.

L'hémoglobine, dont la fonction essentielle est le transport d'O_2, est composée de 4 sous-unités globine (2α et 2β) et d'hème, constitué de protoporphyrine IX et d'un atome de fer central – la ferriprotoporphyrine IX (FP IX). Dans la vacuole digestive, la globine est dégradée par des protéases parasitaires (plasmepsines, falcipaïne, falcilysine et dipeptidyl aminopeptidase) en acides aminés dont seulement 16% sont utilisés par le parasite (Krugliak et *al.*, 2002). L'excès d'acides aminés est rapidement exporté par la voie des NPP (Mauritz et *al.*, 2009). La FP IX, toxique pour le parasite dépourvu d'hème oxygénase, est détoxifiée en un cristal inerte, l'hémozoïne ou pigment malarique. Cette détoxification se fait en partie par

oxydation de FP(Fe^{2+}) en FP(Fe^{3+}) sous l'effet de l'acidité vacuolaire (pH 5). La libération d'électrons par l'oxydation du Fe génère de l'H_2O_2 toxique pour *Plasmodium*, même si le parasite possède des systèmes de défense contre les radicaux libres (Atamna et *al*., 1995) comme la superoxide dismutase (SOD) et la catalase. Ces deux dernières enzymes préviennent des dommages du stress oxydatif en détoxifiant les ions superoxydes et l'H_2O_2 (Becker et *al*., 2004).

Au-delà de l'apport en acides aminés, la digestion de l'hémoglobine, très consommatrice d'énergie, permettrait au parasite de maintenir une stabilité osmotique au sein de l'érythrocyte et de libérer de la place dans la cellule hôte pour sa croissance (Lew et *al*., 2003).

3.1.2. La mitochondrie plasmodiale

REVUE

M. Torrentino-Madamet, J. Desplans, C. Travaillé, Y. Jammes and D. Parzy

Microaerophilic respiratory metabolism of Plasmodium falciparum mitochondrion as a drug target

Current Molecular Medicine 2010, 10, 29-46

Résumé

La mitochondrie de *P. falciparum* est un organite qui présente des caractéristiques structurales et physiologiques différentes des mitochondries d'autres eucaryotes. Il existe des différences considérables dans les propriétés mitochondriales des stades sexués et asexués. Une des hypothèses est l'adaptation du parasite aux différents environnements, en particulier les grandes différences de taux d'O_2 entre l'homme et le moustique.

Dans cette revue, nous présentons une synthèse de données récentes sur l'ultrastructure, le génome et la physiologie de la mitochondrie. Nous tentons de clarifier le rôle de la mitochondrie dans l'environnement intra-érythrocytaire et nous nous focalisons particulièrement sur les voies métaboliques mitochondriales qui concernent la phosphorylation oxydative, incluant le cycle d'acide tricarboxylique, la biosynthèse *de novo* des pyrimidines via la dihydroorotate déhydrogénase et sur les particularités de la chaîne de transfert des électrons. D'autre part, nous relatons certaines caractéristiques de la mitochondrie plasmodiale comme la perte de pyruvate déhydrogénase, l'insensibilité de la NAHD-déhydrogénase à la rotenone, ainsi que la potentielle existence d'une oxydase alternative et de protéines de découplage.

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Microaerophilic Respiratory Metabolism of *Plasmodium falciparum* Mitochondrion as a Drug Target

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Abstract: The *Plasmodium falciparum* mitochondrion is an organelle that presents structural and physiological characteristics different from mitochondria in other eukaryotes. Moreover, there are substantial differences in the properties of asexual and sexual mitochondria. One of the reasons is the adaptation of the parasite to different environments, in particular the great differences in oxygen tension between the host and the mosquito.

In this review, we present a synthesis of the recent data on the ultrastructure, the genome and the physiology of the mitochondrion. We try to clarify the mitochondrial role in the intraerythrocytic environment and particularly focus on mitochondrial metabolic pathways that relate to oxidative phosphorylation, including the tricarboxylic acid cycle, *de novo* pyrimidine biosynthesis *via* dihydroorotate dehydrogenase and the particularities of the electron transport chain. In addition, we provide details on certain characteristics like the lack of pyruvate dehydrogenase, the existence of a rotenone-insensitive NADH-dehydrogenase, the possible existence of an alternative oxidase, and uncoupled proteins.

Such unique particularities of parasite mitochondria could be promising targets for development of a new therapy. The elucidation of the role of this organelle in microaerophilic respiratory metabolism and the association of antimalarial drugs with hyperbaric oxygen therapy might provide new treatments for infection by *P. falciparum*.

Keywords: *Plasmodium falciparum*, mitochondrion, microaerophilic, respiratory metabolism, TCA, mitochondrial electron transport chain, oxidative stress.

INTRODUCTION

Plasmodium falciparum (*P. falciparum*) is a protozoan parasite responsible for the most severe form of human malaria. This infection causes 2.7 million deaths annually, most of them in African children under the age of five years [1]. The expansion drug resistance remains a serious problem, and the development of new antimalarial drugs is urgent [2].

Because the energy metabolism of *P. falciparum* is quite different from that of the mammalian host, the enzymes of the energy transducing pathways in the parasite are promising targets for antimalarial drugs. In particular, significant advances have been made in our understanding of the importance of *P. falciparum* mitochondria [3, 4]. In parasite cells, mitochondria adapt morphologically and physiologically to the environmental conditions of hosts, particularly O_2 pressure.

In this review, we try to clarify the role of the parasite mitochondrion in the intraerythrocytic environment with regard to the specific and principally microaerophilic respiratory metabolism of the parasite, and to provide potential targets for the development of novel malaria chemotherapies.

ULTRASTRUCTURE AND MORPHOLOGY IN ASEXUAL AND SEXUAL STAGES OF *P. FALCIPARUM*

P. falciparum parasites are unicellular eukaryotes that possess a dynamic organelle called the mitochondrion that undergoes a series of ultrastructural changes during the life cycle. As others organelles (apicoplast, food vacuole), the mitochondrion is located in cytoplasm. There are apparent variations in the number, morphology and size of mitochondria between the asexual and sexual gametocytic stages [5].

Separate studies using epifluorescence microscopy and transmission electron microscopy (MET) of *P. falciparum* have shown a single mitochondrion with a double-membrane in human intraerythrocytic parasites [6]. It grows from a single, small organelle into a highly branched structure in later-stage parasites. The trophozoite mitochondrion was described as circular in shape with a double membrane, whereas it elongates and becomes proportionately wider in the mature

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1566-5240/10 $55.00+.00

trophozoite stage. The mitochondrion appears capable of fusion and frequently (but not always) associates with the plasma membrane. Contrary to gametocytes, trophozoite mitochondria have few tubular-like cristate structures formed from the inner membranes, and they have a low density matrix, suggesting limited metabolic activity of the organelle [7]. During trophozoite-schizont stage transition, the mitochondrion initiates branching. During schizogony, cytokinesis begins, and the predominantly branched mitochondrion is apparently divided by fission to become two daughter organelles. Upon division, each mitochondrion segregates in order to become incorporated into the newly formed merozoites [8]. This organelles partition has been well modelised by the Van Dooren lab [6].

Acristate structures evolve into cristate structures in the sexual gametocytic stage. Both male and female gametocytes contain six mitochondrial organelles of typical appearance [9]. The morphologic changes of *P. falciparum* mitochondrion in mosquitoes lead to different biochemical properties such as the appearance of succinate dehydrogenase activity. This morphologic transformation of the mitochondrion occurs with the change of host to the oxygen-rich mosquito [10].

The MET has permitted to note that the apicoplast and mitochondrion have an intimate physical association during specific stages of the parasite intraerythrocytic life cycle, probably to facilitate metabolite exchange. Both organelles have an endosymbiotic origin from when an alpha-proteobacterium became incorporated into a proto-eukaryote [11-13].

The apicoplast division precedes mitochondrial division during schizogony. However, in the long intraerythrocytic cycle, the mitochondrion and the apicoplast are closely bound to each other. The two organelles are associated during the ring and trophozoite stages. In the trophozoite stage, there is apposition between the mitochondrion and apicoplast, with the mitochondrion forming a lengthened tubular organelle and the apicoplast remaining a rounded organelle. In the schizonte stage, the mitochondrion is slightly elongated with respect to the apicoplast and presents numbers of contact points with the elongated apicoplast. In the merozoite stage, the two organelles segregate as a pair into daughter merozoites [6].

This association between the mitochondrion and apicoplast is required for metabolic interaction between mitochondrial- and apicoplast-localised enzymatic steps such as the haem biosynthetic pathway [14, 15].

THE MITOCHONDRIAL GENOME OF *P. FALCIPARUM*

The 6-kb mitochondrial DNA (mtDNA) of *P. falciparum* is the smallest mtDNA known. During and after the process of endosymbiotic acquisition, parasite mitochondria give up most of their genes to the host cell nucleus, suggesting a tight nuclear regulation of mitochondrial activity and respiratory metabolism [16, 17]. With respect to the apicoplast genome, this small DNA also differs in its simplicity. mtDNA is formed from linear element repetitions in tandem, which encode only three subunits of the mitochondrial electron transport chain (mETC): subunits I and III of cytochrome c oxidase (cox I and cox III of complex IV) and a cytochrome b subunit of ubiquinol-cytochrome c reductase (complex III) and fragmented ribosomal RNA genes [5, 18, 19]. Fragments are separately transcribed and bind to themselves in order to reconstitute seemingly functional ribosomal subunits without covalent bonds (Fig. **1**). Other subunits of the respiratory chain and tRNAs, which are encoded by mtDNA in other organisms, are probably encoded by nuclear DNA or possibly the apicoplast [20, 21] and imported into the mitochondrion, as are the majority of mitochondrial proteins [22]. Interestingly, cytochrome oxidase II (Cox II of complex IV) is encoded by two independent nuclear genes, cox2a (chromosome 13) and cox2b (chromosome 14) in *P. falciparum*, whereas in eukaryotes, the cox II subunit is encoded by a single gene in the mitochondrial genome. The cox2a and cox2b genes encode the N-term domain and C-term domain of the Cox II protein, respectively. A lateral transfer of these genes from the green alga nucleus to the *P. falciparum* nuclear genome has been suggested, but coordinated expression of the cox2a and cox2b genes and assembling of complex IV have not been studied in *P. falciparum* [18]. Lack of conventional NADH dehydrogenase subunits results from the absence of complex I in *P. falciparum* parasites [23, 24].

Like the *P. falciparum* nuclear genome, mtDNA is T-rich. Sequence conservation is quite high, with 90% of identity among the species *P. falciparum, P. vivax, P. yoelii,* and *P. gallinaceum*, for which complete mitochondrial genome sequences are available [19]. Replication of the mitochondrial genome occurs according to a phage-like mechanism of a rolling-circle in *P. falciparum* and undergoes extensive recombination, similar to the mtDNA element of some other lower eukaryotes. MtDNA synthesis has effect at the same time as chromosomal DNA replication throughout the asexual cycle of the parasite in the host cell. Transcripts of mtDNA genes are more abundant during the late trophozoite and schizont stages than the ring and early trophozoite stages [18]. Both gene dosage and transcription play an important role in the expression of the mtDNA during parasitic development in the blood stages. Little is known regarding chromosomal and mitochondrial DNAs during gametocytogenesis, exflagellation and gametogenesis of the sexual stage parasite. Lines of evidence on stage specific expression of actin, tubulin, and rRNA genes during gametocytogenesis of the parasite have become available for the chromosomal genes [5, 18, 19]. The products of these prokaryote-derived genes, as well as mitochondrial-targeted gene products of eukaryotic origin, are thought to be trafficked back to the mitochondrion [25].

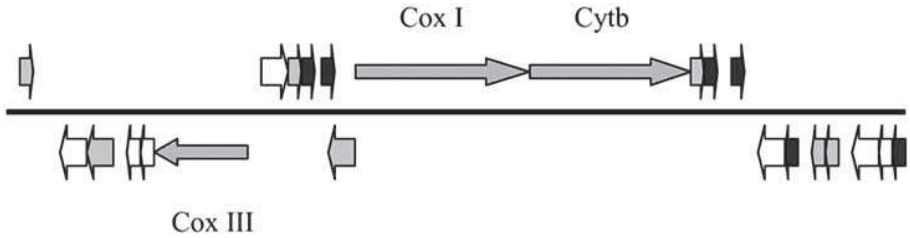

Fig. (1). Schematic map of *P. falciparum* mitochondrial genome.

The total length of *P. falciparum* mitochondrial genome is 5967 bp. Mitochondrial sequences are drawn to scale, with coding sequence on either the forward or reverse strand indicated above or below the line, respectively. Linear element repetitions are shown in tandem, only 3 genes (CoxI, Cytb and CoxIII) encode subunits of the electron transport chain (diagonal motif arrow). Fragments of ribosomal RNA genes are detailed, those similar to lsrRNA (white arrow), those similar to ssrRNA (grey arrow) and those which are currently unassigned (black arrow). Complete sequence genome is available with accession number NC 002375.

MITOCHONDRIAL-TARGETED PROTEINS AND MITOCHONDRIAL PROTEIN IMPORT MACHINERY

Indeed, the great majority of genes required for mitochondrial functions are encoded by the nuclear genome and imported into the mitochondrion following synthesis in the cytoplasm. Complete sequencing of the *P. falciparum* genome has permitted through homology searching to determine genes that have a putative role in the *P. falciparum* mitochondrion [26].

In *P. falciparum*, mitochondrial targeting signals are located at the N-terminus of proteins, although examples of internal and C-terminal targeting motifs are also known. Studies of mechanism of mitochondrial traffic have shown that the mETC involved in the formation of the transmembrane potential is essential for the passage of these proteins through the mitochondrial membrane [22]. As in other organisms, N-terminal mitochondrial targeting signals in *P. falciparum* are positively charged and contain an Arg, an enrichment in Ala and Ser and few negatively charged residues. These residues form amphiphilic α-helices [27]. Mitochondrial transit peptides (mTPs) interact with multiprotein translocase complexes at the outer and inner mitochondrial membranes [28]. These interactions facilitate protein translocation across the mitochondrial membranes into the mitochondrial matrix. The machinery for the outer membrane is called the Tom complex (Translocator outer membrane), and the machinery for the inner membrane is the Tim complex (Translocator inner membrane). Bioinformatic analysis indicates that the various translocases, including the Tom and Tim proteins involved in mitochondrial targeting are conserved in *P. falciparum* with some differences in the regulation of these processes. Once in the matrix, mTPs are removed by a mitochondrial peptidase process [29] (Table **1** and Fig. **2**).

A new neural network system (*Plas*Mit, http://gecco.org.chemie.uni-frankfurt.de) for prediction of mTPs in *P. falciparum* sequences was developed, based on the relative amino acid frequency in the first 24 N-terminal amino acids with an 84% rate of correct prediction. Based on 5,334 annotated genes in the *P. falciparum* genome, 381 (7.1%) mitochondrial genes have been predicted [30]. This is close to the number of mitochondrial proteins in other organisms. Most of these sequences are identified as hypothetical proteins or proteins with hypothetical function [18]. With TargetP and MitoProtII methods, only 246 proteins have been targeted to the mitochondrion after translation. With this new tool, it will be possible to begin assembling metabolic pathways that putatively occur in the mitochondrion [30] (Fig. **3**).

Nevertheless, the discovery of delivery of *P. falciparum* gene products to different intracellular compartments, probably through expression of more than one protein isoform, results in questions about the abundance of such genes in the whole genome. For example, the majority of the nuclear-encoded falcilysin protein is apparently trafficked into the apicoplast, and an additional minor fraction is probably targeted to the mitochondrion [31].

RESPIRATORY METABOLISM

In eukaryotic organisms, the dominant role for the mitochondrion is cellular respiration. This process is dependent on the presence of oxygen for production of ATP, which is used as a source of chemical energy [32, 33]. Glucose broken down in the cytosol through glycolysis is eventually degraded in the mitochondrial tricarboxylic acid (TCA) cycle to CO_2. The TCA cycle release electrons (*via* NADH) that drive the respiratory chain embedded in the inner mitochondrial membrane, consuming oxygen and generating water [34]. The electron carriers pump protons into the mitochondrial matrix, creating an electrochemical gradient that is used to drive ATP synthase. In addition to supplying cellular energy, eukaryote mitochondria are involved in many other functions, such as transient storage of calcium, apoptosis or programmed cell death, thermogenesis, haem and steroid synthesis, as well as the control of the cell cycle and cell growth [35].

However, the function of the *P. falciparum* mitochondrion remains unclear. The *P. falciparum* mitochondrion, in both developmental stages, is implicated in ATP production [9], but despite the identification of almost all the subunits of the ATP

Table 1. Genes Implicated in the Mitochondrial Import Machinery

Accession Number	Sequence Name	Sources
Complexe TOM		
PFF0825c	TOM40, Mitochondrial import receptor subunit	
PFE1230c	TOM22, receptor protein	Hypothetical protein
Complexe TIM		
PF13_0300	TIM23	
PF14_0328	TIM17, Mitochondrial import inner membrane translocase subunit	Putative
PF07_0110	TIM50 homologue	Hypothetical protein
PF11_0265	TIM44, Mitochondrial import inner membrane translocase	Putative
MAL81.14	YidC homologue Oxa1	
PFL2065c	TIM10, Mitochondrial import inner membrane translocase subunit	Putative
PF14_0208	TIM13, Mitochondrial import inner membrane translocase subunit	Hypothetical protein
PF13_0358	TIM9, Mitochondrial import inner membrane translocase	Putative
PFL0430w	TIM10, Mitochondrial import inner membrane translocase homologue	Putative
PFF1330c	TIM22, Mitochondrial import inner membrane translocase subunit	Putative
Chaperone network of the mitochondrial matrix		
PF11_0351	Heat shock protein 70 homologue, DnaK molecular chaperone	
PF11_0258	Co-chaperone GrpE	Putative
PF07_0103	Pam18, Chaperone DnaJ domain	Putative
CAE01413	Heat shock protein 10	
PF10_0153	Heat shock protein 60, mitochondrial chaperonin	
PFI0985c	Chaperone protein, DnaJ domain	
MAL13P1.162	DNAJ-like protein	Putative
PFD0465w	DNAJ homologue	
PF14_0197	Possible DNL zinc finger protein similar to Hep1 required for stabilization of mtHSP70 chaperones	Hypothetical protein
PF11_0188	Heat shock protein 90	
PFL0740c	10 kd chaperonin	Putative
Mitochondrial processing		
PFE1155c	Mitochondrial processing peptidase alpha subunit	Putative
PFI1625c	Organelle processing peptidase beta subunit	Putative
MAL13P1.184	Mitochondrial intermediate processing peptidase	Putative

The transport across the mitochondrial compartments requires the concerted action of a number of translocases and chaperone network of the mitochondrial matrix. The function sources have been assigned on the basis of sequence similarity to orthologue genes (putative) as well as domain identification for computer gene (hypothetical protein).

synthase, the contribution of the mitochondrion to the ATP pool is considered to be minimal [4]. Intraerythrocytic stages of malaria parasites have been considered for a long time to rely primarily on anaerobic glycolysis for energy production and to possess mitochondria that lack a functional TCA cycle and oxidative phosphorylation. With almost total completion of the malaria genome project, complete sets of genes encoding the enzymes of the TCA cycle and the mETC have been identified [25] (Table **2**). Nevertheless, the implication of this biochemical pathway is undetermined.

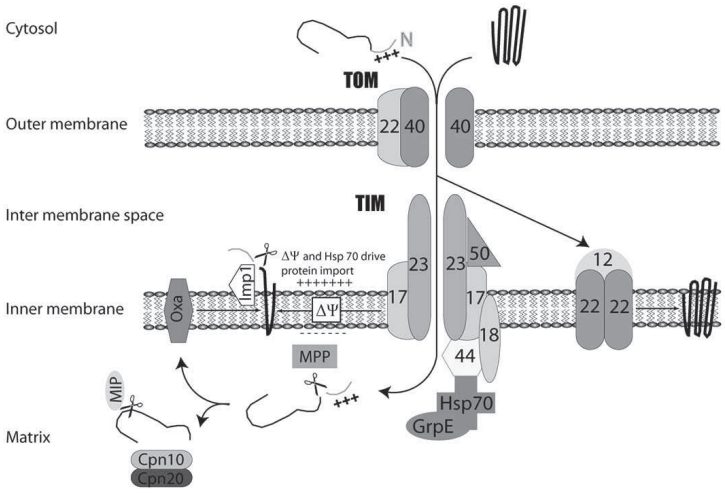

Fig. (2). Multiprotein translocase complexes in the *P. falciparum*.

Mitochondrial proteins interact with multiprotein translocase complexes *via* mitochondrial transit peptides to facilitate their translocation across the mitochondrial membranes through the mitochondrial matrix. Two complexes are implicated in the translocation mechanism : the translocator outer membrane complex (TOM) with Tom40 and Tom22 and the translocator inner membrane complex (TIM) with Tim23 as core protein. This process is regulated by Tim17 and Tim50, and also implies a motor apparatus based to the matrix side of the Tim complex that include mitochondrial Hsp70 and its co-chaperones Tim44, GrpE and Pam18. After translocation into the matrix, some mitochondrial proteins use an additional targeting signal that directs them back to the inner membrane or intermembrane space. This targeting mechanism is mediated by the YidC homologue Oxa1.
This pathway “Import of proteins into the mitochondrion” originates from the Hebrew University of Jerusalem web site http://sites.huji.ac.il/malaria.

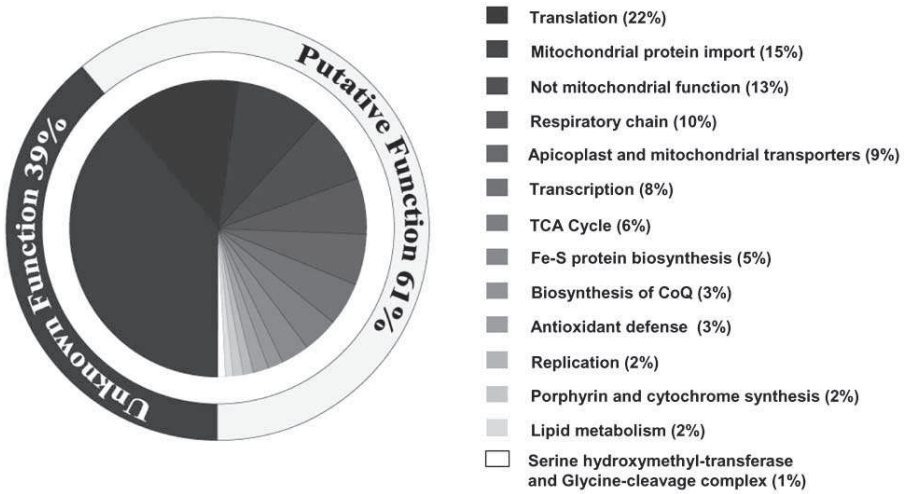

Fig. (3). Functional classification of genes with mitochondrial signal sequences.

Using the *Plas*Mit, genes encoding mitochondrial-targeted proteins have been predicted. Mitochondrial metabolic networks have been reconstructed from the list of these genes. In addition to functions responsible for mitochondrial DNA and mitochondrial-encoded proteins expression, and 39% of genes with unknown functions, different metabolic pathways implicated in the physiology of the mitochondrion have been identified. These networks have premised to construct of pathways that putatively occur in the mitochondrion.

The Mitochondrial Respiratory Chain in the Asexual Blood Stage

The malaria parasite has a functional respiratory chain and an oxygen-requiring system that is necessary for growth and survival [9, 36-38]. Genes encoding respiratory chain proteins have been shown to be present in the genome of malaria parasites. *P. falciparum* may have a biochemically active mETC containing four enzymatic activities: an alternative NADH dehydrogenase of complex I, succinate dehydrogenase of complex II, ubiquinol-cytochrome c oxidoreductase of complex III and cytochrome c oxidase of complex IV. The parasite genome also codes an ATP synthase of the respiratory chain complex V [26, 39] (Fig. **4**).

Table 2. Summary of Respiratory Metabolism for the Mitochondria of *P. falciparum*

Metabolic Pathways and Related Identified Genes	Accession Number	References
Mitochondrial TCA cycle		
Citrate synthase mitochondrial precursor, putative	PF10_0218	[38]
Citrate synthase-like protein, putative	PFF0455w	[38]
Isocitrate dehydrogenase (NADP) mitochondrial precursor	PF13_0242	[78, 93]
2-oxoglutarate dehydrogenase, e1 component, mitochondrial precursor	PF08_0045	[26, 94]
3-methyl-2-oxobutanoate dehydrogenase (lipoamide), putative	PFE0225w	[54, 94]
Branched-chain alpha keto-acid dehydrogenase E1-alpha subunit	PF13_0070	[26]
ATP-specific succinyl-CoA synthase beta subunit, putative	PF14_0295	[95]
Succinyl-CoA synthase alpha subunit	PF11_0097	[95]
Succinate dehydrogenase Fe-S subunit	PFL0630w	[96]
Dihydrolipoamide succinyltransferase, putative	PF13_0121	[96]
Flavoprotein subunit of succinate dehydrogenase	PF10_0334	[97-99]
Fumarate hydratase class I, putative	PFI1340w	[100]
Irp-like protein, aconitate hydratase	PF13_0229	[100]
Lipoate-protein ligase, activator of 2-oxoglutarate dehydrogenase	MAL8P1.37	[101, 102]
Lipoate-protein ligase a; activator of 2-oxoglutarate dehydrogenase	PF13_0083	[54]
Dihydrolipoamide dehydrogenase	PFL1550w	
Dihydrolipoamide acyltransferase, putative	PFC0170c	
Mitochondrial electron transport		
Rotenone-insensitive NADH dehydrogenase, putative	PFI0735c	[39, 90, 103]
FAD-dependent glycerol-3-phosphate dehydrogenase, putative	PFC0275w	[104]
Malate quinone oxidoreductase, putative	PFF0815w	[105]
Ubiquinol cytochrome c oxidoreductase, Fe-S protein 3, putative	PF14_0373	[50]
Ubiquinol-cytochrome c reductase complex subunit, putative	PF10_0120	[106]
Ubiquinol-cytochrome c reductase hinge protein, putative	PF14_0248	[7, 50, 107]
Cytochrome c, putative	PF14_0038	[7, 108]
Cytochrome c1 precursor, putative	PF14_0597	[109]
BCS1-like protein required for the assembly of the cytochrome bc(1) complex, putative	PFF0155w	[5, 50]
Cytochrome c oxidase, putative	PFI1375w	[18, 110]
Cytochrome c oxidase subunit, putative	PFI1365w	[111, 112]
Cytochrome c oxidase subunit 2, putative	PF13_0327	[7, 107]
Cytochrome c oxidase subunit II precursor, putative	PF14_0288	[7, 107]
Cg8, Cytochrome c oxidase subunit IV	PF07_0032	
Cytochrome c oxidase assembly protein	PF14_0721	
Cytochrome c oxidase assembly protein, putative	PF14_0331	
Cytochrome c oxidase assembly protein (haem A: farnesyltransferase), putative	PFE0970w	[7, 107]
Protein similar to Oxa1 involved in cytochrome oxidase assembly, hypothetical protein	MAL8P1.14	[113]
Cytochrome c2 precursor, putative	MAL13P1.55	[113]
ATP synthase beta chain mitochondrial precursor, putative	PFL1725w	[114]
ATP synthase gamma chain mitochondrial precursor, putative	PF13_0061	[115]
ATP synthase F1 alpha subunit, putative	PFB0795w	[80]
ATP synthase delta subunit mitochondrial, putative	MAL13P1.47	[66, 116]
ATP synthase delta subunit mitochondrial, hypothetical protein	PF11_0485	
ATP synthase F1 epsilon subunit, mitochondrial putative	MAL7P1.75	
ATP synthase subunit c F0 (ATP9) proteolipid subunit, putative	MAL7P1.340	
Dihydroorotate dehydrogenase, mitochondrial precursor	PFF0160c	

(Table 2). Contd…..

Metabolic Pathways and Related Identified Genes	Accession Number	References
Cytochrome synthesis		
Cytochrome c haem lyase, putative	PFL1185c	[117, 118]
Cytochrome c1 haem lyase, putative	PFL0180w	[119]
Cg3 similar to SCO1 copper transport/insertion protein required for cytochrome c oxidase assembly	PF07_0034	
Antioxidant defense		
Thioredoxin peroxidase	PFL0725w	[71, 120-122]
Thioredoxin, putative	MAL13P1.225	[101, 121, 123-126]
Thioredoxin, putative	PFI1250w	[120, 125, 127, 128]
PfGLP-1, 1-cys-glutaredoxin-like protein-1	PFC0205c	[129]
Fe-superoxide dismutase 2 (SOD2), putative	PFF1130c	[130, 131]
Glutathione peroxidase	PFL0595c	[71, 132]
Glutathione reductase	PF14_0192	[133-135]

During the asexual stage, based on oxygen consumption by free parasites of *P. falciparum* [9, 37] and *P. yoelii yoelii* [4], the mETC of malaria parasites was found to be functional; and consequently, the different respiratory complexes I, II, III, and IV were predicted. Evidence of the function of a classical respiratory pathway in *P. falciparum* includes the existence of a mitochondrial transmembrane potential [8] and substrate-dependent mitochondrial oxygen consumption [7].

Maintenance of electropotential across the mitochondrial inner membrane is furthermore a critical function of the mETC. It has been suggested in the literature that the exclusive function of the mETC is to regenerate coenzyme Q (CoQ) as a substrate for DHOD in order to generate orotate, an intermediate of pyrimidine biosynthesis [40, 41]. Others have suggested an alternative model in which electron flux upstream of cytochrome *bc1* is essential for maintenance of the mitochondrial transmembrane potential and parasite survival [42]. At least five mitochondrial dehydrogenases (a rotenone-insensitive NADH dehydrogenase, glycerol 3-phosphate dehydrogenase, dihydroorotate dehydrogenase (DHOD), succinate dehydrogenase and malate quinone oxidoreductase) generate reduced CoQ, which in turn is re-oxidised by complex III to provide the mETC activity [7, 43]. The native ubiquinone of *P. falciparum* is CoQ8, but because it is too hydrophobic, it cannot be used as an exogenous substrate [39].

Complex I (NADH Dehydrogenase)

Krungkrai *et al*. suggest the presence of an NADH dehydrogenase complex I by observing that glutamate, an NADH-linked substrate, stimulates ADP phosphorylation in trophozoites permeabilised with digitonin. The existence of an NADH dehydrogenase was also demonstrated in isolated mitochondria of *P. falciparum* human parasites and *P. berghei* mouse parasites by testing the effects of the specific inhibitor rotenone on oxygen consumption and enzyme activity [44]. This inhibition is substrate-dependent. In the presence of NADH, complex I is insensitive to rotenone, whereas it is sensitive in the presence of succinate. Likewise, the oxidative phosphorylation in *P. falciparum* mitochondrion is stimulated in the presence of dihydroorotate (DHO). Thus, the synthesis of pyrimidine bypasses the respiratory chain in which the DHOD transfers electrons to the respiratory chain *via* CoQ, cytochrome c and oxygen. The parasite enzyme requires both NADH and CoQ for maximal catalysis. According to Krungkrai, the *P. falciparum* complex I may contain a NADH dehydrogenase activity, that function only for transhydrogenation from NADH to CoQ in the inner mitochondrial membrane [44].

In 2006, contrary to the recently completed malaria genome project, Biagini revealed that the *P. falciparum* mitochondrion lacks the conventional rotenone-sensitive complex I (or NADH dehydrogenase) found in most mammalian mitochondria and instead contains an alternative complex I (or type II NADH dehydrogenase - PfNDH2). Alternative NADH dehydrogenases have been described in some detail for plants, fungi, and bacteria. Ralph demonstrated evidence of a rotenone-insensitive NADH dehydrogenase in *P. falciparum* in 2005 [45]. Type II NADH dehydrogenases are rotenone insensitive and are not proton pumping. However, they nevertheless provide a mechanism for removal of excess reducing power to balance the redox state of the cell and are able to recycle mitochondrial matrix or cytosolic NAD(P)H, respectively depending on the localisation on the internal or the external face of the inner mitochondrial membrane [39].

Complex II (Succinate-Ubiquinone Reductase/ Quinol Fumaratereductase)

The unique property of parasitic complex II is that in aerobic respiration, the flavoprotein subunit (Fp) and the iron sulfur protein subunit (Ip) form a catalytic hydrophilic complex that serves to catalyse electron

transfer from succinate to quinone (SDH activity). In contrast, during anaerobic respiration, this complex catalyses electron transfer from quinol to fumarate (FRD activity). The Fp/Ip complex is bound to the matrix side of the inner mitochondrial membrane *via* two small membrane-anchoring proteins containing haem *b* (cytochrome *b* subunits: CybL and CybS). Furthermore, the CybL/CybS subunits seem to be essential for the electron transfer between the catalytic complex and quinone species [22, 46, 47].

Complex III or bc1 (Ubiquinol-Cytochrome c Oxidoreductase)

In eukaryotic organism, the coenzyme ubiquinol-cytochrome c oxidoreductase of complex III transfers protons across the membrane, producing a proton gradient. This complex III plays a critical role in biochemical generation of ATP (oxidative phosphorylation) [32, 33].

The gene encoding mitochondrial ubiquinol-cytochrome c oxidoreductase was characterised from gametocytic and intraerythrocytic stages of human malarial parasites cultivated *in vitro* [48].

Three inhibitors of ubiquinol-cytochrome *c* oxidoreductase, myxothiazole, antimycin A and atovaquone, collapse mitochondrial membrane potential and inhibit respiration [3, 43]. However, complex III of *P. falciparum* seems more sensitive to atovaquone, based on measurement of the membrane potential of the mitochondrion. Moreover, the erythrocytic stages and the late gametocytes are more atovaquone sensitive than the early gametocytes [37].

An alternative but unlikely hypothesis could be that the complex III is non-essential, but that the collapse of the membrane potential triggers some kind of programmed death similar to that seen in metazoans [49].

Complex IV (Cytochrome c Oxidase)

In eukaryote mETC, the enzyme cytochrome c oxidase of complex IV takes part in establishing of proton gradient , used to synthesise ATP by the ATP synthase [32, 33].

In *P. falciparum*, cytochrome c oxidase was purified from the mitochondria of parasites cultivated *in vitro* and was found to be sensitive to cyanide inhibition [50].

Complex V (ATP Synthase)

In eukaryotes, the mETC establishes a proton gradient that induces ATP generation to organise the respiratory pathway. The mETC and oxidative phosphorylation catalysed by ATP synthase are thus coupled by a proton gradient across the inner mitochondrial membrane [32, 33]. The mitochondrial synthesis of ATP requires a regular supply of ADP and inorganic phosphate (P_i). ADP is imported *via* the ATP/ADP carrier in exchange for a molecule of ATP. This import is dependent on the proton gradient generated by the mETC. The biochemical link between ATP synthase, the ATP/ADP transporter and the phosphate carrier is structural, with these proteins coming together to form an 'ATP synthasome' [25].

In *P. falciparum,* the ADP/ATP transporter adenylate translocase is expressed during the asexual stages and has been localised at the inner mitochondrial membrane, supporting an active role of the mitochondrion in ATP generation by oxidative phosphorylation [51A]. Genes encoding the F_0 and F_1 subunits of ATP synthase have also been identified in the *P. falciparum* genome [18]. The *P. falciparum* phosphate carrier is expressed in both asexual and gametocyte stages of the parasite and has been shown to be functional in heterologous mitochondria, suggesting the mode of import is similar to that seen in other organisms [25].

In many eukaryotes, when electron transport is disrupted, mitochondrial membrane potential can be maintained for a period by the reverse action of F_0F_1 ATP synthase using ATP hydrolysis to pump protons. In *P. falciparum*, this mechanism can be functional because 5-subunits of F_1 subcomplex have been reported in its genome and recently, Mogi and Kita have identified four F_0 subunits of the mitochondrial F_0F_1 ATP synthase [51B].

PHYSIOLOGY OF THE MITOCHONDRION

The TCA Cycle

In eukaryotes, the TCA cycle occurs under aerobic conditions and is the site of oxidative decarboxylation of pyruvate into acetyl-CoA by pyruvate dehydrogenase [34].

Thanks to the Malaria Genome Project, TCA cycle enzymes including malate dehydrogenase, isocitrate dehydrogenase and a complete pyruvate dehydrogenase complex have been identified in *P. falciparum* [26]. However, the pyruvate dehydrogenase complex in *P. falciparum* was targeted to the apicoplast. We have previously seen that the mitochondrion and the apicoplast are closely associated by enzymatic interaction. With the absence of a mitochondrial pyruvate dehydrogenase complex, it seems that the apicoplast was required for acetyl-CoA production through fatty acid biosynthesis and the acetyl-CoA source cannot result directly from glycolysis. In addition, after the diffusion of the pyruvate through the large and non-specific porins of the mitochondrial outer membrane, a specific transporter is necessary. Such a transporter does not exist for acetyl-CoA. It therefore appears that the TCA cycle of *P. falciparum* is disconnected from acetyl-CoA production. Nevertheless, it is particularly important to determine the source of mitochondrial acetyl-CoA in order to understand the metabolism of this organelle [25, 26].

However, lack of a mitochondrial pyruvate dehydrogenase complex suggests that energy generation in *P. falciparum* is considerably different from pathways described in other eukaryotes [45]. It is not clear if the oxidation of glycolytic products is used

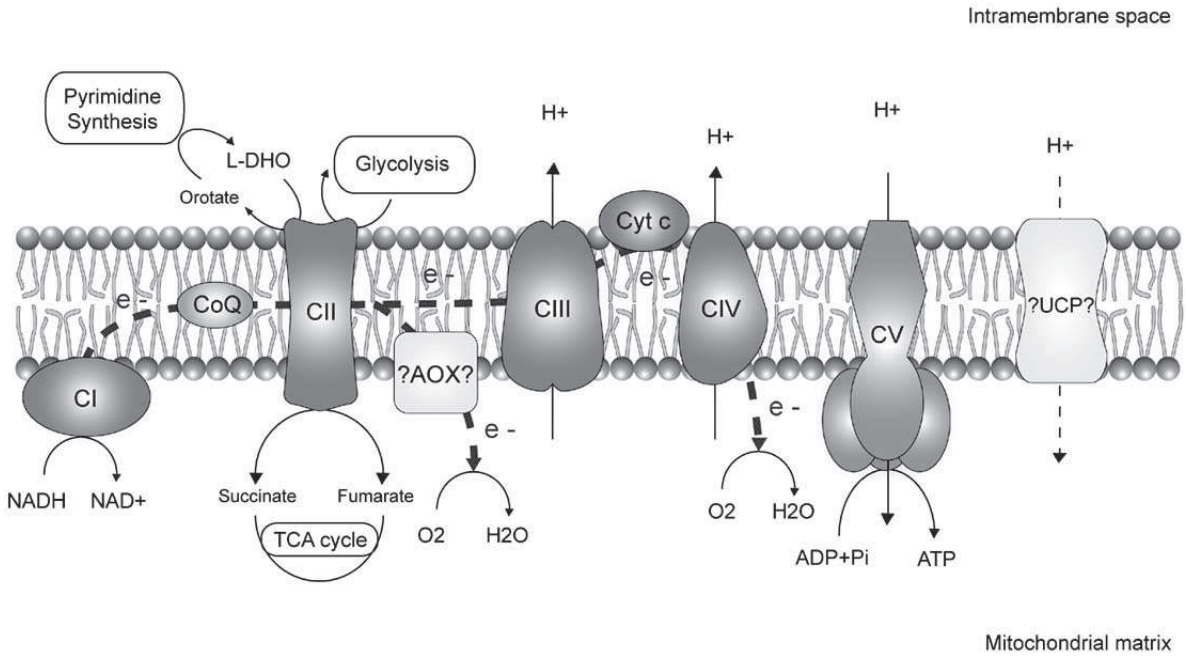

Fig. (4). *P. falciparum* mitochondrial respiratory chain.
The three particularities of *P. falciparum* respiratory chain are an alternative NADH dehydrogenase (CI), a hypothetical alternative oxidase (AOX) and putative uncoupled protein (UCP). The electron transfer is made through the different complexes *via* coenzyme Q (CoQ), establishing the transmembrane potential gradient used to produce ATP. Electron flow (e -) is shown in dotted lines.
NAD: nicotinamide adenine dinucleotide hydride, NADH: nicotinamide adenine dinucleotide hydride, L-DHO: L-dihydroorotate, TCA: tricarboxylic acid, Cyt c: Cytochrome c, complex I (CI, NADH dehydrogenase), complex II (CII, succinate-ubiquinone reductase/quinol fumaratereductase), complex III (CIII, ubiquinol-cytochrome c oxidoreductase), complex IV (CIV, cytochrome c oxidase), complex V (CV, ATP synthase).

for energy production or for the generation of substrates for biosynthetic pathways [4]. If a complete TCA cycle can function in *P. falciparum*, the main function of the cycle may be to produce succinyl-CoA to be used in haem biosynthesis. At the intraerythrocytic stage, the functionality of the TCA cycle remains unclear. Recent studies on malaria parasites have demonstrated the capacity of the mitochondrion in trophozoites to sustain oxidative phosphorylation and calcium transport in the presence of TCA cycle intermediates [25]. The mitochondrial TCA cycle in asexual stages may have functions in redox regulation through the production of NADPH that may be used in the mETC, thus contributing to energy generation in the mitochondrion. The TCA cycle and mETC are directly connected on two enzymatic levels [25]. The succinate dehydrogenase (SDH) complex, located on the inner membrane of the mitochondrion, is the only enzyme that participates in both the mitochondrial TCA cycle and the mETC (at level of complex II). SDH converts succinate to fumarate as part of the TCA cycle. This reaction also converts FAD to $FADH_2$. In the mETC, SDH transfers electrons from succinate to ubiquinol. The other enzymatic reaction that generates metabolite intermediaries for the mETC is malate dehydrogenase (MDH). MDH reduces NAD for the generation of oxaloacetate, and electrons from $NADH_+$ are donated to CoQ through complex I of the respiratory chain [34].

De novo Pyrimidine Biosynthesis: Dihydroorotase Dehydrogenase

P. falciparum parasites require purines and pyrimidines for nucleic acid synthesis during mature stages. Uridine 5'-monophosphate *de novo* biosynthesis is catalysed by six discrete enzymatic activities in malaria parasites. Of these, only the DHOD is localised in the inner mitochondrial membrane and classified into two families based upon amino acid sequence, substrate/cofactor dependence, and cellular localisation. The *P. falciparum* DHOD would belong to family 2, where the membrane-bound enzymes (family 2) transfer electrons to CoQ, chemically coupling pyrimidine biosynthesis to the respiratory chain [52]. The cytosolic enzymes (family 1) utilise fumarate or NAD+ as the terminal electron acceptor. *P. falciparum* DHOD, active in monomeric form, is a flavin-dependent mitochondrial enzyme that catalyses oxidation of L-DHO to L-orotate coupled to the reduction of CoQ to ubiquinol, the single redox reaction in *de novo* pyrimidine biosynthesis [53]. The enzyme may participate in limited O_2 consumption on the parasite during intraerythrocytic stages through the simple electron transport system without any oxidative phosphorylation in the mitochondrion. During

intraerythrocytic stages, *P. falciparum* DHOD has high specific activity in mature trophozoites and schizonts [53].

The *P. falciparum* erythrocytic cycle seems to maintain an active mETC in order to serve only one metabolic function: regeneration of the CoQ that is required as the electron acceptor for DHOD [52]. Transgenic *P. falciparum* parasites expressing *Saccharomyces cerevisiae* DHOD, which does not require CoQ as an electron acceptor, were completely resistant to inhibitors of respiratory chain. Thus, acquisition of only one enzyme can make mETC nonessential in the erythrocytic stages. Because the mETC in *P. falciparum* is essential for the establishment of transmembrane potential, the alternative DHOD (proguanil-sensitive) pathway becomes apparent only when electron transport is inhibited [40, 43].

PARTICULARITY OF *P. FALCIPARUM* MITOCHONDRION

The *P. falciparum* mitochondrion presents four original aspects: (i) a pyruvate dehydrogenase (PDH) complex, (ii) an alternative PfNDH2 complex I, (iii) a hypothetical alternative oxidase and (iv) putative uncoupled proteins.

The *P. falciparum* Mitochondrion Lacks a Pyruvate Dehydrogenase Complex

We have seen previously that the only PDH on *P. falciparum* is located exclusively in the apicoplast, where it presumably contributes to production of the acetyl-CoA needed for fatty acid synthesis. Thus, the absence of a mitochondrial PDH suggests that the TCA cycle probably plays a small role in the generation of high energy electrons for ATP synthesis [54]. Energy generation on *P. falciparum* is significantly different from pathways described in other eukaryotes.

An extramitochondrial source of electrons is suggested by the discovery of an NADH-dehydrogenase [45].

PfNDH2

We have seen previously that the membrane-bound NADH dehydrogenase that faces the mitochondrial matrix appears to have been replaced by the rotenone-insensitive NADH- dehydrogenase or PfNDH2 [45].

The inhibitory profile of PfNDH2 revealed that the enzyme activity was sensitive to diphenylene iodonium chloride (DPI) and diphenyl iodonium chloride (IDP), known inhibitors of alternative NADH dehydrogenases. The failure of PfNDH2 function resulted in a depolarisation of the mitochondrion, leading to parasite death. Thus, this enzyme is likely to be a critical component of the mETC [39]. Since the intraerythrocytic parasites are supposed to have a rather inactive TCA cycle, it seems that PfNDH2 oxidises NAD(P)H from the cytosol. A major source of cytosolic NADH is glycolysis, but the function of lactate dehydrogenase essentially renders this process redox neutral. There are additional substantial sources of NADH from general biosynthetic processes during cell growth that may provide reducing power for PfNDH2 [39].

In the *P. falciparum* mitochondrion, there is no proton pumping function ascribed to PfNDH2; however, there are other components contributing to the respiratory chain, such as succinate dehydrogenase (or complex II), glycerol-3-phosphate dehydrogenase (G3PDH), DHOD and malate quinone oxidoreductase (MQO). Nevertheless, these components contribute little to the overall flux to the complex III and complex II operates in reverse creating a branched respiratory chain [55].

Hypothesis of the Presence of an Alternative Oxidase (AOX)?

From oxygen consumption measurements of the *P. falciparum* mETC, Murphy provided evidence of a branched respiratory pathway in which electrons are transferred directly from ubiquinol to oxygen [56]. He hypothesised that *P. falciparum* parasites utilise a branched respiratory chain pathway, consisting of a classical cyanide-sensitive branch and an alternative cyanide-resistant branch. In the presence of high concentrations of cyanide, only 75% of the *P. falciparum* parasite oxygen consumption was found to inhibited. The cyanide-resistant branch of parasite oxygen consumption was completely inhibited by two inhibitors: propyl gallate and salicylhydroxamic acid (SHAM), which are specific toward the AOX activities in other organisms. In other organisms and notably in plants, the branched respiratory pathway plays a role in controlling oxidative stress. Such a role would be a major benefit to *P. falciparum*, which is known to be sensitive to oxidising conditions [56].

In plants containing both an alternative respiratory chain and the classical mETC, the AOX does not appear to contribute directly to the mitochondrial transmembrane potential or the energy balance of the cell. It can, however, contribute indirectly by accepting electrons from enzymes that donate electrons to CoQ to the preservation of TCA cycle turnover. AOX has been shown to contribute to the survival of plant cells under conditions in which the cytochrome chain is overloaded or blocked [57].

Nevertheless, no gene encoding the AOX has been detected in the *P. falciparum* genome. The sequence diversity of the gene might not be detected by sequence homology, or the alternative oxidase activity might be catalysed by another unknown protein [18]. As we have seen above, most of the mitochondrial proteins are identified as hypothetical proteins.

Existence of Uncoupled Proteins (UCP)?

In eukaryotes, under some conditions, protons can re-enter in the mitochondrial matrix without contributing to ATP synthesis. This process is known as proton leak

or mitochondrial uncoupling and is due to the facilitated diffusion of protons into the matrix [58]. The inner mitochondrial membrane of eukaryote cells contains a large amount of thermogenin (an uncoupling protein), which dissipates the proton transmembrane electrochemical gradient and bypasses ATP synthase. Therefore, it acts as uncoupler by forming an alternative pathway for the flow of protons back to matrix. This results in energy consumption and thermogenesis rather than ATP production [58].

Antibodies against the UCP have been used in immunoblotting and have been shown to have cross-reactivity with proteins of similar size in *P. falciparum* free-parasites, suggesting the presence of one or more uncoupling proteins in these cells [38]. However, the mETC of the *P. falciparum* mitochondrion is different from that typical mitochondria. There is no observed ATP turnover by complex V, but a transmembrane potential exists across the *P. falciparum* inner mitochondrial membrane. There must be a proton leak in the membrane that is sufficient in conductance to complete the circuit. It is not yet clear whether the leak is a result of the basal proton conductance in the membrane or a result of a specific UCP. Alternatively, it is possible that a minimal ATP flux is present and is sufficient to close the circuit [55]. It is unclear how this putative *P. falciparum* UCP is operating or regulated, but its presence might indicate that the proton circuit of the parasite mitochondrion is closed by enabling re-entry of protons *via* the UCP [55].

Apoptosis

The apoptosis is defined by dependence on caspase activation, leading to nuclear condensation, mitochondrial depolarisation, and cell death. Apoptosis symbolises an essential role of eukaryote mitochondria [59].

The apoptosis in the malaria mitochondrion is not well documented. During apoptosis, mitochondria undergo permeabilisation after death stimuli, resulting in loss of transmembrane potential. Using protonophore m-chlorophenyl hydrazone (CCCP) as a positive control, Meslin *et al*. observed a disruption of the transmembrane potential similar to that obtained with chloroquine and atovaquone, which is known to depolarise the parasite mitochondrion. Thus, these studies provide evidence for apoptotic death of *P. falciparum* in erythrocytic stages. In addition, a putative metacaspase-dependent pathway has been found to be involved in the process [60].

Kumar have also described apoptosis in *P. falciparum,* studying the effect of bilirubin on the parasite asexual stages. These searches suggest that bilirubin significantly induces oxidative stress in the parasite and increases formation of reactive oxygen species (ROS), which activate the mitochondrial pathway of apoptosis or cause mitochondrial dysfunction. Thus, bilirubin induces an apoptosis-like phenomenon on the malaria parasite and reduces the mitochondrial transmembrane potential to cause cell death [61].

Recently, by measuring the conventional parameters for apoptosis, it has been claimed that death caused by most of the antimalarials is not associated with classical apoptosis. Atovaquone is the exception, which reduces mitochondrial transmembrane potential and respiration, vital parameters for mitochondrial pathway of apoptosis [61].

P. FALCIPARUM MITOCHONDRION: MICROAEROPHILIC METABOLISM

Microaerophilic organisms are organisms that require oxygen for their life cycle, but only a low percentage (3%-5% O_2 for *P. falciparum*). Their growth is slow at ambient oxygen concentrations [62]. *P. falciparum* can be easily cultivated *in vitro* in a candle jar, a hermetic container where a lit candle creates a carbon dioxide-rich, oxygen-poor atmosphere. This parasite can make ATP by aerobic respiration if oxygen is present, but it is also capable of switching to glycolysis for energy generation [63].

All published findings support the hypothesis that the *P. falciparum* mitochondrion has a low respiratory metabolism: (i) mitochondrion of asexual stages of *P. falciparum* contains few cristae, (ii) parasite cultures grow optimally at low oxygen tension, (iii) until recently, some mitochondrial enzymes such as mitochondrial pyruvate dehydrogenase of the TCA cycle were unidentified, and (iv) levels of mETC enzymatic activity appear to be much lower than in eukaryotic cells [64]. Recently studies on mETC showed the loss of conventional complex I, a new indication in support of microaerophilic metabolism [39]. *In vivo*, the malaria parasite life cycle involves two hosts: female *Anopheles* mosquitoes and humans. Parasite multiplication in the mosquito is known as the sexual stage. While in human, the parasites undergo asexual multiplication in erythrocytes. Throughout all intraerythrocytic stages, the *P. falciparum* parasite is submitted to different environmental constraints and especially O_2 pressure. Indeed, in human, the parasite circulates in venous blood at 5% O_2 pressure while in arterial blood, and particularly at the level of lungs, the O_2 pressure is at 13% [65]. Furthermore, in mosquito, the malaria parasite is exposed to 21% O_2 pressure in salivary glands. This implies an adaptive response of the malaria parasite to survive. *In vitro*, at the erythrocytic stages, the parasite is known as a microaerophilic organism that uses O_2, to a limited degree (0.5%-5% O_2), which is required for maximal growth. The O_2 utilisation by the parasite is not involved in energy production but principally in *de novo* pyrimidine biosynthesis *via* DHOD [53]. This microaerobic metabolism is a paradox because parasites live in oxygen-carrier erythrocytes. *P. falciparum* culture exposure under 21% O_2 had no lethal effect on parasites without morphologic alteration of parasites, but it did increase the length of schizogony [62].

In eukaryotic organisms, the process of cellular respiration, also known as aerobic respiration, is dependent on the presence of O_2. When O_2 is limited, the glycolytic products are metabolised by aerobic respiration, a process that is independent of the mitochondrion [34]. Thus, *P. falciparum* has aerobic metabolism in gametocytes in that it shifts from glycolysis towards mitochondrial respiration and possibly oxidative respiration. In the human, sporozoites and merozoites respond to a microaerobic metabolism and are totally dependent on anaerobic glycolytic ATP production [64]. During erythrocytic stages, the parasite produces energy mainly through anaerobic glycolysis rather than through mitochondrial respiration. Consequently, the mETC functions differently. However, the limited utilisation of O_2 during the intraerythrocytic stage suggests that *P. falciparum* parasites are microaero-philic and homolactating fermenting organisms [50]. These findings suggest that the primary function of oxygen in *P. falciparum* might not be to act as the final electron acceptor in the mitochondria but rather to act as a substrate of metalloprotein oxygenases.

Additionally, the fact that fumarate partially inhibited DHO-dependent respiration suggests that complex II succinate ubiquinone reductase/quinol fumarate reductase in the erythrocytic stage cells of *P. falciparum* functions as a quinol fumarate reductase. This enzymatic activity may allow recovery of anaerobic microorganisms [66]. This *P. falciparum* specific feature is an evolutionary adaptation to microaerophilic conditions. Complex II plays a unique role in mammalian-type mitochondria as a component of the TCA cycle as well as the mETC, and it is therefore a direct link between major systems for energy metabolism. There may also be an alternative electron transport branch pathway, including an anaerobic function of complex II [56, 57].

More recently, Fisher described a predictive model of alternative PfNDH2 complex I as an evolutionary adaptation to a microaerobic lifestyle that enables uncoupled oxidation of NADH. The alternative complex I activity of generating reducing power is unregulated with respect to membrane potential and is crucial to the explanation of why many organisms have adopted type II NADH:dehydrogenase in their mETC [55]. This model of an alternative PfNDH2 as microaerophilic adaptation has several advantages: (i) a reduction of mitochondrial transmembrane potential in the absence of extensive ATP synthesis; (ii) a reduction of mitochondrial superoxide generation and (iii) a mechanism for the deregulated oxidation of cytosolic NADH [55]. These findings encourage the re-evaluation of mitochondrial metabolism function in *P. falciparum,* including CoQ biosynthesis, Fe-S protein biosynthesis, maintenance of Ca^{2+} homeostasis, membrane potential maintenance and antioxidant defence.

Consequently, microaerophilic metabolism in *P. falciparum* mitochondria can constitute a physiological adaptation to avoid the toxic effect of ROS generation in oxygen-rich environments and to avoid oxidative stress produced by haemoglobin degradation in blood-feeding parasites [55, 64].

P. FALCIPARUM MITOCHONDRION AND OXIDATIVE STRESS

Sources of Reactive Oxygen Species: The Mitochondrion and the Food Vacuole

Oxidative stress is an unavoidable consequence of aerobic metabolism. The respiratory chain in mitochondrion, especially complexes I and III, is a major site of production of ROS. At physiological oxygen levels, about 1-5%, oxygen is reduced by the mETC as a superoxide anion that can be converted into other ROS, such as hydrogen peroxide and the highly toxic hydroxyl radical. To avoid the toxic consequences of ROS generation, all aerobic organisms have developed antioxidant defence systems [67].

P. falciparum , a haematophagous parasite, digests haemoglobin, resulting in the release of large amounts of haem prosthetic group. Part of this haem is eventually decomposed, either enzymatically to hemozoin, or non-enzymatically through reduced glutathione resulting in free iron. Therefore, ROS produced in mitochondria interact with the pro-oxidant products of haemoglobin digestion, free iron and haem [64]. On *P. falciparum* , mitochondrial respiration, although slow, and haemoglobin digestion are pathways for production of ROS and are potentially synergetic. To attenuate oxidative stress, parasites have developed a preventive antioxidant defence in reducing oxygen consumption and lowering ROS production by the parasite antioxidant defence system [64] (Fig. **5**).

Antioxidant Defence Systems

To maintain a low intracellular level of ROS, malaria parasites have developed a major functional antioxidant defence system that includes glutathione S-transferase (GST), superoxide dismutase (SOD) as well as a thioredoxin system and a glutathione system [68, 69]. Notably, *P. falciparum* possesses neither a classical catalase [70] nor a classical glutathione peroxidase, but it does possess peroxidases with a diversity of substrate specificities [71].

The activity of the mETC is much lower than in mammalian cells, but it leaks superoxide anions that must be detoxified. *P. falciparum* possesses a Mn-SOD (SOD2) that may be associated with the mitochondrion [72]. The SODs, a family of metalloproteins, catalyse the dismutation of superoxide anions, forming hydrogen peroxide and oxygen. Hydrogen peroxide itself needs to be reduced to water and oxygen to prevent the oxidation of other cellular components. This reaction is catalysed by a mitochondrial peroxiredoxin-linked detoxification system that utilises haem-peroxidase catalase, glutathione-dependent peroxi-

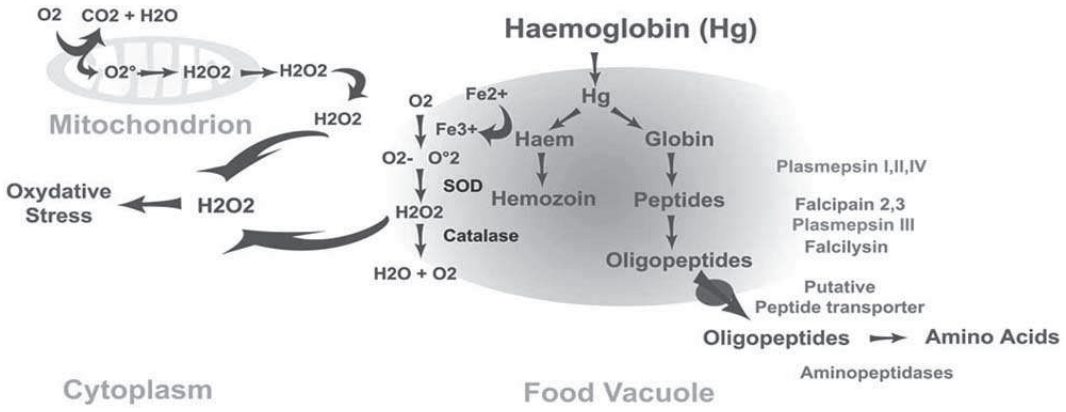

Fig. (5). Source of oxidative stress and antioxidant defence systems in *P. falciparum*.

Oxidative stress is an unavoidable consequence of aerobic metabolism in opposition to glycolisis. The mitochondrial respiratory chain product H_2O_2 and the haemoglobin digestion result in the release of large amounts of haem prosthetic group. These two pathways are source of reactive oxygen species and are potentially synergetic. To maintain intracellular low levels of ROS, parasites have developed major functional antioxidant defence implicating a mitochondrial superoxide dismutase (SOD) and catalase ingested from the host cell.

dases (GSH-Px) and thioredoxin-dependent peroxidases (Trx-Px). GSH-Px and Trx-Px obtain their reducing equivalents from two distinct redox systems, the glutathione and the thioredoxin redox systems [73-75]. The biochemical and structural features of the mitochondrial peroxiredoxin and thioredoxin of *P. falciparum* have been analysed, and their mitochondrial localisation has been demonstrated. The presence of a potential peroxidase such as PfTPx-2 in mitochondria, and a potential mitochondrial SOD2, is consistent with an ROS production of a respiratory chain in the mitochondrion of *P. falciparum* [76]. The presence of the mitochondrial peroxidase is imperative for the malaria parasite to maintain the integrity of the organelle because it is likely that, through the respiratory chain and the action of mitochondrial SOD, hydrogen peroxide is generated. Therefore, this mitochondrial peroxiredoxin-linked reduction of H_2O_2 is a crucial defence that protects the organelle from oxidative damage. Other redox active molecules like lipoic acid possibly lead to the reduction of Trx2 [77].

Recent findings have shown that lipoic acid plays a pivotal role in the defence against mitochondrial oxidative damage. Even if a peroxiredoxin and a thioredoxin have been annotated in the GenBank, the mitochondrion apparently does not contain Trx-reductase, which initiates the thioredoxin redox cascade. Therefore lipoic acid, an essential cofactor of mitochondrial alpha-ketoacid dehydrogenase complexes, provides reducing equivalents for the thioredoxin-dependent detoxification of hydroperoxides. The presence of a mitochondrial alpha-ketoacid dehydrogenase complex has been verified in *P. falciparum* intraerythrocytic stages. In agreement with this distribution, *P. falciparum* possesses organelle-specific pathways that supply the parasite with lipoic acid. Thus, lipoic acid is involved in the defence against ROS [75].

Another protein involved in maintaining the mitochondrial redox environment is an NADP+-dependent isocitrate dehydrogenase that was shown to be upregulated at both the transcriptional and protein levels when erythrocytic stages of *P. falciparum* were exposed to oxidative stress [78]. The role of this enzyme as an integral part of the TCA cycle remains uncertain, although 2-oxoglutarate clearly could feed into the TCA cycle in addition to being used for other biosynthetic and metabolic processes [75].

Equilibration of oxidative stress is crucial for the parasite because in many cells oxidative stress may lead to the induction of apoptosis.

THE MITOCHONDRION AS A POTENTIAL TARGET

Some unique properties of the *P. falciparum* mitochondrion suggest that enzymes of the mETC could be exploited as chemotherapeutic targets for drug development [47, 79, 80]. The importance of the mitochondrion in the growth of the asexual intraerythrocytic stage of *P. falciparum* is indicated by the antimalarial activity of drugs that act on the electron transfer system [3, 36, 81].

Various inhibitors of the mETC have been utilised in *in vitro* chemosensitivity assays. Table **3** indicates the results of *in vitro* assays with different mitochondrial inhibitors and their respective enzymatic targets. The sensitivity of the *P. falciparum* mETC to mitochondrial inhibitors differs substantially according to the *P. falciparum* strain studied [41]. This mitochondrial inhibitors allowed to elucidate the physiological function of the *P. falciparum* mitochondrion. Except for atovaquone, they could not be use in a therapeutic intention due to their cellular toxicity.

The rotenone is a specific inhibitor of the NADH dehydrogenase of complex I of the mETC, inhibiting

the electron transfer from iron-sulfur centers in complex I to ubiquinone. It results in the inability to produce ATP from the oxidation of NADH. Rotenone is classified by the World Health Organization as moderately hazardous. It is mildly toxic to humans and other mammals [3, 44].

The antimycin A binds to the Qi site of the cytochrome c oxidoreductase, inhibits the electron transfer *via* the ubiquinol-cytochrome c oxidoreductase complex and disrupts the proton gradient across the inner membrane. This disruption of the proton gradient prevents the production of ATP as protons are unable to flow through the ATP synthase complex. The inhibition of complex III by the antimycin A result in the formation of large quantities of ROS [3, 4, 50].

The myxothiazol, a antibiotic from the myxobacterium, is a methoxyacrylamide derivative. This specific inhibitor of ubiquinol-cytochrome c oxidoreductase prevents electron transfer from Fe-S center to CoQ, the myxothiazol effect is similar to that the antimycine A [3, 50].

The atovaquone is a hydroxy-1,4-naphthoquinone that inhibits electron transfer by fixing selectively the cytochrome *b* without affecting the host mitochondrial functions. The drug is a structural analogue of CoQ, which accepts electrons from dehydrogenase enzymes and passes them to electron transport cytochromes. This analogue inhibits the binding of CoQ-complex III at the Qo cytochrome domain and the electron transfer from CoQ to cytochrome bc_1 complex [82]. Moreover, CoQ carries electrons from DHOD to the *P. falciparum* respiratory chain *via* cytochrome bc_1 complex. In the absence of electron transfer, the mitochondrial transmembrane potential collapses, and without DHOD oxidisation, the pyrimidines biosynthesis is inhibited. Because plasmodia are unable to scavenge pyrimidines for DNA synthesis and to synthesise them *de novo*, the inhibition of DHOD results in parasite death [3].

To prevent emergence and spread of *P. falciparum* resistance, it is necessary to use drug in combination for therapeutic and chemoprophylaxis [83-85]. The combination resulted in protection of each drug from the development of resistance and reduced the overall transmission of malaria [86]. In combination therapy, the synergistic or additive potential of individual drugs is exploited. The atovaquone is already currently used in epidemic regions in combination with proguanil. This drug has been commercially available from GlaxoSmithKline since 2000 as Malarone™, used for malaria treatment and prophylaxis. The proguanil acts as a mitochondrial sensitiser and demonstrated synergistic activity *in vitro* even against atovaquone-resistant *P. falciparum* isolates. This effect of proguanil may be the result of its enhancement of atovaquone collapse of the mitochondrial transmembrane potential. The appearance of atovaquone resistance is a high natural frequency of cytochrome *b* mutants [87]. Specific mutations (Y268S, Y268C) have been shown to confer resistance *in vivo,* but there are other mechanisms of resistance that remain unknown [88]. The atovaquone has been also used in combination with doxycycline [81, 82]. As both drugs suppress *de novo* pyrimidine biosynthesis in parasites, an *in vitro* potentiation has been detected between atovaquone and doxycycline. In other hand, the quinolone such as 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ), structural analogue of CoQ, functions probably by interfering with the ubiquinol binding site of the complexe I [89]. The atovaquone combined with HDQ resulted in synergetic effect suggests that HDQ affects the mETC [90]. The HDQ was described as a high-affinity inhibitor of alternative NADH dehydrogenases. Moreover, the combination of atovaquone and SHAM, specific inhibitors of respiratory chain and alternative respiratory pathway respectively, also inhibits parasite growth synergistically [57]. However, such AOX has not been identified in *P. falciparum*, and the genome sequence has failed to reveal a gene encoding an orthologue of such enzyme.

A new therapeutic support in malaria treatment is the association of antimalarial drugs and hyperbaric oxygen therapy (HBO). This has been successfully used against bacterial and fungal infections. HBO therapy was observed to alter the levels of parasitaemia of mice infected with a non-cerebral line of *P. berghei* [91]. However, the HBO effects on the entire curve of parasitaemia and on the clinical symptoms were not further investigated. Moreover, HBO is well tolerated in humans and animals and can prevent cerebral malaria and death by neuroprotective mechanisms. This new therapy generates promising perspectives. HBO could act alone or in combination with recently discovered neuroprotective or anti-inflammatory molecules to improve cerebral malaria [92].

CONCLUSION

The *P. falciparum* mitochondrion is not an independent organelle but rather integrated into numerous functional, metabolic, and signalling networks with other cellular compartments, particularly the apicoplast. Moreover, a growing number of mitochondrial proteins possess a dual cellular localisation.

At the evolutionary scale, apicomplexan parasites appear to be in the process of minimising mitochondrial contributions to their physiology, maybe for redox balance. In intraerythrocytic stages of *P. falciparum* , mETC can be rendered unnecessary by the acquisition of transgenic DHOD. It would seem that the only function of mtDNA, which is the provision of a few subunits of the mETC, could be made superfluous by lateral gene transfers that bypass the need for electron transport [43, 53].

In despite of the part played by the *P. falciparum* TCA cycle in energy generation, the parasite mitochondrion is nevertheless crucial for several metabolic pathways, indicating that this organelle plays

Table 3. Comparison of *In Vitro* Assay of Different Mitochondrial Electron Transport Chain Inhibitors

Cible	Inhibitor	IC50 (µM)	Strain and Reference
Complex I	Rotenone	27.02 +/-3.5	Strain TM6 [39]
		51 +/-3.8	Strain 3D7 (Personal data)
Alternative complex I	HDQ	0.014 +/- 0.002	Isolate FCBR [90]
	DPI	0.24 +/-0.03	Strain TM6 [39]
		0.12 +/-0.02	Strain 3D7 (Personal data)
	IDP	5.99 +/-0.36	Strain TM6 [39]
		5.3 +/- 0.8	Strain 3D7 (Personal data)
Complex II	Thenoyltrifluoroacetone	2.10-4	Isolates T_9 and KT_3 [37]
Complex III	Antimycin A	0.013	Strain D6 [41]
		2.8 +/- 0.8	Strain 3D7 (Personal data)
	Myxothiazole	0.033	Strain D6 [41]
	Atovaquone	0.001+/- 0.0002	Strain 3D7 [82]
		0.002 +/- 0.003	Strain 3D7 (Personal data)
Complex V	DCCD	5.6 +/- 0.59	Strain L-3 [136]
DHODH	5-fluoroorotate	0.018	Strain D6 [41]
AOX	SHAM	247 +/- 6	Strain NF54 [56]
		246 +/- 4	Strain 3D7 (Personal data)
	Propyl gallate	24 +/- 3	Strain NF54 [56]
		33 +/- 5	Strain 3D7 (Personal data)

HDQ / 1-hydroxy-2-dodecyl-4(1*H*)quinolone.
DPI / diphenylene iodonium chloride.
IDP / phenyl iodonium chloride.
DCCD / N,N'-dicyclohexylcarbodimide.
SHAM / Salicylhydroxamic acid.

a role in anabolism. Recent findings have complicated earlier paradigms regarding energy production [55]. Several studies indicate that the mitochondrion possesses an active mETC, oxidative phosphorylation and an electrochemical gradient across the inner mitochondrial membrane.

Much work remains to be done to clarify the role of the mitochondrion on *P. falciparum*. Existing works have focused on the asexual intraerythrocytic stages because of the simplicity of making *in vitro* cultures, but the characterisation of other stages is likely to be rewarding. Particular interest in the comprehension of the *P. falciparum* metabolism may be essential, especially the study of the metabolic interconnection between the apicoplast and the mitochondrion, for development of therapeutic methods against the parasite.

ABBREVIATIONS

AOX = alternative oxidase
CCCP = chlorophenylhydrazone
CoQ = coenzyme Q
CoxII = cytochrome oxidase II
DCCD = N,N'-dicyclohexylcarbodimide
DHODH = dihydroorotate dehydrogenase
DPI = diphenylene iodonium chloride
Fp subunit = flavoprotein subunit
G3PDH = glycerol-3-phosphate dehydrogenase
GSH-Px = glutathione-dependent peroxidase
GST = glutathione S-transferase
HBO = hyperbaric oxygen therapy
coxI = cytochrome c oxidase I
cox III = cytochrome c oxidase III
IDP = phenyl iodonium chloride
Ip subunit = iron-sulfur protein subunit
L-DHO = L-dihydro-orotate
L-OA = L-orotate
LpIA = lipoic acid protein ligase
LsrRNA = Large subunits of ribosomal RNA
MDH = malate dehydrogenase

MET = transmission electron microscopy
mETC = mitochondrial electron transport chain
MQO = malate quinone oxidoreductase
mtDNA = mitochondrial DNA
mTPs = mitochondrial transit peptides
PDH = pyruvate dehydrogenase
PfNDH2 = type II NADH dehydrogenase
P_i = inorganic phosphate
P. falciparum = *Plasmodium falciparum*
ROS = reactive oxygen species
rRNA = ribosomal RNA
SDH = succinate dehydrogenase
SHAM = salicylhydroxamic acid
SOD = superoxide dismutase
SsrRNA = Small subunits of ribosomal RNA
TCA cycle = tricarboxylic acid cycle
Tim = Translocator inner membrane
Tom = Translocator outer membrane
tRNA = transfer RNA
Trx = thioredoxin
Trx-Px = thioredoxin-dependent peroxidase
UCP = uncoupled protein

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Au cours du cycle intra-érythrocytaire, la mitochondrie et l'apicoplaste sont en étroite relation physique (Figure 8) et vont subir pendant la schizogonie érythrocytaire un processus de division simultanée (Figure 9).

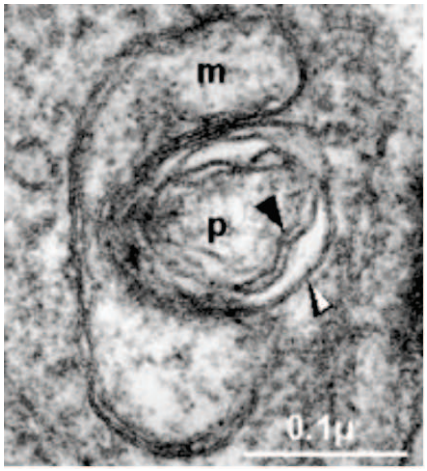

Figure 8. Microscopie électronique de l'étroite relation physique entre la mitochondrie (m) et l'apicoplaste (a) de P. falciparum (Maréchal et Cesbron-Delauw, 2001).

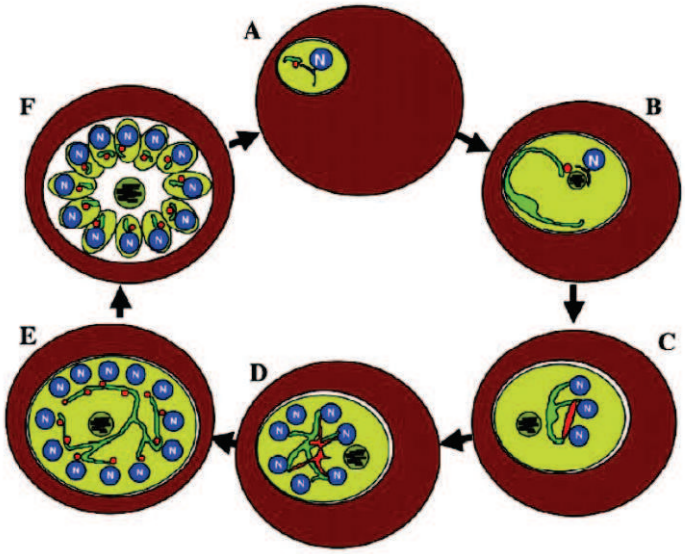

Figure 9. Division simultanée du noyau, de la mitochondrie (verte) et de l'apicoplaste (rouge) (Van Dooren et al., 2005)

Cette proximité se traduit par l'existence de voies métaboliques partagées entre les deux organites.

La mitochondrie, siège de consommation d'O_2, joue un rôle dans l'équilibre du stress oxydatif qui pourrait être attribué en partie à la voie de l'oxydase alternative décrite par Murphy et *al*. en 1997. Cette voie alternative, très bien définie chez les plantes, joue un rôle de protection du stress oxydatif en diminuant le taux de ROS mitochondrial. Cette fonction oxydase alternative, permettant le transfert direct d'électrons de l'ubiquinone à l'O_2 sans passer par les complexes III et IV, est assurée par des protéines AOX mitochondriales et chloroplastiques (PTOX) (Atteia et *al.* 2004). Les homologies de séquences du génome nucléaire de *P. falciparum* avec les plantes ainsi que l'origine végétale de l'apicoplaste, nous permettent d'émettre l'hypothèse de l'existence d'une fonction AOX.

L'enzyme de cette voie alternative, l'oxydase alternative, est inhibée spécifiquement par diverses drogues dont l'acide salicylhydroxamique (SHAM) qui est capable de supprimer la consommation d'O_2 résiduelle résistante à l'action du cyanure.

Seules quelques études concernent l'effet du SHAM chez le parasite :

- 25% de la consommation d'O_2 de *P. falciparum* reste insensible à de fortes concentrations de cyanure mais est totalement inhibée par le SHAM, inhibiteur spécifique de l'oxydase alternative (Murphy et *al*., 1997),

- le SHAM potentialise l'effet inhibiteur de l'atovaquone sur la croissance *in vitro* de *P. falciparum* et une action synergique a été décrite entre l'atovaquone et le n-propyl gallate, autre inhibiteur de la voie de respiration alternative (Murphy et Lang-Unnash.,1999),

- le pourcentage de la consommation d'O_2 inhibé par le SHAM est diminué par une forte concentration d'atovaquone (10 µM) (Suswam et *al*., 2001),

- hypothèse de l'existence d'une voie de l'oxydation alternative chez *P. falciparum*. (Krungkrai et *al*., 2004).

Actuellement, aucun gène codant pour une oxydase alternative plasmodiale n'a pu être identifié suggérant plusieurs possibilités : (i) une divergence des séquences ne permettant pas son identification par les algorithmes d'homologie ou (ii) une activité oxydase catalysée par une protéine actuellement inconnue.

3.2. Source exogène de ROS, les différentes pressions partielles d'oxygène dans la circulation sanguine

In vitro, les travaux de Trager et Jensen en 1976 ont montré que les conditions optimales de culture sont réalisées en condition microaérophile (5% CO_2, 5% O_2, 90% N_2). Cette adaptation des parasites à un faible taux d'O_2 est paradoxale pour un organisme qui se

développe dans l'érythrocyte. L'air ambiant (21% O_2) est donc considéré comme hyperoxique pour le parasite. Mais *in vivo*, le parasite est soumis à de grandes variations de pressions partielles d'oxygène (pO_2) lors de son passage dans la circulation sanguine et plus particulièrement, dans les capillaires pulmonaires. Dans l'organisme, le transfert de l'O_2 entre le milieu ambiant et son utilisateur final, la mitochondrie, obéit au phénomène de «cascade». En effet, la pO_2 de l'air alvéolaire est de 105 mmHg (16% d'O_2), celle du sang artériel de 95 mmHg (13% d'O_2) alors que celle du sang veineux et des capillaires tissulaires n'est que de 40 mmHg (5% d'O_2) (Tsai et *al*., 2003). Au niveau des mitochondries, la pression de «travail» est seulement de 2 à 4 mmHg et cette valeur constitue une valeur critique inférieure. Lorsque le parasite arrive au niveau des capillaires pulmonaires, l'enrichissement en pO_2 est considérable ; en moins d'une seconde, la pO_2 croît de 40 à 95 mmHg (Figure 10).

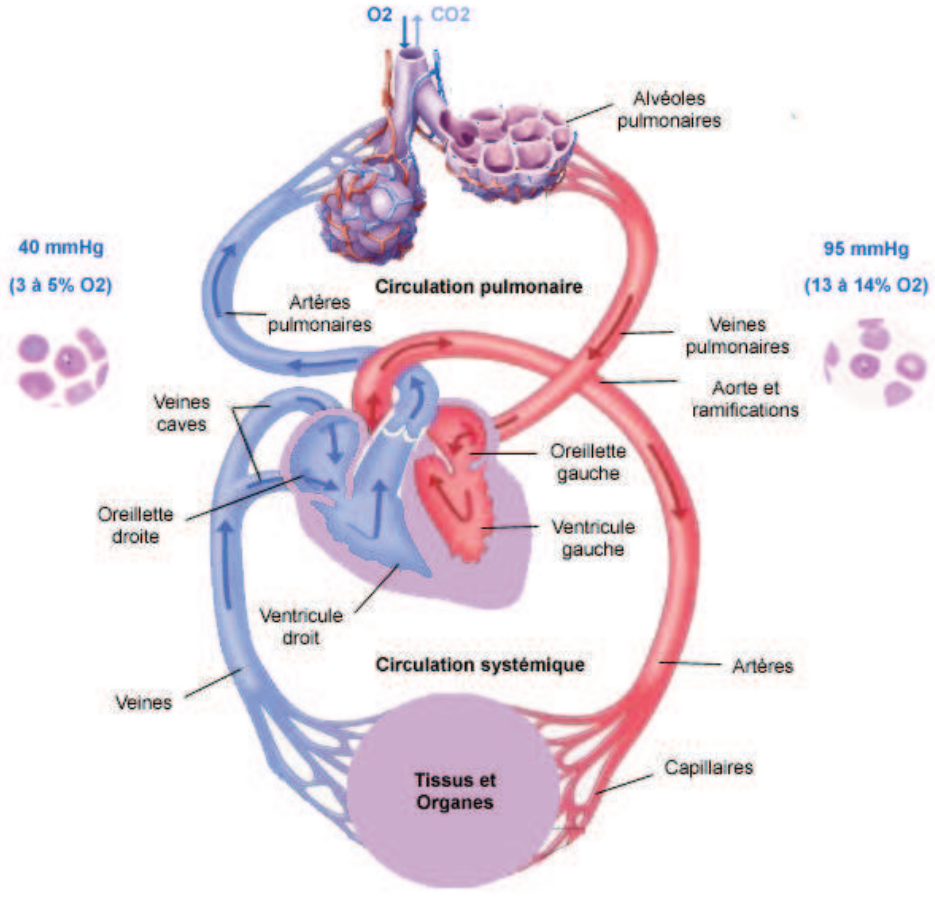

Figure 10. Variation des pressions partielles d'O_2 et de CO_2 (en mmHg) au cours de la circulation pulmonaire et de la circulation systémique.

La durée de ce séjour en milieu riche en oxygène depuis les alvéoles pulmonaires jusqu'aux capillaires tissulaires peut atteindre plusieurs minutes. Cette durée peut être augmentée par les phénomènes de cytoadhérence et de séquestration des hématies parasitées dans les capillaires, mécanismes de l'aggravation de l'accès palustre. Au niveau des poumons, la cytoadhésion est le fait d'hématies parasitées par des formes jeunes du parasite associées à de nombreux neutrophiles, monocytes et lymphocytes (Maguire et *al*., 2005), contrairement à ce qui peut être observé au niveau des vaisseaux cérébraux où les formes séquestrées sont principalement des trophozoïtes âgés et des schizontes (MacPherson et *al*., 1985). Dans cet environnement riche en oxygène, *P. falciparum* se trouve exposé à un stress oxydatif impliquant des ROS. Au niveau moléculaire, les ROS joueraient, outre le rôle de molécule signal du stress, un signal intrinsèque du développement et de la croissance (Noctor et *al*., 2007). D'autres études avancent également un rôle des ROS dans la réponse de l'hôte et dans la pathogénicité du paludisme (Clark et Cowden, 2003 ; Becker et *al*., 2004).

Les modifications de la pO_2 jouent-elles un rôle dans la maturation du parasite lors de son passage pulmonaire ?

Existe-t-il des voies métaboliques spécifiquement plasmodiales activées par ces variations de pO_2 ?

Dans la littérature, le nombre de publications concernant l'effet de l'oxygène sur le développement de *P. falciparum* est très limité :

- existence d'un effet de l'O_2 sur des cultures asynchrones de *P. falciparum* (Scheibel *et al.*, 1979),
- une plus grande difficulté à adapter les isolats de *P. falciparum* à la culture à des taux élevés d'O_2 (Takagi et Waki, 1987),
- une variation de l'effet de l'O_2 sur *P. falciparum* en fonction du type d'hématies utilisé (Orjih *et al.*, 2005),
- l'activité antipaludique des dérivés de l'artémisinine semble majorée par l'augmentation des taux d'O_2 au sein des cultures parasitaires (Krungkrai et Yuthavong, 1987).

Ces observations suggèrent que l'O_2 a un effet sur le développement du parasite et que les molécules actives via un stress oxydatif pourraient agir en synergie avec des taux élevés d'O_2.

OBJECTIFS

Ces travaux de thèse s'inscrivent dans un projet de recherche intitulé « Paludisme et microcirculation »; parmi les objectifs, il m'a été confié l'étude de l'influence du taux d'O_2 et de l'effet d'un inhibiteur de l'oxydase alternative sur le métabolisme de *P. falciparum.*

L'adaptation du parasite à un stress environnemental comme l'hyperoxie (21% O_2) ou la présence de drogue (SHAM) requiert une dynamique transcriptionnelle et traductionnelle qui se traduit par une adaptation métabolique. Plusieurs études ont déjà montré que l'expression des gènes et des protéines était modulée en réponse à différents stress (Oakley et *al.*, 2007; Radfar et *al.*, 2008; Prieto et *al.*, 2008; Hu et *al.*, 2010). L'émergence des technologies à haut débit «transcriptomique» et «protéomique» dans le domaine du paludisme offre l'opportunité d'envisager une approche combinée de ces deux techniques comparatives pour identifier les voies métaboliques impliquées dans l'adaptation du parasite à un stress.

L'étude de l'effet des variations environnementales sur la croissance et le métabolisme de *P. falciparum* a été réalisée *in vitro* sur la souche de référence 3D7. Cette étude a été menée à travers trois approches:

1. Déterminer l'effet de l'hyperoxie sur le développement de la souche 3D7 de *P. falciparum* par observation phénotypique sur frottis sanguins ;

2. Etudier l'effet de l'hyperoxie sur le transcriptome (puce à ADN) et le protéome (électrophorèse 2D différentielle) de *P. falciparum* et d'identifier les voies métaboliques impliquées dans la réponse à ce stress oxydatif ;

3. Etudier le métabolisme énergétique de *P. falciparum* et plus particulièrement, l'effet du SHAM, inhibiteur de la voie alternative de la respiration afin de tenter d'identifier le ou les acteurs de cette voie par les approches de bioinformatique et d'immuno-détection.

RÉSULTATS

ARTICLE 1

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Influence of oxygen on asexual blood cycle and susceptibility of *Plasmodium falciparum* to chloroquine : requirement of a standardized in vitro assay

Malaria Journal 2007, 6 :44

"Influence of oxygen on asexual blood cycle and susceptibility of Plasmodium falciparum to chloroquine : requirement of a standardized in vitro assay"

Problématique :

Quel est l'effet de l'hyperoxie sur l'aspect phénotypique de la souche 3D7 de *P. falciparum* ?

Des taux élevés d'oxygène modifient-ils le profil de chimiosensibilité de la souche 3D7 ?

L'objectif principal de cette étude est d'évaluer l'influence de l'oxygène sur la croissance *in vitro* de *P. falciparum* et sur la concentration inhibitrice à 50% (CI_{50}) de la chloroquine. Deux souches ont été utilisées : 3D7 chloroquino-sensible et W2 chloroquino-résistante.

Le passage à 21% d'O_2 n'induit pas d'effet délétère sur la croissance des souches 3D7 et W2. Nous observons, par contre, une augmentation de la durée de la schizogonie à 21% d'O_2, respectivement de 4 heures et 1 heure pour les souches 3D7 et W2.

L'effet de l'O_2 sur la CI_{50} de la chloroquine, pour 136 isolats de *P. falciparum* transmis au laboratoire entre février 2004 et décembre 2005, a été évalué par test *in vitro* isotopique. Les CI_{50} de la chloroquine à 10% d'O_2 sont significativement plus élevées que celles à 21% d'O_2, respectivement avec une moyenne de 173.5 nM et 121.5 nM ($p < 0.0001$). En particulier, sur les 63 isolats chloroquino-résistants ($CI_{50} > 100$ nM) à 10% d'O_2, 17 ont été retrouvés sensibles à la chloroquine à 21% d'O_2, avec une CI_{50} inférieure à 100 nM.

Au vue de ces résultats, il est donc nécessaire de standardiser les tests *in vitro,* et tout particulièrement la composition gazeuse du milieu, afin que les résultats des différentes équipes sur l'évaluation de la sensibilité des parasites aux antipaludiques puissent être comparés.

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Influence of oxygen on asexual blood cycle and susceptibility of *Plasmodium falciparum* to chloroquine: requirement of a standardized *in vitro* assay

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Abstract

Objective: The main objective of this study was to assess the influence of gas mixtures on *in vitro* *Plasmodium falciparum* growth and 50% inhibitory concentration (IC_{50}) for chloroquine.

Methods: The study was performed between February 2004 and December 2005. 136 *Plasmodium falciparum* isolates were used to evaluate gas mixtures effect on IC_{50} for chloroquine by isotopic microtest. The oxygen effect on asexual blood cycle of 3D7 and W2 clones was determined by thin blood smears examination and tritiated hypoxanthine uptake.

Results: From 5% O_2 to 21% O_2 conditions, no parasiticide effect of O_2 concentration was observed *in vitro* on the clones 3D7 and W2. A parasitostatic effect was observed during the exposure of mature trophozoïtes and schizonts at 21% O_2 with an increase in the length of schizogony. The chloroquine IC_{50} at 10% O_2 were significantly higher than those at 21% O_2, means of 173.5 nM and 121.5 nM respectively ($p < 0.0001$). In particular of interest, among the 63 isolates that were *in vitro* resistant to chloroquine ($IC_{50} > 100$ nM) at 10% O_2, 17 were sensitive to chloroquine ($IC_{50} < 100$ nM) at 21% O_2.

Conclusion: Based on these results, laboratories should use the same gas mixture to realize isotopic microtest. Further studies on comparison of isotopic and non-isotopic assays are needed to establish a standardized *in vitro* assay protocol to survey malaria drug resistance.

Background

Drug resistance of *Plasmodium falciparum*, the most deadly human malaria parasite with nearly 500 millions of new clinical cases each year [1], makes malaria control more difficult [2,3]. There are basically three approaches to the assessment of the antimalarial drug susceptibility of *P. falciparum*: *in vivo* assays as defined by the World Health Organization [4], *in vitro* assays and molecular markers of resistance [5].

In a number of laboratories surveying malaria drug resistance, *in vitro* tests are performed using the uptake of a radiolabelled nucleic acid precursor [^{3}H]-hypoxanthine [6] as a marker of parasite growth. Others methods can be used: the WHO schizont maturation tests by optical microscopy (Mark III) with pre-dosed plates [7], which was based on the method of Rieckmann *et al* [8] and of Wernsdorfer [9], a flow cytometric analysis of propidium iodide incorporation into parasite, which permits a stage-specific evaluation of antimalarial compounds [10], and colorimetric assays with the measurement of Histidine Rich Protein II (HRP2) by an enzyme-linked immunosorbent assay (ELISA) [11,12] and the DELI-microtest (Double-site Enzyme-linked Lactate dehydrogenase Immunosorbent assay) [13,14].

Many factors can influence the results of the chemosusceptibility tests [15]: the initial parasitaemia, the haematocrit, the time of incubation, the time point when [^{3}H]-hypoxanthine is added, the use of serum substitutes and the gas mixture. Laboratories using isotopic microtest to monitor drug resistance work at different oxygen tensions: 3% O_2 [10], 5% O_2 [11,12], 10% O_2 [16], in candle jars [13,14] (which corresponds to approximately 15% O_2 [17]) and 21% O_2 [15] (in CO_2 incubators). WHO recommends the use of a candle jar in their *in vitro* microtests (Mark III). But all have adopted the same threshold for the resistance to antimalarial compounds under different oxygen tensions. The aim of this study was to evaluate the influence of oxygen on the asexual blood cycle and the *in vitro* chemosusceptibility of *P. falciparum* to chloroquine in order to contribute to the establishment of a standardized *in vitro* assay protocol.

Methods

Isolates of P. falciparum

Between February 2004 and December 2005, 136 *P. falciparum* isolates were obtained from patients attending the North Hospital in Marseille [18] (France). Venous blood was collected into Vacutainer ACD tubes (Becton Dickinson, Rutherford, NJ) before treatment and transported at 4°C to the laboratory in Marseilles, that is associated to the French National Malaria Reference Center. Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) and examined to determine parasite density. Samples with parasitaemia ranging from 0.01% to 6.2% were used to test drug sensitivity. Parasitized erythrocytes were washed three times in RPMI 1640 medium (Invitrogen, Paisley, United Kingdom). If parasitaemia exceeded 0.8%, infected erythrocytes were diluted to 0.5–0.8% with uninfected erythrocytes and resuspended in culture medium to a haematocrit of 1.5%. Susceptibility to chloroquine was determined after suspension in RPMI 1640 medium. The suspensions were supplemented with 10% human serum (AbCys, Paris, France) and buffered with 25 mM HEPES (Sigma, St. Louis, MO) and 25 mM $NaHCO_3$ (Sigma). Isolates were used for 60-hr experiments at two different gas mixtures under 10% O_2, 5% CO_2, 85% N_2 in a CO_2 water jacketed incubator series II (Model 3141, Forma Scientific, Inc.) or 21% O_2, 5% CO_2, 74% N_2 in a CO_2 incubator (Model MCO-17 AIC, Sanyo).

Parasite clones

Chloroquine sensitive 3D7 clone and chloroquine resistant W2 clone (MR4 Resource Center) were used in this study. They were maintained in continuous culture as previously described [19] at 6% haematocrit using type O+ (3D7) or type A+ (W2) human erythrocytes in the same conditions as described above at 37°C under a 5% CO_2, 5% O_2, 90% N_2 gas mixture and a humidity of 95%. Culture medium was changed every day, viability and parasitaemia of cultured parasites were calculated by light microscopy analysis of blood smear stained with RAL® 555 (Réactifs RAL, France), (5,000 erythrocytes counted per blood smear). Blood smear pictures were performed using a digital camera (Digital Camera DXM 1200, Nikon), and analyzed with the software Lucia 4.8 (Nikon). Parasite synchronization was performed by sorbitol treatment (D-Sorbitol, ICN Biomedicals) as previously described [20]. To obtain tightly synchronized cultures, two sorbitol treatments were carried out 12 h apart. Approximately 48 h after the initial synchronization, the cultures were synchronized again to eliminate any residual schizonts. Clonality was verified every month using PCR genotyping of polymorphic genetic markers (msp1, msp2 and microsatellite loci [21,22]).

Drug

Chloroquine diphosphate was obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Two-fold serial dilutions of chloroquine were prepared in sterile, distilled water. Final concentrations ranging from 5 nM to 3200 nM were distributed in triplicate into Falcon 96-well flat-bottom plates (Becton Dickinson, Franklin Lakes, N.J.), which were dried.

Oxygen effects on asexual blood cycle of P. falciparum

Synchronous cultures of 3D7 or W2 were divided in three subcultures at 2% initial parasitaemia for 3D7 and 0.5% for W2 and 3% haematocrit under three different oxygen

tensions 5% O_2, 10% O_2 and 21% O_2 in three different experiments with duplicates. Culture mediums were not replaced during the experiments. Blood smears were carried out at different times to evaluate parasitaemia and percentages of different stages of parasite (5,000 erythrocytes were counted per blood smear in blind by two different examiners). The same experiments (three different experiments with duplicates) were undertaken with 3D7 parasites with only ten hours exposures of rings stages, trophozoïtes stages or schizonts stages under 21% O_2, 5% CO_2, 74% N_2 gas mixture and under 5% O_2, 5% CO_2, 90% N_2 gas mixture in a CO_2 water jacketed incubator series II (Model 3131, Forma Scientific, Inc.) during the rest of the cycle to assess a stage susceptibility of oxygen effect.

Tritiated hypoxanthine uptake

200 µl of the suspension of no parasitized and parasitized erythrocytes (1% parasitaemia, 2% haematocrit) was distributed in 96-well plates (three different experiments with six measurements). Parasite growth was assessed by adding 1 µCi of [^{3}H]-hypoxanthine with a specific activity of 14.1 Ci/mmol (Perkin Elmer, Meriden, NJ) to each well. Plates were incubated for different periods and at different parasites stages at 37°C under 21% O_2, 5% CO_2, 74% N_2 or 10% O_2, 5% CO_2, 85% N_2 gas mixture and a humidity of 95%. Duplicate wells were used to make thin blood smears to evaluate parasitaemia and parasite stages. Immediately after incubation the plates were frozen and then thawed to lyse erythrocytes. The content of each well was collected on standard filter microplates (Unifilter™ GF/B, Perkin Elmer) and washed using a cell harvester (Filtermate™ Cell Harvester, Packard, Meriden, NJ). Filter microplates were dried and 25 µl of scintillation cocktail (Microscint™ O, Perkin Elmer) was placed in each well. Radioactivity incorporated (in counts per minute, cpm) by the parasites was measured using a scintillation counter (Top Count™, Perkin Elmer).

Drug susceptibility assays

The isotopic micro drug susceptibility test used in this study was performed as described previously [23]. The 50% inhibitory concentration (IC_{50}), i.e. the drug concentration corresponding to 50% of the uptake of [^{3}H]-hypoxanthine by the parasites in drug-free control wells, was determined by nonlinear regression analysis of log dose-response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean IC_{50} with 95% confidence intervals. The cut-off value for *in vitro* resistance to chloroquine is 100 nM [24].

Statistical analysis

The different results were analyzed with the software STATA 9.0 using a Chi-square test for related samples, a Student's T test or a Wilcoxon signed-rank test (as needed) for related samples. Differences were considered statistically significant when $p < 0.05$.

Results

In order to determine the effect of oxygen on asexual blood stages of *P. falciparum*, synchronous cultures of 3D7 were exposed to 5, 10 and 21% O_2 during two cycles of parasites. As shown in Figure 1A, there were no significant difference between parasitaemia or parasite stage distribution under 5 and 10% O_2 during all the experiment. On the contrary, from 35 h to the rest of experimental points, a significant difference between parasite stages was observed (Figure 2), for example at 40 h, there were 42% of rings and 44.9% of schizonts under 5% O_2 and 21.7% of rings and 67.6% of schizonts under 21% O_2 ($p < 0.001$, Chi-square test). After the complete reinvasion around 60 h, parasitaemia were the same in the three experimental conditions and no morphologic alteration were observed by light microscopy (Figures 3A and 3B). These results have shown that parasite exposure under 21% O_2 had no lethal effect on parasites, but increased the length of schizogony. Moreover, only mature trophozoïtes and schizonts were susceptible to an effect of exposure to 21% O_2 (Figure 4).

The delay was also observed with W2 clone (Figure 1B), with a briefer schizogony (8 hours for W2 and 11 hours 3D7) and a higher multiplication index for W2 (% of infected red blood cells by rings after reinvasion/% of infected red blood cells by rings at the beginning of the previous cycle), (8 for W2 and 3 for 3D7).

No significant difference were observed in tritiated hypoxanthine uptake between 10 and 21% O_2 with the two clones 3D7 and W2 (Figures 5A and 5B), ($p = 0.087$ and $p = 0.76$ respectively). The maximum of incorporation (around 2,500 cpm with 3D7 and 4,500 cpm with W2) was achieved at 50 hours with a steady-state until 60 hours for 3D7 and W2 in the two experimental conditions. 3D7 and W2 chloroquine IC_{50} were evaluated under 5, 10 and 21% O_2 (three experiments). No significant difference were observed with 3D7, the IC_{50} were 15.8 nM [10.8–20.7], 14.1 nM [12.4–16.9], 17.6 nM [14.1–21.1] under 5% O_2, 10% O_2 and 21% O_2 respectively. On the contrary, with W2 clone, IC_{50} under 21% O_2 was significantly lower than IC_{50} under 5% O_2 and 10% O_2, 83.3 nM [15–168], 299 [228–369] and 277 [192–322] respectively ($p < 0.05$, Student's T test).

One hundred and thirty six *Plasmodium falciparum* isolates were used to evaluate chloroquine susceptibility at 10% O_2 and 21% O_2. Three tests at 10% O_2 and 13 tests at 21% O_2 were not interpretable, because of lack of significant difference in cpm between control-wells and higher chloroquine concentration wells. Chloroquine susceptibility

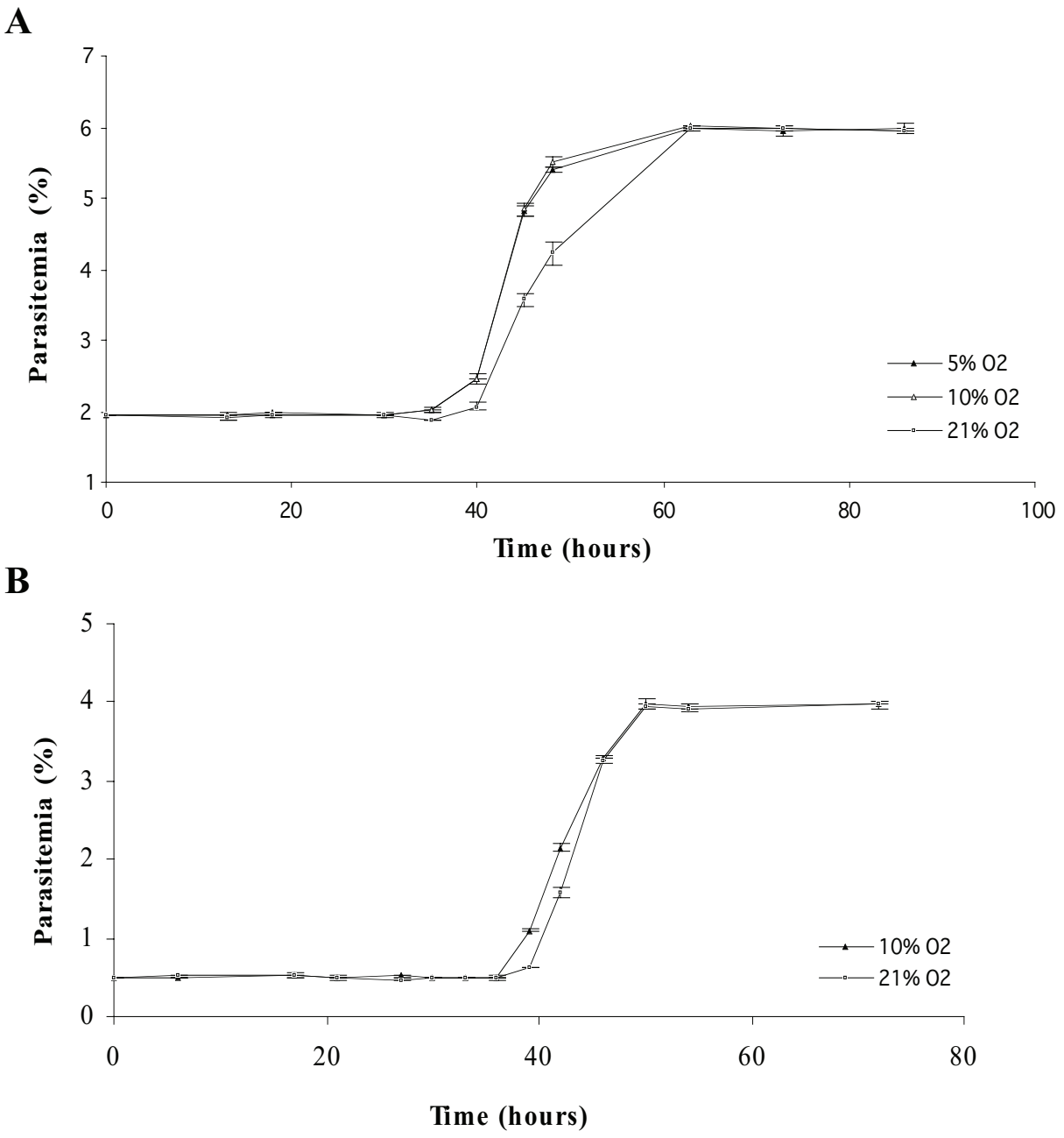

Figure 1
Evolution of asexual parasitaemia of synchronous cultures of *Plasmodium falciparum* under different oxygen tensions. Each point represents the mean ± standard deviation of three experiments. **A**: 3D7 clone. **B**: W2 clone.

of 120 isolates was finally tested at 10 and 21% O_2 (Figure 6A). The differences between the chloroquine IC_{50} of the 120 isolates at 10% O_2 and at 21% O_2 were statistically significant ($p < 0.0001$, Wilcoxon signed-rank test), means of 173.5 nM with a standard deviation of 168.4 and 121.5 nM with a standard deviation of 106.7 respectively. The median and interquartile (Q25% & Q75%) values of the chloroquine IC_{50} at 10% O_2 and IC_{50} at 21% O_2 of the 120 isolates were respectively 127 nM, 30 nM, 258 nM at 10% O_2 and 85.9 nM, 36 nM, 168 nM at 21%

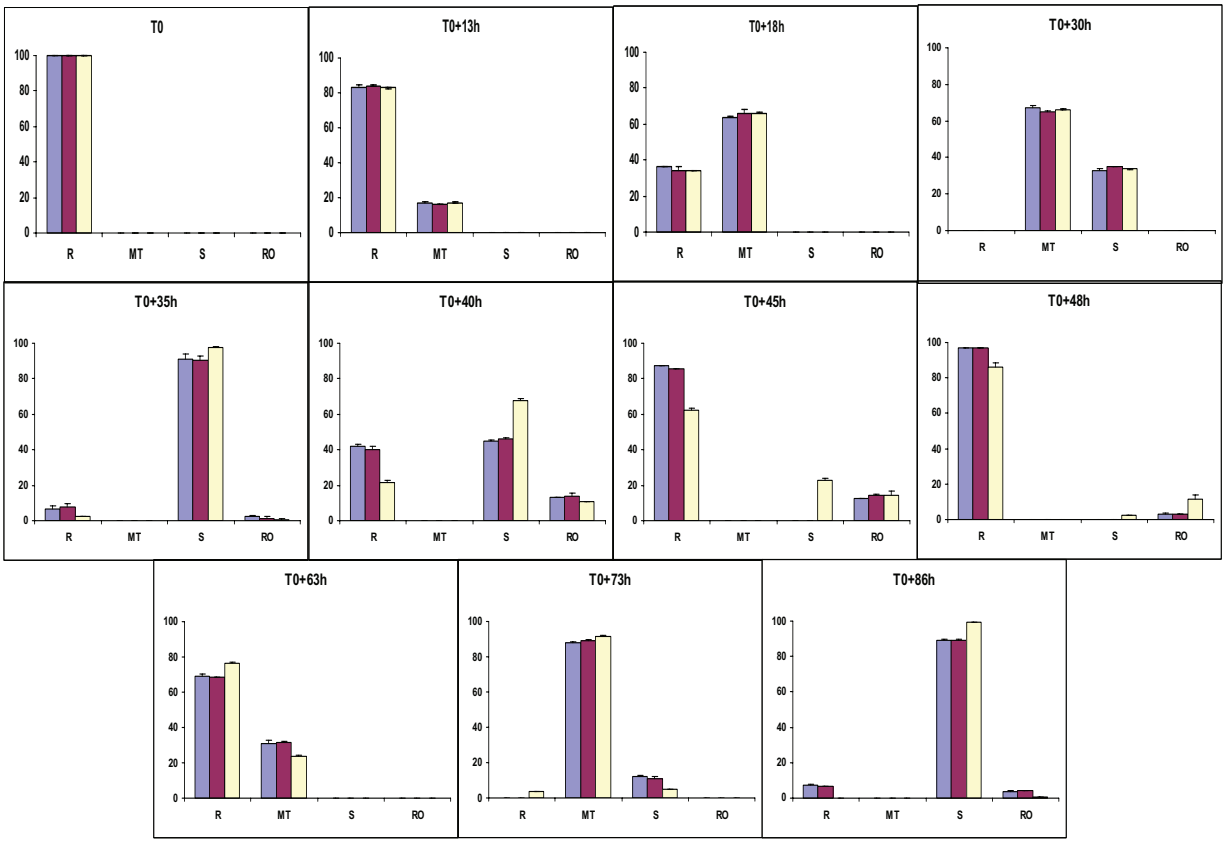

Figure 2
Percentages evolution of asexual stages of synchronous culture of 3D7 *Plasmodium falciparum* clone under different oxygen tensions during 86 hours. Each percentage represents the mean ± standard deviation of three experiments. R: Ring. MT: Mature Trophozoïte: S: Schizont. RO: Rosace. Blue histograms correspond to 5% O_2; Purple histograms correspond to 10% O_2. Yellow histograms correspond to 21% O_2.

O_2. In particular of interest, 52.5% of isolates had chloroquine IC_{50} > 100 nM at 10% O_2 although 42.5% of the same isolates had chloroquine IC_{50} > 100 nM at 21% O_2. Moreover, among the 63 isolates which had chloroquine IC_{50} > 100 nM at 10% O_2, 17 had chloroquine IC_{50} < 100 nM at 21% O_2 (Figure 6B) (i.e. 1/3) although five isolates only had chloroquine IC_{50} > 100 nM at 21% O_2 among the 57 isolates which had chloroquine IC_{50} < 100 nM at 10% O_2 (Table 1), (Chi-square test for paired samples, $p < 0.025$).

There were no significant difference between the observed control cpm at 10 (mean 12,607, standard deviation 13,425) and 21% O_2 (mean 13,302, standard deviation 14,662).

Discussion

The first works about oxygen effects on *P. falciparum* asynchronous cultures [17] had shown that microaerophilic environment allowed an optimal development of parasites. Their growth was impossible in strict anaerobic conditions. *P. falciparum* possesses a functional mitochondrial respiratory chain with oxygen consumption [25]. It has been shown that there is some protector effect of CO_2 at high oxygen concentration [17] through the medium pH which stability (between 7.2 et 7.45) is required for parasite growth [26]. The analysis of the different parasites stages distribution according to the time and the oxygen concentration has allowed us to reveal a possible slow down of cellular cycle without morphologic alteration of parasites under 21% O_2.

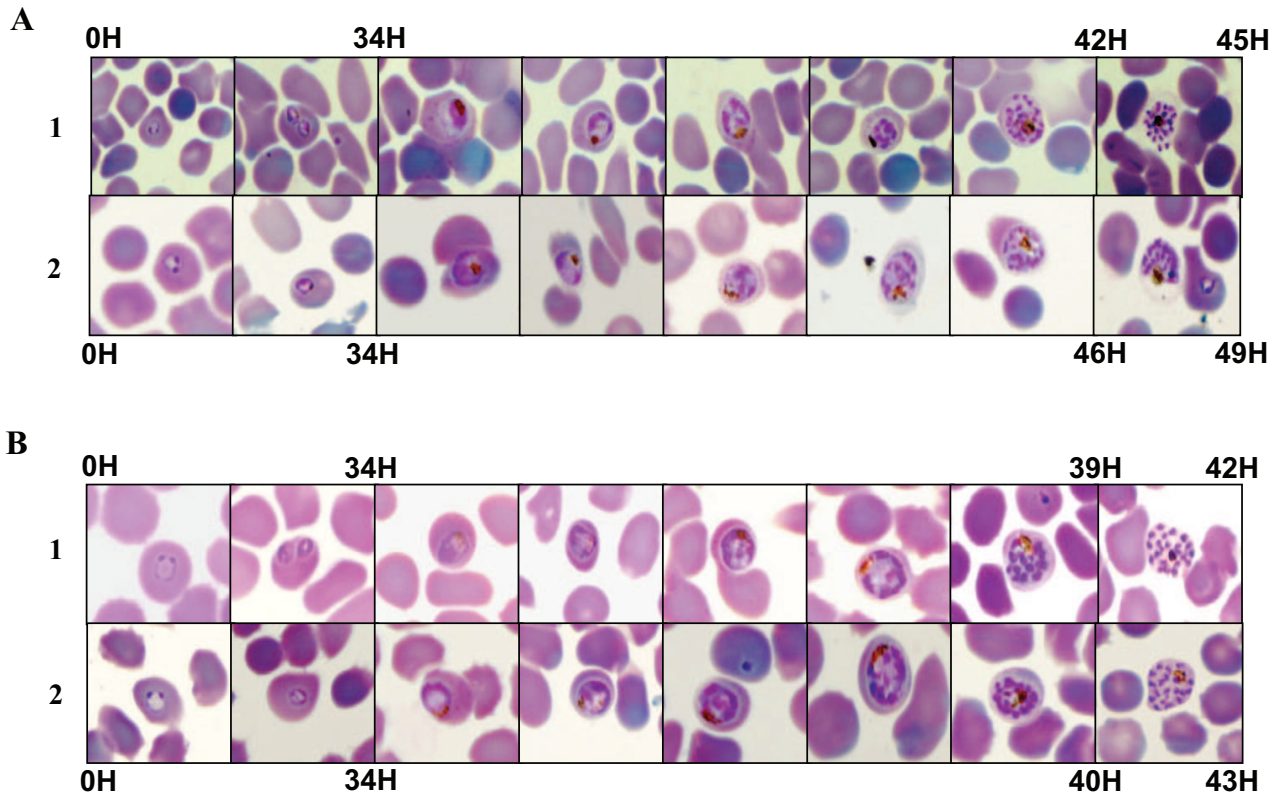

Figure 3
Cell cycle evolution of asexual blood stages of *Plasmodium falciparum*. **A**: 3D7 clone. **B**: W2 clone. **1**: 5% O_2. **2**: 21% O_2.

In the present study, the exposure to 21% O_2 did not change parasitaemia growth rate after complete reinvasion. Previous studies [27] had shown the same absence of oxygen effect on synchronous cultures parasitaemia during four days. Here, it has been shown that the exposure of 3D7 and W2 *P. falciparum* clones to 21% O_2 had parasitostatic effect by lengthening the schizogony. Mature stages had a particular susceptibility to high oxygen concentration. Moreover, these results justified to test drug susceptibility after a 60 hours period of incubation with [^{3}H]-hypoxanthine. At the end of trophozoïtes stage, DNA replication begins and is followed by a succession of S phases during the schizogony [28]. Under 21% O_2, the nucleic acids synthesis decreased from 30 hours for the two *P. falciparum* clones comparing to 5% O_2 exposure. Under 5% O_2, a low [^{3}H]-hypoxanthine incorporation took place for the first twenty hours, probably corresponding to RNA synthesis in rings, followed by an increase between 20 and 30 hours in possible relation with the beginning of DNA replication at late tropho-

Table 1: Repartition of drug susceptibility of *Plasmodium falciparum* isolates at 10% O_2 and 21% O_2.

		21% O_2	
		CQ[a] IC_{50}[b] < 100 nM	CQ IC_{50} > 100 nM
10% O_2	CQ IC_{50} < 100 nM	52	5
	CQ IC_{50} > 100 nM	17	46

[a] CQ: chloroquine.
[b] IC_{50}: inhibitory concentration 50% (nM).

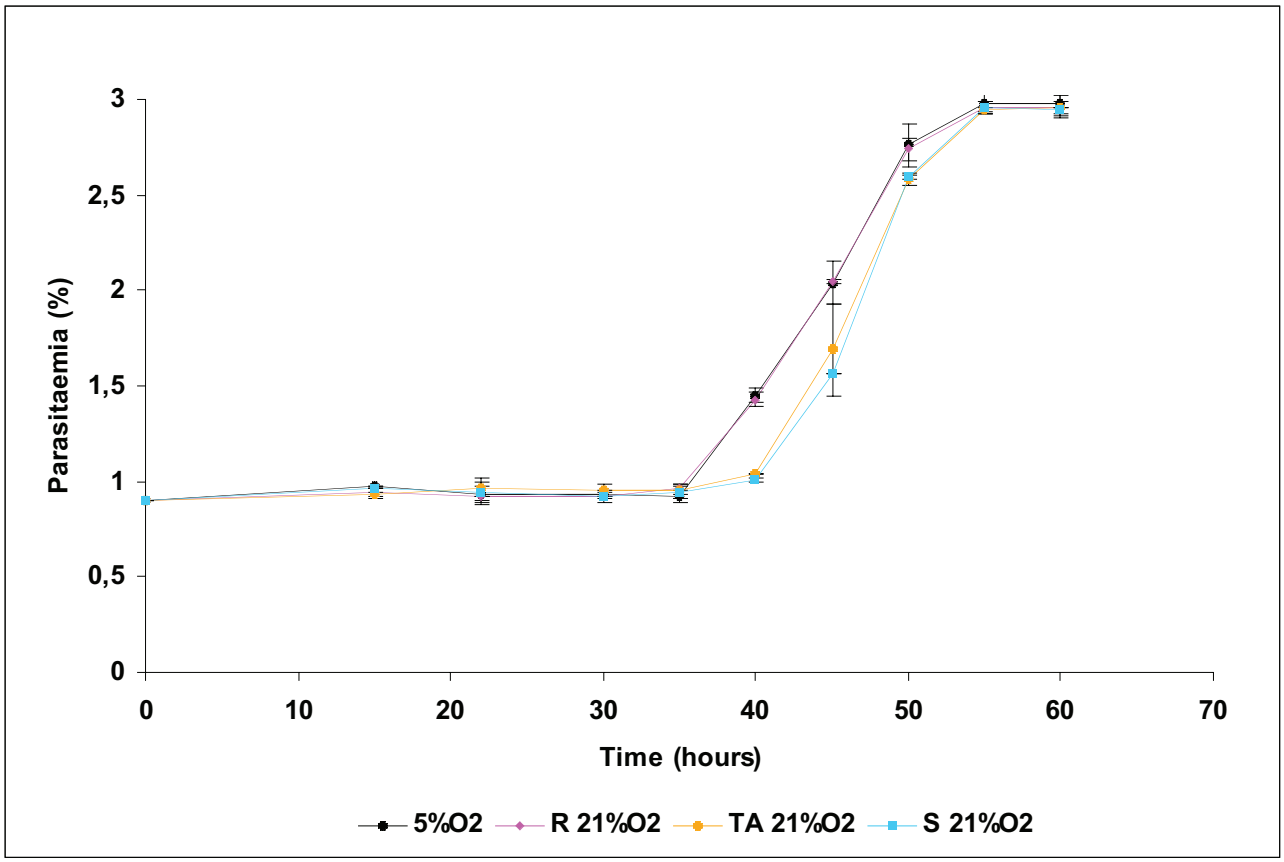

Figure 4
Evolution of asexual parasitaemia of synchronous cultures of *Plasmodium falciparum* exposed to 21% O_2 during ten hours at ring, trophozoïte or schizont stage. Each point represents the mean ± standard deviation of three experiments.

zoïtes stage [29]. Between 30 and 40 hours, during schizogony, DNA synthesis increased and the peak was achieved at 50 hours [30].

In the present study, the chloroquine IC_{50} at 10% O_2 were significantly higher than those at 21% O_2. A previous study did not show oxygen dependent effects of chloroquine on *P. falciparum* in culture [31], but in that experiment only four strains were tested. In the present work, among the 63 isolates which had chloroquine $IC_{50} > 100$ nM at 10% O_2, 17 had chloroquine $IC_{50} < 100$ nM at 21% O_2. The effect of gas mixture on the results of chloroquine chemosusceptibility should led different laboratories involved in malaria resistance survey to adapt a resistance threshold for each gas mixture or to use the same conditions to perform isotopic microtests.

Conclusion

Several factors influencing the results of the chemosusceptibility tests (the initial parasitaemia, the haematocrit, the time of incubation, the time point when [^{3}H]-hypoxanthine is added, the use of serum substitutes) have already been investigated by Basco [15,32]. The present data suggest the importance of the gas mixture on isotopic microtest results for chloroquine. Further studies are needed to evaluate gas mixture impact on isolates susceptibility to other antimalarial compounds and their correlation with molecular markers of resistance and *in vivo* evaluation of drug efficacy. Other investigations about the preparation of drug solutions, the storage of pre-dosed culture plates are required before *in vitro* drug sensitivity assay can become a standardized tool for laboratories to validate the threshold for resistance in respect to the clinical responses and molecular markers.

Conflict of interest

The author(s) declare that they have no competing interests.

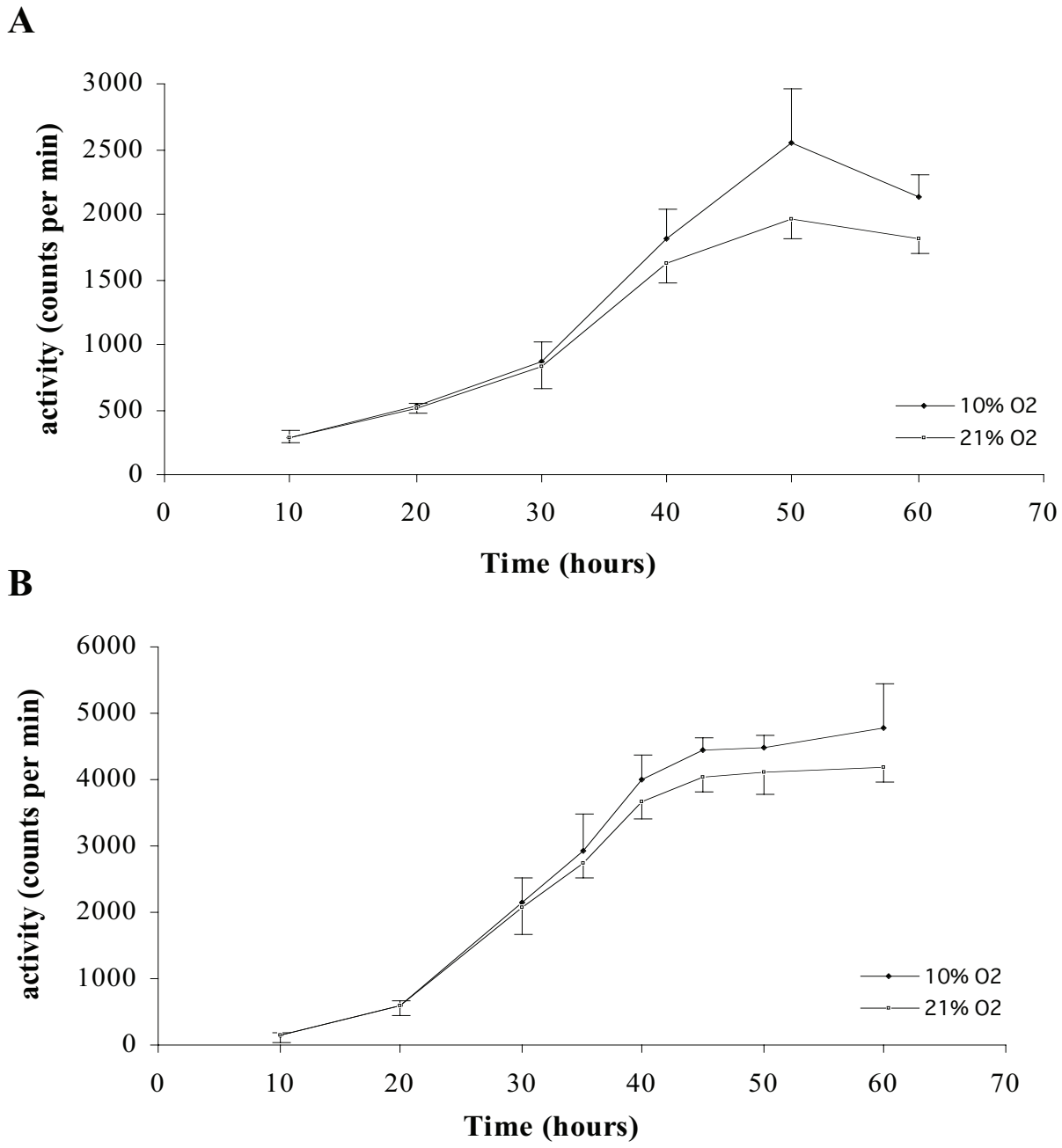

Figure 5
Tritiated hypoxanthine uptake of synchronous *Plasmodium falciparum* cultures under 10% O_2 and 21% O_2. Each point represents the mean ± standard deviation of three experiments. **A**: 3D7 clone. **B**: W2 clone.

Authors' contributions

SB contributed to the design and execution of the study, data analysis and prepared the first draft of the manuscript. PP contributed to the data collect and drafting of the manuscript. TF contributed to the design of the study and drafting of the manuscript. MMF contributed to the execution of the study and writing of the manuscript. EB contributed to the execution of the study. JM contributed

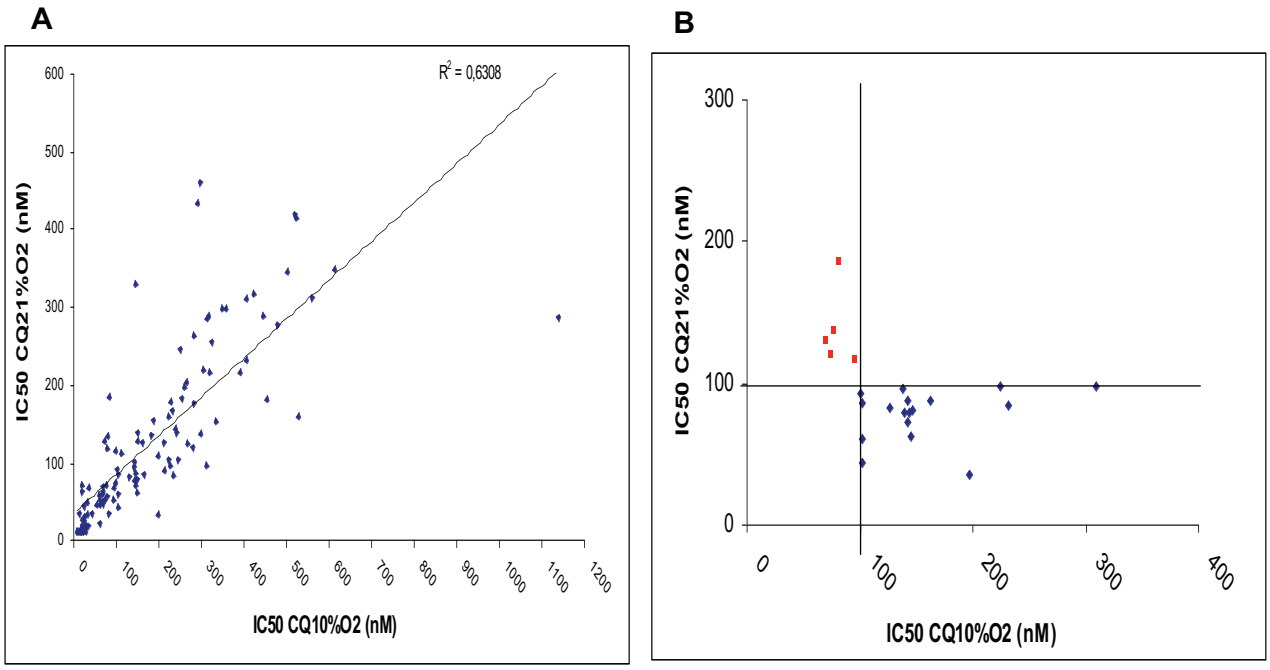

Figure 6
Chemosusceptibility of *Plasmodium falciparum* isolates to chloroquine under 10% O_2 and 21% O_2. **A**: chloroquine IC_{50} of 120 isolates. **B**: red plots corresponds to isolates with a switch from susceptible to chloroquine at 10% O_2 to resistant at 21% O_2; blue plots corresponds to isolates with a switch from resistant at 10% O_2 to susceptible at 21% O_2.

to the execution of the study. JPD contributed to the data collect and the intellectual content of the manuscript. DP contributed to the study design. PM contributed to the data collect and the intellectual content of the manuscript. CR contributed to the study design as well as data analysis and writing of the manuscript. BP contributed to the study design, data analysis and writing of the manuscript.

Disclaimer

The views and opinions are those of the authors and do not purport to represent those of the French Ministry of Defense.

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Conclusion et Perspectives :

L'hyperoxie induit un retard de cycle par augmentation de la durée de la schizogonie. Mais ces modifications phénotypiques s'accompagnent-elles de perturbations transcriptomiques et protéomiques de *P. falciparum* ?

RÉSULTATS

ARTICLE 2

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Global response of Plasmodium falciparum to hyperoxia : a combined transcriptomic and proteomic approach

Malaria Journal 2010, in press

"Global response of Plasmodium falciparum to hyperoxia : a combined transcriptomic and proteomic approach"

Problématique :

Quel est l'effet *in vitro* de l'hyperoxie sur le transcriptome et le protéome de *P. falciparum* afin d'identifier les voies métaboliques impliquées dans la réponse au stress oxydatif ?

Comme nous l'avons évoqué dans l'introduction, *P. falciparum* est soumis à différentes conditions environnementales, particulièrement à des pressions partielles d'oxygène qui fluctuent chez les différents hôtes.

Afin d'étudier l'adaptation de *P. falciparum* à 21% O_2, une approche combinée utilisant des techniques de transcriptome et de protéome a été réalisée. Dans ces conditions d'hyperoxie, des variations significatives transcriptomiques et protéomiques ont été identifiées au stade trophozoïte, expliquant ainsi les modifications phénotypiques observées au stade schizonte.

Ces analyses différentielles sont décrites dans l'article suivant.

Global response of *Plasmodium falciparum* to hyperoxia: a combined transcriptomic and proteomic approach

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Abstract

Background

Over its life cycle, the *Plasmodium falciparum* parasite is exposed to different environmental conditions, particularly to variations in O_2 pressure. For example, the parasite circulates in human venous blood at 5% O_2 pressure and in arterial blood, particularly in the lungs, at 13% O_2 pressure. Moreover, the parasite is exposed to 21% O_2 levels in the salivary glands of mosquitoes.

Methods

To study the metabolic adaptation of *P. falciparum* to different oxygen pressures during the intraerythrocytic cycle, a combined approach using transcriptomic and proteomic techniques was undertaken.

Results

Even though hyperoxia lengthens the parasitic cycle, significant transcriptional changes were detected in hyperoxic conditions in the late-ring stage. Using PS 6.0^{TM} software (Ariadne Genomics) for microarray analysis, this study demonstrate up-expression of genes involved in antioxidant systems and down-expression of genes involved in the digestive vacuole metabolism and the glycolysis in favour of mitochondrial respiration. Proteomic analysis revealed increased levels of heat shock proteins, and decreased levels of glycolytic enzymes. Some of this regulation reflected post-transcriptional modifications during the hyperoxia response.

Conclusions

These results seem to indicate that hyperoxia activates antioxidant defence systems in parasites to preserve the integrity of its cellular structures. Moreover, environmental constraints seem to induce an energetic metabolism adaptation of *P. falciparum*. This

study provides a better understanding of the adaptive capabilities of *P. falciparum* to environmental changes and may lead to the development of novel therapeutic targets.

Background

Plasmodium falciparum is a protozoan parasite responsible for the most severe form of human malaria. This infection causes between 708,000 and 1,003,000 human deaths each year, most of them occurring in African children under the age of five years [1]. Several anti-malarial agents are used for malaria treatment and prophylaxis in endemic regions. However, the expansion of drug-resistance remains a serious problem. To develop new anti-malarial drugs, a better understanding of *P. falciparum* biology is required [2]. Some unique properties of the *P. falciparum* mitochondrion indicate that its respiratory metabolism could be exploited to generate chemotherapeutic targets [3]. Indeed, atovaquone, a mitochondrial cytochrome *bc1* complex inhibitor, is currently used in combination with proguanil (Malarone™, GlaxoSmithKline) for malaria treatment and prophylaxis [4,5].

In vitro, *P. falciparum* growth is maximal in limited oxygen content (0.5%-5.0% O_2), so the parasite is considered as a microaerophilic organism. *In vivo*, the parasite life cycle involves two hosts: the mosquito and the human, during which the parasite undergoes different cellular morphological changes and experiences oxygen pressure variations. The passage from one host to another implies metabolic adaptation and changes in the ultrastructural and physiological organization of mitochondria [6-8]. In humans, the parasite is exposed to varying oxygen pressures, which can reach up to 13% O_2 in lung capillaries [9]. In mosquitoes, the parasite is exposed to 21% O_2 levels in salivary glands. To adapt to these environmental constraints, the parasite has developed metabolic adaptations essential for it survival. During the intraerythrocytic cycle, two metabolic pathways are the major sources of ROS (superoxide anions, hydrogen peroxide and hydroxyl radicals) on *P. falciparum* - the mitochondrial respiratory chain and

haemoglobin digestion [10]. In the presence of oxygen, *P. falciparum* can produce ATP by aerobic respiration and through glycolysis. Accordingly, microaerophilic metabolism may be a metabolic adaptation to prevent oxidative stress generation [10,11]. The parasite also consumes haemoglobin in its digestive vacuole for protein biosynthesis. This metabolic pathway is a source of superoxide anions and ferriprotoporphyrin IX (FIX) accumulation [12], and thus ROS produced in the mitochondria could interact with these products of haemoglobin digestion and increase oxidative damage to the parasite cells. Therefore, *P. falciparum* has developed a preventive defence system to reduce cellular damage.

To identify metabolic pathways involved in the hyperoxia response, the effect of oxygen on *P. falciparum* was studied using high-throughput transcriptomic and proteomic analyses in the late-ring stage. These approaches were designed to minimize non-specific responses [13], and they revealed that a stress response occurs following parasite exposure to hyperoxia and that *P. falciparum* modifies the metabolism of two organelles (the mitochondrion and the digestive vacuole) as a metabolic adaptation to this environmental challenge.

Methods

Plasmodium falciparum in vitro culture

The 3D7 *P. falciparum* strain (the reference strain used for the genome sequencing project) was obtained from the Malaria Research and Reference Reagent source centre (MR4, managed by the American type culture collection). Parasitized human red blood cells (RBC type A+) were maintained in culture in RPMI 1640 medium (Invitrogen,

Paisley, United Kingdom) supplemented with 10% human serum and buffered with 25 mM HEPES (Sigma-Aldrich, St Louis, MI, USA) and 25 mM $NaCO_3$ (Sigma) and in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 [14]. The haematocrit was maintained at 6% and the parasitaemia at 3-5%. Culture medium was changed every day. Strain clonality was verified every month using PCR genotyping of polymorphic genetic markers (*msp1*, *msp2* and microsatellite loci) [15,16].

Plasmodium falciparum culture synchronization

To obtain tightly synchronized parasite cultures, several synchronization steps were successively employed. First, parasitized erythrocytes were treated with D-sorbitol (ICN Biomedicals, Inc., CA, United States of America) as previously described [17]. This step enriched cultures in the ring parasite stage. Secondly, in the next parasitic cycle, schizonts were selected using CS columns on a VarioMACS unit (Miltenyi Biotec, Germany) according to standard procedures [18]. Five hours later, the culture was treated with 5% D-sorbitol to eliminate parasites in mature stages (schizonts). These successive parasite synchronization steps allowed us to obtain parasites tightly synchronized in the ring stage (the window from 0 to 5 h after parasite invasion of the erythrocyte). The synchronized parasites were maintained in standard culture conditions before treatment.

Hyperoxia exposure of *P. falciparum* cultures

Tightly synchronized cultures (ring stage aged between 4-9 hours) were split and subjected to two different conditions, either a normoxic atmosphere (5% O_2, 5% CO_2, 90% N_2 gas mixture) or a hyperoxic atmosphere (21% O_2, 5% CO_2, 74% N_2 gas mixture) at 37°C in two series II incubators (Model 3131, Forma Scientific, Inc.). Culture conditions were maintained over eight hours for transcript analysis (parasites aged

between 12-17 hours) and for 12 hours for protein analysis (parasites aged between 16-21 hours). For each culture condition, four biological replicates were performed. Viability, parasitaemia and erythrocytic cycle stage proportions were monitored daily by examining blood smears stained with RAL® 555 (RAL, Martillac, France). Blood smears were taken at 0, 24, 32, 36, 48, and 78 hours.

RNA extraction

After incubation under normoxic or hyperoxic atmospheric conditions, total RNA from parasitized erythrocytes was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA extracts were treated with 1 U of RNase-free DNase I (Applied Biosystems, CA, United States of America) and quantified using a NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, United States of America). The integrity of the RNA was controlled with an RNA nano chip (2100 Bioanalyzer, Agilent Biotechnologies, Wilmington, DE). Samples were immediately used or stored at - 80°C.

Microarray experiments and analysis

A 2X11k custom *P. falciparum* whole genome microarray was designed and manufactured using SurePrint Inkjet technology® (Agilent Technologies). In brief, the microarray was composed of 10,128 sixty-mer oligonucleotides representing 5,364 coding sequences located in the chromosomal, apicoplastic and mitochondrial genomes. Additional probes were added to control for quality. Labelling and hybridization was carried out following the manufacturer's protocol (Two-Color Microarray-Based Gene Expression Analysis, Agilent Technologies). Briefly, starting with 300 ng of total RNA, fluorescent cRNA (antisense) was generated using the Low RNA Input Fluorescent Amplification Kit (Agilent Technologies) and either cyanine 3-labeled CTP (Cy-3) or

cyanine 5-labeled CTP (Cy-5) fluorescent dyes (PerkinElmer Life Sciences, Boston, MA). Dye swap hybridization was performed for 17 h at 60°C using the *In situ* Hybridization Kit Plus (Agilent Technologies). All processing steps were performed in an ozone-controlled environment ($[O_3]$ < 2 ppb) to avoid ozone-induced degradation of cyanine dyes on microarray slides. Slides were scanned at 5µm resolution with a G2505B DNA microarray scanner (Agilent Technologies). Image analysis and intra-array signal correction was performed using the Agilent Feature Extractor Software A.9.1.3. Data processing, analysis and visualization were performed using the Resolver software 7.1 (Rosetta Inpharmatics). An error model-based transformation pipeline was used to map replicate reporters to genes, perform inter-array normalization and calculate fold changes (FC) as described elsewhere [19]. Using these FC values, a gene set enrichment analysis (GSEA) was performed using the Mann-Whitney-U-test enrichment algorithm in the PathwayStudio software 6.0 (PS 6.0^{TM}, Ariadne Genomics). The GSEA procedure determines whether the behaviour of an *a priori* set of genes shows significant concordance across two different biological states. This GSEA analysis focuses on groups of genes that share common biological function in revealing differential levels of each transcript. A gene network was generated based on information extracted from the literature using Medscan^{TM} and the *P. falciparum*-specific database PS 6.0^{TM} and using the "physical or regulatory connections" parameter between genes. Fold change values of microarray data were imported into PS 6.0^{TM} and used to interpret the pathway with gene regulation networks.

Real-time quantitative RT-PCR

cDNA was synthesized from total RNA (DNA-free) with random hexamers using the High-Capacity cDNA Archive Kit (Applied Biosystems). Primers with a melting

temperature between 55 to 60°C were designed to yield a 94- to 146-bp product preferentially containing an exon/intron boundary. Specific primer sequences used for the qRT-PCR are summarized in Additional data (Additional file 1). Real-time PCR was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems) in a 25-µL reaction volume with the Power SYBR Green® PCR Master Mix Kit (Applied Biosystems). Each sample was assayed in triplicate and analysed with the ABI PRISM Sequence Detection System software Version SDS 2.2.1 (Applied Biosystems). Amplification of the *18S rRNA* sequence served as the internal control for normalization. At the end of each reaction, cycle threshold (Ct) was manually set to the level that reflected the best kinetic PCR parameters, and melting curves were acquired and analysed. Relative quantification analysis was performed using the 2-•• Ct method where •• Ct = $(Ct_{target} - Ct_{18S\ rRNA})_H$- $(Ct_{target} - Ct_{18S\ rRNA})_N$., and the data reflect changes in target gene expression between two experimental conditions (N: normoxic and H: hyperoxic groups) [20].

Protein sample preparation and CyDye labelling

After incubation under normoxic or hyperoxic atmospheric conditions, parasitized erythrocytes were washed three times in PBS medium (Invitrogen) and lysed in cold H_2O-saponin (0.1%, Sigma) for 10 min. The lysate was then centrifuged at 1500 g for 5 min. The supernatant was discarded and the pellet containing free parasites was recovered by washing in PBS medium followed by a centrifugation step (1500 g for 5 min). The free parasites were washed until the supernatant became colourless. The pellet was then suspended in 4% (w/v) CHAPS (Sigma) and disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific, Illkirch, France) five times for 60 seconds on ice at maximum amplitude. The lysate was then centrifuged at 16000 g for 15 min. The supernatant was

further precipitated with acetone 100% (Sigma). The protein concentration for each sample was estimated using the BioRad Lowry-based DC assay (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. Total proteins were suspended in standard cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 (Sigma)) to obtain a protein concentration adjusted to 2.5 µg/µL. Protein samples were minimally labelled with CyDye according to the manufacturer's protocols (GE Healthcare, Piscataway, NJ) [21]. The mixture of labelled proteins was then separated by two-dimensional (2D) electrophoresis.

Two-dimensional electrophoresis, image analysis and in-gel digestion

Isoelectric focusing (IEF) was performed on 18-cm 3-10 linear IPG strips (GE Healthcare). Destreak buffer containing 1% (v/v) IPG buffer 3-10 was used for overnight rehydration of IPG strips. The samples were applied at the acidic end of the IPG strip using a cup-loading technique. IEF was carried out on a Ettan IPGphor II (GE Healthcare) electrophoresis unit at 20°C for a total of 45 kVh (ramp to 300 V in 3 hrs, ramp to 1000 V in 6 hrs, ramp to 8000 V in 3 hrs, hold at 8000 V for 4 hours). IPG strips were equilibrated in equilibration buffer containing 50 mM Tris-HCl, pH 8.6, 6 M urea, and 2% SDS and 30% glycerol supplemented with 1% (w/v) DTT for 15 min at room temperature, followed by protein alkylation (carbamidomethylation) in the same equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 15 min at room temperature. IPG strips were then placed on the top of 10% uniform polyacrylamide gels. Strips were overlaid with 0.5% agarose in 1x running buffer containing bromophenol blue and the proteins were further separated by SDS-PAGE (10 W per gel) at 20°C in an Ettan DALT Six electrophoresis system (GE Healthcare). After electrophoresis, the gels with Cydye-labelled proteins were directly imaged using a

Typhoon™ Trio Image scanner (GE Healthcare UK). The intensity was adjusted to ensure that the maximum volume of each image was within 60,000 - 80,000 U. Analysis of 2-D DIGE was performed with DeCyder 6.5 software (GE Healthcare) using the differential in-gel analysis (DIA) and the biological variation analysis (BVA) modules. Protein spots that were expressed differentially between two experimental conditions (|ratio|• 1.5, *p*• 0.05 *t-Test*) were marked with master gel numbers. Based on DeCyder v6.5 analysis, spots of interest from gels stained with Imperial Blue Stain (Pierce) were excised and digested using a Shimadzu Xcise automated gel processing platform (Shimadzu Biotech, Kyoto, Japan) as described previously [22] and stored at -20°C.

Mass spectrometry analysis

The samples were analysed by nanoscale capillary liquid chromatography-tandem mass spectrometry (nano LC-MS/MS). Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima, Waters, MA). Chromatographic separation was conducted on a reversed-phased capillary column (Atlantis™ dC18, 3 µm, 75 µm x 150 mm Nano Ease™, Waters, MA) with a 180-200 nl min^{-1} flow. The gradient profile consisted of a linear gradient from 95% A (H_2O, 0.1% HCOOH) to 60% B (80% ACN, 0.1% HCOOH) in 60 min followed by a linear gradient to 95% B in 10 min. Mass data acquisitions were piloted by MassLynx 4.0 software using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electro-spray capillary voltage was set to 3.2 kV, the cone voltage was set to 30 V, and the source temperature was set to 80°C. The MS survey scan was m/z 400-1300 with a scan time of 1 s and an interscan time of 0.1 s. When the intensity of a peak rose above a threshold of 15 counts, tandem

mass spectra were acquired. Normalized collision energies for peptide fragmentation were set using the charge-state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 s and an interscan time of 0.1 s. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimized for various mass ranges and charges of precursor ions. Mass data collected during a nano LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay and no deisotoping to generate peak lists in the micromass pkl format. Pkl files were then fed into the local search engine Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were searched against the *Homo sapiens* (218356 sequences) and *P. falciparum* (13110 sequences) National Center for Biotechnology Information non-redundant (NCBInr) protein database (March, 2010). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was <0.2 Da. All identified proteins had a Mascot score greater than 29 and 38 respectively for *P. falciparum* and *Homo sapiens*, corresponding to statistically significant ($p \bullet 0.05$ *t-Test*) identification. Identifications were considered valid when they contained at least two peptide sequences per protein. If a single peptide sequence was identified per one protein, the mascot score and sequence coverage were taken into account (Additional file 2).

Results

Effect of hyperoxia on *P. falciparum* and experimental design

To determine the effects of hyperoxia on asexual blood stage *P. falciparum* parasites, tightly synchronized cultures of the 3D7 strain were exposed to normoxic (5% O_2) and hyperoxic (21% O_2) conditions for two life cycles. The proportion of parasitaemia and erythrocytic cycle stages were monitored by blood smears in triplicate at different times: 0, 24, 32, 36, 48, and 78 hours (Figure 1). In normoxic condition, the 3D7 *P. falciparum* strain had a life cycle of 45 hours with entry into schizogony at approximately 32 hours. Parasite exposure to 21% O2 increased the length of parasitic cycle and decreased the parasitaemia, but it did not alter the parasites morphology (Figure 1). At 48 hours, the second parasitic cycle began for parasites exposed to 5% O_2 (nearly all of them were at ring stage (100%)), while the majority of parasites exposed to 21% O_2 remained in the schizont stage (90% schizonts and 10% ring). Thus, hyperoxia induces a delay of *P. falciparum* cell cycle of four hours as previously described [23]. After reinvasion during the following cycle (third cycle), hyperoxia exposure did not change parasitaemia and parasites had a normal life cycle without excess lethality (unpublished data). These results seem to indicate a biological adaptation of the parasite to hyperoxia. To study the effects of hyperoxia on *P. falciparum*, complementary high-throughput transcriptomic and proteomic approaches were used. Transcriptome and proteome profiles from parasitized RBCs exposed to normoxic (5% O_2) or hyperoxic (21% O_2) atmospheric conditions were compared. The results were controlled by the experimental design on two levels: (i) the percentage of atmospheric oxygen and (ii) the timing of the parasitic stage. First, hyperoxic exposure was chosen at 21% O_2 in light of the above data [23]. Second, transcriptome and proteome experiments were performed at the late-ring stage, after RBC

reinvasion with a synchronization window of four hours. This stage allowed us to avoid the effects of cycle delay.

Plasmodium falciparum **response to hyperoxia treatment: microarray analysis**

To investigate the response of *P. falciparum* to hyperoxia, the 3D7 strain was cultured *in vitro* under normoxic (5% O_2) and hyperoxic (21% O_2) atmospheric conditions. Three biological replicates in each group were performed and comparisons were made with a dye-swap experimental scheme. The raw microarray data are available in the Gene Expression Omnibus database [platform GPL9482 and samples from GSM466802 to GSM466807, 2010 [24]]. Among 5,364 coding sequences represented on the microarray, 219 genes were significantly altered following hyperoxia exposure (p• 0.01, Student's *t-Test*, |FC|• 1.5, Additional file 3), among which 114 were up-expressed and 105 were down-expressed. Based on the selected 219 genes, a GSEA (FDR • 0.05**)** was performed [25], and this allowed us to define 9 functional groups that were significantly altered (following hyperoxia treatment) (Additional file 4 and Table1). Among these functions, "DNA repair," "Vacuolar acidification" and "Response to oxidative stress" were previously reported to be involved in the hyperoxia response [10,26-30].

GSEA data were first integrated to create a gene network based on information extracted from the literature using Medscan™ and PS 6.0™ (Ariadne Genomics). Next, the expression levels of genes included in the gene network were assigned using PS 6.0™ and microarray expression data. This programme gives a dynamic view of metabolism during the hyperoxia response (Figures 2A and 2B). Thus, PS 6.0 analysis clustered 28 modulated-expression genes in five metabolic groups labelled "Energetic metabolism," "Protein folding," "Signal Transduction," "DNA repair" and "Translation" (Figure 2A).

This transcriptomic analysis allowed us to identify up-regulated genes involved in DNA repair and protein folding and down-regulated genes linked to PKA-dependent signal transduction and glycolysis (Figure 2A). Additionally, PS 6.0 software revealed an alteration of an ATP-dependent sub-network: specifically, up-regulation of the mitochondrial ATP synthase complex and down-regulation of the V-type ATPase complex (Figure 2B).

To confirm the GSEA data, eight genes presenting significant variations in expression were quantified using real-time qRT-PCR (Additional file 1). These genes, which were involved in glycolysis [PlasmoDB: *PF14_0598, PF10_0245*], antioxidant metabolism [PlasmoDB: *PF14_0187, PF11_0087*], signal transduction [PlasmoDB: *PFL1110c, PFI1685w*] and ATP synthase activity [PlasmoDB: *PF10_0059, MAL7P1.13*] were chosen according to their essential functions in response to hyperoxia. qRT-PCR was performed on total RNA extracted from the same three samples used for microarray analysis. Correlation coefficients for each specific-gene standard curve were always > 0.99 (unpublished data). Gene amplification was normalized by *18S rRNA* [PlasmoDB: *MAL7_18Sa*] levels as previously described [31]. The two analyses (qRT-PCR and microarray) yielded consistent results for all the genes evaluated (Figure 3).

Plasmodium falciparum response to hyperoxia treatment: proteomic analysis

To identify *P. falciparum* proteins involved in the hyperoxia response, 2D-DIGE experiments coupled to MS were performed. Four independent cultures of *P. falciparum* cultivated under normoxic and hyperoxic conditions were included in this analysis. After protein separation on 2-DE, each gel was individually imaged and all gel images were analysed using the DeCyder 6.5 software. Among 1840 protein spots matched, 33 spots

were significantly modulated (|FC|≥1.5, $p \bullet 0.05$ *t-Test*) following hyperoxia treatment (14 and 19 spots were up- and down-modulated, respectively; Figure 4). All spots were successfully identified by MS and corresponded to 14 *Homo sapiens* and 19 *P. falciparum* proteins (Table 2 and Additional file 5). However, some proteins were detected in more than one spot, indicating different isoforms. Indeed, only six proteins were identified for *Homo sapiens* and 13 for *P. falciparum* (Table 2). These results indicated that hyperoxia induced protein modulations at two levels: namely, protein expression and post-translational modification. Among the four spots detected (spot numbers 1301, 1314, 1326, and 1331) as Pf-Hsp70 protein [PlasmoDB: PF08_0054, GenBank: gi|124512406], only two isoforms were significantly up-regulated in hyperoxic conditions (Table 2 and Figure 5). These results indicated that the hyperoxia response could induce post-translational regulation of several parasite proteins.

To determine the metabolic pathways perturbed following hyperoxia, the identified proteins were classified using the NCBI COG database. The proportion of modulated proteins involved in each functional category was determined as follows. For *P. falciparum*, six functional categories were found to be altered, among which were chaperone-assisted protein folding, translation, antioxidant metabolism and glycolysis, which were already identified in transcriptomic analysis. For *Homo sapiens*, the identified proteins were classified into three functional categories: antioxidant metabolism, glycolysis and O_2 transporter (Table 2).

The 2D-DIGE analyses also indicated the accumulation of some proteins involved in digestive vacuole metabolism such as human catalase [GenBank: gi|4557014] and beta-globin [GenBank: gi|183817]. The accumulation of beta-globin suggests proteases

inhibition of the beta-globin degradation pathway. To explore this hypothesis, the transcripts of four genes [PlasmoDB: *PF14_0077, PF11_0161, PF11_0165, PF11_0162*] were quantified using real-time qRT-PCR as described above (Additional file 1). All genes involved in digestive vacuole metabolism were found to be down-expressed (Figure 6).

Discussion

In vivo, the *P. falciparum* parasite is subjected to varying oxygen levels throughout its life cycle (*i.e.*, from 5% O_2 in human venous blood to 13% O_2 in the human lungs and 21% O_2 in mosquito salivary glands). In the mosquito, the metabolic adaptation of parasite to oxygen-rich environment involved mitochondrial and physiological differences [32]. These oxygen variations imply that metabolic adaptation of *P. falciparum* is crucial for it survival. During malaria complications such as acute respiratory distress syndrome, late-ring stage parasites are susceptible to sequestration in pulmonary capillaries and are thus exposed to hyperoxic conditions [33]. These sequestered young parasites could be exposed to higher oxygen levels than the physiologically relevant O_2 tension. Additionally, Blanco *et al* reported that hyperbaric oxygen therapy (HBO, 100% O_2) has a beneficial effect on malaria syndrome evolution [34]. Indeed, a better understanding of the metabolic adaptation of the malaria parasite to hyperoxia could help to develop new anti-malarial drug treatments that could be used in association with HBO treatment. To study the global response of *P. falciparum* to hyperoxia, a dual high-throughput approach combining microarray and 2D-DIGE analysis was used on parasite cultures under hyperoxic conditions (*e.g.*, 21% O_2).

Accordingly Hsp90, Hsp70, GAPDH, and elongation factor 1 and 2 were found to be altered at the transcript and protein levels under hyperoxia.

Since the development of high-throughput technologies, few studies have been published regarding the transcriptome and proteome of *P. falciparum* in response to environmental constraints or drug treatments [35-40]. Since hyperoxia induces a *P. falciparum* cycle delay, the sample collections were performed before the phenotypic effect. Although the majority of genes had a periodic expression profile [41], RNA transcription is maximal between 18 and 24 hours of the parasitic cycle [42]. In the present study, sample collections were performed at the late-ring parasite stage. Moreover, as a delay exists between mRNA and protein accumulation [43-45], the time of exposure was also taken into account and a four hours delay was chosen between mRNA and protein sampling. It has been suggested that there may be a discrepancy between *P. falciparum* transcriptomic and proteomic responses [35,39]. Preliminary microarray experiments were performed with transcripts from synchronized *P. falciparum* in the same experimental scheme, but the samples were collected after four hours of hyperoxia treatment. These microarray analyses indicated that 176 transcripts were significantly deregulated (|FC|• 1.5, p• 0.01, Student's *t-Test*), and some genes significantly deregulated were involved in the early antioxidant response, such as 1-cys peroxidoxin [PlasmoDB: PF08_0131], Fe-superoxide dismutase [PlasmoDB: PF08_0071] and thioredoxin peroxidase [PlasmoDB: MAL7P1.159] (Additional file 6). This early stress response is generally observed in stress condition does not reflect a specific hyperoxia adaptation [30,46]. Here, despite significant transcript variations (p• 0.01, Student's *t-Test*), gene fold-changes observed were low (|FC|• 1.5) under hyperoxia. The low-level changes observed in the *P. falciparum* transcriptome could be explained by tight gene regulation [47,48] or by post-transcriptional regulation of most *P. falciparum* genes [43-45]. Consequently, analysis of

the parasite's adaptive response to hyperoxia requires the use of extremely successful bioinformatic tools for microarray data interpretation such as PS 6.0 software [44,49], and this analysis was completed using a highly sensitive proteomic approach such as 2D-DIGE.

It is generally accepted that one of the first effects of hyperoxia is ROS overproduction (superoxide anions (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-)), which is generated by metabolism, and particularly, by respiratory metabolism [50]. DNA, lipid, and protein alterations by ROS may be lethal to malaria parasites. Thus, to fight oxidative stress, *P. falciparum* has developed an adaptive defence response including repair mechanisms for nucleic acids and proteins [29,51]. Despite high ROS defence system expression under normoxia, transcriptomic analysis suggests an up-regulation of ROS defence systems [29]. Five genes involved in DNA repair were found to be up-regulated during hyperoxia. Among them, two sub-unities of *DNA primase* [PlasmoDB: *PF14_0366* and *PFI0530c*] and *replication protein A1* [PlasmoDB: *rpa1*, *PFI0235w*] have been described to be involved in chromosomal replication [52,53]. *Rpa1* was reported also to interact with *rad51* [PlasmoDB: *PF11_0087*] in nucleosomes during replication to correct DNA mismatches [51,54]. And *Hsp40* [PlasmoDB: DNAJ homologue, *PFF1415c*] is associated with DNA repair and the replication machinery [55]. These observations suggest that DNA repair enzymes maintain the integrity of the parasitic genome under high oxygen pressure.

Protein oxidation caused by ROS is circumvented by diverse functions such as regulation of the redox state and modulation of protein stability [56]. Chaperone proteins, Hsps known as stress response proteins, further assure this protection. Several proteins

involved in chaperone activity including Hsp40 [PlasmoDB: DNAJ homologue; *PFF1415c*], Hsp60 [PlasmoDB: *PF10_0153*], Hsp70 [PlasmoDB: *PF11_0351* and PF08_0054], Hsp90 [PlasmoDB: *PFL1070c* and PF07_0029], and protein 14-3-3 [PlasmoDB: MAL8P1.69] were found up-regulated under hyperoxia. As described by Akide-Ndunge *et al* [57], *Hsp60* is up-regulated under oxidative stress like hyperoxia and its expression is coordinated with antioxidant enzymes in a stage-dependent manner, suggesting thus that Hsp up-regulation is implicated in ROS removal. Elsewhere, Pf-Hsp70 forms a functional network in the mitochondrial matrix with DNAJ, Hsp60 and prohibitin to be involved in post-translational modification of proteins [56]. Isoforms of Hsp70 were detected following hyperoxia exposure, which may also correspond to post-translational modification as previously predicted [58,59]. These Hsps, which act as sensors of environmental conditions, are involved in adaptation mechanism by post-translational modification [56]. However, the role of these post-translational modifications on regulation of protein expression in *P. falciparum* is little known [38,39]. Nevertheless, the chaperone activity of Hsps seems regulate during the hyperoxia response and facilitate *P. falciparum* adaptation to hyperoxic environments.

Under hyperoxia, down-regulation of glycolytic enzymes (glucose-6-phosphate isomerase [PlasmoDB: PF14_0341] and glyceraldehyde-3-phosphate dehydrogenase [PlasmoDB: GAPDH, PF14_0598]) was detected in this study. Additionally, three enzyme involved in *de novo* pyrimidine biosynthesis were found up-regulated such as carbonic anhydrase [GenBank: gi|4502517], *carbamoyl-phosphate synthetase* [PlasmoDB: *PF13_0044*] and *aspartate carbamoyltransferase* [PlasmoDB: *PF13_0240*] [60]. This last, up-expressed in microarray analysis, produces dihydroorotate oxidase (DHO), an essential substrate of mitochondrial respiratory chain complex II [61]. This pyrimidine pathway is essential for

nucleic acid synthesis to repair DNA lesions caused by the oxidative stress. Microarray data indicated that *alpha and epsilon ATP synthase F1 subunits* [PlasmoDB: *PFB0795w* and *MAL7P1.75*] were up-regulated, indicating that parasites seem to produce ATP through mitochondrial respiration. Mitochondrial F_0F_1 ATP synthase is composed of two subcomplexes, F_0 and F_1. F_1 is composed of five subunits, and these have been reported in the *P. falciparum* genome [62]. Recently, Mogi and Kita have identified four F_0 subunits of *P. falciparum* ATP synthase [63,64] and Mather *et al.* support the existence of ATP synthase activity [65]. Two transcripts of ATP synthase F_0 subcomplexe, *a* subunit (ATP6) and *b* subunit (ATP4), were up-regulated under hyperoxia exposure, which is in favour of a mitochondrial respiration. To adapt to hyperoxia, *P. falciparum* seems switch from anaerobic glycolysis to aerobic respiratory metabolism (Figure 7).

In addition to mitochondrial metabolism, the digestive vacuole is another ROS source organelle in *P. falciparum* during haemoglobin digestion [10]. Haemoglobin digestion is optimal at an acidic pH, which is also necessary for protease activity. To provide the acidic environment in the digestive vacuole, haemoglobin digestion stimulates ATP consumption by the V-type H^+-ATPase pump [66-68]. In microarray analysis, the V-type ATPase complex [PlasmoDB: *MAL13P1.271, PF13_0034, PFE0965c, PF11_0412, PF13_0227, PF13_0065*], a membrane transporter, was found to be down-regulated under hyperoxia. Therefore, *P. falciparum* exposed to hyperoxia could generate a pH change in the digestive vacuole responsible for vacuolar protease activity. Additionally, beta-globin accumulation occurred in hyperoxic conditions. This beta-globin accumulation could result from a decline in protease activity. To test these two hypotheses, transcripts of the proteases involved in beta-globin degradation were quantified. Reduced expression of four genes involved in haemoglobin degradation into

AAs (*plasmepsin 2* [PlasmoDB: *PF14_0077*], *falcipain 2 putative* [PlasmoDB: *PF11_0161*], *falcipain 2 precursor* [PlasmoDB: *PF11_0165*] and *falcipain 3* [PlasmoDB: *PF11_0162*]) was validated using qRT-PCR. In 2002, Oliveira *et al* hypothesized that blood-feeding parasites reduced their mitochondrial function to compensate for ROS generation from the digestive vacuole [10].

Moreover, catalase [GenBank: gi|4557014], a human protein, was found concentrated in the digestive vacuole [69]. As the *P. falciparum* genome does not contain a catalase gene, the parasite may import human catalase to detoxify H_2O_2 generated by oxidation of haem under stress conditions. Collectively, metabolism in the *P. falciparum* digestive vacuole would be perturbed in response to hyperoxia, and ROS production would be slowed (Figure 7).

Conclusions

Two complementary analytic approaches were used to investigate the response of *P. falciparum* to hyperoxia; (i) a transcriptomic study allowed us to detect whole parasite transcripts, and (ii) a proteomic study identified proteins significantly altered via post-translational modifications and accumulated host proteins. Based on all these results and according to published data mining [29,46], a schematic representation of the adaptive response of *P. falciparum* following hyperoxia exposure was proposed (Figure 7). In order to prove this representation, further biochemical approaches would be required.

Hyperoxia exposure induces metabolic adaptations in *P. falciparum*. These adaptations seem to involve, at least, two parasite organelles, the digestive vacuole and the

mitochondrion, both sources of ROS production. To preserve parasite integrity from oxidative stress, all these data suggest that the glycolysis pathway is suppressed in favour of respiratory metabolism and that digestive vacuole metabolism is slowed. Campanale *et al* demonstrated that stress caused by haemoglobin digestion modulates the glycolytic pathway [70]. Highly active mitochondria release H_2O_2, which interacts with pro-oxidant products (free iron and haem) in the digestive vacuole. These two ROS sources could be potentially synergistic. The equilibrium of oxidative stress is vital for the parasite; indeed, Hsps could be regulated to facilitate adaptation of parasite to environmental stress as observed in many organisms [56].

The knowledge of the metabolic pathways involved in stress responses to environmental conditions is fundamental to understanding the mechanisms of parasite adaptation. This study provides a starting point for investigations into new anti-malarial treatments, particularly drugs associated with hyperbaric oxygen therapy [34], which has been successfully used to treat other infections [71].

List of abbreviations:

2D-DIGE: 2D-differential-gel-electrophoresis; 2-DE: 2-Dimensional electrophoresis; AA: amino acid; ACN: acetonitrile; BVA: biological variance analysis; Ct: cycle threshold; CQ: chloroquine; Cy-2: cyanine 2; Cy-3: cyanine 3; Cy-5: cyanine 5; DHA: dihydroartemisinin; DHO: dihydroorotate; DHOD: dihydroorotate dehydrogenase; DIA: differential in-gel analysis; FC: fold change; FIX: ferriprotoporphyrin IX; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase; GSEA: Gene Set Enrichment Analysis; *gst*: glutathione S-transferase; H_2O_2: hydrogen peroxide; HBO: hyperbaric oxygen therapy; *hsp*: heat shock protein; IEF: isoelectric focusing; MS: mass spectrometry; Nano LC-MS/MS: nanoscale capillary liquid chromatography-tandem mass spectrometry; O^-_2: superoxide anions; OH^-: hydroxyl radicals; O_3: ozone; *P. falciparum: Plasmodium falciparum*; PMT: photomultiplier tube; NPPs: new permeation pathways; qRT-PCR: quantitative real time PCR; Q-TOF: quadrupole orthogonal acceleration time-of-flight; RBC: red blood cells; ROS: reactive oxygen species, *rpa1*: replication protein A1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MTM conceived the study and the design, carried out microarray and proteomic studies, participated in bioinformatics analyses and wrote the manuscript. LA carried out proteomic studies, conducted proteomic statistical analyses and revised the manuscript. JD helped in the design of molecular studies and revised the manuscript. YL participated in the microarray design and conducted statistical and bioinformatics analyses. MB, MP and PF carried out mass spectrometry identifications. YJ conceived the study and edited

the manuscript. DP initiated the project, designed the method, participated in the analyses and revised the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Lengthening of *P. falciparum* cell cycle following hyperoxia exposure. Phenotypic changes in *P. falciparum* asexual blood stages were observed during two cycles under normoxic (A) and hyperoxic (B) conditions. The parasitaemia and parasitic stages were evaluated by blood smears at different times: 0, 24, 32, 36, 48, and 78 hours. The different intraerythrocytic stages and their corresponding percentages are indicated as follows: ring (R), trophozoite (T) and schizont (S).

Figure 2. Gene networks involved in the metabolic adaptation to hyperoxia in tightly synchronized *P. falciparum* cultures. Using PS 6.0^{TM} software and microarray expression data, gene networks were built and genes modulated in response to hyperoxia are represented. (A) Representation of metabolic interrelations related to adaptative hyperoxia exposure. *Energetic metabolism: aspartate carbamoyltransferase* (P13_0240), *carbamoyl-phosphate synthetase* (PF13_0044), *glutamine-fructose-6-phosphate transaminase* (PF10_0245), *glucose-6-phosphate dehydrogenase* (PF14_0511), *acetyl-CoA synthetase* (PFF1350c), *gapdh* (PF14_0598) - *Signal transduction: regulatory sub-unit of cAMP-dependent protein kinase* (PFL1110c), *catalytic sub-unit of cAMP-dependent protein kinase* (PFI1685w), *ser thr protein kinase* (PF13_0085) - *Translation: citrate synthase* (PF10_0218), *translation elongation factor 1 alpha 1* (PF13_0305), *proteasome 26S subunit* (MAL13P1.343), *dihydrolipoamide dehydrogenase* (PFL1550w), *translation elongation factor 2* (PF14_0486), *adaptor-related protein complex 1* (PF13_0062), *polymerase RNA I* (PF11_0358) - *DNA repair: DNA primase* (PF14_0366 and PFI0530c), *rpa1* (PFI0235w) - *Protein folding: ferredoxin reductase* (PF11_0407), *Hsp10* (PFL0740c), *Hsp60* (PF10_0153), *Hsp70* (PF11_0351 and PF08_0054), *Hsp90* (PFL1070c and PF07_0029), *prohibitin* (PF08_0006), *DnaJ*

(PFF1415c). (B) Representation of ATP-dependent gene sub-networks altered in hyperoxic conditions. *V-type ATPase:V-type ATPase putative* (MAL13P1.271), *vacuolar ATP synthase subunit h putative* (PF13_0034), *vacuolar ATP synthetase putative* (PFE0965c), *vacuolar ATP synthase subunit F putative* (PF11_0412), *vacuolar ATP synthase subunit D putative* (PF13_0227), *vacuolar ATP synthase catalytic subunit a* (PF13_0065) - *Mitochondrial ATP synthase F1: ATP synthase subunit putative* (PF14_0615), *mitochondrial ATP synthase F1 epsilon subunit* (MAL7P1.75), *mitochondrial ATP synthase F1 alpha subunit putative* (PFB0795w). Red and blue colors correspond respectively to up- and down-regulated genes compared between hyperoxic to normoxic conditions.

Figure 3. Validation of microarray data by qRT-PCR. Data from qRT-PCR and a microarray of eight selected genes were compared between hyperoxic and normoxic conditions. Adjacent bars correspond to the mean $\log_2$ (fold change) and respective standard deviation and present qRT-PCR and microarray results in gray scale for the respective gene. The abbreviations and their corresponding gene IDs (PlasmoDB accession numbers) are indicated below the graphic. Crude values of mean fold changes are presented in the table. The same samples were used for the qRT-PCR and microarray experiments. *gapdh*: glyceraldehyde-3-phosphate dehydrogenase - *gf6p*: glutamine-fructose-6-phosphate transaminase - *gst*: glutathione S-transferase - *pka*: protein kinase A - *atp6*: mitochondrial ATP synthase F_0 a subunit – *atp4*: mitochondrial ATP synthase F_0 b subunit.

Figure 4. Alterations of the *P. falciparum* proteome under hyperoxic exposure. The proteins from *P. falciparum* parasites cultivated under normoxic (A) or hyperoxic (B)

conditions were labelled with Cy3 and Cy5, respectively, and separated by 2-DE using a 10% homogeneous SDS polyacrylamide gel with a pH range from 3 to 10. As determined by DeCyder software, protein spots that were down- (A) or up- (B) regulated following hyperoxic exposure (|FC| 1.5, p 0.05) are marked with master numbers (Table 2 and Additional file 5). Bold and italic numbers correspond, respectively, to proteins identified from *P. falciparum* and *Homo sapiens*. Areas of gels containing a high density of spots down- and up-modulated are enlarged.

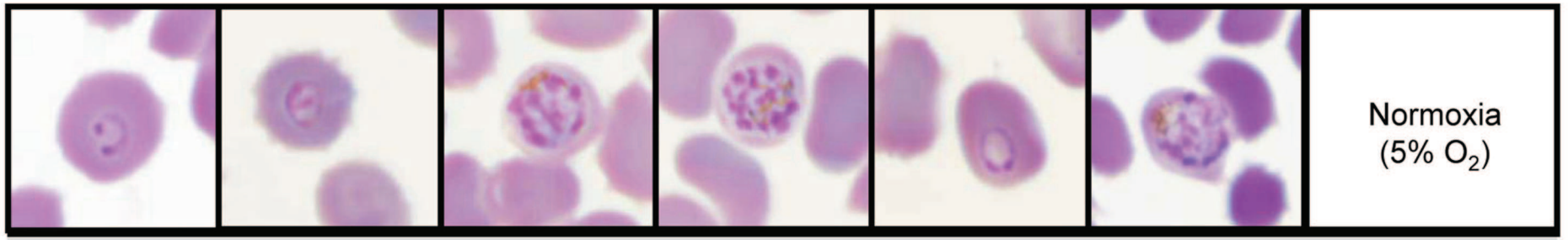
Figure 5. Selective post-translational modification of Pf-Hsp70 following hyperoxia exposure. (A) Enlarged 2D-DIGE gel images and their corresponding three-dimensional profiles are shown for a series of four protein spots identified as Pf-Hsp70. The amount of protein is proportional to the volume peak. Numbers correspond to master gel and significant deregulated spots (*i.e.*, 1314 and 1326) are reported in the table 2. (B) A graphic quantification of the four spots corresponding to Pf-Hsp 70 under normoxic (light-gray bars) and hyperoxic conditions (dark-gray bars). Spot numbers are specified at the bottom. Adjacent bars correspond to the mean standard abundance and respective standard deviation. Fold change and *p*-values are indicated at the bottom for each spot. A.U.: arbitrary units, FC: fold change.

Figure 6. Analysis of the beta-globin digestion pathway. qRT-PCR and microarray data of transcripts involved in digestive vacuole metabolism were compared between hyperoxic and normoxic conditions. Adjacent bars correspond to the mean $\log_2$ (fold change) and respective standard deviation and present qRT-PCR and microarray results in gray scale for the respective gene. The abbreviated names and their corresponding gene ID (PlasmoDB accession numbers) are indicated below the graphic. Crude values of

mean fold changes are presented in the table. The same samples were used for the qRT-PCR and microarray experiments.

Figure 7. A schematic representation of *P. falciparum* metabolic adaptation to hyperoxia exposure. Metabolic pathways based on the Ginsburg Pathway [72] for glycolysis (cytoplasmic), respiratory chain (mitochondria, blue and yellow) and haemoglobin digestion (digestive vacuolar, orange) are shown. The gene IDs (PlasmoDB) are reported for each enzymatic reaction. Human protein names are underlined. Up- and down-regulated genes and reactive oxygen species (ROS) production are indicated in red and blue characters, respectively.

A

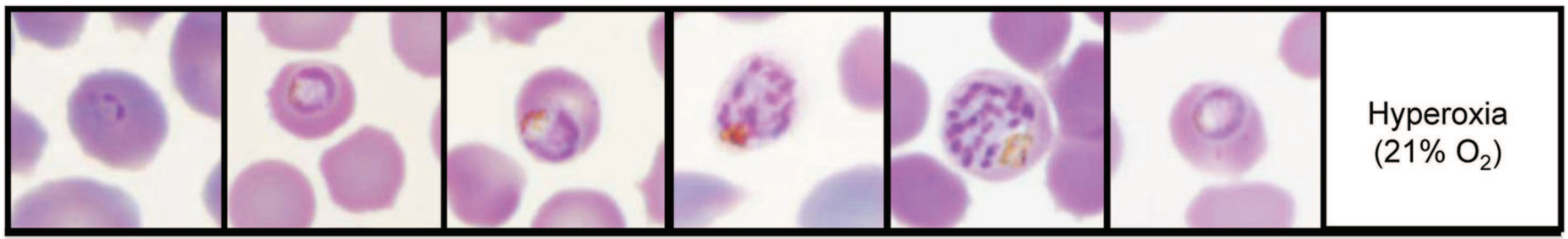


0.5	0.5	0.6	0.6	1.8	2.0	Parasitemia (%)
0	24	32	36	48	78	Time (hours)
98% R 2% S	99% T 1% R	98% S 2% T	98% S 2% R	97% R 3% S	96% S 4% T	Stages (%)

Normoxia ($5\%\ O_2$)

First Cycle | Second Cycle

B



0.5	0.5	0.5	0.7	0.7	1.2	Parasitemia (%)
0	24	32	36	48	78	Time (hours)
98% R 2% S	98% T 2% R	99% T 1% S	96% S 4% T	90% S 10% R	94% T 6% S	Stages (%)

Hyperoxia ($21\%\ O_2$)

First Cycle | Second Cycle

Figure 1

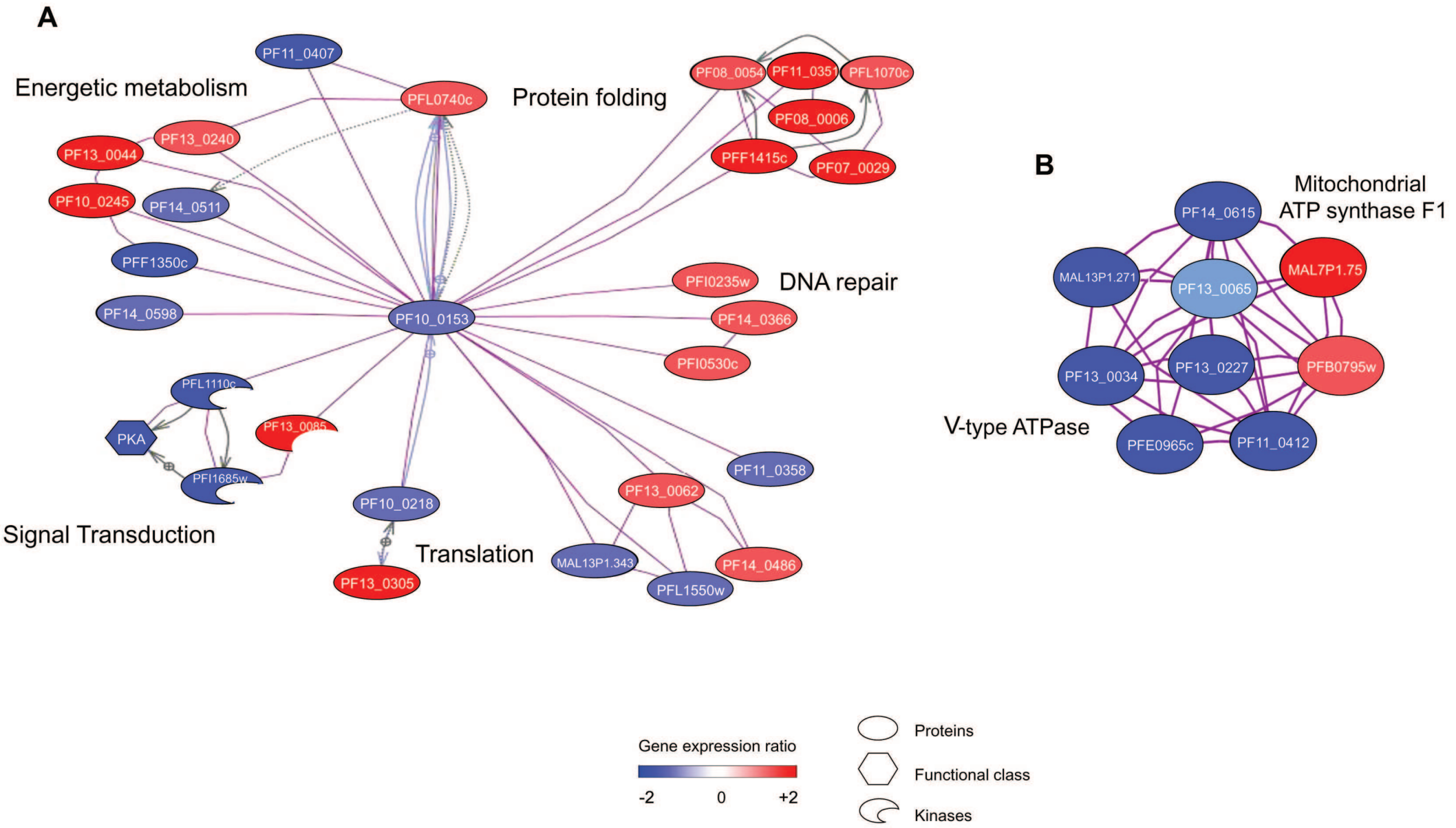
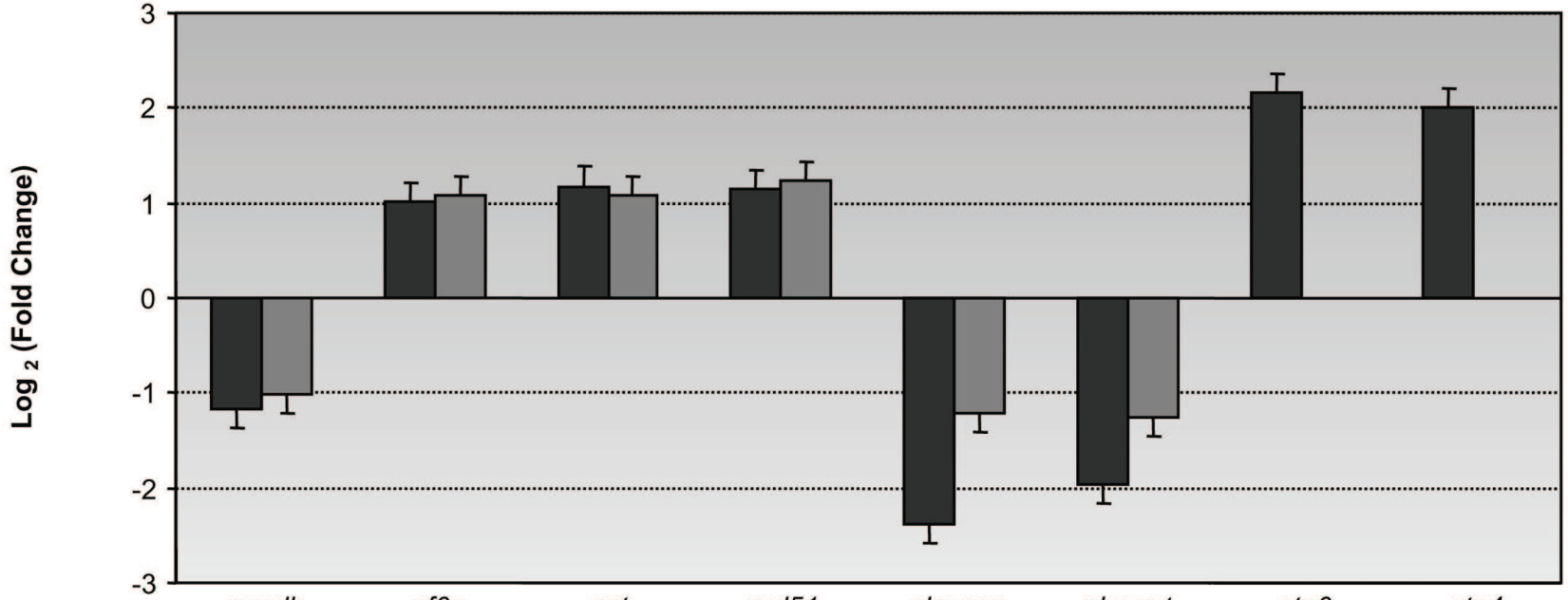
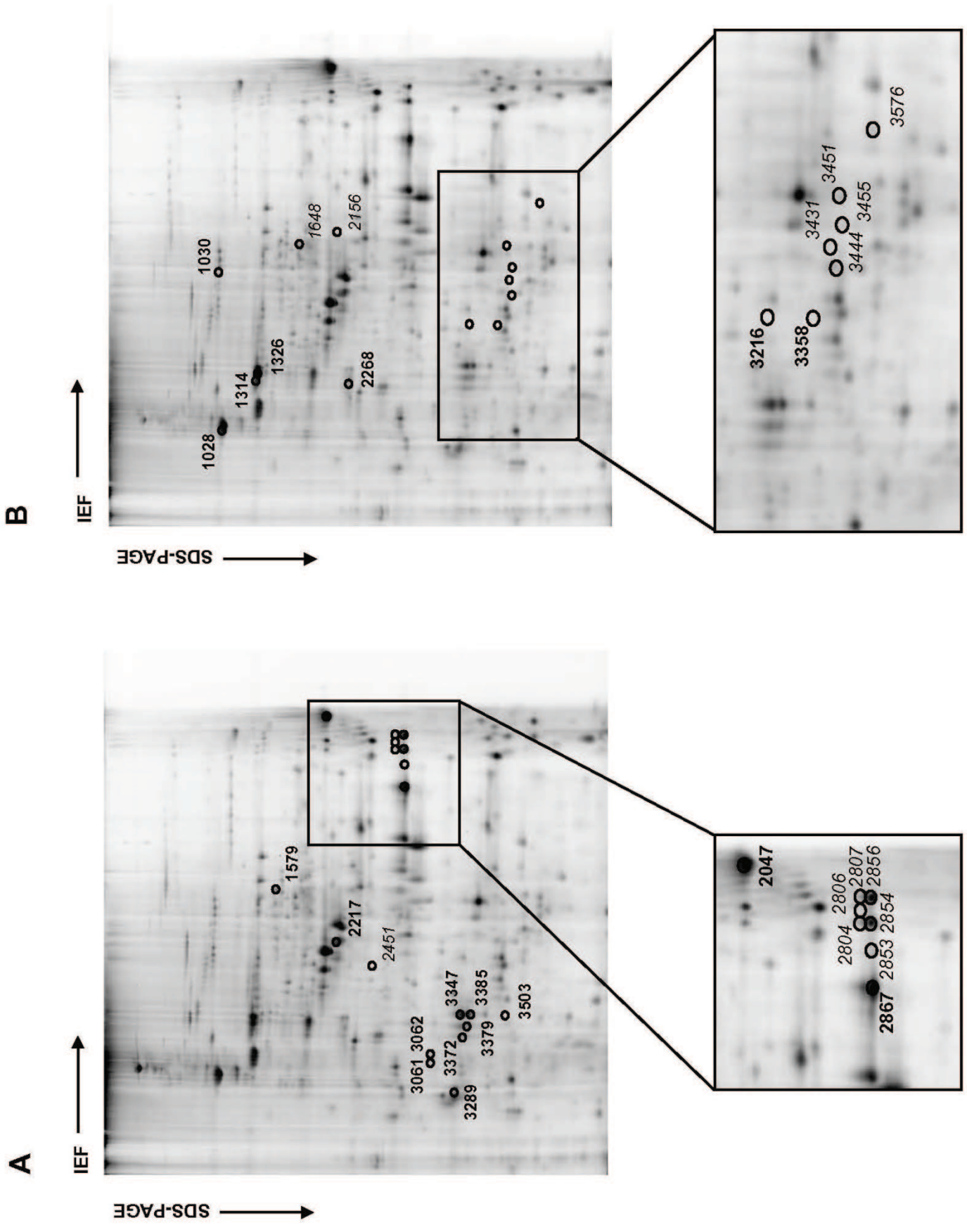

A
Energetic metabolism
PF11_0407
PFL0740c
PF13_0240
PF13_0044
PF10_0245
PF14_0511
PFF1350c
PF14_0598
Protein folding
PF08_0054
PF11_0351
PFL1070c
PF08_0006
PFF1415c
PF07_0029
DNA repair
PFI0235w
PF14_0366
PFI0530c
PF10_0153
PFL1110c
PKA
PF13_0085
PFI1685w
Signal Transduction
PF10_0218
Translation
PF13_0305
PF11_0358
PF13_0062
MAL13P1.343
PF14_0486
PFL1550w
B
Mitochondrial
ATP synthase F1
PF14_0615
MAL13P1.271
PF13_0065
MAL7P1.75
PF13_0034
PF13_0227
PFB0795w
V-type ATPase
PFE0965c
PF11_0412
Gene expression ratio
-2
0
+2
Proteins
Functional class
Kinases

Figure 2



	gapdh (PF14_0598)	gf6p (PF10_0245)	gst (PF14_0187)	rad51 (PF11_0087)	pka reg (PFL1110c)	pka cat (PFI1685w)	atp6 (PF10_0059)	atp4 (MAL7P1.13)
qRT-PCR	-1,16	1,02	1,18	1,15	-2,38	-1,96	2,16	2,01
Microarray	-1,02	1,09	1,09	1,24	-1,21	-1,26	N.D.	N.D.

Figure 3


A
IEF
SDS-PAGE
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2451
3061
3062
3289
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3379
3385
3503
2047
2804
2806
2807
2856
2853
2854
2867
B
IEF
SDS-PAGE
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1326
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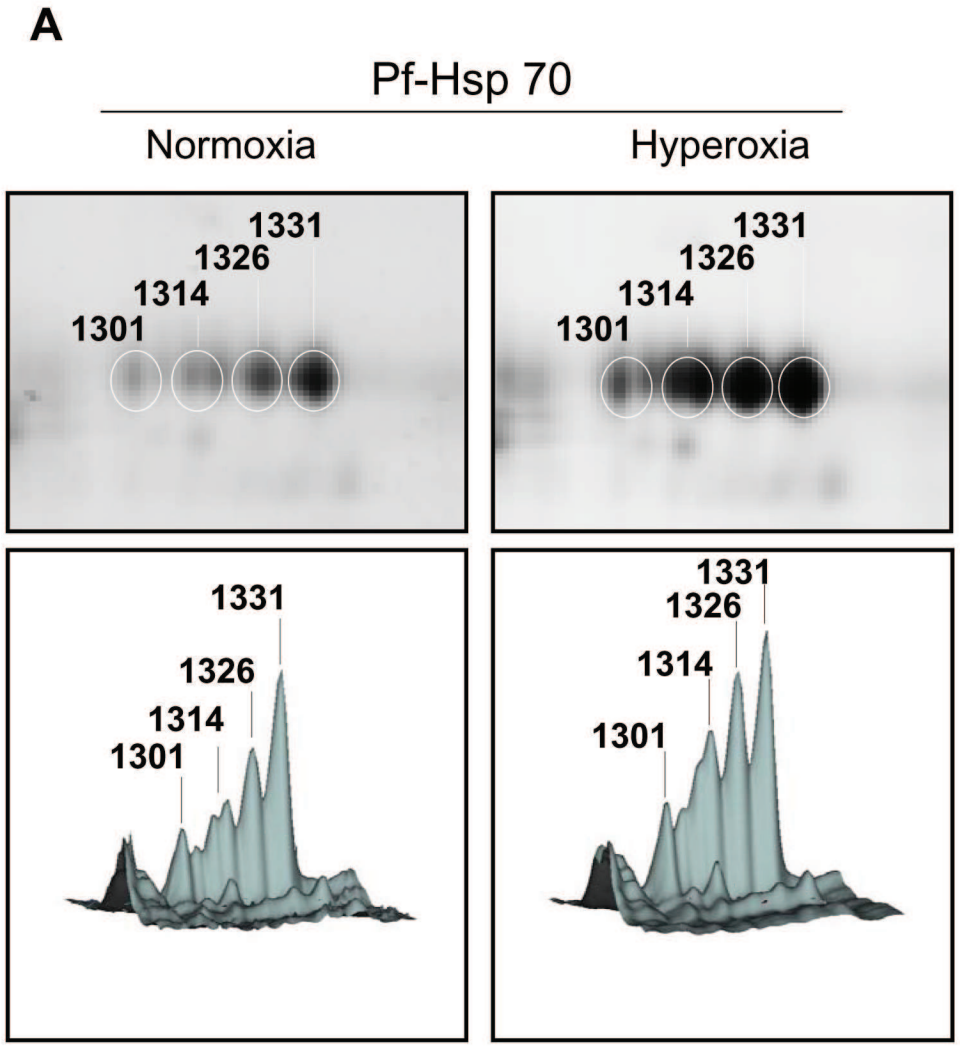
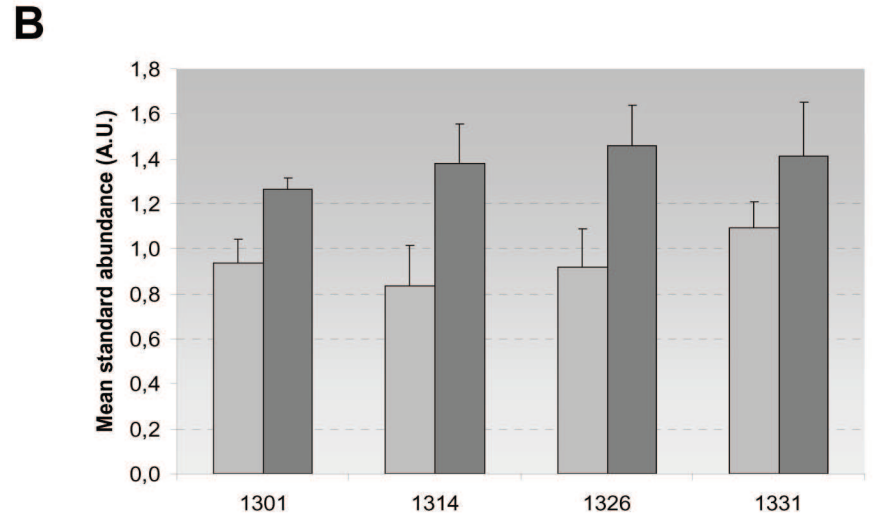
2268
3216
3358
3431
3444
3451
3455
3576

Figure 4





Fold Change	1.35	1.65	1.58	1.3
p-value	0.069	0.03	0.026	0.087

Figure 5

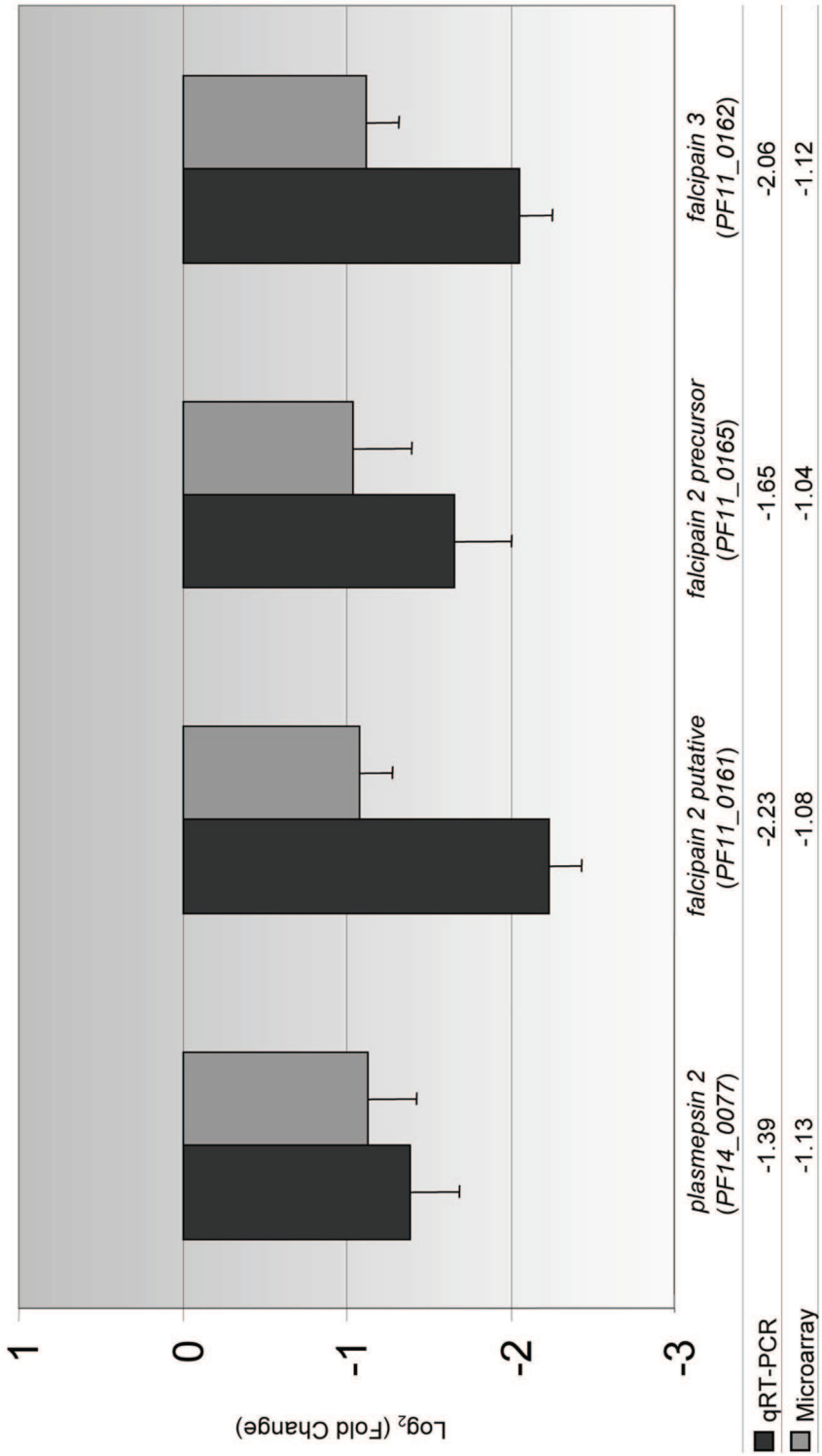

1
0
-1
-2
-3
Log2 (Fold Change)
plasmepsin 2 (PF14_0077)
falcipain 2 putative (PF11_0161)
falcipain 2 precursor (PF11_0165)
falcipain 3 (PF11_0162)
qRT-PCR -1.39 -2.23 -1.65 -2.06
Microarray -1.13 -1.08 -1.04 -1.12

Figure 6

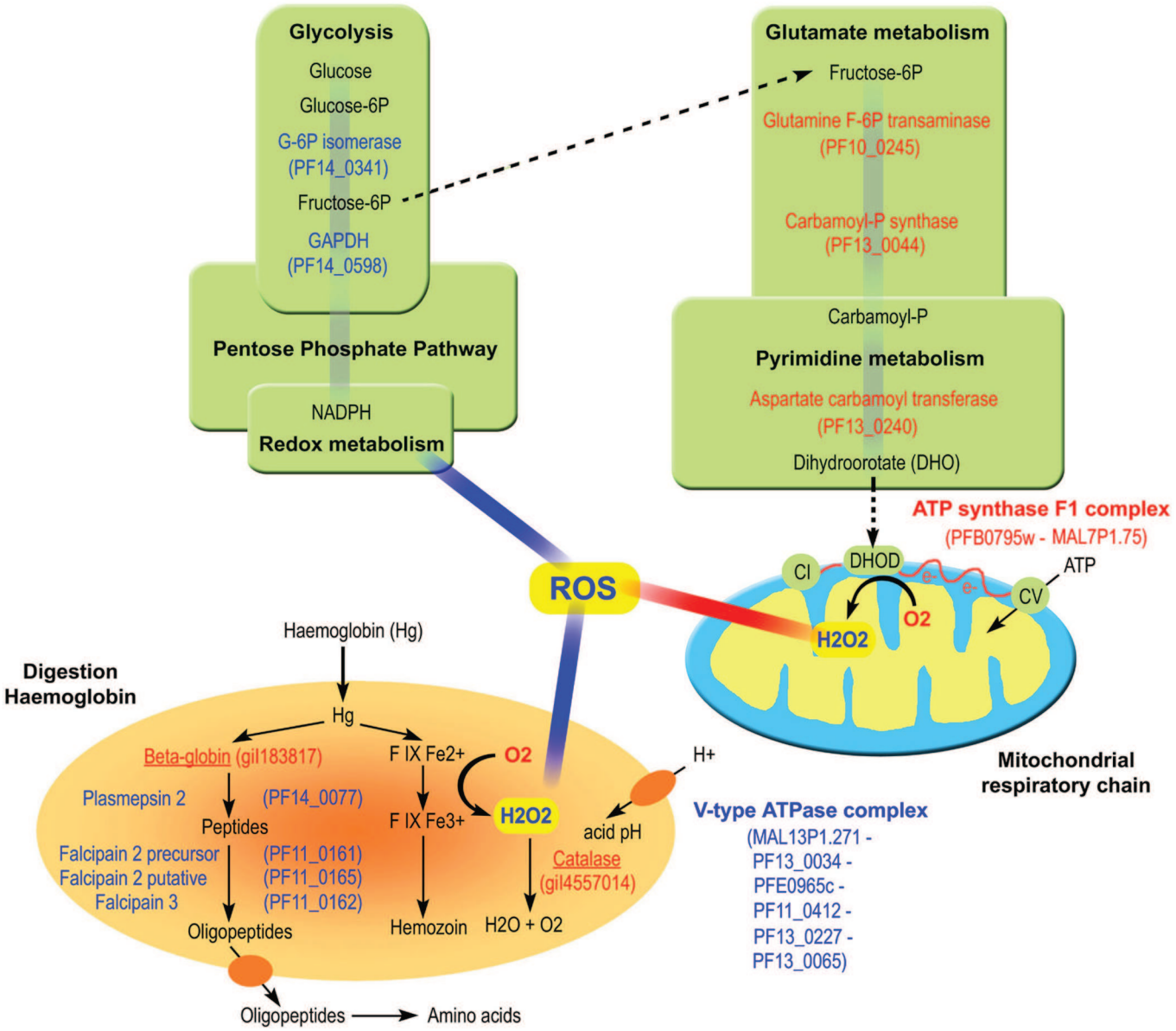

Glycolysis
Glucose
Glucose-6P
G-6P isomerase
(PF14_0341)
Fructose-6P
GAPDH
(PF14_0598)
Pentose Phosphate Pathway
NADPH
Redox metabolism
Glutamate metabolism
Fructose-6P
Glutamine F-6P transaminase
(PF10_0245)
Carbamoyl-P synthase
(PF13_0044)
Carbamoyl-P
Pyrimidine metabolism
Aspartate carbamoyl transferase
(PF13_0240)
Dihydroorotate (DHO)
ATP synthase F1 complex
(PFB0795w - MAL7P1.75)
ROS
CI
DHOD
ATP
CV
O2
H2O2
Mitochondrial
respiratory chain
Haemoglobin (Hg)
Digestion
Haemoglobin
Hg
Beta-globin (gil183817)
F IX Fe2+
O2
H+
Plasmepsin 2
(PF14_0077)
Peptides
F IX Fe3+
H2O2
acid pH
Falcipain 2 precursor
Falcipain 2 putative
Falcipain 3
(PF11_0161)
(PF11_0165)
(PF11_0162)
Catalase
(gil4557014)
Oligopeptides
Hemozoin
H2O + O2
Oligopeptides
Amino acids
V-type ATPase complex
(MAL13P1.271 -
PF13_0034 -
PFE0965c -
PF11_0412 -
PF13_0227 -
PF13_0065)

Figure 7

Table 1. Biological functions perturbed following hyperoxia exposure on *P. falciparum*.

Functional group[a]	**Number of entities**[b]	***p*-value**[c]
GPI anchor biosynthesis	29	0.0006
DNA repair	15	0.0072
Vacuolar acidification	11	0.0255
Actin filament organization	12	0.0296
Nucleosome assembly	12	0.0490
Regulation of cell shape	6	0.0519
Leading strand elongation	10	0.0533
Lysosomal H+ import	26	0.0589
Response to oxidative stress	17	0.0799

[a]Gene Set Enrichment Analysis (GSEA) using the Mann-Whitney-U-test enrichment algorithm in PathwayStudio software indicated functional groups significantly altered ($p < 0.08$). For each functional group, the [b]number of genes included and corresponding [c]*p*-values are listed.

Table 2. Proteins identified by differential 2-D DIGE analysis following hyperoxia exposure of *P. falciparum*.

gi numberc	Gene ID	Protein name	MW (kDa)	pI	Master spot number	Significance (Mascot score)	Average ratio	*t-Test*
			P. falciparum					
Translation								
gi|124512420	MAL8P1.69	14-3-3 protein homologue	29.86	4.96	3289	425	-1.54	0.0078
gi|124513850	PF13_0304	elongation factor 1 alpha	49.16	9.12	2047	130	1.60	0.0090
gi|8918238	PF14_0486	elongation factor 2	85.03	6.30	1030	67	1.72	0.0070
gi|124810293	PF14_0655	RNA helicase-1	45.62	5.48	2268	105	1.53	0.040
Parasitophorous vacuolar membrane Transporter								
gi|124810348	PF14_0678	exported protein 2	33.62	5.10	3061	88	-1.92	0.00093
					3062	180	-2.06	0.0046
Glycolysis								
gi|124809201	PF14_0341	glucose-6-phosphate isomerase	67.61	6.78	1579	36	-1.63	0.00092
gi|124810131	PF14_0598	glyceraldehyde-3-phosphate dehydrogenase	37.08	7.59	2867	695	-1.69	0.033
Chaperone-assisted protein folding								
gi|124512406	PF08_0054	heat shock protein 70	74.39	5.51	1314	257	1.65	0.03
					1326	137	1.58	0.026
gi|505340	PF07_0029	heat shock protein 86	86.77	4.91	1028	412	1.50	0.0052
Amino acids metabolism								
gi|86170756	PFF0435w	ornithine aminotransferase	47	6.47	2217	96	-2.14	0.0081
gi|124513590	MAL13P1.214	phosphoethanolamine	31.31	5.43	3347	145	-2.05	0.0055
		N-methyltransferase			3372	252	-1.96	0.00089
					3379	253	-2.17	0.00058
					3503	52	-1.62	0.00056
					3385	452	-2.71	0.00026

Proteasome-mediated proteolysis								
gi|124512686	MAL8P1.142	proteasome beta-subunit	31.08	6.00	3216	58	1.78	0.015
gi|124513790	MAL13P1.270	proteasome subunit	27.50	6.17	3358	142	1.55	0.049
		Homo sapiens						
Oxygen transporter								
gi|183817		beta-globin	19.21	6.28	3451	165	7.71	0.020
					3576	102	1.91	0.011
Antioxidant metabolism								
gi|4502517		carbonic anhydrase I	28.91	6.59	3431	173	4.18	0.0095
					3444	155	3.47	0.0019
					3455	224	4.02	0.00038
gi|4557014		catalase	59.95	6.9	1648	200	6.47	0.012
gi|16306550		selenium binding protein 1	52.93	5.93	2451	73	-1.64	0.041
gi|168985379		flotillin 1	39.81	6.03	2156	157	1.64	0.040
Glycolysis								
gi|31645		glyceraldehyde-3-	36.20	8.26	2804	199	-1.94	0.0042
		phosphate dehydrogenase			2806	181	-1.99	0.012
					2807	132	-1.84	0.0069
					2853	112	-2.17	0.0027
					2854	122	-2.25	0.0032
					2856	96	-2.20	0.0019

The proteins were identified by mass spectrometry following in gel trypsin digestion. The spot numbers correspond to the same numbers in figure 4. The Mascot gi number of the spots, their gene ID (PlasmoDB), their name, the theoretical MW and *pI* values, as well as the corresponding Mascot score are listed for MS/MS analysis (scores greater than 29 for *P. falciparum* and 38 for *Homo sapiens* are considered significant ($p \leq 0.05$)). Paired average volume ratio (hyperoxic versus normoxic conditions) and *p*-values (*t-Test*) were obtained using Decyder software. MW: molecular weight.

Additional Material files

Additional file 1
Primers sequence using real-time qRT-PCR.

Additional file 2
Single-Peptide-Based Protein Identifications.

Additional file 3
Raw data microarray at 8 hours time point under hyperoxia versus normoxia conditions on synchronized parasites.

Additional file 4
List of altered genes following hyperoxia treatment using GSEA data and PS 6.0 software.

Additional file 5
MS/MS peptide sequences, respective gi number, gene ID and master spot number of proteins identified from the differential 2-D DIGE analysis following hyperoxia exposure of P. falciparum.

Additional file 6
Raw data microarray at 4 hours time point under hyperoxia versus normoxia conditions on synchronized parasites.

Additional Files

Les «Additional Files» concernant cet article 2 sont consultables en annexe.

Données complémentaires non soumises à publication :

Materials and methods

Hemozoin dosage

The inhibitory activity on hemozoin formation was evaluated using a quantitative in vitro spectrophotometric microassay. After tight synchronisation of the parasites, erythrocytes were resuspended in RPMI 1640 medium supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM NaHCO3. Two sets of controls were employed simultaneously. One contained only uninfected RBCs and another contained parasite lysate treated with dihydroartemisinin (DHA), an inhibitor of hemozoin formation. Hemozoin production was evaluated in the parasite lysate extracts under normoxic or hyperoxic conditions in triplicate for time periods of 0-20-40 hours. At the end of the incubation time, hemozoin production in each assay was measured in the parasite lysate extracts as described previously (Orjih and Fitch, 1993). Briefly, the pellet containing the hemozoin was washed once by resuspending it in 10 mL of 20 mM Tris-buffered solution pH 7.2 and by centrifuging it at 4°C for 30 min at 27000 g. The hemozoin pellet was solubilised in 2 mL of 2.5% SDS buffered with 25 mM Tris to pH 7.8 and left for 2 h at room temperature. After centrifugation for 30 min at 27000 g, the pellet was recovered in 1.8 mL of SDS/Tris-buffered solution and 0.2 mL of 1 N NaOH. The mixture was vigorously vortexed and incubated for two hours at room temperature to convert hemozoin into ferriprotoporphyrin IX (FIX). The absorbance of each well was measured at 405 nm corresponding to hemozoin depolymerisation (Orjih and Fitch, 1993).

Results

Analysis of the haemoglobin digestion pathway

During parasite infection, haemoglobin is digested for the production of oligopeptides and the formation of hemozoin including beta-globin and FIX as intermediary metabolites. 2D-DIGE analyses indicated the accumulation of some proteins involved in haemoglobin digestion such as human catalase (gi|4557014) and beta-globin (gi|183817). The accumulation of beta-globin suggests inhibition of the haemoglobin digestion pathway. To test this hypothesis, FIX polymerisation (i.e., hemozoin) was quantified by spectrophotometric analysis as previously described (Orjih and Fitch, 1993 ; Campanale et *al.*, 2003). Under normoxic (5% O2) and hyperoxic (21% O2) conditions, hemozoin formation was not perturbed, reflecting an absence of inhibitory FIX polymerisation following hyperoxic stress (Figure 11). As a control, FIX polymerisation was quantified in parasites cultured under DHA pressure, an inhibitor of hemozoin formation (Figure 11). These results indicated that FIX polymerisation was not altered in hyperoxic conditions.

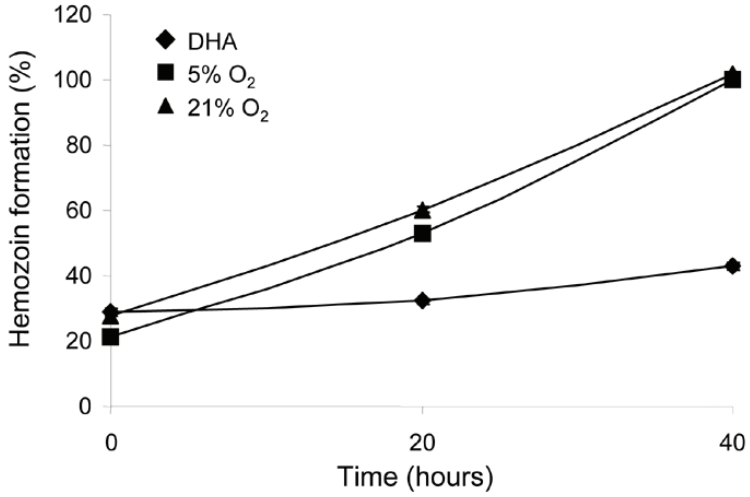


Figure 11. Analysis of the haemoglobin digestion pathway.

Dosage of hemozoin formation under normoxic (5% O2) and hyperoxic (21% O2) exposure. Ferriprotoporphyrin IX polymerisation was evaluated using a quantitative in vitro spectrophotometric microassay. A P. falciparum culture under normoxic conditions was used as the negative control, and dihydroartemisinin (DHA), an inhibitor of hemozoin formation, was used as the positive control. The values correspond to averages of three assays and standard deviations are also indicated.

Discussion

In addition to changes in mitochondrial metabolism, the digestive vacuole, another ROS source organelle in *P. falciparum* during haemoglobin digestion, was altered by hyperoxia (Oliveira and Oliveira, 2002). During the intraerythrocytic cycle, *P. falciparum* ingests up to 65% of the host cell haemoglobin in the digestive vacuole as an amino acid (AA) source and maintains osmotic stability by FIX polymerisation (Foley and Tilley, 1998). The "new permeation pathways" (NPPs) avoids osmotic stress and are very intricately involved in the neutralisation of toxic FIX and iron (Lew et *al.*, 2003 ; Mauritz et *al.*, 2009). Haemoglobin digestion is optimal at an acidic pH, which is also necessary for protease activity. To provide the acidic environment in the digestive vacuole, haemoglobin digestion stimulates ATP consumption by the V-type H+-ATPase pump (Hayashi et *al.*, 2000 ; Saliba et *al.*, 2003 ; Beyenbach and Wieczorek, 2006). In our microarray analysis, the V-type ATPase complex (MAL13P1.271, PF13_0034, PFE0965c, PF11_0412, PF13_0227, PF13_0065), a membrane transporter, was found to be down-regulated under hyperoxia. Therefore, *P. falciparum* exposed to hyperoxia could generate a pH change in the digestive vacuole responsible for vacuolar protease activity. Additionally, beta-globin accumulation occurred in hyperoxic conditions. This beta-globin accumulation could result from a decline in haemoglobin degradation into AAs by the proteases. Our previously results showed a down-expression of four transcripts of proteases involved in haemoglobin degradation into AAs (plasmepsin 2 (PF14_0077), falcipain 2 putative (PF11_0161), falcipain 2 precursor (PF11_0165) and falcipain 3 (PF11_0162)) by qRT-PCR. Surprisingly, the level of hemozoin formation was unchanged in hyperoxic conditions. Indeed, in the hyperoxic environment, the haemoglobin digestion pathway alteration seems attributable to down-regulation of proteases involved in AA production. Previously, Krugliak et *al.* reported that the small AA part of haemoglobin digestion is converted into parasite protein (Krugliak et *al.*, 2002). In 2002, Oliveira et *al.* hypothesised that blood-feeding parasites reduced their mitochondrial function to compensate

for ROS generation from the digestive vacuole (Oliveira and Oliveira, 2002). Our results indicated that hemozoin formation, an important ROS source, is maintained even when the respiratory chain is activated, as suggested by up-regulation of ATP synthase. Recently, Mauritz et *al.* demonstrated that hemozoin formation seems fundamental for osmotic stability preservation of parasitised RBCs (Lew et *al.*, 2003 ; Lew et *al.*, 2004 ; Mauritz et *al.*, 2009). *P. falciparum* parasites need thus to activate the enzymatic function of vacuolar ROS remov*al.* Effectively, catalase (gi|4557014), a human protein, was found concentrated in the digestive vacuole. Collectively, metabolism in the *P. falciparum* digestive vacuole would be perturbed in response to hyperoxia, and ROS production would be slowed by the catalase.

Conclusion et Perspectives :

L'hyperoxie induit des perturbations du transcriptome et du protéome de *P. falciparum* permettant au parasite de s'adapter à ce stress oxydatif. En plus de l'activation des systèmes de défense antioxydants pour neutraliser la production de ROS, les voies métaboliques plasmodiales impliquées dans cette réponse sont la glycolyse, la digestion de l'hémoglobine et la respiration mitochondriale. Le métabolisme énergétique semble donc jouer un rôle important dans l'adaptation du parasite à l'hyperoxie. L'effet d'un inhibiteur spécifique de l'oxydation alternative (le SHAM) serait-il délétère pour les parasites en condition hyperoxique ?

RÉSULTATS

ARTICLE 3

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Proteomic analysis revealed alterations of the *Plasmodium falciparum* metabolism following salicylhydroxamic acid exposure

Malaria Journal 2010, submitted

"Proteomic analysis revealed alterations of the Plasmodium falciparum metabolism following salicylhydroxamic acid exposure"

Problématique :

Quel est l'effet du SHAM, inhibiteur de la voie alternative de la respiration sur le métabolisme de *P. falciparum* ?

Existe-t-il un gène ou une protéine AOX chez *P. falciparum* ?

Alors que chez l'homme, le métabolisme respiratoire est caractérisé par la chaîne mitochondriale de transfert d'électrons, certains organismes (plantes supérieures, levures, et parasites comme *Trypanosoma*) présentent une chaîne respiratoire alternative. Cette dernière met en jeu une enzyme : l'oxydase alternative (AOX). Même si aucun gène codant pour l'AOX n'a encore été identifié chez *P. falciparum*, un inhibiteur spécifique de cette enzyme (acide salicylhydroxamique, SHAM) présente un effet sur la respiration de *P. falciparum* d'après des mesures de consommation d'oxygène.

Pour évaluer l'effet du SHAM sur la croissance *in vitro* du parasite, nous avons déterminé la CI_{50} du SHAM pour la souche 3D7 et observé les altérations phénotypiques de *P. falciparum* après exposition au SHAM et/ou hyperoxie. Une approche protéomique a été réalisée pour étudier les voies métaboliques altérées après exposition au SHAM.

Ce sont ces travaux que détaille l'article suivant.

Proteomic analysis revealed alterations of the *Plasmodium falciparum* metabolism following salicylhydroxamic acid exposure

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Abstract

Background: Although human respiratory metabolism is characterised by the mitochondrial electron transport chain, some organisms (higher plants, yeast, and apicomplexa such as *Trypanosoma*) present a "branched respiratory chain" pathway. This branched pathway includes both a classical and an alternative respiratory chain. The latter involves an enzyme called the alternative oxidase (AOX). Although the *Plasmodium falciparum* AOX is not yet identified, a specific inhibitor of this enzyme (SHAM : salicylhydroxamic acid) showed a drug effect on *Plasmodium falciparum* (*P. falciparum*) respiratory function using oxygen consumption measurements.

Methods: To evaluate the SHAM effect on *in vitro* parasite growth, we have determined the SHAM IC_{50} for the 3D7 strain and observed the phenotypic alterations of *P. falciparum* after SHAM or/and hyperoxia exposure. A proteomic approach was used to study the metabolic pathways altered following SHAM exposure.

Results: The combinatory effect of SHAM and hyperoxia seems deleterious for the *in vitro* growth of *P. falciparum*. After SHAM exposure, 28 proteins were significantly deregulated using a fluorescent 2D differential gel electrophoresis (2D DIGE). Among these deregulated proteins, some were particularly involved in energetic metabolism of *P. falciparum*.

Conclusions: Our results indicated that SHAM appears to activate glycolysis metabolism, and also to decrease the stress defence systems. These data provide a better understanding of parasite biology and may lead to develop new anti-malarial drug treatments that could be used in association with hyperbaric oxygen therapy.

Background

The malaria parasite *P. falciparum* possesses a mitochondrion with specific structural and physiological features [1-4]. Unlike most other eukaryotes, malarial mitochondrial electron transport chain (mETC) activity appears to be primarily involved in *de novo* pyrimidine biosynthesis through the activity of dihydroorotate dehydrogenase (DHOD) rather than through an ATP source [4-6]. Intraerythrocytic stages of malaria parasites have been considered for a long time to rely primarily on anaerobic glycolysis for ATP production. But recently five subunits of the F_1 subcomplex and four F_0 subunits of the mitochondrial F_0F_1 ATP synthase have been reported in the *P. falciparum* genome, supporting the fact that oxidative phosphorylation could be functional [7]. As parasite respiratory metabolism is distinct from human respiration, major complexes of the mETC are targeted by the commonly used antimalarial drugs and can be exploited for chemotherapy [8]. As a consequence, diverse inhibitors of the mETC have been used in *in vitro* chemosensitivity assays and validated the importance of the mitochondrion in the growth of the asexual intraerythrocytic stage of *P. falciparum* [9,10].

The evidence of the physiological function of mitochondrial complexes is indicated by the antimalarial activity of drugs that act on the mETC. Therefore, study of the rotenone drug effect underlined the divergence of the first component (Complex I) of the *P. falciparum* mETC [11-13]. The eukaryotic complex I is classically composed of a rotenone-sensitive NADH dehydrogenase. In contrast, *P. falciparum* encodes a rotenone-insensitive alternative complex I (type II NADH dehydrogenase, PfNDH2). This complex I is comparable to type II NADH dehydrogenase found into plants. Not surprisingly, parasite mitochondria have an endosymbiotic origin probably from algal symbiont into a proto-eukaryote [14-16]. Moreover, other mitochondrial drugs as atovaquone inhibit electron transfer at the level of the *bc*1 complex (Complex III) by interfering with the ubiquinol oxidation site of cytochrome *b*. The drug effect is lethal for the parasite, presumably by interruption of essential links to *de novo* pyrimidine biosynthesis and to collapse of the mitochondrial transmembrane potential [17,18]. This drug is already currently used in endemic regions in combination with proguanil (Malarone™, GlaxoSmithKline) [19]. On the other hand, the combination of atovaquone and SHAM, specific inhibitors of AOX, inhibits parasite growth synergistically [20,21]. The AOX, which is absent from mammals, has been most extensively studied in higher plants. The plant respiratory pathway also includes an alternative respiratory chain that is not coupled to ATP production, and the classical mETC. The AOX does not appear to contribute directly to mitochondrial transmembrane potential or energy balance of the cell [22,23]. However, it can contribute indirectly by accepting electrons from enzymes that provide electrons to coenzyme Q for the preservation of TCA cycle turnover and the balance of carbon metabolism and electron transport. From the mitochondrial

transmembrane potential and oxygen consumption measurements of the *P. falciparum* mETC, the evidence of an alternative respiratory pathway in which electrons are transferred directly from coenzyme Q to oxygen was underlined [24]. The results of this study supposed that *P. falciparum* parasites used a branched respiratory chain pathway, consisting of a cyanide-sensitive classical respiratory pathway and a cyanide-resistant respiratory alternative pathway. The cyanide-resistant pathway of parasite oxygen consumption was completely inhibited by two inhibitors, propyl gallate and SHAM, which are specific inhibitors of AOX activity in other organisms [25-27]. The AOX gene has been characterised in several organisms such as plants, yeast, bacterium and notably, from others apicomplexa such as *Trypanosoma* and *Cryptosporidium parvum* (*C. parvum*) [27-29]. Nevertheless, until now, no gene encoding AOX has been detected in the *P. falciparum* genome but 60% of the predicted genes could not be assigned to orthologous functions.

To study the SHAM effect on the intraerythrocytic stages, we have performed *in vitro* chemosusceptibility assays and observed the *in vitro* growth of *P. falciparum* under SHAM and/or hyperoxia exposure. In the present work, a 2D-DIGE approach combined with mass spectrometry (MS) analysis was used to define the consequences of SHAM on the *P. falciparum* proteome. The deleterious effect of this drug on the *in vitro* parasite growth in hyperoxia, the involved metabolic pathways and its mode of action on glycolysis are discussed, supporting our hypothesis that the energetic metabolism may indeed provide an attractive chemotherapeutic target.

Methods

In vitro chemosusceptibility assay

The *in vitro* antimalarial activity of SHAM was determined using the isotopic semi-micro-test method measuring [^{3}H] hypoxanthine incorporation in parasite nucleic acids derived from the method of Desjardins [30]. Culture conditions and test procedures were done as previously described [31]. The range of drug concentrations used was from 0.5 to 600 μM. The IC_{50}, *i.e.*, the drug concentration corresponding to 50% of the uptake of [^{3}H] hypoxanthine by the parasite in drug-free control wells, was determined by nonlinear regression analysis of log dose-response curves.

Highly synchronised *P. falciparum* cultures, SHAM and hyperoxia pressure

The 3D7 *P. falciparum* strain was cultured in human A+ erythrocytes as previously described [32]. The cultures were tightly synchronised (with 4 hours interval) using combined D-sorbitol and CS columns on a VarioMACS according to standard procedures [33,34]. To test the SHAM effects on *P. falciparum* parasites (3% parasitemia and 6% haematocrit), the drug was added to 25 ml culture medium at IC_{50} (250 μM) and the parasites were further incubated for 12 hours and harvested. For each proteomic experiment, 4 biological replicates were performed.

To evaluate the combinatory effect of SHAM pressure and hyperoxia, cultures were adapted to hyperoxic conditions (21% O_2, 5% CO_2, 74% N_2 gas mixture) and regularly synchronised during six *P. falciparum* parasites cycles before the addition of SHAM. The effects of SHAM alone and combined SHAM/hyperoxia were observed at 0, 24, 32, 48 and 78 hours (0 hours corresponding to SHAM addition at the ring stage). Viability, parasitemia and morphology were monitored daily by examining blood smears stained with RAL® 555 (RAL, Martillac, France) from SHAM addition at the ring stage.

Sample preparation

After SHAM pressure, parasitised erythrocytes (late-ring stages aged 16-20 hours) were washed three times in PBS medium (Invitrogen) and lysed in cold H_2O-saponin (0.1%, Sigma, St Louis, MI) for 10 min. The lysate was then centrifuged at 1500 g for 5 min. The supernatant was discarded and the pellet containing free parasites was recovered by washing in cold PBS medium followed by a centrifugation step (1500 g for 5 min). The free parasites were washed until the supernatant became colourless. The pellet was then suspended in 4% (w/v) CHAPS (Sigma) and disrupted by ultrasonication (Vibracell 72412, Bioblock Scientific, Illkirch, France)

times for 60 seconds on ice at maximum amplitude. The lysate was then centrifuged at 16,100 g for 15 min. The supernatant was further precipitated with 100% acetone (Sigma). The protein concentration for each sample was estimated using the BioRad Lowry-based DC assay (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. Total proteins were suspended in standard cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base, pH 8.5 (Sigma)) to obtain a protein concentration adjusted to 2.5 µg/µL. Protein samples were minimally labelled with CyDye according to the manufacturer's protocols (GE Healthcare, Piscataway, NJ) [35].

Briefly, protein extracts (50 µg) were labelled with 400 pmol of CyDye, freshly dissolved in anhydrous dimethyl formamide (Sigma) and incubated on ice for 30 min in the dark. The reaction was quenched with 1 µL of free lysine (10 nM, Sigma) by incubating for 10 min on ice. An equal volume of 2x sample buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 10 mM DTT and 1% (v/v) IPG Buffer 3-10 (GE Healthcare)) was added to the CyDye-labelled sample. The mixture of labelled proteins was then separated by 2D-differential-gel-electrophoresis (2D-DIGE).

Two-dimensional electrophoresis of parasite proteins, image analysis and in-gel digestion

Isoelectric focusing (IEF) was performed on 18-cm pH 3-10 linear IPG strips (GE Healthcare). Destreak buffer containing 1% (v/v) IPG buffer pH 3-10 was used for overnight rehydration of IPG strips. The samples were applied at the acidic end of the IPG strip using a cup-loading technique. IEF was carried out on an Ettan IPGphor II (GE Healthcare) electrophoresis unit at 20°C for a total of 45 kVh (ramp to 300 V in 3 hrs, ramp to 1000 V in 6 hrs, ramp to 8000 V in 3 hrs, hold at 8000 V for 4 hrs). IPG strips were equilibrated in a equilibration buffer containing 50 mM Tris-HCl, pH 8.6, 6 M urea, 2% SDS and 30% glycerol supplemented with 1% (w/v) DTT for 15 min at room temperature, followed by protein alkylation (carbamidomethylation) in the same equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 15 min at room temperature. IPG strips were then placed on the top of 10% uniform polyacrylamide gels. Strips were overlaid with 0.5% agarose in 1x running buffer containing bromophenol blue, and the proteins were further separated by SDS-PAGE (10 W per gel) at 20°C in the Ettan DALT Six electrophoresis system (GE Healthcare). After electrophoresis, the gels with Cydye-labelled proteins were directly imaged using a Typhoon™ Trio Image scanner (GE Healthcare UK).

After 2D-DIGE, gels were scanned on the Typhoon™ Trio Image scanner (GE Healthcare UK) at different excitation wavelengths (Cy3, 580 BP 30/green (532 nm); Cy5, 670 BP 30/red (633 nm); Cy2, 520 BP

40/blue (488 nm)). The intensity was adjusted to ensure that the maximum volume of each image was within 60,000-80,000 U. Analysis of 2-D DIGE was performed using DeCyder 6.5 software (GE Healthcare) according to the manufacturer's recommendations. Briefly, the differential in-gel analysis (DIA) module was used to detect the intra-gel spots and the biological variation analysis (BVA) module to match different gels using the in-gel standard. The paired *t*-test was used for statistical analysis of the data. Protein spots that were expressed differentially between two experimental conditions ($|ratio| \geq 1.5$, $p \leq 0.05$ *t*-test) were marked with master gel numbers. Based on DeCyder v6.5 analysis spots of interest were excised from gels using a Shimadzu Xcise automated gel processing platform (Shimadzu Biotech, Kyoto, Japan). Excised spots were prepared as described previously and then stored at -20°C before analysis by MS [36].

Mass spectrometry analysis

The samples were analysed by nanoscale capillary liquid chromatography-tandem mass spectrometry (nano LC-MS/MS). Purification and analysis were performed on a C18 capillary column using a CapLC system (Waters, Milford, MA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima, Waters, MA). Chromatographic separation was conducted on a reversed-phased capillary column (Atlantis^{TM} dC18, 3 µm, 75 µm x 150 mm Nano Ease^{TM}, Waters, MA) with a 180-200 nl min-1 flow. The gradient profile consisted of a linear gradient from 95% A (H_2O, 0.1% HCOOH) to 60% B (80% ACN, 0.1% HCOOH) in 60 min followed by a linear gradient by 95% B in 10 min. Mass data acquisitions were piloted by MassLynx 4.0 software using automatic switching between MS and MS/MS modes. The internal parameters of Q-TOF were set as follows. The electro-spray capillary voltage was set to 3.2 kV, the cone voltage was set to 30V, and the source temperature was set to 80°C. The MS survey scan was m/z 400-1300 with a scan time of 1 s and an interscan time of 0.1 s. When the intensity of a peak rose above a threshold of 15 counts, tandem mass spectra were acquired. Normalised collision energies for peptide fragmentation were set using the charge-state recognition files for +2 and +3 peptide ions. The scan range for MS/MS acquisition was from m/z 50 to 1500 with a scan time of 1 s and an interscan time of 0.1 s. Fragmentation was performed using argon as the collision gas and with the collision energy profile optimised for various mass ranges and charges of precursor ions. Mass data collected during a nano LC-MS/MS analysis were processed using ProteinLynx Global Server 2.2 software (Waters) with the following parameters: no background subtraction, smooth 3/2 Savitzky Golay and no deisotoping to generate peak lists in the micromass pkl format. Pkl files were then fed into a local search engine Mascot Daemon v2.2.2 (Matrix Science, London, UK). The data were searched against the *Homo sapiens*

(218356 sequences) and *P. falciparum* (13110 sequences) National Center for Biotechnology Information non-redundant (NCBInr) protein databases (March 15, 2010). Search parameters allowed for one missed tryptic cleavage site, the carbamidomethylation of cysteine, and the possible oxidation of methionine; precursor and product ion mass error tolerance was < 0.2 Da. All identified proteins had a Mascot score greater than 34 and 43 for *P. falciparum* and *Homo sapiens*, respectively, corresponding to statistically significant identification ($p < 0.05$).

Results

Effect of SHAM on *P. falciparum* asexual blood cycle

Consistent with previously published results [20,37], SHAM inhibited growth of the 3D7 strain with an average IC_{50} of 250 µM. To determine the SHAM effect on asexual blood stages of *P. falciparum*, this drug was added at the IC_{50} concentration on synchronous cultures of 3D7 at the ring stage. The parasitemia and parasitic stage percentages were monitored during two life cycles by blood smears at 0 (corresponding to SHAM addition at the ring stage), 24, 32, 48 and 78 hours in three independent experiments. Even though the parasitemia of SHAM-treated cultures was low at 48 hours compared to untreated parasites (Fig. 1A and 1B, 1.8% parasitemia versus 0.4% respectively), SHAM treatment induced no morphological changes and no lengthening of the parasitic cycle. However, at the second cycle, the parasites have a normal life cycle without lethality after complete reinvasion. Thus, SHAM perturbed the parasite growth at the IC_{50} concentration but does not cause phenotypic changes.

SHAM and hyperoxia combinatory effect on the asexual blood cycle of *P. falciparum*

To study the deleterious effect of SHAM on asexual blood stages of *P. falciparum* in hyperoxia, 21% O_2-adapted cultures of 3D7 were exposed to IC_{50} SHAM in three independent experiments. The parasitemia and percentages of the different stages of parasites were evaluated by blood smears at 0 (corresponding to SHAM addition at the ring stage), 24, 32, 48 and 78 hours. In *in vitro* culture under hyperoxic conditions, the 3D7 *P. falciparum* strain had a life cycle of 49 hours (Fig. 1C). Parasite exposure to 21% O_2 and SHAM pressure resulted in morphologic alterations involving cellular degeneration during the first 24 hours (Fig. 1D). These results suggest that SHAM addition to 3D7 *P. falciparum* strain under oxidative stress had lethal effects from the beginning of the parasitic cycle. Consequently, the SHAM/hyperoxia combinatory effect appeared to result in cellular death, suggesting that the adaptive pathway for hyperoxia was disturbed under SHAM pressure.

SHAM-alteration to the *P. falciparum* proteome

To study the SHAM effect on *P. falciparum*, a proteomic approach was performed in synchronised parasites exposed to IC_{50} SHAM. To identify altered *P. falciparum* proteins, 2D-DIGE experiments coupled to MS were performed. Four independent cultures of untreated and SHAM-treated *P. falciparum* were included in this analysis. After protein separation by 2-DE using pH 3-10 IPG strips and homogeneous 10% SDS-PAGE, each gel was individually imaged, and all gel images were analysed using the DeCyder 6.5 software. Among

3220 matched protein spots, 28 spots were differentially modulated (|FC| ≥ 1.5, $p \leq 0.05$ *t*-test) between the control and SHAM treatment (16 and 12 spots were up- and down-modulated, respectively, Fig. 2). The corresponding protein spots were excised from gels, analysed by LC-MS/MS, and searched against *P. falciparum* and *Homo sapiens* databases (NCBInr). Among the differentially modulated spots, 28 were identified by MS that correspond to 16 *Homo sapiens* and 12 *P. falciparum* specific proteins (Table 1). However, some proteins were detected in more than one spot (*e.g.*, 4 for glyceraldehyde-3-phosphate dehydrogenase, 3 for catalase), suggesting the presence of different isoforms. Indeed, the number of distinct proteins identified were 10 for *Homo sapiens* and 8 for *P. falciparum* (Table 1).

To determine the metabolic pathways perturbed under SHAM pressure, the identified proteins were classified using the NCBI COG database (Table 1). Among the functional categories, some proteins were predicted to play a role in glycolysis, chaperone-assisted protein folding and redox metabolism. Among the antioxidant proteins, human catalase, carbonic anhydrase I and carbonic anhydrase II were down-regulated in SHAM-treated *P. falciparum*.

Discussion

SHAM affects *P. falciparum* growth

Previous studies have examined the effects of cyanide, a complex IV inhibitor, on *P. falciparum* respiratory metabolism using a polarographic assay and showed that 25% of the parasite oxygen consumption was residual in the presence of high concentrations of cyanide [37]. These results indicated that the parasite exploits a cyanide-insensitive alternative respiratory pathway. This residual oxygen consumption was totally inhibited by SHAM, a specific inhibitor of AOX enzymatic activity [12]. These physiological observations suggested the existence of a *P. falciparum* AOX and were supported by the ability of SHAM to potentiate the activity of atovaquone, a complex III inhibitor, in *in vitro* chemosusceptibility assays [20].

Our results indicated that the SHAM diminished the replication rate of *P. falciparum* in a microaerophyllic atmosphere. Under hyperoxia conditions, SHAM exposure has a deleterious effect on parasite survival. Consequently, as described in plants [22,23], SHAM action appears to prevent the diminution in the levels of reactive oxygen species (ROS), which are exceedingly produced in hyperoxic conditions by the mETC [38].

SHAM alters the *P. falciparum* proteome

To investigate the metabolic pathways altered by the antimalarial activity of SHAM, a comparative study on the *P. falciparum* proteome was performed using 2D-DIGE coupled to MS for identification. Previous studies reported that the rate of mitochondrial O_2 consumption during the ring stage was less than that during the trophozoite stage [39]. The experimental design (parasite stage, drug dose, incubation time) was chosen based on these last results. In this study, SHAM was exposed at the IC_{50} concentration to ring stage parasites for 12 hours. The AOX protein, involved in the mitochondrial alternative respiratory pathway, is inhibited specifically by SHAM (coenzyme Q analogue). Because AOX reduces O_2, its activity should be dependent on the oxygen concentration and also on the coenzyme Q concentration and its redox state [22]. This alternative pathway could provide a regulatory function for excess electrons when the mETC is saturated (as during hyperoxia) and thus, decrease ATP production (unpublished data). Additionally, this “electron leak” mechanism may improve potential repression of the TCA cycle by elevated levels of NADH and ATP [23].

Most SHAM-altered proteins are involved in glycolysis and antioxidant metabolism. The major implication of glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase, enolase, and phosphoglycerate mutase) suggests the need to sustain the major energy dependence of the parasite

on glycolysis in agreement with pentose phosphate cycle activation. Although the function of the *P. falciparum* mitochondrion remains unclear, its contribution to ATP production is considered to be minimal in asexual stages. *P. falciparum* rely principally on anaerobic glycolysis for energy production [4]. Therefore, the parasites are dependent on glycolysis in a microaerophilic environment while in hyperoxia conditions, we have shown that the respiratory metabolism is favoured at the expense of glycolysis (unpublished data). This observation could explain the lethal effect of SHAM in hyperoxic conditions. The reducing equivalents generated during glycolysis are reoxidised through glycerol-3-phosphate (G3PDH) in the cytosol and by PfNDH2 in the mitochondria [40,41]. In plants, the alternative respiratory pathway involves an alternative NADH dehydrogenase, coenzyme Q and AOX [22]. Mitochondrial AOX exists under two forms, covalent and noncovalent homodimers [42]. The oxidised covalent form of the enzyme is inactive, whereas reducing the disulfide bond generated by NADH activates the enzyme, possibly mediated by a glutathione/thioredoxin coupling system [43]. The end products of *P. falciparum* glycolysis are pyruvate, lactate and glycerol. Interestingly, glycolysis activation under SHAM pressure could be the result of a metabolic adaptation in a microaerophilic environment. Firstly, pyruvate is an allosteric activator of AOX counterbalancing the AOX inhibition by SHAM. Under conditions of partial respiration, accumulation of pyruvate would result in increased NADH, which could potentially activate AOX [23]. Secondly, lactate, via L-lactate dehydrogenase, may be a substrate for the mETC under stress conditions [40]. Finally, glycerol production by the glycerol-3-phosphate permitted the reoxidation of NADH and the activity of the mETC [44] (Fig. 3). In a microaerophilic environment, the activation of glycolysis is concordant with the role of glycolysis metabolism in the reoxidation of NADH [45], an essential co-factor of the alternative respiratory pathway in *T. brucei* [25] and *C. parvum* [41]. Indeed, *T. brucei brucei* survive using an anaerobic pathway while TbAOX is inhibited [25]. Collectively, these results showed that SHAM, via its action on glycolysis metabolism, appears to play a role in NAD/NADH balance. The mechanism of regulation of AOX activity involved several compounds, including those in redox states (NAD/NADH) and allosteric effectors (pyruvate) [46]. Furthermore, diverse observations appear to show that AOX activity will be regulated by a fine metabolic control of feed-back regulation as described in higher plants [23]. Thus, the existence of a AOX function in *P. falciparum* could be beneficial for the survival of the parasite in rich-oxygen environments as in the salivary glands of mosquitoes or perhaps in the human lungs.

SHAM treatment leads to an inhibition of antioxidant proteins. Among the proteins identified, some were originated from the human erythrocyte host and correspond to host proteins imported by the parasite or proteins arising from host–parasite interaction. Consequently, cytosolic catalase and carbonic anhydrase I were

down-regulated under SHAM pressure. While *P. falciparum* lacks the antioxidant enzyme catalase, host catalase was imported into the food vacuole and accomplished H_2O_2 detoxification [47]. Thus, the down-importation of catalase could be attributed to a SHAM effect against oxidative damage by diminishing H_2O_2 production. Carbonic anhydrase has been identified as a major intracellular peroxidation target in erythrocytes and could be attributed to oxidative stress induced by SHAM in the parasitised erythrocytes [48].

In our study, a number of identified proteins (Hsp90, enolase, catalase, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase) have been previously found as potential glutaredoxin target proteins [49]. In higher plants, the reduction of AOX in its active form involved a glutathione/thioredoxin coupling system, suggesting a SHAM effect on feed-back regulation of AOX activity [43].

The other deregulated proteins were particularly involved in protein processing (heat shock protein, chaperonin containing TCP1), as well as translation (elongation factor 2), consequences of a general stress state of the parasite. Chaperone-assisted protein folding was described to contribute to protein folding, assembly and translocation, inducing expression of proteins in these pathways under several types of cellular stress [50].

Conclusions

Although the hypothesis of AOX-like is still unclear in *P. falciparum*, SHAM, an AOX specific inhibitor, disturbed the *in vitro* growth of *P. falciparum* only in hyperoxic conditions. Additionally, recent studies sustained the existence of the alternative respiratory pathway on *P. falciparum* illustrated by the identification of alternative NADH dehydrogenase, mostly described in some detail for plants [12,51]. Although the *P. falciparum* AOX gene was not yet identified, the metabolic pathways involved in SHAM response illustrates the existence of a similar AOX function. The failure of a comparative sequence analysis is mainly due to the extreme AT bias (80%) of the parasite genome or the high gene variability between other species and *P. falciparum* [52]. Nevertheless, an AOX-like function could play a role in the oxidative stress defence and could be a major benefit to *P. falciparum*, which is shown sensitive to hyperoxic conditions. Indeed, the respiratory metabolism was the predominant source of ROS on *P. falciparum* and partial inhibition of this metabolism led to a decrease of ROS. This hypothesis is in agreement with a publication reporting the AOX protective role in preventing ROS production [22].

Several studies on the use of mETC inhibitors underlined the crucial role of respiratory metabolism to parasite survival [9]. But, much work remains to scrutinise the specific biochemical pathways of the *P. falciparum* mitochondria [8]. This unique particularity of the alternative respiratory pathway could be a promising target for development of a new therapy as the association of antimalarial drugs and hyperbaric oxygen therapy.

Table 1: Proteins identified from the 2-D DIGE analysis in SHAM-treated *P. falciparum*.

gi number	Gene ID	Protein Name	MW (kDa)	pI	Master spot number	Significance (Mascot score)	Av. Ratio	t-test
P. falciparum								
Glycolysis								
gi|124810131	PF14_0598	glyceraldehyde-3-phosphate dehydrogenase	37.06	7.59	2448	155	1.58	0.070
					2455	67	1.64	0.024
					2495	47	1.66	0.057
					2497	178	1.36	0.19
gi|124513266	PF13_0141	L-lactate dehydrogenase	34.33	7.12	2478	146	1.96	0.056
					2518	175	1.72	0.099
gi|124802328	PF10_0155	enolase	49.02	6.21	2816	57	1.86	0.28
gi|124804024	PF11_0208	phosphoglycerate mutase	29.89	8.31	3074	48	-2.55	0.13
Translation								
gi|124809712	PF14_0486	elongation factor 2	94.54	6.36	852	346	1.50	0.039
			94.54	6.36	858	279	1.42	0.051
Chaperone assisted protein folding								
gi|124806075	PFL1070c	endoplasmin homolog (Hsp90), putative	95.30	5.28	816	1217	1.53	0.065
Merozoite invasion								
gi|3821945	PFI1475w	merozoite surface protein1	41.84	6.69	1098	64	2.20	0.078
Homo sapiens								
Redox metabolism								
gi|4502517		carbonic anhydrase I	28.91	6.59	2957	136	-2.26	0.13
					2960	160	-1.63	0.37
					2976	241	-3.72	0.031
gi|4557395		carbonic anhydrase II	29.28	6.87	3039	135	-2.26	0.092
Chaperone assisted protein folding								
gi|14124984		Chaperonin containing TCP1, subunit 3 (gamma)	60.93	6.10	1323	96	1.49	0.0043
gi|48762932		chaperonin containing TCP1, subunit 8 (theta)	60.15	5.42	1388	218	1.46	0.067
Hemoglobin digestion								
gi|4557014		catalase	59.94	6.90	1456	191	-3.75	0.033
					1457	219	-3.63	0.029
					1474	185	-3.29	0.055

gi|189054178	beta-globin	66.15	7.62	2719	267	-2.68	0.0032
				2750	244	-2.15	0.0071
				2782	154	-4.32	0.0067
				2808	112	-3.93	0.0021
Pentose phosphate cycle							
gi|5803187	transaldolase 1	37.68	6.36	2478	83	1.96	0.056
DNA replication							
gi|15029922	RNH1 protein	50.10	4.83	1878	114	1.58	0.024
Integral membrane proteins							
gi|62088410	spectrin, beta, erythrocytic variant	269.04	5.23	586	532	1.52	0.010
gi|1845265	ankyrin	204.54	5.63	610	92	1.47	0.057

The spot number corresponds to the same numbers as indicated in the figure 2. The Mascot gi number of the spots, their gene ID (gene corresponding as found in PlasmoDB), their name, the theoretical MW and pI values, as well as the corresponding Mascot score are listed for MS/MS analysis (scores, greater than 34 for *P. falciparum* and 43 for *Homo sapiens*, are considered as significant ($p<0.05$)). Paired average volume ratio (experiment SHAM versus control) and p-values (*t*-test) were obtained using Decyder software.
MW: molecular weight.

List of abbreviations

2D-DIGE: 2D-differential-gel-electrophoresis

2D-E: dimensional electrophoresis

ACN: acetonitrile

AOX: alternative oxidase

BVA: Biological Variance Analysis

C. parvum: Cryptosporidium parvum

Cy-2: cyanine 2

Cy-3: cyanine 3

Cy-5: cyanine 5

DHOD: dihydroorotate dehydrogenase

DIA: differential in-gel analysis

FC: Fold Changes

G3PDH: glycerol-3-phosphate

Hsp: heat shock protein

IEF: Isoelectric focusing

MS: mass spectrometry

mETC: mitochondrial electron transport chain

NCBInr: National Center for Biotechnology Information non-redundant

nano LC-MS/MS: nanoscale capillary liquid chromatography-tandem mass spectrometry

P. falciparum: Plasmodium falciparum

PfNDH2: type II NADH dehydrogenase

PBS: phosphate buffer saline

PMT: photomultiplier tube

Q-TOF: quadrupole orthogonal acceleration time-of-flight

ROS: reactive oxygen species

SHAM: Salicylhydroxamic acid

Trypanosoma brucei: *T. brucei*

Authors' contributions

MTM conceived the study and the design, carried out proteomic studies, participated in data analyses and wrote the manuscript. LA carried out proteomic studies, conducted proteomic statistical analyses and revised the manuscript. CT helped in the parasite culture. MP, MB and PF carried out mass spectrometry identifications. YJ conceived the study and edited the manuscript. DP initiated the project, designed the method, participated in the analyses and revised the manuscript. All authors read and approved the final manuscript.

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All authors declare that they have no conflicts of interest.

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Figure 1: Morphologic alterations of *P. falciparum* following SHAM pressure.

Phenotypic changes of *P. falciparum* asexual blood stages were observed during two parasite cycles under normoxia (A), SHAM pressure (B), hyperoxia (*i.e.*, 21% O_2) (C) and SHAM/hyperoxia conditions (D). The parasitemia and parasitic stages were evaluated by blood smears at 0, 24, 32, 48 and 78 hours. After SHAM addition following hyperoxic exposure, significant morphological alterations were observed during the two cycles of parasites. The different intraerythrocytic stages and their corresponding percentages are indicated as follows: ring (R), trophozoites (T), schizonts (S) and parasites death (D).

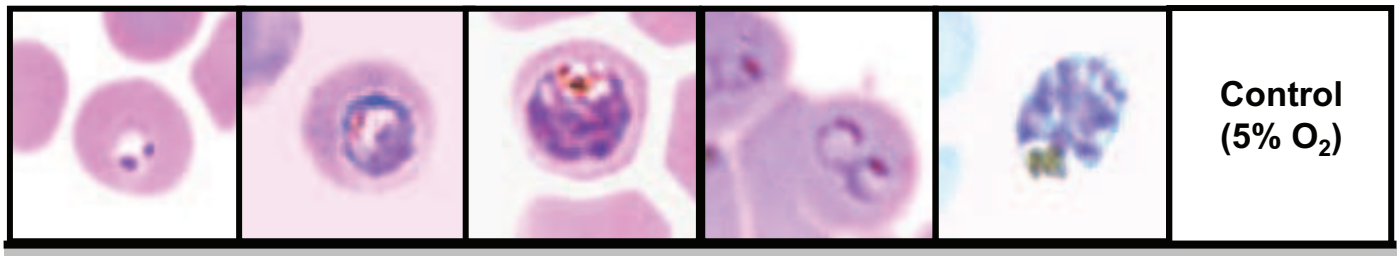
Figure 2: SHAM effect on *P. falciparum* 3D7 strain proteome.

Representative data from a 2D-DIGE experiment using a 10% homogenous SDS polyacrylamide gel with pH range from 3 to 10 are shown. The proteins from untreated *P. falciparum* parasites or treated parasites with 250 µM SHAM were labelled with Cy3 and Cy5, respectively. As determined by DeCyder software, protein spots that were up- and down-expressed on *P. falciparum* under SHAM treatment ($|FC| \geq 1.5$, $p \leq 0.05$ *t*-test) were marked with master numbers (table 1). Bold and italicised numbers correspond, respectively, to identified proteins from *P. falciparum* and *Homo sapiens*.

Figure 3: A schematic representation of the *P. falciparum* metabolic pathways perturbed under SHAM treatment.

Glycolysis and mitochondrial respiratory chain pathways based on the Ginsburg Pathway [http://sites.huji.ac.il/malaria] are represented.

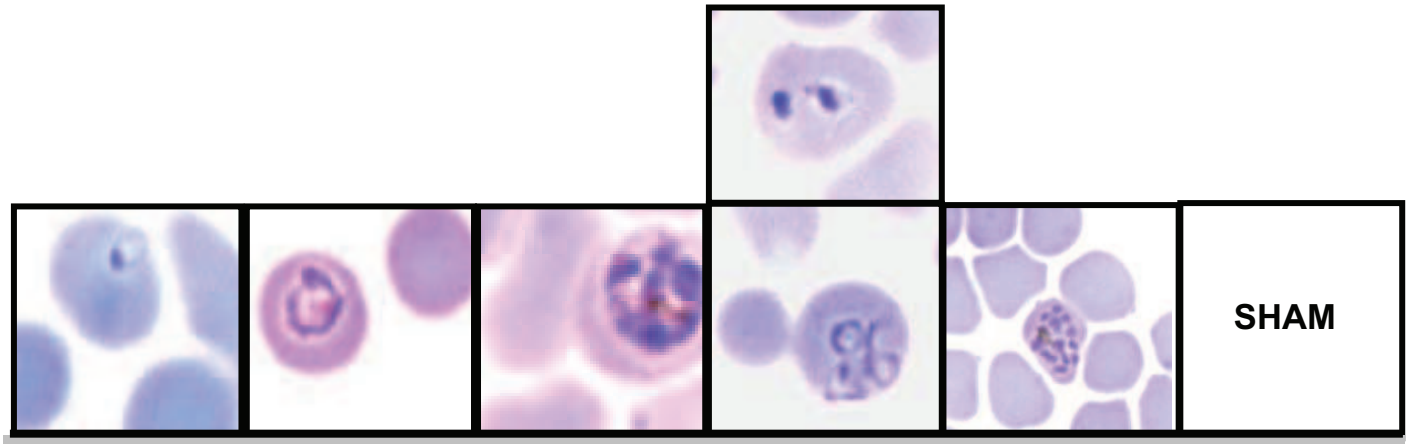
A



0.5	0.5	0.6	1.8	1.9	**Parasitemia (%)**
0	24	32	48	78	**Time (hours)**
98% R 2% S	99%T 1% R	98% S 2% T	97% R 3% S	96% S 4% T	**Stages**

Control ($5\%\ O_2$)

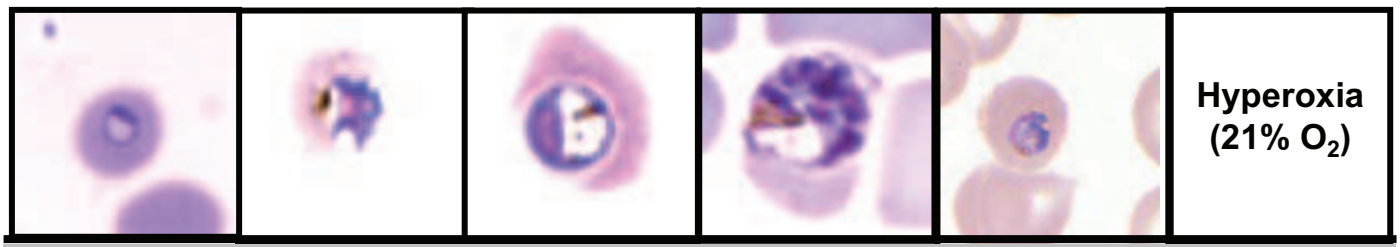
B



0.5	0.5	0.5	0.4	1.2	**Parasitemia (%)**
0	24	32	48	78	**Time (hours)**
98% R 2% S	97% T 3% S	98% S 2% T	54% D 43% R/3% S	87% S 13% T	**Stages**

SHAM

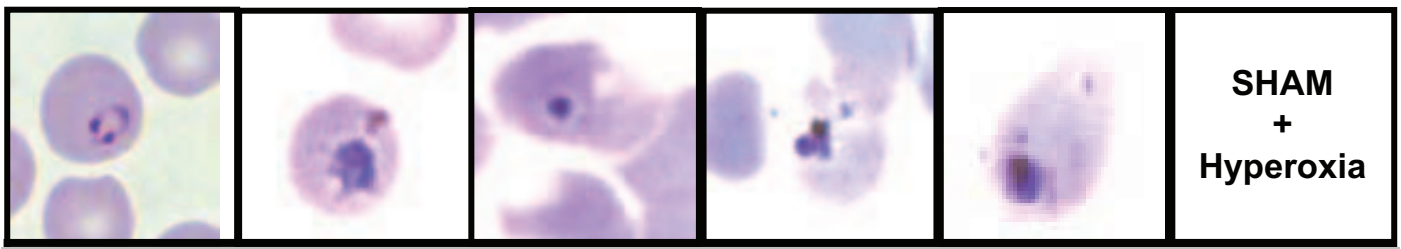
C



0.5	0.5	0.5	0.7	1.2	**Parasitemia (%)**
0	24	32	48	78	**Time (hours)**
98% R 2% S	98% T 2% R	99% T 1% S	90% S 10% R	94% T 6% S	**Stages**

Hyperoxia ($21\%\ O_2$)

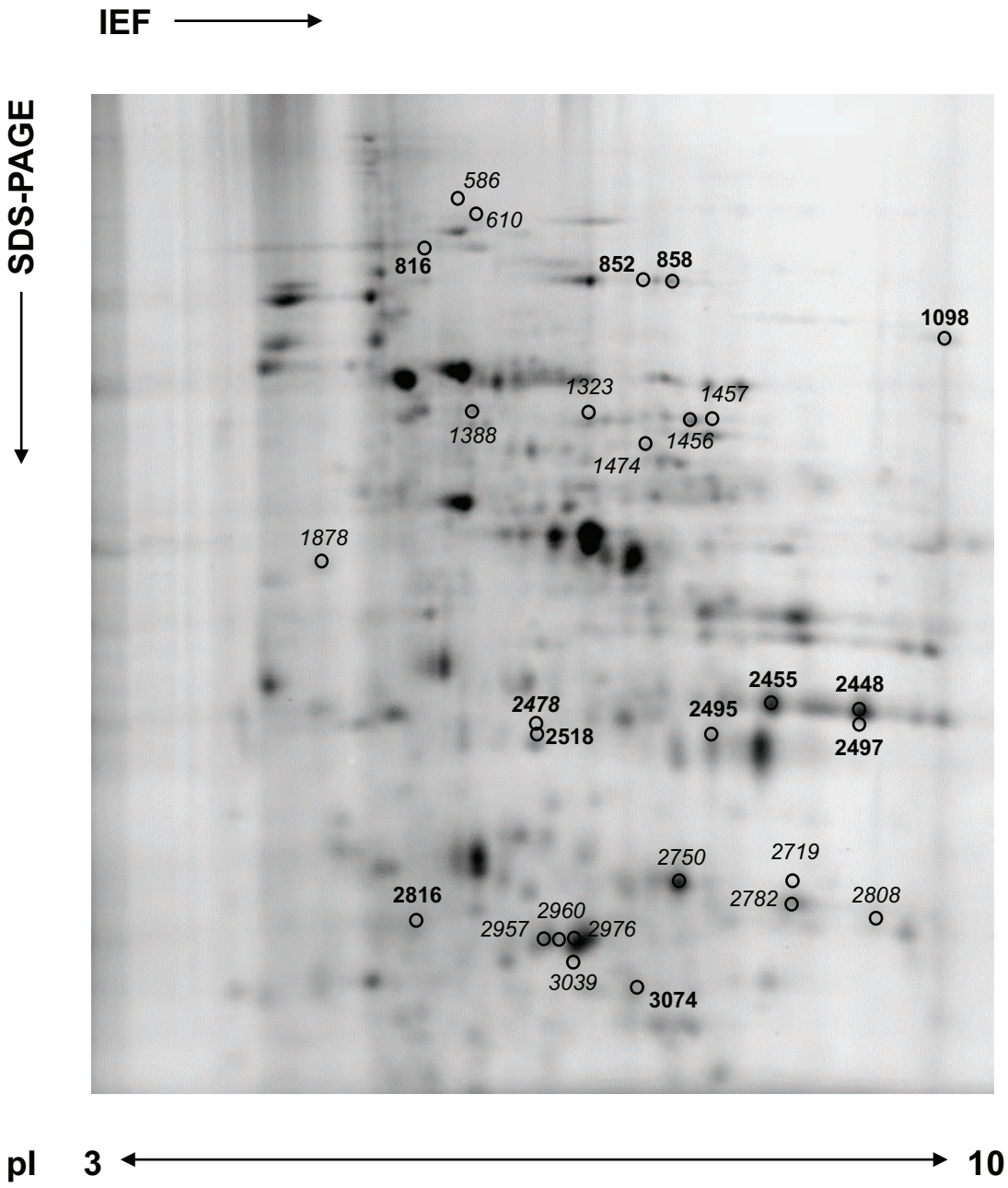
D



0.5	0	0	0	0	**Parasitemia (%)**
0	24	32	48	78	**Time (hours)**
98% R 2% S	100% D	100% D	100% D	100% D	**Stages**

SHAM + Hyperoxia

Figure 1


IEF
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2808
2960
2957
2976
3039
3074
pI
3
10

Figure 2

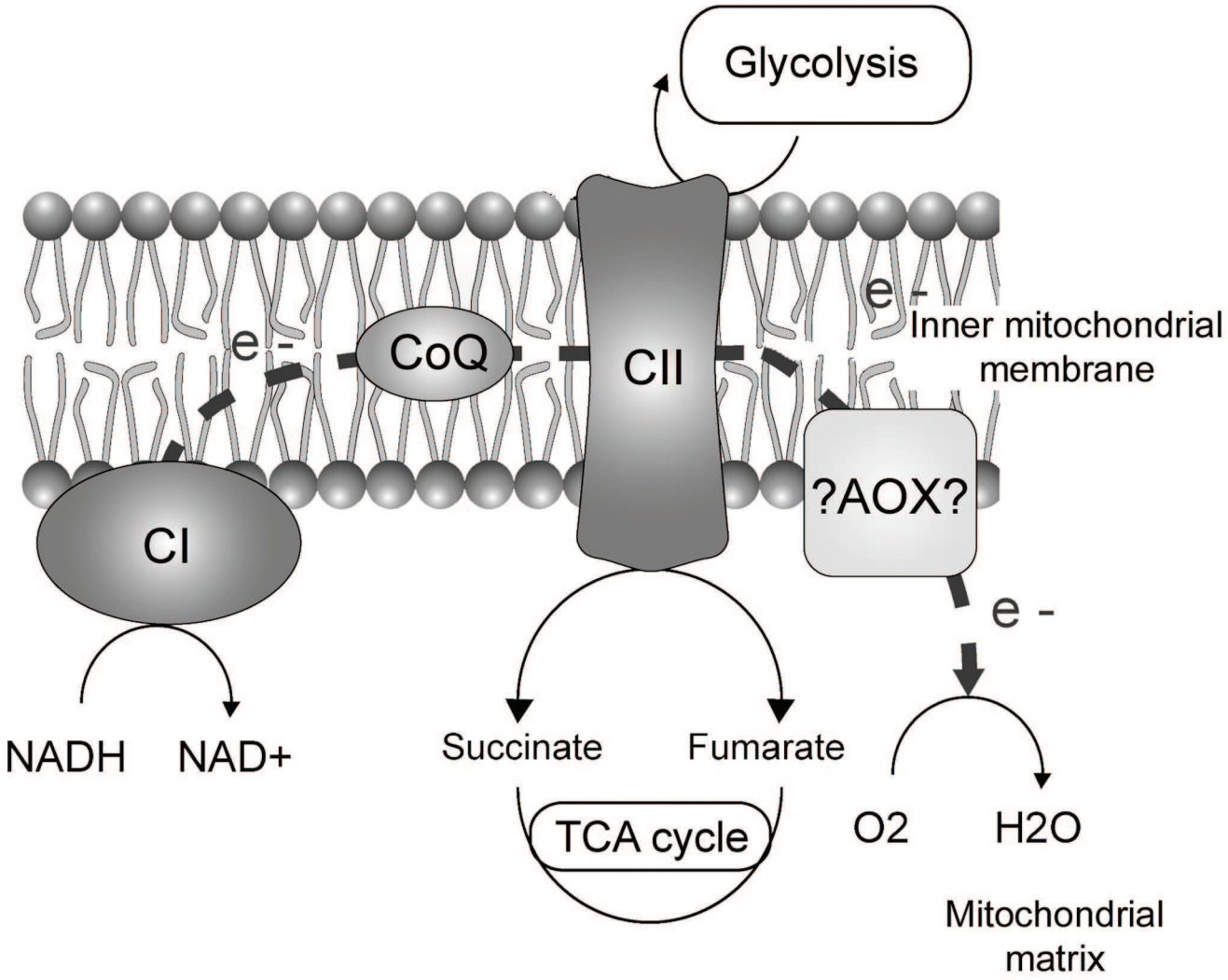
Glycolysis
e -
Inner mitochondrial membrane
e -
CoQ
CII
?AOX?
CI
e -
NADH
NAD+
Succinate
Fumarate
TCA cycle
O2
H2O
Mitochondrial matrix

Figure 3

Conclusion et Perspectives :

Le SHAM a un effet délétère sur les parasites en condition hyperoxique. Le principal métabolisme impliqué dans cette réponse est la glycolyse, responsable de l'équilibre des équivalents réducteurs (NADH). La fonction AOX, cible du SHAM, jouerait un rôle essentiel dans l'adaptation du parasite à l'hyperoxie. Chez *P. falciparum,* les alignements de séquence n'ayant pas permis d'identifier un gène AOX, pouvons-nous identifier sa protéine ?

Données complémentaires non soumise à publication :

Materials and methods

Western blot analysis

Western blotting with fluorescence-based methods including the ECL Plus western blotting detection system (GE Healthcare) was used to detect both the total protein expression profile (CyDye) and the specific immunoreactive proteins (fluorescent antibody). The same protein samples used for 2D-DIGE were labelled with Cy3 Cyanine dye. For 1-D and 2-D western blot analysis, respectively, 10 µg of total proteins and 25 mg of each Cy-labelled protein sample were reduced in Tris buffer containing dithiothreitol (1% w/v, Sigma) (Laemmli 1970). A 2-DE was performed for the 2-D western blot. Gels were transferred onto a nitrocellulose membrane (0.45-µm, GE Healthcare) using a semi-dry blotting system at 200 mA for 30 min (Towbin 1992). Membranes were saturated with 5% non-fat dried milk in phosphate buffer saline (PBS) containing 0.05% v/v tween-20 for 1 h. Western blot analysis was carried out with rabbit anti-TbAOX polyclonal antibody (*Trypanosoma brucei brucei* anti-AOX antibody, kindly provided by Minu Chaudhuri et *al.* (Chaudhuri et *al.* 1998)) that were diluted at 1/1000 in saturation buffer (PBS containing 0.05% v/v Tween-20 with 5% non-fat dried milk, 4°C). After overnight incubation, membranes were incubated with goat anti-rabbit IgG horse radish peroxidase (HRP) conjugated antibody (1/5000, Beckman Coulter, San Jose, CA, USA) and revealed using an ECL Plus western blotting detection system (GE Healthcare). All manipulations were performed in the dark. The gel, just after the 2-DE and western blot, was scanned using a TyphoonTM Trio Image scanner (GE Healthcare UK) at different excitation wavelengths (580 BP 30/green (532 nm) for Cy3 and 520 BP 40/blue (488 nm) for ECL Plus). Specific immunoreactive proteins were excised from gels and prepared for analysis by MS as previously described.

Results

Identification of protein recognised by Trypanosoma brucei brucei anti-AOX antibodies

To identify a homologue of AOX protein in *P. falciparum*, a 2-D immunoproteomic approach was performed using *Trypanosoma brucei brucei* anti-AOX polyclonal antibody (TAO). Firstly, this antibody was validated by 1-D western blotting to ensure its specificity and its cross-reactivity with orthologue proteins such as *Arabidopsis thaliana* AOX (*A. thaliana*). The antigenicity of TAO antibody against AOX was confirmed in *A. thaliana* protein samples, which was used as a positive control. The protein molecular weights obtained with this polyclonal antibody are similar to those expected, illustrating the existence of different protein isoforms and indicating that TAO antibody will be useful for the identification of the *P. falciparum* AOX (Elthon et *al.* 1989 ; Finnegan et *al.* 1999). However,

for the *P. falciparum* total protein sample, only one band was detected at expected molecular weight with TAO antibody under standard culture conditions (Figure 12).

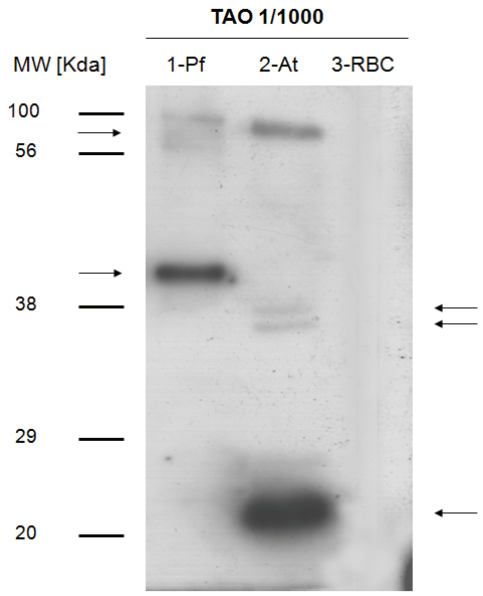

Figure 12 : Identification of specific protein spots recognised by Trypanosoma brucei brucei anti-AOX antibody.

Western blotting was performed to accurately detect immunoreactive proteins using antibodies against Trypanosoma brucei brucei AOX protein. Validation of TAO antibody specificity and cross-reactivity in P. falciparum (line 1), Arabidopsis thaliana (line 2) and RBC (line 3) total proteins. The arrows indicate the antigenic proteins identified by TAO antibody.

Secondly, a 2-D western blot combined with MS was performed to characterise *P. falciparum* proteins detected by TAO antibody. A major difficulty of this approach is to match perfectly antigenic spots detected on the 2-D western blot with their counterparts on the preparative gel, matched spots were further analysed by MS. For this reason, a 2-D western blot using a fluorescence-based method was performed. Consequently, following blot digitalisation at Cy3 and ECL Plus wavelengths, two images corresponding, respectively, to the total protein expression profile and the antigenic protein pattern were obtained. The superimposition of these two images allowed us to accurately excise spots of interest on a preparative gel run in parallel. The protein profile was used as the "internal standard" to perform a perfect match between the blot and the preparative gel. Five immunoreactive spots were recognised on the 2-D western blot by TAO antibody in normal culture conditions (Figure 13) and under hyperoxic conditions (data not shown). These five antigenic spots were excised and submitted for MS identification. The five protein spots were successfully identified using *P. falciparum* protein databases (NCBInr), three correspond to the 70 kDa heat shock protein (Hsp70) and two to hypothetical proteins (Table 3).

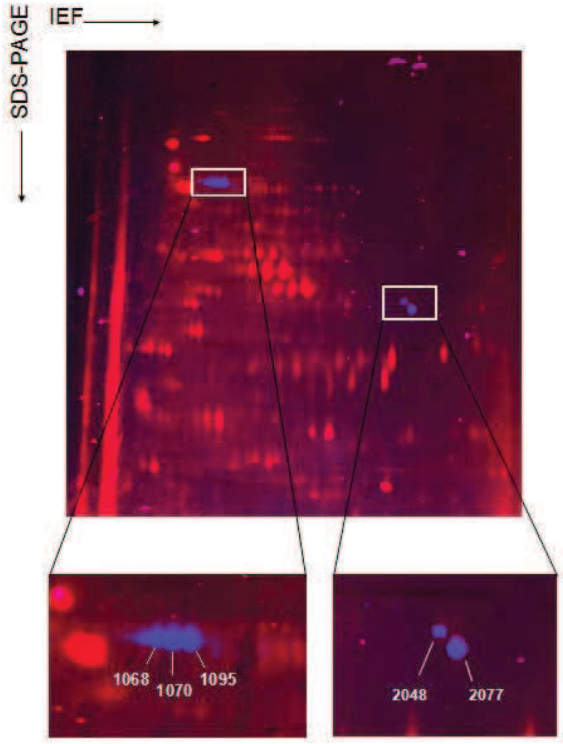

Figure 13 : Identification of specific protein spots recognised by Trypanosoma brucei brucei anti-AOX antibody.

2-D western blotting was performed to accurately detect immunoreactive proteins using antibodies against Trypanosoma brucei brucei AOX protein. Merged images of 2-D western blot pattern of the same protein sample used in figure 12 pre-labelled with Cy3 (red) and revealed with a goat anti-rabbit IgG HRP-conjugate against Trypanosoma brucei brucei AOX (blue). On the 2-D western blot pattern using a fluorescence-based method, immunoreactive protein spots detected are boxed. Box gels areas containing the immunoreactive spots are enlarged. The numbers indicate the antigenic spots identified by mass spectrometry (spot numbers : 1068-1070-1095-2048-2077, see table 3).

Table 3. P. falciparum proteins identified from the western blot 2-D analysis using *Trypanosoma brucei brucei* anti-AOX antibody.

gi number	Gene ID	Protein Name	MW (kDa)	pI	Master spot number	Significance (Mascot score)
gi|124512406	PF08_0054	heat shock 70 kDa protein	74.38	5.51	1068	205
gi|124512406	PF08_0054	heat shock 70 kDa protein	74.38	5.51	1070	257
gi|124512406	PF08_0054	heat shock 70 kDa protein	74.38	5.51	1095	346
gi|124513134	MAL13P1.102	hypothetical protein	89.1	9.07	2048	39
gi|124511866	MAL7P1.77	hypothetical protein	61.14	7.12	2077	38

The proteins were identified by mass spectrometry following in gel trypsin digestion. The spot number corresponds to the same numbers as indicated in the figure 13. The Mascot gi number of the spots, their gene ID (gene corresponding as found in PlasmoDB), their name, the theoretical MW and pI values, as well as the Mascot score are listed for MS/MS analysis (scores, greater than 34, are considered as significant ($p<0.05$)). MW : molecular weight.

On the other hand, an immunoprecipitation assay was performed with this TAO antibody in culture atmospheric and hyperoxic conditions; and the proteins identified by MS were defined as hypothetical proteins (PFF0445w and MAL7P1.167, Table 4).

Table 4. *P. falciparum* proteins identified from the immunoprecipitation assay using *Trypanosoma brucei brucei* anti-AOX antibodies.

gi number	Gene ID	Protein Name	MW (kDa)	pI	Coverage (%)	Significance (Mascot score)
gi|86170764	PFF0445w	hypothetical protein	72.45	6.57	1	35
gi|124512136	MAL7P1.167	hypothetical protein	33.19	9.28	1	35

The proteins were identified by mass spectrometry following in gel trypsin digestion. The Mascot gi number of the proteins, their gene ID (gene corresponding as found in PlasmoDB), their name, the theoretical MW and pI values, as well as the Mascot score are listed for MS/MS analysis (scores, greater than 34, are considered as significant ($p<0.05$)). MW : molecular weight.

Discussion

Research on the P. falciparum alternative oxidase (AOX)

The AOX is an ubiquinol oxidase, branched upstream from the ubiquinol-cytochrome c oxidoreductase (complex III), where it sustains cyanide-resistant respiration. Evolutionarily related to other di-iron proteins, this protein has a large distribution among eukaryotic lineages (Suzuki et *al.* 2004 ; Vanlerberghe et *al.* 1997). Using monoclonal antibodies or comparative sequence analysis, AOX has been identified in a wide variety of plants and also in some algae, fungi, eubacteria and protists (Elthon et *al.* 1989). Plant AOX is a low molecular weight mitochondrial protein (approximately 30-40 kDa) and can be localised in two cellular compartments : mitochondria (plants, *Trypanosoma brucei brucei*, *Cryptosporidium parvum*) (Atteia et *al.* 2004 ; Chaudhuri et *al.* 1998 ; Roberts C. W. et *al.* 2004) and plastids (plants) (Atteia et *al.* 2004). In plant mitochondria, AOX is a disulfide-linked dimmer protein that is active in the reduced state in the presence of a-keto acids (e.g., pyruvate) (Oliver et *al.* 2008 ; Sluse et *al.* 1998). Thus, long thought to be specific to higher plants, AOX is now being located in many non-photosynthetic eukaryotes. For example, AOX protein (TbAOX) has been identified in *Trypanosoma brucei brucei* (*T. brucei brucei*) and this protein exists as a monomer on the mitochondrial membrane (Chaudhuri et *al.* 1998 ; Chaudhuri et *al.* 2005). TbAOX appears to play an important role in energy metabolism and maintaining redox balance, the *T. brucei brucei* alternative respiratory pathway appears to be beneficial to cope with environmental factors such as oxygen fluctuation (Chaudhuri et *al.* 2006). Such a role would be a major benefit to *P. falciparum*, which is subjected to different O2 pressure during its human life cycle.

In 2002, the *P. falciparum* genome was completely sequenced and represented a new starting point in molecular biological research (Winzeler 2008). However, 60% of the predicted genes could not be assigned to orthologous functions and were assigned as hypothetical proteins. To identify a *P. falciparum* AOX gene in our laboratory, we recently performed a BLAST search (15 August 2010) by comparative sequence analysis of the published sequences from plants, fungi and parasites. However, no AOX was identified in the *P. falciparum* genome, including the mitochondria and apicoplast (vegetal origin) genomes. The *P. falciparum* genome is AT-rich and possesses numerous sequences supposed to be originated from algae by gene transfers (Funes et *al.* 2002 ; Waller et *al.* 2003). For this

reason, the research of the *P. falciparum* AOX gene is not incoherent because AOX is largely described on plants. The failure of this approach is mainly due to the extreme AT bias (80%) of the parasite genome or the high gene variability between other species and *P. falciparum* (Paila et *al.* 2008).

Alternatively, a proteomic approach was performed by 2D western blot using polyclonal antibody raised against TbAOX (Chaudhuri et *al.* 1998). Even if these antibodies specifically recognised *A. thaliana* AOX, no immunoreactive band had been identified as *P. falciparum* AOX by MS. Three identified protein spots corresponded to Hsp70 in culture atmospheric conditions. Several reasons can been hypothesised : (i) polyclonal aspect of antibody, (ii) copurification of TbAOX and Hsp70 proteins to produce antibodies (Pastorino et *al.* 2008 ; Wurtz et *al.* 2009), and (iii) the recognition of similar epitopes between TbAOX and *P. falciparum* Hsp70 by TAO antibody. However, two hypothetical proteins (MAL13P1.102 and MAL7P1.77, Table 3) were recognised by 2D western blot using TAO antibody and two others (PFF0445w and MAL7P1.167, Table 4) by immunoprecipitation assay with this same TAO antibody. But the current annotation of these hypothetical proteins does not allow identifying them as potential oxidases.

The possible subcellular localisation of *P. falciparum* AOX from the mitochondria or apicoplast suggested that AOX could be under-represented in *P. falciparum* total protein. Indeed, it is possible that the *P. falciparum* AOX like was undetectable in unfractionated samples. To enrich the sample in specific protein, the identification could be carried out on protein extracts of the fractions enriched with mitochondria/apicoplasts or different conditions inducing AOX up-expression due to relative abundance of this protein. This last suggestion appears most adept for studying mitochondrial protein in regard to the number of mitochondria per parasite (1 for intraerythrocytic stage and 6 for gametocytes) (Torrentino-Madamet M. et *al.* 2010).

De récentes études supportent l'existence d'une voie respiratoire alternative chez *P. falciparum* comme illustrée par l'identification d'une NADH déhydrogénase alternative, largement décrite dans le règne végétal (Biagini et *al.,* 2006 ; Fisher et *al.,* 2007). Cependant, des questions demeurent : existe-t-il une protéine AOX chez *P. falciparum* ? Quel est le rôle de cette enzyme ou d'une enzyme SHAM-sensible encore inconnue dans le métabolisme de *P. falciparum* ?

DISCUSSION et PERSPECTIVES

La vie des plasmodies est une véritable gageure, car outre une prolifération considérable (de 18 à 36 cellules filles en 48 heures dans la schizogonie érythrocytaire à plusieurs milliers dans la schizogonie hépatocytaire), outre la nécessité de s'introduire dans différents types cellulaires, ces cellules doivent s'adapter à de multiples stress environnementaux. Au cours de ce travail de thèse, nous nous sommes intéressés aux conséquences de l'hyperoxie sur un organisme réputé microaérophile (Scheibel et *al.*, 1979).

Chez les eucaryotes, les mitochondries sont le site de synthèse d'une grande quantité d'ATP utilisée par les cellules. Cette synthèse d'ATP est assurée par l'oxydation des substrats représentés essentiellement par les acides gras et le pyruvate, produit terminal de la glycolyse. Acide gras et pyruvate sont transformés, dans la mitochondrie, en acétyl-coenzyme A, substrat du cycle de Krebs. Ils sont totalement dégradés et des coenzymes (NAD^+ et FAD^+) sont réduites. Ces coenzymes se régénèrent grâce à la chaîne respiratoire et permettent simultanément la synthèse d'ATP. Par contre, chez *P. falciparum*, la principale source d'ATP proviendrait de la glycolyse du fait de sa microaérophilie (Fry et *al.*, 1990). Mais tous les gènes codant pour les enzymes du cycle de Krebs ont été identifiés et une chaîne de phosphorylation oxydative semblerait fonctionnelle (Gardner et *al.*, 2002 ; Mather et *al.*, 2010).

Ainsi, la phosphorylation oxydative plasmodiale jouerait particulièrement plusieurs rôles qui permettraient :

- de générer un potentiel de membrane nécessaire au transport des métabolites et des protéines à travers la membrane mitochondriale ;

- de réoxyder de nombreuses enzymes du métabolisme pour permettre le fonctionnement des différentes voies métaboliques, comme la voie de biosynthèse *de novo* des pyrimidines ;

- de réguler la production de ROS.

Chez les eucaryotes, le potentiel transmembranaire permet la synthèse d'ATP via l'ATP synthase par transfert de protons, notion contestée chez *P. falciparum* due à l'échec de l'identification de la totalité des gènes de l'ATP synthase. Nous avons mis en évidence une surexpression des transcrits des différentes sous-unités de cette enzyme en condition d'hyperoxie. La découverte récente des gènes de quatre sous-unités de l'ATP synthase (dont ATP4 et ATP6), nous a permis de vérifier que la majorité des transcrits du complexe ATP synthase était effectivement surexprimée, laissant supposer un rôle fonctionnel de cette

enzyme dans l'adaptation à l'hyperoxie (Mogi et Kita, 2009). Cet état physiologique a été décrit *in vivo* par l'équipe de Daily ainsi que par notre laboratoire sur des prélèvements sanguins de patients impaludés (données non publiées). L'analyse transcriptionnelle de parasites issus de prélèvements sanguins montre une induction d'un groupe de gènes associé à la phosphorylation oxydative avec une répression des gènes de la glycolyse (Daily et *al.*, 2007). Ces données sont en accord avec notre hypothèse d'adaptation métabolique. Les parasites présenteraient différents états énergétiques associés aux différentes pressions partielles d'oxygène chez l'hôte.

Certaines particularités fonctionnelles de la mitochondrie plasmodiale permettraient au parasite de s'adapter rapidement à ces différents niveaux d'oxygène. En effet, le complexe II, comme chez les autres eucaryotes, possède une activité succinate-ubiquinone réductase (condition aérobique), mais peut également avoir une fonction quinol-fumarate réductase absente chez les organismes aérobies et présente chez les bactéries anaérobies (Takashima et et *al.*, 2001 ; Kita et et *al.*, 2001). L'activité du complexe II est indispensable pour la régénération de l'ubiquinone (ou coenzyme Q), accepteur d'électrons de la dihydrorotate déshydrogénase, enzyme essentielle pour la synthèse *de novo* des pyrimidines (Painter et *al.*, 2007) (Figure 14). Ces notions pourraient expliquer l'absence d'effets délétères dans nos études phénotypiques en condition hyperoxique.

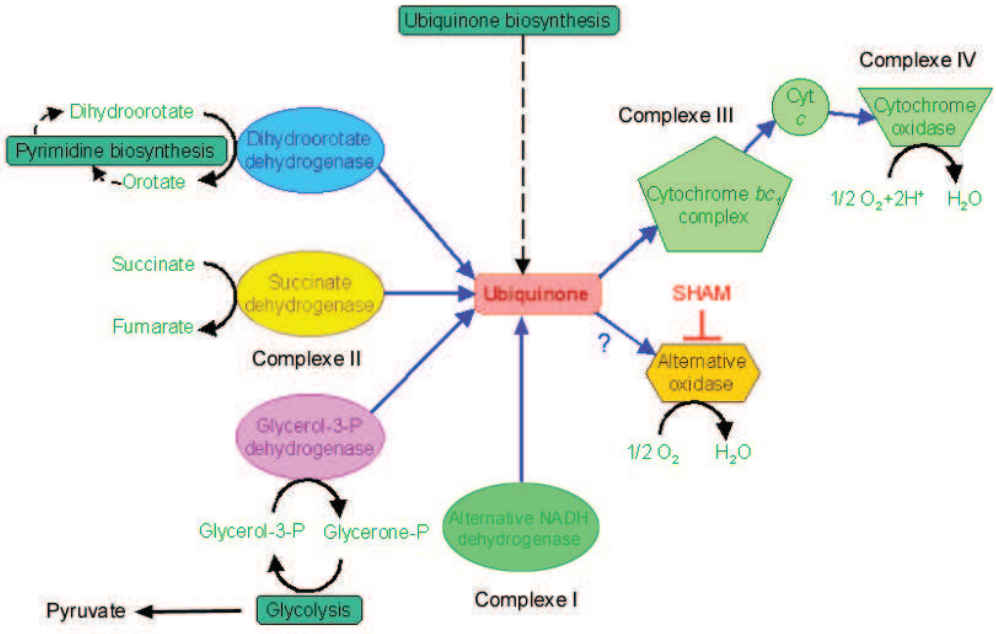

Figure 14. Flux d'électrons mitochondrial chez P. falciparum.
(Ginsburg [http ://sites.huji.ac.il/malaria/], 2006)

L'ubiquinone est une molécule très ubiquitaire, localisée dans les endo-membranes cellulaires. Agent antioxydant puissant, elle empêche la formation de ROS et prévient ainsi de l'oxydation membranaire et de la peroxydation des lipides. Chez les organismes sensibles à l'oxydation comme *P. falciparum*, l'équilibre des ROS est primordial mais encore largement inexploré (Golenser et *al.*, 1991).

La mitochondrie apparaît alors essentielle comme régulateur dans la réponse au stress oxydatif (Oliveira et *al.*, 2002). Chez *P. falciparum*, la mitochondrie et la vacuole digestive sont à l'origine de la production de ROS (Figure 15).

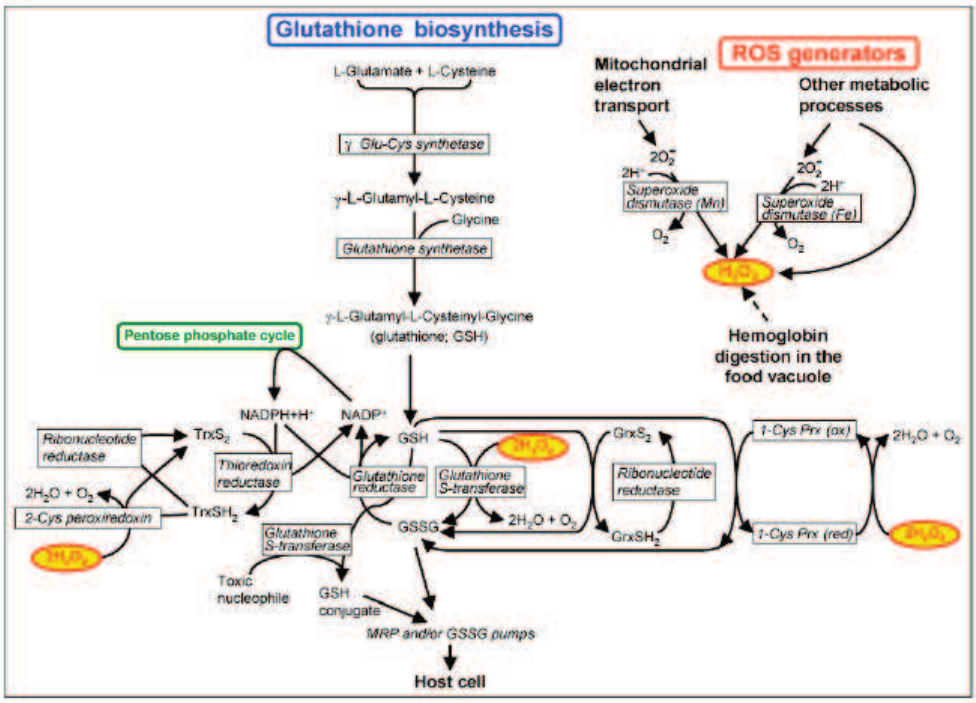

Figure 15. Sources de stress oxydatif chez P. falciparum.
(Bozdech et al., 2004)

Dans notre étude, le stress oxydatif induit par l'hyperoxie entraîne des perturbations du métabolisme énergétique telles qu'une inhibition de la glycolyse en faveur de la respiration mitochondriale et un ralentissement du métabolisme de la vacuole digestive. L'action combinée sur les métabolismes de la mitochondrie et de la vacuole digestive permet de maintenir un faible taux de ROS en condition hyperoxique. L'action sur la vacuole digestive entraîne un déficit d'apport en acides aminés pouvant expliquer le retard de croissance au moment de la schizogonie. La surexpression des protéines chaperonnes («heat-shock»), observée dans nos analyses de protéomique, complètent la panoplie des moyens de protection

contre les ROS. Cette famille de protéines «heat-shock», incluant la Hsp70, joue un rôle essentiel dans les processus de translocation, de stabilisation et d'assemblage des protéines. Ces protéines interviennent dans la réparation des dommages oxydatifs induits au niveau de l'ADN et des protéines par un stress oxydatif (Acharya et *al.*, 2007).

Un autre mécanisme de lutte contre les ROS a été décrit dans le règne végétal et est dépendant d'une protéine AOX qui permet le transfert direct d'électrons de l'ubiquinone à l'O_2 sans passer par les complexes III et IV (Sluse et Jarmuszkiewicz, 1998). Ce mécanisme a été identifié également chez certains protistes comme *Trypanosoma brucei brucei* (Chaudhuri et *al.*, 2006). Son rôle physiologique serait de recycler rapidement le pool d'ubiquinone quand l'activité respiratoire est élevée, et ainsi de protéger la mitochondrie du stress oxydatif en limitant la formation de ROS (Sluse et Jarmuszkiewicz, 1998). Ce système est indispensable pour la survie de *T. brucei brucei* chez l'hôte puisqu'il est totalement dépendant de la glycolyse pour la production d'ATP et pour réoxyder les équivalents réducteurs (NADH) produits pendant la glycolyse (Figure 16).

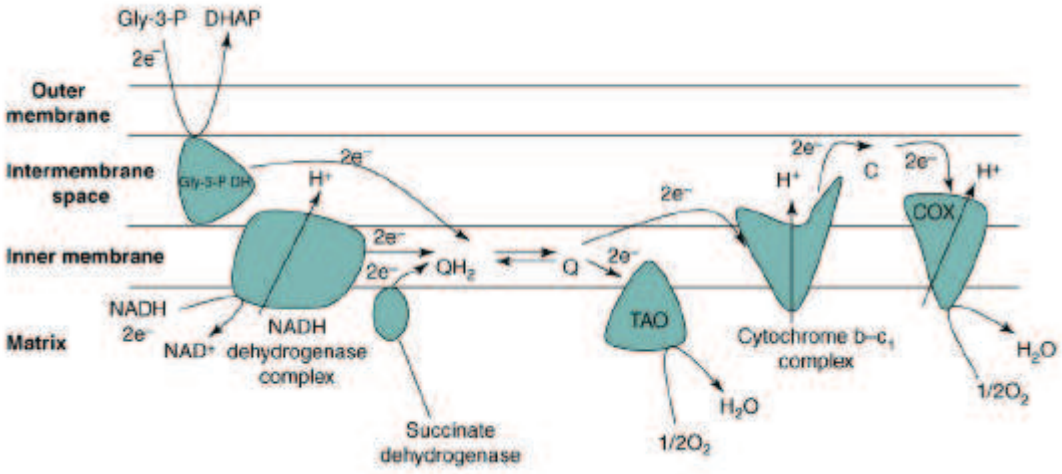

Figure 16. Flux d'électron mitochondrial chez Trypanosoma brucei brucei. (Chaudhuri et al., 2006)

Chez *P. falciparum*, l'existence d'une fonction AOX a été mise en évidence par différentes études (Murphy et Lang-Unnasch, 1999 ; Murphy et *al.*, 1997 ; Suswan et *al.*, 2001). Cette fonction AOX permettrait au parasite d'échapper aux variations de taux d'O_2 imposées par l'environnement dans lequel il évolue. La présence d'une telle voie jouerait un rôle primordial dans la biologie du parasite très sensible au stress oxydatif. A ce jour, aucune protéine AOX plasmodiale n'a été identifiée. Mais nos résultats apportent des arguments en

faveur de l'existence d'une fonction AOX. Le traitement des cultures par le SHAM, inhibiteur spécifique de l'AOX, a un effet totalement délétère sur les parasites maintenus en hyperoxie. Une fonction AOX permettrait au parasite de s'adapter à un environnement hyperoxique en maintenant un taux de ROS compatible avec sa survie.

Ce travail de thèse nous a permis de mettre en place les techniques de transcriptomique et protéomique au sein de notre laboratoire. La maîtrise du cycle érythrocytaire du parasite et plus particulièrement, une fine synchronisation des cultures, nous a permis de circonvenir aux problèmes dus au retard de cycle pouvant perturber les analyses transcriptionnelles.

Un élément marquant de la biologie du parasite a été mis en évidence comme l'importance de l'ATP dans la réponse hyperoxique au niveau de la mitochondrie et de la vacuole digestive.

Il reste néanmoins un énorme travail :

- d'analyse protéomique de la mitochondrie plasmodiale que nous avons initiée par les techniques de fractionnement d'organites en électrophorèse en flux (Free Flow Electrophoresis, FFE) ;
- d'analyse par les techniques d'imagerie en temps réel qui permettent de visualiser la morphologie et les comportements mitochondriaux en présence de différentes drogues ;
- de validation biochimique de la voie de phosphorylation oxydative (dosage du lactate et de l'ATP) dans le cycle érythrocytaire de *P. falciparum* ;
- et enfin, d'élucidation de l'identification des molécules responsables de la fonction AOX et du rôle de l'apicoplaste.

P. falciparum présente des voies métaboliques qui lui sont spécifiques par rapport aux autres eucaryotes, et par conséquent font de la mitochondrie plasmodiale une cible thérapeutique attractive. L'association d'antipaludiques spécifiques du métabolisme mitochondrial (exemple l'atovaquone) avec une oxygéno-thérapie hyperbarique permettrait d'envisager une nouvelle thérapie pour l'infection à *P. falciparum*.

Les connaissances sur la biologie de *P. falciparum* sont un pas essentiel dans la lutte contre le paludisme.

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PUBLICATIONS

Publications

Dans le cadre de cette thèse

1. Briolant, S., Parola, P., Fusai, T., Madamet-Torrentino, M., Baret, E., Mosnier, J., Delmont, J.P., Parzy, D., Minodier, P., Rogier, C., and Pradines, B. (2007). **Influence of oxygen on asexual blood cycle and susceptibility of Plasmodium falciparum to chloroquine: requirement of a standardized in vitro assay.** *Malar Journal* **6**, 44.

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Congrès et Présentations

Dans le cadre de cette thèse

Congrès des Doctorants « Sciences de l'Environnement »
Agora des Sciences à Marseille -13 et 14 Mars 2007
Prix du Poster « Influence des conditions environnementales sur le transcriptome de *Plasmodium falciparum* »

Journée de la Recherche
Faculté de Pharmacie de Marseille - 28 Mars 2007
Présentation Orale « Etude du métabolisme respiratoire de *Plasmodium falciparum* »

Congrès de Bioinformatique
Museum d'Histoire Naturelle à Paris
21 et 22 Janvier 2009

6éme Congrès Européen de Médecine Tropicale et Maladies Internationales
Vérone – Italie
6 au 10 Septembre 2009

Annexes

Additional Files de l'article 2

"Global response of Plasmodium falciparum to hyperoxia : a combined transcriptomic and proteomic approach"

Additional file 1
Primers sequence using real-time qRT-PCR.

Additional file 2
Single-Peptide-Based Protein Identifications.

Additional file 3
Raw data microarray at 8 hours time point under hyperoxia versus normoxia conditions on synchronised parasites.

Additional file 4
List of altered genes following hyperoxia treatment using GSEA data and PS 6.0 software.

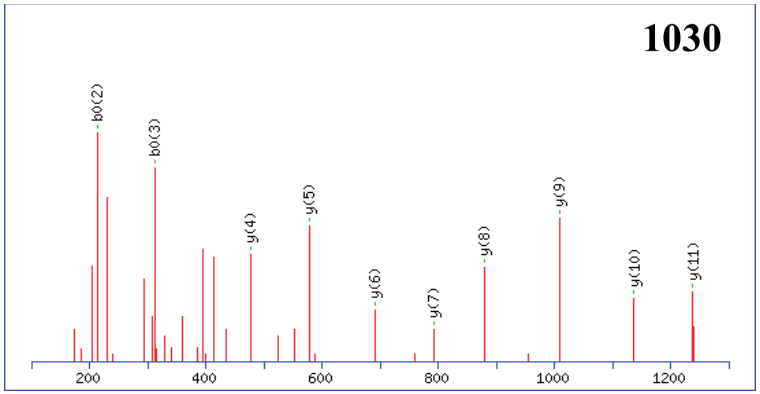
Additional file 5
MS/MS peptide sequences, respective gi number, gene ID and master spot number of proteins identified from the differential 2-D DIGE analysis following hyperoxia exposure of P. falciparum.

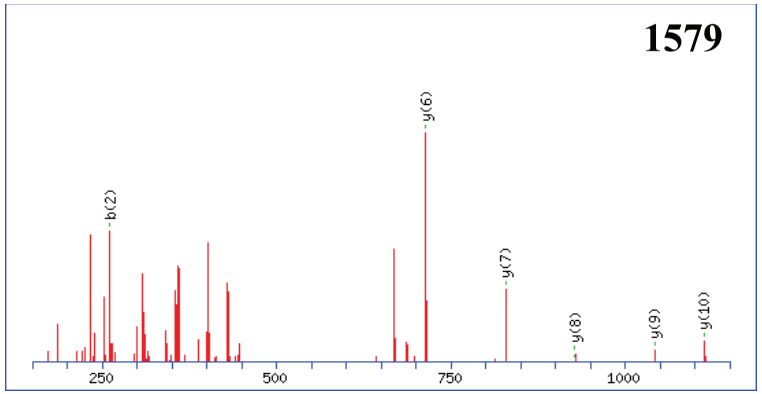
Additional file 6
Raw data microarray at 4 hours time point under hyperoxia versus normoxia conditions on synchronised parasites.

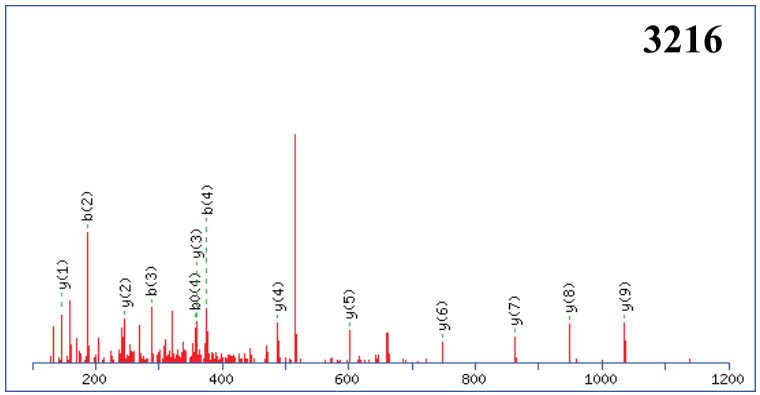
Additional file 1: Primers sequence using real-time qRT-PCR.

Accession Number [a]	Name Primers	Forward (F) and reverse (R) sequencing primers
MAL7_18Sa	*rRNA18S*/F	5' GCTGACTACGTCCCTGCCC 3'
	rRNA18S/R	5' ACAATTCATCATATCTTTCAATCGGTA 3'
PF14_0077	*plasmepsin 2*/F	5' TTAGATGTTATCAAAGTCCCATTCTTACC 3'
	plasmepsin 2/R	5' CAGGTTCTAATGTGTATTTACCATTTTCTG 3'
PF11_0161	*falcipain 2p61*/F	5' GCAGCTTATGATTGGAGATTACATAGTG 3'
	falcipain 2p61/R	5' AGCATATTGTGATTCTACGGAACCTATACT 3'
PF11_0165	*falcipain 2p65*/F	5' AGAAGGTATTTTCGATGGAGAATGTG 3'
	falcipain 2p65/R	5' TGTTGTCCCCATGAGTTCTTAATTATATAA 3'
PF11_0162	*falcipain 3*/F	5' GAGAATGTGGAGCAGCACCAA 3'
	falcipain 3/R	5' CAGATCCCCATGGTTTTTAATGATATAA 3'
PF14_0598	*gapdh*/F	5' GAAGGTCCACTTAAAGGAATCTTAGGAT 3'
	gapdh/R	5' TTCATGTCAAAGATTGATGATCTGTTATC 3'
PF10_0245	*gf6p*/F	5' AGAACTGGCTTATATACATTGTGAAGGTTT 3'
	gf6p /R	5' TTACAGGGATATTGTCTTCACCACCTA 3'
PF14_0187	*gst*/F	5' AACGGTGATGCTTTTGTTGAATT 3'
	gst /R	5' GCTTTGAGCTAATATCAAATCTCCAA 3'
PF11_0087	*rad51*/F	5' TAACCAAGTCGTTGCCAAGGT 3'
	rad51/R	5' CATGAGCTATTATGTTTCCACCTATAGG 3'
PFL1110c	*pkareg*/F	5' GGAGAATTAGCTCTTCTCAAAAATAAACC 3'
	pkareg /R	5' GTCCTAATAATCTTTTGAAACTTTTTCTATCTAAATA 3'
PFI1685w	*pkacat*/F	5' AGAAATTTTATTGAACGTCGGACAT 3'
	pkacat /R	5' AGGGTTCATTCGCATAAAAAGG 3'
PF10_0059	*atp6/F*	5' AAGCAAAACAATATTCTTCTTATCGATT 3'
	atp6/R	5' TGTGTAAAGATTTTGATTTGTCATAGGAA 3'
MAL7P1.13	*atp4/F*	5' ATTTACAGTAAACAATTTTCTCTCAGTCAAA 3'
	atp4/R	5' ATACTGAAATGTAAGCCACTTAATCTTTTC 3'

[a] Accession Number from Plasmodium Genome Resource database [www.plasmodb.org]
Foodnotes: *gapdh*: glyceraldehyde-3-phosphate dehydrogenase - *gf6p*: glutamine-fructose-6-phosphate transaminase - *gst*: glutathione S-transferase - *pka*: Protein kinase A - *atp6*: mitochondrial ATP synthase F0 *a* subunit – *atp4*: mitochondrial ATP synthase F0 *b* subunit.







Spot number	Protein name	Accession no. (NCBI)	Sequence identified	precursor m/z and charge observed	Ions score	Expect
1030	elongation factor 2 [Plasmodium falciparum]	gi|8918238	ETVTEESTITCLGK	1566.76565	67	5.50E-06
1579	Glucose-6-phosphate isomerase [Plasmodium falciparum]	gi|120743	FLANVDPNDVNR	1372.67145	36	9.40E-03
3216	proteasome beta-subunit [Plasmodium falciparum]	gi|124512686	DATSSNFIQIVK	1321.70925	58	5.30E-05

Additional file 2: Single-Peptide-Based Protein Identifications.

Additional file 3: Raw data microarray at 8 hours time point under hyperoxia versus normoxia conditions on synchronised parasites.

Accession Number	Sequence Names	FC E1	FC E2	FC E3	*p*-value *t-test*	FC Average	Standard Deviation
PFA0440w	hypothetical protein. conserved	1,078	1,052	1,081	0,00771	1,070	0,0161
PF14_0449	hypothetical protein	1,077	1,105	1,084	0,00323	1,089	0,0144
PF11_0415	hypothetical protein	-1,431	-1,366	-1,240	0,00264	-1,346	0,0968
PF14_0705	hypothetical protein	1,334	1,153	1,229	0,00913	1,239	0,0910
PFF1400w	RAP protein. putative	1,170	1,162	1,127	0,00115	1,153	0,0226
PF14_0451	mitochondrial ribosomal protein S14 precursor. putative	-1,216	-1,179	-1,097	0,00678	-1,164	0,0605
PF13_0292	hypothetical protein	-1,191	-1,285	-1,158	0,00318	-1,211	0,0660
PFD1090c	clathrin assembly protein. putative	1,049	1,037	1,032	0,00240	1,039	0,0091
PF11_0268	hypothetical protein	-1,150	-1,183	-1,143	0,00959	-1,159	0,0214
PF11_0254	hypothetical protein	1,104	1,098	1,087	0,00035	1,097	0,0087
PF14_0338	hypothetical protein	1,239	1,238	1,133	0,00886	1,203	0,0609
PF07_0131	erythrocyte membrane protein 1 (PfEMP1) pseudogene	1,280	1,212	1,173	0,00240	1,222	0,0541
PF10_0197	hypothetical protein	1,077	1,050	1,068	0,00166	1,065	0,0135
MAL13P1.159	hypothetical protein. conserved	-1,077	-1,097	-1,154	0,00758	-1,109	0,0401
PF14_0474	hypothetical protein	-1,147	-1,173	-1,079	0,00575	-1,133	0,0484
PF13_0155	hypothetical protein. conserved	1,149	1,275	1,170	0,00415	1,198	0,0675
PFD0395c	hypothetical protein. conserved	-1,141	-1,251	-1,141	0,00533	-1,178	0,0636
PFI1440w	hypothetical protein. conserved	1,193	1,255	1,251	0,00039	1,233	0,0345
PFC0335c	hypothetical protein. conserved	1,089	1,141	1,155	0,00252	1,128	0,0352
PF14_0198	glycine -- tRNA ligase. putative	1,040	1,058	1,064	0,00814	1,054	0,0127
PFL1350w	RNA pseudouridylate synthase. putative	-1,321	-1,194	-1,317	0,00230	-1,277	0,0724
PF10_0319	hypothetical protein	1,365	1,318	1,369	0,00100	1,351	0,0285
MAL13P1.350	hypothetical protein. conserved	1,251	1,322	1,259	0,00367	1,277	0,0390
PFC0490w	dolichyl-diphospho-oligosaccharide protein	1,277	1,280	1,113	0,00985	1,223	0,0956
PF07_0125	hypothetical protein. conserved	1,150	1,140	1,202	0,00400	1,164	0,0334
PF13_0307	hypothetical protein. conserved	1,066	1,103	1,121	0,00306	1,097	0,0278
PFF0600w	hypothetical protein. conserved	1,137	1,140	1,083	0,00293	1,120	0,0321
PF10_0170	hypothetical protein	-1,049	-1,049	-1,072	0,00195	-1,056	0,0135
PFE0500c	hypothetical protein. conserved	1,286	1,323	1,198	0,00149	1,269	0,0643
MAL8P1.50	hypothetical protein. conserved	1,191	1,128	1,145	0,00172	1,155	0,0325
PFE0130c	hypothetical protein. conserved	1,137	1,130	1,135	0,00513	1,134	0,0035
PFB0535w	GDP-fructose:GMP antiporter. putative	1,146	1,159	1,082	0,00600	1,129	0,0412

PF14_0136	hypothetical protein. conserved	-1,103	-1,080	-1,115	0,00169	-1,099	0,0179
MAL8P1.48	small nuclear ribonucleoprotein. putative	-1,212	-1,204	-1,191	0,00062	-1,202	0,0104
PF11_0525	hypothetical protein	1,102	1,186	1,115	0,00431	1,134	0,0452
PFF0920c	hypothetical protein. conserved	1,049	1,077	1,077	0,00188	1,068	0,0162
PFL0990w	hypothetical protein. conserved	-1,166	-1,197	-1,144	0,00288	-1,169	0,0264
PF10_0047	RNA binding protein. putative	1,232	1,246	1,326	0,00209	1,268	0,0508
PF13_0326	actin-depolymerizing factor. putative	-1,213	-1,199	-1,108	0,00552	-1,173	0,0571
PF13_0293	hypothetical protein. conserved	-1,099	-1,109	-1,104	0,00004	-1,104	0,0050
MAL13P1.90	hypothetical protein. conserved	1,201	1,269	1,117	0,00763	1,196	0,0759
PFB0700c	hypothetical protein	-1,142	-1,131	-1,103	0,00089	-1,125	0,0200
PF10_0041	U5 small nuclear ribonuclear protein. putative	1,047	1,062	1,082	0,00293	1,064	0,0176
PF14_0107	hypothetical protein. conserved	1,061	1,092	1,096	0,00533	1,083	0,0191
PF11_0470	hypothetical protein	1,100	1,102	1,161	0,00561	1,121	0,0349
PFA0550w	hypothetical protein. conserved	1,115	1,142	1,112	0,00888	1,123	0,0164
PF14_0359	hypothetical protein. conserved	1,128	1,178	1,152	0,00308	1,153	0,0255
PF07_0062	GTP-binding translation elongation factor tu family	1,245	1,138	1,250	0,00419	1,211	0,0633
PF11_0281	hypothetical protein	-1,085	-1,128	-1,174	0,00500	-1,129	0,0448
PFD0940w	hypothetical protein. conserved	-1,225	-1,215	-1,177	0,00058	-1,206	0,0253
PFL0070c	hypothetical protein. conserved in P. falciparum	1,178	1,187	1,130	0,00088	1,165	0,0308
PF14_0297	ecto-nucleoside triphosphate diphosphohydrolase 1	1,121	1,119	1,193	0,00618	1,144	0,0420
PF11_0485	mitochondrial ATP-synthase. delta subunit putative	1,082	1,068	1,082	0,00058	1,077	0,0081
PF07_0108	hypothetical protein. conserved	1,067	1,035	1,051	0,00510	1,051	0,0159
PFI1415w	Serine/Threonine protein kinase. putative	1,105	1,068	1,147	0,00954	1,107	0,0393
PF11_0240	dynein heavy chain. putative	1,133	1,155	1,213	0,00172	1,167	0,0412
PFL0355c	hypothetical protein. conserved	-1,152	-1,207	-1,169	0,00160	-1,176	0,0284
PFE0310c	hypothetical protein. conserved	1,068	1,070	1,130	0,00728	1,089	0,0353
PF10_0260	hypothetical protein	1,146	1,103	1,119	0,00145	1,123	0,0219
PFE1170w	hypothetical protein	-1,150	-1,158	-1,170	0,00009	-1,159	0,0102
PF10_0031	hypothetical protein	-1,156	-1,213	-1,126	0,00500	-1,165	0,0444
PF13_0269	glycerol kinase. putative	1,124	1,104	1,080	0,00133	1,103	0,0221
MAL13P1.83	karyopherin	1,054	1,047	1,050	0,00172	1,051	0,0035
PFB0380c	hypothetical protein	1,114	1,083	1,091	0,00856	1,096	0,0160
PF13_0230	hypothetical protein. conserved	1,274	1,216	1,156	0,00416	1,215	0,0587
MAL13P1.17	hypothetical protein. conserved	-1,176	-1,180	-1,178	0,00182	-1,178	0,0018
PF10_0243	hypothetical protein	1,516	1,302	1,256	0,00567	1,358	0,1384
PFC1110w	VARC pseudogene	-1,043	-1,049	-1,064	0,00117	-1,052	0,0107

PFI1470c	leucine-rich repeat protein 8. LRR8	1,112	1,149	1,104	0,00191	1,122	0,0242
PFB0080c	hypothetical protein	-1,099	-1,137	-1,075	0,00427	-1,104	0,0311
PFB0460c	hypothetical protein	1,197	1,307	1,163	0,00644	1,223	0,0754
PFF0820w	hypothetical protein. conserved	-1,081	-1,075	-1,055	0,00425	-1,070	0,0135
PF14_0718	hypothetical protein. conserved	-1,537	-1,256	-1,394	0,00399	-1,395	0,1403
PF10_0219	hypothetical protein	-1,064	-1,100	-1,102	0,00181	-1,089	0,0214
PF14_0160	hypothetical protein	1,104	1,115	1,114	0,00005	1,111	0,0061
PF10_0336	hypothetical protein	1,305	1,267	1,151	0,00753	1,241	0,0801
PF11_0153	hypothetical protein	1,101	1,070	1,084	0,00616	1,085	0,0155
PF14_0051	hypothetical protein. conserved	1,122	1,130	1,116	0,00071	1,123	0,0073
MAL13P1.264	hypothetical protein. conserved	-1,078	-1,127	-1,100	0,00191	-1,102	0,0246
PF14_0412	hypothetical protein	1,123	1,173	1,074	0,00898	1,123	0,0495
PF10_0351	hypothetical protein	-1,464	-1,374	-1,222	0,00410	-1,353	0,1221
PF14_0112	POM1. putative	1,140	1,109	1,172	0,00344	1,140	0,0312
PFI0120c	protein kinase. FIKK family	-1,169	-1,174	-1,176	0,00011	-1,173	0,0039
PFL0150w	origin recognition complex 1 protein	1,079	1,110	1,095	0,00131	1,095	0,0160
PF13_0165	hypothetical protein. conserved	1,161	1,161	1,079	0,00980	1,134	0,0477
PFC0340w	DNA polymerase delta small subunit. putative	1,356	1,395	1,188	0,00546	1,313	0,1100
PF13_0077	DEAD box helicase. putative	1,125	1,082	1,103	0,00136	1,104	0,0212
PFE0100w	hypothetical protein. conserved	1,061	1,086	1,104	0,00259	1,083	0,0215
PF14_0680	hypothetical protein	1,327	1,228	1,251	0,00270	1,268	0,0515
PFL2265c	hypothetical protein. conserved	-1,057	-1,070	-1,104	0,00438	-1,077	0,0241
PF13_0037	DEAD box helicase. putative	1,139	1,128	1,081	0,00891	1,116	0,0310
MAL8P1.34	hypothetical protein. conserved	1,167	1,182	1,242	0,00860	1,197	0,0396
PF07_0105	exonuclease i. putative	1,284	1,231	1,292	0,00805	1,269	0,0332
PFE0650c	hypothetical protein. conserved	-1,175	-1,213	-1,187	0,00540	-1,192	0,0197
PF10_0118	hypothetical protein	1,141	1,186	1,232	0,00180	1,186	0,0456
MAL8P1.103	hypothetical protein. conserved	-1,091	-1,102	-1,057	0,00399	-1,083	0,0236
PFE1235c	hypothetical protein	1,105	1,138	1,070	0,00632	1,105	0,0339
MAL13P1.34	hypothetical protein. conserved	-1,343	-1,405	-1,443	0,00430	-1,397	0,0505
PFL0540w	mannosyltransferase. putative	1,219	1,243	1,489	0,00925	1,317	0,1495
PF11_0455	hypothetical protein	-1,186	-1,200	-1,240	0,00258	-1,209	0,0280
MAL13P1.28	hypothetical protein. conserved	-1,147	-1,257	-1,130	0,00710	-1,178	0,0688
PF11_0482	hypothetical protein	1,097	1,059	1,058	0,00402	1,071	0,0222
MAL13P1.107	hypothetical protein. conserved	1,271	1,310	1,538	0,00482	1,373	0,1440
PF11_0053	PfSNF2L	-1,114	-1,162	-1,119	0,00223	-1,132	0,0265

PF08_0044	Plasmodium falciparum protein kinase 1	-1,104	-1,149	-1,063	0,00828	-1,106	0,0430
PFB0335c	cysteine protease. putative	-1,182	-1,178	-1,207	0,00080	-1,189	0,0155
MAL7P1.100	serine/threonine protein kinase. Pfnek-4	1,162	1,120	1,139	0,00043	1,140	0,0208
PFL0325w	hypothetical protein. conserved	1,108	1,153	1,253	0,00940	1,172	0,0742
PF13_0050	hypothetical protein. conserved	1,213	1,194	1,221	0,00141	1,209	0,0137
PFF1245c	hypothetical protein. conserved	1,236	1,111	1,176	0,00691	1,174	0,0625
PF11_0289	hypothetical protein	-1,250	-1,128	-1,208	0,00395	-1,196	0,0619
PF11_0117	replication factor C subunit 5. putative	1,267	1,250	1,134	0,00643	1,217	0,0725
PFA0555c	UMP-CMP kinase. putative	-1,190	-1,178	-1,278	0,00609	-1,215	0,0547
PFE0560c	hypothetical protein. conserved	-1,120	-1,135	-1,073	0,00664	-1,109	0,0323
PF08_0047	hypothetical protein. conserved	-1,091	-1,073	-1,105	0,00859	-1,090	0,0162
PF11_0228	hypothetical protein	-1,232	-1,228	-1,375	0,00589	-1,279	0,0839
MAL13P1.275	NLI interacting factor-like phosphatase. putative	-1,187	-1,246	-1,111	0,00602	-1,181	0,0678
MAL13P1.62	hypothetical protein	-1,190	-1,156	-1,118	0,00280	-1,155	0,0359
PFL2005w	replication factor c subunit 4	1,110	1,075	1,113	0,00561	1,099	0,0208
PFD0720w	hypothetical protein. conserved	-1,344	-1,335	-1,245	0,00299	-1,308	0,0546
PF14_0352	ribonucleoside-diphosphate reductase. large subunit	1,048	1,067	1,058	0,00058	1,058	0,0093
PFC0695w	hypothetical protein. conserved	-1,260	-1,202	-1,169	0,00351	-1,210	0,0458
PF11_0393	hypothetical protein. conserved	-1,148	-1,092	-1,189	0,00916	-1,143	0,0487
MAL8P1.106	hypothetical protein. conserved	1,060	1,055	1,094	0,00613	1,070	0,0210
PFL1155w	GTP cyclohydrolase I	1,073	1,058	1,078	0,00088	1,070	0,0107
PF07_0124	hypothetical protein. conserved	1,089	1,058	1,112	0,00980	1,087	0,0270
PFD0410c	hypothetical protein	1,126	1,112	1,065	0,00800	1,101	0,0318
PFI0715w	Zinc binding protein. putative	-1,177	-1,146	-1,135	0,00051	-1,153	0,0219
PFF0475w	hypothetical protein	-1,076	-1,124	-1,081	0,00271	-1,094	0,0263
PF10_0407	dihydrolipoamide acetyltransferase. putative	1,097	1,101	1,105	0,00587	1,101	0,0038
PFB0360c	cysteine protease. putative	-1,180	-1,096	-1,224	0,00845	-1,167	0,0654
PF13_0106	hypothetical protein. conserved	1,090	1,140	1,111	0,00449	1,114	0,0251
PF13_0271	ABC transporter. (heavy metal transporter family)	-1,087	-1,071	-1,063	0,00968	-1,074	0,0123
PFE0275w	hypothetical protein. conserved	-1,261	-1,140	-1,335	0,00913	-1,245	0,0986
MAL8P1.83	eukaryotictranslation initiation factor. putative	-1,105	-1,150	-1,181	0,00635	-1,145	0,0385
PFL2395c	dimethyladenosine transferase. putative	1,128	1,156	1,166	0,00861	1,150	0,0196
PFI0660c	protease. putative	-1,066	-1,102	-1,076	0,00904	-1,081	0,0182
PF13_0191	hypothetical protein. conserved	1,142	1,134	1,135	0,00231	1,137	0,0042
PF13_0276	membrane-associated histidine rich protein 2	-1,204	-1,221	-1,129	0,00216	-1,184	0,0488
PF10_0262	hypothetical protein	-1,181	-1,146	-1,084	0,00622	-1,137	0,0494

PF14_0626	dynein beta chain. putative	-1,050	-1,052	-1,084	0,00554	-1,062	0,0191
PF10_0087	diphthine synthase	-1,102	-1,080	-1,087	0,00770	-1,089	0,0112
PFL1750c	hypothetical protein. conserved	1,158	1,209	1,086	0,00760	1,151	0,0614
PF11_0350	hypothetical protein	-1,180	-1,068	-1,148	0,00951	-1,132	0,0573
PFF0925w	hypothetical protein. conserved	-1,181	-1,096	-1,096	0,00950	-1,124	0,0489
PF14_0507	hypothetical protein	-1,111	-1,104	-1,077	0,00084	-1,097	0,0181
PFD1155w	erythrocyte binding antigen-165	-1,248	-1,419	-1,339	0,00204	-1,336	0,0857
PFD0680c	ubiquitin carboxyl-terminal hydrolase a. putative	1,106	1,115	1,077	0,00316	1,099	0,0200
PFI0285w	hypothetical protein. conserved	-1,105	-1,215	-1,196	0,00471	-1,172	0,0590
PFL0900c	arginyl-tRNA synthetase. putative	-1,128	-1,111	-1,109	0,00643	-1,116	0,0108
MAL7P1.6	hypothetical protein. conserved in P.falciparum	1,058	1,046	1,056	0,00757	1,053	0,0068
PF13_0193	MSP7-like protein	-1,278	-1,299	-1,166	0,00423	-1,248	0,0713
PF14_0015	aminopeptidase. putative	-1,030	-1,026	-1,049	0,00571	-1,035	0,0124
PF10_0052	hypothetical protein	1,159	1,220	1,231	0,00970	1,204	0,0387
PFC0275w	FAD-dependent glycerol-3-phosphate dehydrogenase	1,112	1,106	1,063	0,00909	1,094	0,0268
PF07_0051	erythrocyte membrane protein 1 (PfEMP1)	-1,166	-1,166	-1,104	0,00601	-1,145	0,0358
PF14_0621	hypothetical protein	-1,257	-1,194	-1,136	0,00865	-1,196	0,0603
PF10_0335	hypothetical protein	1,084	1,079	1,116	0,00883	1,093	0,0204
PFL1030w	hypothetical protein. conserved	1,120	1,100	1,074	0,00783	1,098	0,0232
PFI0230c	bacterial histone-like protein. putative	-1,167	-1,178	-1,241	0,00537	-1,195	0,0396
PF14_0324	hypothetical protein. conserved	1,079	1,135	1,148	0,00350	1,121	0,0368
PF13_0062	clathrin-adaptor medium chain. putative	1,098	1,059	1,064	0,00301	1,074	0,0212
PFL1830w	ubiquitin-like protein. putative	-1,172	-1,123	-1,206	0,00183	-1,167	0,0417
PFL1525c	pre-mRNA splicing factor RNA helicase. putative	-1,104	-1,158	-1,135	0,00146	-1,132	0,0274
PFE1270c	hypothetical protein. conserved	1,112	1,082	1,072	0,00914	1,089	0,0205
PFC0065c	alpha/beta hydrolase protein. putative	-1,190	-1,122	-1,280	0,00824	-1,197	0,0794
PF11_0408	hypothetical protein	1,135	1,176	1,159	0,00076	1,157	0,0208
PF10_0258	hypothetical protein	1,104	1,100	1,089	0,00054	1,098	0,0081
PF11_0196	hypothetical protein	1,100	1,108	1,148	0,00126	1,119	0,0259
PFB0935w	cytoadherence linked asexual protein 2	-1,288	-1,348	-1,162	0,00660	-1,266	0,0947
PFD1110w	hypothetical membrane protein. conserved	-1,155	-1,095	-1,101	0,00272	-1,117	0,0333
PFB0570w	SPATR-like protein. putative	-1,151	-1,245	-1,291	0,00900	-1,229	0,0714
PFF1395c	glutamyl-tRNA(Gln) amidotransferase subunit B	1,226	1,168	1,098	0,00666	1,164	0,0640
PFI1780w	hypothetical protein	-1,133	-1,175	-1,272	0,00576	-1,193	0,0714
PF11_0324	hypothetical protein	1,117	1,104	1,153	0,00111	1,125	0,0255
MAL13P1.342	hypothetical protein. conserved	-1,293	-1,306	-1,561	0,00496	-1,387	0,1509

PF10_0268	merozoite capping protein 1	1,079	1,093	1,067	0,00070	1,080	0,0131
PF13_0019	sodium/hydrogen exchanger. Na+. H+ antiporter	-1,066	-1,073	-1,107	0,00407	-1,082	0,0221
PF13_0349	nucleoside diphosphate kinase b; putative	1,075	1,062	1,096	0,00369	1,078	0,0172
PF14_0428	histidine -- tRNA ligase. putative	-1,086	-1,128	-1,115	0,00968	-1,110	0,0217
PF10_0278	BRIX domain containig protein. putative	1,253	1,250	1,138	0,00496	1,214	0,0655
PF07_0033	Cg4 protein	1,118	1,166	1,228	0,00383	1,171	0,0555
PFB0310c	merozoite surface protein 4	-1,183	-1,184	-1,190	0,00000	-1,186	0,0037
PF10_0352	merozoite surface protein. putative	-1,423	-1,363	-1,269	0,00831	-1,352	0,0775
PF10_0256	hypothetical protein	1,079	1,063	1,040	0,00458	1,061	0,0197
PF14_0577	hypothetical protein	1,210	1,176	1,342	0,00710	1,243	0,0878
PFL1435c	myosin d	-1,193	-1,194	-1,130	0,00150	-1,172	0,0364
PF13_0173	hypothetical protein. conserved	-1,513	-1,578	-1,283	0,00895	-1,458	0,1549
PFE0460c	hypothetical protein. conserved	-1,139	-1,095	-1,111	0,00235	-1,115	0,0224
PF10_0341	hypothetical protein. conserved	1,137	1,083	1,117	0,00651	1,112	0,0274
PF10_0295	hypothetical protein	-1,102	-1,103	-1,096	0,00772	-1,100	0,0037
PF13_0227	vacuolar ATP synthase subunit D. putative	-1,140	-1,162	-1,160	0,00106	-1,154	0,0125
PF10_0055	hypothetical protein	-1,222	-1,204	-1,324	0,00176	-1,250	0,0648
PF10_0286	hypothetical protein	1,132	1,146	1,124	0,00217	1,134	0,0111
PFL1455w	hypothetical protein. conserved	1,352	1,265	1,462	0,00634	1,360	0,0985
PFE0085c	hypothetical protein. conserved	-1,135	-1,287	-1,255	0,00522	-1,226	0,0802
PF10_0198	hypothetical protein	1,056	1,084	1,043	0,00657	1,061	0,0213
PFI0925w	gamma-glutamylcysteine synthetase	-1,106	-1,102	-1,103	0,00001	-1,104	0,0023
PFE0155w	hypothetical protein. conserved	1,104	1,121	1,131	0,00043	1,119	0,0137
PFF0385c	hypothetical protein. conserved	1,136	1,133	1,145	0,00041	1,138	0,0061
PF13_0142	u6 snRNA-associated sm-like protein. putative	-1,147	-1,142	-1,070	0,00943	-1,119	0,0430
PFL1760w	hypothetical protein. conserved	-1,189	-1,151	-1,081	0,00685	-1,140	0,0548
MAL13P1.43	hypothetical protein. conserved	-1,148	-1,112	-1,091	0,00906	-1,117	0,0289
PF14_0031	hypothetical protein	1,085	1,091	1,069	0,00038	1,082	0,0110
PF11_0382	ribosomal protein S9. putative	-1,200	-1,248	-1,186	0,00040	-1,211	0,0325
PFI0450c	apoptosis-related protein. putative	-1,143	-1,115	-1,176	0,00140	-1,145	0,0308
PFI0105c	protein kinase. FIKK family	-1,256	-1,324	-1,347	0,00109	-1,309	0,0473
PFL2300w	hypothetical protein. conserved	-1,223	-1,203	-1,315	0,00317	-1,247	0,0594
PF10_0141	cdk7. putative	-1,077	-1,084	-1,132	0,00483	-1,097	0,0299
PFC0710w	inorganic pyrophosphatase. putative	1,081	1,138	1,083	0,00430	1,101	0,0321
PFE0195w	cation transporting P-ATPase	1,192	1,223	1,106	0,00591	1,174	0,0605
PFA0470c	cold-shock protein. putative	-1,338	-1,247	-1,308	0,00202	-1,297	0,0464

PF11_0427	dolichyl-phosphate b-D-mannosyltransferase. putative	-1,252	-1,232	-1,194	0,00045	-1,226	0,0296
MAL7P1.108	hypothetical protein. conserved	-1,256	-1,186	-1,412	0,00793	-1,284	0,1159
MAL13P1.38	hypothetical protein. conserved	-1,120	-1,113	-1,084	0,00090	-1,106	0,0194
PFI1280c	protein kinase. putative	-1,155	-1,151	-1,081	0,00521	-1,129	0,0419
PF13_0197	Merozoite Surface Protein 7 precursor. MSP7	-1,531	-1,526	-1,268	0,00385	-1,441	0,1504
PF13_0309	hypothetical protein. conserved	-1,192	-1,137	-1,080	0,00806	-1,136	0,0564
MAL13P1.301	protein with aminophospholipid-transporting P-ATPase and guanyl cyclase domains	1,090	1,081	1,121	0,00211	1,097	0,0214

Additional file 4: List of altered genes following hyperoxia treatment using GSEA data and PS 6.0 software.

Accession Number	Type	Name	Connectivity	Local Connectivity	Indegree	Outdegree
PF14_0511	Protein	glucose-6-phosphate dehydrogenase	773	2	1	1
PF10_0153	Protein	heat shock 60kDa protein 1 (chaperonin)	647	31	3	28
PF08_0054	Protein	heat shock 70kDa protein 1A	1547	5	2	3
PF10_0218	Protein	citrate synthase	288	4	1	3
PFL1070c	Protein	heat shock protein 90kDa beta (Grp94), member 1	507	3	1	2
MAL13P1.221	Protein	ornithine carbamoyltransferase	148	3	0	3
PF10_0245	Protein	glutamine-fructose-6-phosphate transaminase 1	228	3	0	3
PF14_0598	Protein	glyceraldehyde-3-phosphate dehydrogenase	794	1	0	1
PKA	Functional Class	cAMP-dependent protein kinase, protein kinase A	221	3	2	1
PFF1350c	Protein	acyl-CoA synthetase short-chain family member 2	118	2	0	2
PF08_0006	Protein	prohibitin	167	1	0	1
PF13_0305	Protein	eukaryotic translation elongation factor 1 alpha 1	302	2	1	1
PFL1110c	Protein	protein kinase, cAMP-dependent, regulatory, type I, alpha	241	6	0	6
PF14_0486	Protein	eukaryotic translation elongation factor 2	252	1	0	1
PFL1550w	Protein	dihydrolipoamide dehydrogenase	162	3	0	3
PFI0235w	Protein	replication protein A1, 70kDa	147	1	0	1
MAL13P1.343	Protein	proteasome 26S subunit, non-ATPase	100	3	0	3
PF13_0044	Protein	carbamoyl-phosphate synthetase 1, mitochondrial	367	3	0	3
dnaJ	Protein	DnaJ (Hsp40) homolog, subfamily A, member 2	175	5	0	5
PFL0740c	Protein	heat shock 10kDa protein 1 (chaperonin 10)	149	11	4	7
PF11_0407	Protein	ferredoxin reductase	87	2	0	2
PF07_0029	Protein	heat shock protein 90kDa alpha (cytosolic)	122	3	0	3
PF11_0351	Protein	heat shock 70kDa protein 9 (mortalin)	125	2	0	2
PFI1685w	Protein	protein kinase, cAMP-dependent, catalytic, alpha	91	4	1	3
PF11_0358	Protein	polymerase (RNA) I polypeptide B, 128kDa	104	2	0	2
PF13_0085	Protein	protein kinase, cAMP-dependent, catalytic, beta	45	3	0	3
PF13_0062	Protein	adaptor-related protein complex 1, mu 1 subunit	42	4	0	4
PF14_0366	Protein	primase, DNA, polypeptide 1 (49kDa)	37	2	0	2
PFI0530c	Protein	primase, DNA, polypeptide 2 (58kDa)	21	2	0	2

Additional file 5: MS/MS peptide sequences, respective gi number, gene ID and master spot number of proteins identified from the differential 2-D DIGE analysis following hyperoxia exposure of *P. falciparum*.

gi number	Gene ID	Master spot number	MS/MS Peptide Sequences
		P. falciparum	
Translation			
gi|124512420	MAL8P1.69	3289	R.CDCTYR.S K.LAEQAER.Y R.YDEMADAMR.T R.TLVEQCVNNDKDELTVEER.N R.IISSVEQK.E K.NVAATYR.K K.LIPNTSESESK.V R.AFDDAITEFDNVSEDSYK.D K.DSTLIMQLLR.D
gi|124513850	PF13_0304	2047	R.TLIEALDTMEPPK.R K.IGGIGTVPVGR.V R.GYVASDTK.N K.FLNIDSK.I K.VVEENPK.A R.QTIAVGIIK.S K.KEPGAVTAK.A
gi|8918238	PF14_0486	1030	R.ETVTEESTITCLGK.S
gi|124810293	PF14_0655	2268	R.ELAQQIQK.V K.LFILDEADEMLSR.G K.KDELTLEGIR.Q K.DELTLEGIR.Q R.VLVTTDLLAR.G

Parasitophorous vacuolar membrane Transporter			
gi|124810348	PF14_0678	3061	K.DPISLTIK.D R.EIVGDNTIEK.K R.EIVGDNTIEKK.T
		3062	R.EIVGDNTIEK.K R.EIVGDNTIEKK.T K.LRQDPSLIVAK.I R.QDPSLIVAK.I
Glycolysis			
gi|124809201	PF14_0341	1579	R.FLANVDPNDVNR.A
gi|124810131	PF14_0598	2867	K.LGINGFGR.I K.DPSQIPWGK.C K.ELASSHLK.G K.KVIMSAPPK.D K.VIMSAPPK.D K.QLIVSNASCTTN.C K.QLIVSNASCTTNCLAPLAK.V R.CALSNIIPASTGAAK.A K.VLPELNGK.L R.VPIGTVSVVDLVCR.L K.YEEVALEIK.K K.KAAEGPLK.G K.AGLALNDNFFK.L
Chaperone-assisted protein folding			
gi|124512406	PF08_0054	1314	R.TTPSYVAFTDTER.L R.NPENTVFDAK.R K.FTESSVQSDMK.H K.NAVITVPAYFNDSQR.Q K.DAGTIAGLNVMR.I R.LVNFCVEDFK.R R.FEELCIDYFR.D R.NTTIPAKK.S R.LSQDEIDR.M K.LQPAEIETCMK.T K.EAESVCAPIMSK.I

		1326	R.TTPSYVAFTDTER.L R.NPENTVFDAK.R K.FTESSVQSDMK.H K.ATAGDTHLGGEDFDNR.L R.LVNFCVEDFK.R R.NTTIPAKK.S R.LSQDEIDR.M K.LQPAEIETCMK.T K.EAESVCAPIMSK.I
gi|505340	PF07_0029	1028	M.STETFAFNADIR.Q R.ELISNASDALDK.I K.LSAEPEFFIR.I K.TNNTLTIEDSGIGMTK.N K.NDLINNLGTIAR.S K.VEDVTEELENAEK.K K.ALLFIPK.R K.GVVDSEDLPLNISR.E K.SGDEMIGLK.E
Amino acids metabolism			
gi|86170756	PFF0435w	2217	R.TLGCVSASTDK.K K.LCENADK.L K.LGAPFLQNLK.E K.GLLCAIEFK.N K.KEPGAVTAK.A
gi|124513590	MAL13P1.214	3347	M.TLIENLNSDK.T K.ILSDIELNENSK.V N.IVNMANER.V K.IIFEANDILTK.E K.EFPENNFDLIYSR.D K.FISLDDGWSR.K
		3372	M.TLIENLNSDK.T K.ILSDIELNENSK.V K.IIFEANDILTK.E K.NVVSK.D
		3379	M.TLIENLNSDK.T K.ILSDIELNENSK.V K.IIFEANDILTK.E
		3503	K.ILSDIELNENSK.V K.IIFEANDILTK.E

		3385	M.TLIENLNSDK.T K.TFLENNQYTDEGVK.V K.ILSDIELNENSK.V N.IVNMANER.V K.IIFEANDILTK.E K.EFPENNFDLIYSR.D K.ENWDDEFK.E K.FISLDDGWSR.K
Proteasome-mediated proteolysis			
gi|124512686	MAL8P1.142	3216	R.DATSSNFIQIVK.V
gi|124513790	MAL13P1.270	3358	K.SSNFAVLAVEK.K K.IVQEFLEK.N K.AIFEVVELSSK.N K.NVEVALLTEK.D
		Homo sapiens	
Oxygen transporter			
gi|183817		3451	K.VNVDEVGGEALGR.L R.FFESFGDLSTPDAVMGNPK V
		3576	K.VNVDEVGGEALGR.L R.FFESFGDLSTPDAVMGNPK V
Antioxidant metabolism			
gi|4502517		3431	K.NGPEQWSK.L K.YSSLAEAASK.A K.ADGLAVIGVLMK.V K.VLDALQAIK.T
		3444	K.YSSLAEAASK.A K.ADGLAVIGVLMK.V K.VLDALQAIK.T
		3455	K.NGPEQWSK.L K.LYPIANGNNQSPVDIK.T K.GGPFSDSYR.L K.YSSLAEAASK.A K.ADGLAVIGVLMK.V K.VLDALQAIK.T

gi|4557014	1648	K.ADVLTTGAGNPVGDK.L K.LNVITVGPR.G K.NLSVEDAAR.L R.DLFNAIATGK.Y K.DYPLIPVGK.L
gi|16306550	2451	K.SQPEPLVVK.G K.LVLPSLISSR.I R.IYVVDVGSEPR.A
gi|168985379	2156	K.EMLAAACQMFLGK.T R.AQQVAVQEQEIAR.R K.ITLVSSGSGTMGAAK.V
Glycolysis		
gi|31645	2804	K.LVINGNPITIFQER.D R.GALQNIIPASTGAAK.A K.LTGMAFR.V R.VPTANVSVVDLTCR.L K.QASEGPLK.G.L
	2806	K.LVINGNPITIFQER.D R.GALQNIIPASTGAAK.A K.LTGMAFR.V R.VPTANVSVVDLTCR.L K.QASEGPLK.G.L
	2807	R.GALQNIIPASTGAAK.A R.VPTANVSVVDLTCR.L K.QASEGPLK.G.L
	2853	K.LVINGNPITIFQER.D K.LTGMAFR.V K.QASEGPLK.G.L
	2854	K.LVINGNPITIFQER.D R.GALQNIIPASTGAAK.A K.LTGMAFR.V K.QASEGPLK.G.L
	2856	R.GALQNIIPASTGAAK.A R.VPTANVSVVDLTCR.L

Additional file 6: Raw data microarray at 4 hours time point under hyperoxia versus normoxia conditions on synchronised parasites.

Accession Number	Sequence Names	FC O1	FC O2	FC O3	FC O4	*p*-value t-test	FC Average	Standard Deviation
PF14_0723	hypothetical protein	-1,228	-1,226	-1,247	-1,214	0.00004	-1,229	0,0135
PFL0820c	hypothetical protein, conserved	-1,196	-1,213	-1,205	-1,183	0.00006	-1,199	0,0132
PFI0475w	small nuclear ribonucleoprotein (snRNP), putative	1,237	1,245	1,255	1,282	0.00009	1,255	0,0198
PFE0985w	hypothetical protein, conserved	-1,087	-1,079	-1,081	-1,070	0.00016	-1,079	0,0068
MAL13P1.48	hypothetical protein, conserved	-1,276	-1,235	-1,244	-1,298	0.00026	-1,263	0,0292
PF11_0497	hypothetical protein	-3,755	-3,389	-3,814	-2,917	0.00027	-3,469	0,4131
PFI1800w	hydrolase, putative	-1,173	-1,172	-1,149	-1,194	0.00027	-1,172	0,0184
PF13_0246	hypothetical protein, conserved	-1,308	-1,267	-1,353	-1,301	0.00028	-1,307	0,0353
PF13_0171	60S ribosomal protein L23, putative	1,485	1,585	1,491	1,427	0.00034	1,497	0,0651
PF11_0438	Ribosomal protein, putative	1,445	1,594	1,507	1,603	0.00041	1,537	0,0751
PFF1050w	nascent polypeptide associated complex alpha chain, putative	1,454	1,500	1,469	1,620	0.00045	1,510	0,0751
PF10_0335	hypothetical protein	1,195	1,171	1,142	1,169	0.00047	1,169	0,0219
PFL0740c	10 kd chaperonin, putative	1,337	1,375	1,267	1,318	0.00048	1,324	0,0447
PFI0375w	ribosomal protein L35, putative	1,362	1,293	1,261	1,333	0.00053	1,312	0,0443
MAL13P1.323	hypothetical protein, conserved	-1,282	-1,201	-1,241	-1,255	0.00053	-1,245	0,0338
PF14_0565	hypothetical protein	-1,060	-1,075	-1,056	-1,064	0.00057	-1,064	0,0084
PFF1065c	hypothetical protein, conserved	-1,139	-1,158	-1,113	-1,142	0.00057	-1,138	0,0188
PF14_0144	mRNA capping enzyme, putative	-1,077	-1,082	-1,085	-1,062	0.00060	-1,077	0,0103
PF13_0213	60S ribosomal subunit protein L6e, putative	1,724	1,641	1,682	1,487	0.00063	1,633	0,1031
PF13_0014	40S ribosomal protein S7 homologue, putative	1,160	1,216	1,168	1,207	0.00068	1,188	0,0277
PFD0720w	hypothetical protein, conserved	1,144	1,102	1,141	1,132	0.00073	1,130	0,0191
PF14_0125	deoxyhypusine synthase	1,113	1,099	1,087	1,122	0.00078	1,105	0,0157
PFD1105w	hypothetical protein	1,215	1,200	1,147	1,188	0.00079	1,188	0,0289
PF13_0177	ATP-dependent RNA helicase, putative	-1,138	-1,097	-1,108	-1,113	0.00081	-1,114	0,0174
PF14_0104	eukaryotictranslation initiation factor 2 gamma subunit, putative	1,094	1,095	1,081	1,118	0.00087	1,097	0,0151
PFL2490c	hypothetical protein, conserved	1,260	1,253	1,232	1,178	0.00088	1,231	0,0374
PFA0380w	serine/threonine protein kinase, putative	-1,275	-1,307	-1,330	-1,221	0.00089	-1,283	0,0472
PF11_0499	hypothetical protein	-3,879	-2,750	-3,720	-2,862	0.00090	-3,303	0,5790
PF08_0131	1-cys peroxidoxin	1,153	1,194	1,164	1,219	0.00093	1,183	0,0300
PFI1075w	hypothetical protein, conserved	1,054	1,078	1,075	1,072	0.00094	1,070	0,0108
PF11_0040	early transcribed membrane protein 11.2, etramp11.2	-1,238	-1,251	-1,312	-1,208	0.00100	-1,252	0,0438
PFI0340c	hypothetical protein, conserved	-1,468	-1,321	-1,314	-1,377	0.00117	-1,370	0,0713

MAL7P1.126	hypothetical protein, conserved	1,297	1,208	1,272	1,209	0.00120	1,246	0,0452
PFE0840c	hypothetical protein, conserved	-1,488	-1,422	-1,514	-1,324	0.00122	-1,437	0,0846
MAL13P1.15	hypothetical protein, conserved	1,239	1,279	1,300	1,195	0.00123	1,253	0,0466
PF14_0676	20S proteasome beta 4 subunit, putative	1,401	1,282	1,306	1,418	0.00126	1,352	0,0679
MAL7P1.5	Maurers Cleft 2 transmembrane domain protein, PfMC-2TM_7.1	1,196	1,209	1,213	1,292	0.00134	1,227	0,0436
PF10_0077	eukaryotictranslation initiation factor 3 subunit 7, putative	1,116	1,110	1,083	1,082	0.00144	1,098	0,0179
PFL0275w	hypothetical protein, conserved	-1,244	-1,189	-1,255	-1,308	0.00146	-1,249	0,0487
PFB0675w	hypothetical protein	-1,146	-1,216	-1,214	-1,232	0.00148	-1,202	0,0384
PF14_0349	histidine triad protein, putative	1,654	1,411	1,444	1,454	0.00158	1,491	0,1103
PFL0060w	hypothetical protein, conserved in P. falciparum	-1,151	-1,129	-1,093	-1,124	0.00158	-1,124	0,0237
PFA0700c	hypothetical protein, conserved in P. falciparum	1,355	1,222	1,269	1,255	0.00161	1,275	0,0569
PFC0845c	ubiquitin--protein ligase, putative	1,093	1,061	1,066	1,084	0.00170	1,076	0,0146
PFC0300c	60S ribosomal protein L7, putative	1,414	1,523	1,368	1,313	0.00171	1,404	0,0892
PF14_0474	hypothetical protein	1,092	1,117	1,072	1,093	0.00176	1,093	0,0183
PF10_0179	PHF5-like protein, putative	1,191	1,208	1,163	1,265	0.00180	1,207	0,0431
PF08_0049	ribonucleoprotein, putative	1,100	1,146	1,133	1,164	0.00184	1,136	0,0273
PF13_0178	translation initiation factor 6, putative	1,323	1,197	1,277	1,326	0.00193	1,281	0,0602
PFB0965c	hypothetical protein	1,106	1,078	1,098	1,129	0.00197	1,103	0,0210
PF14_0225	hypothetical protein	-1,282	-1,254	-1,311	-1,418	0.00200	-1,316	0,0718
PF14_0447	glutaminyl-peptide cyclotransferase	-1,106	-1,178	-1,149	-1,161	0.00204	-1,149	0,0311
PFC0910w	hypothetical protein, conserved	1,489	1,317	1,295	1,383	0.00212	1,371	0,0872
PFI0390c	hypothetical membrane protein, conserved	-1,235	-1,223	-1,154	-1,160	0.00216	-1,193	0,0421
PF11_0203	peptidase	-1,131	-1,102	-1,082	-1,087	0.00230	-1,101	0,0218
PFI0815c	hypothetical protein, conserved	1,153	1,094	1,104	1,130	0.00238	1,121	0,0265
PF11_0433	hypothetical protein	-1,100	-1,095	-1,087	-1,059	0.00238	-1,085	0,0183
PF14_0317	hypothetical protein, conserved	1,180	1,113	1,161	1,197	0.00240	1,163	0,0362
PFC0365w	conserved protein, putative	-1,173	-1,112	-1,173	-1,197	0.00241	-1,164	0,0363
PFL0105w	hypothetical protein, conserved	1,286	1,191	1,219	1,320	0.00249	1,254	0,0597
PFI1115c	pre-mRNA splicing factor protein, putative	1,290	1,237	1,421	1,350	0.00254	1,324	0,0789
PFC0582c	hypothetical protein, conserved	1,367	1,251	1,306	1,208	0.00265	1,283	0,0688
PF11_0447	translation initiation factor eIF-1A, putative	1,186	1,113	1,126	1,168	0.00276	1,148	0,0346
MAL13P1.170	hypothetical protein, conserved	-1,237	-1,175	-1,133	-1,213	0.00282	-1,189	0,0451
PF10_0187	ribosomal protein L30e, putative	1,380	1,579	1,437	1,312	0.00290	1,427	0,1135
PF10_0232	Chromodomain-helicase-DNA-binding protein 1 homolog	-1,046	-1,062	-1,073	-1,080	0.00291	-1,066	0,0150
MAL13P1.250	hypothetical protein, conserved	1,127	1,094	1,088	1,143	0.00292	1,113	0,0265
PF11_0402	hypothetical protein	-1,139	-1,136	-1,194	-1,111	0.00301	-1,145	0,0351

PF14_0175	conserved protein, unknown function	-1,217	-1,229	-1,125	-1,210	0.00306	-1,195	0,0472
PF07_0001	hypothetical protein	1,386	1,321	1,240	1,456	0.00319	1,351	0,0923
PFE0845c	60S ribosomal subunit protein L8, putative	1,079	1,074	1,044	1,075	0.00320	1,068	0,0161
PF14_0048	hypothetical protein	1,179	1,144	1,098	1,126	0.00321	1,137	0,0335
PFL1745c	clustered-asparagine-rich protein	-1,160	-1,139	-1,246	-1,198	0.00327	-1,186	0,0469
PFD0210c	pbs36 homologue	-1,137	-1,212	-1,197	-1,128	0.00327	-1,168	0,0420
MAL13P1.56	m1-family aminopeptidase	-1,178	-1,191	-1,189	-1,105	0.00339	-1,166	0,0410
PF13_0016	methyl transferase-like protein, putative	1,072	1,055	1,075	1,044	0.00343	1,061	0,0149
PFL0255c	uga suppressor tRNA-associated antigenic protein, putative	1,101	1,055	1,086	1,096	0.00347	1,085	0,0206
PFD0355c	Peptidyl-tRNA hydrolase PTH2, putative	1,161	1,221	1,117	1,173	0.00352	1,168	0,0430
PF14_0157	hypothetical protein	1,186	1,196	1,109	1,142	0.00359	1,158	0,0403
PFL0855c	hypothetical protein, conserved	1,061	1,067	1,074	1,104	0.00364	1,076	0,0191
PFL2080c	hypothetical protein, conserved	1,429	1,275	1,287	1,231	0.00367	1,306	0,0854
MAL7P1.94	prefoldin subunit 3, putative	1,212	1,172	1,185	1,303	0.00374	1,218	0,0591
PF14_0407	hypothetical protein	-1,059	-1,082	-1,074	-1,107	0.00378	-1,080	0,0204
MAL8P1.102	hypothetical protein, conserved	1,068	1,108	1,130	1,098	0.00382	1,101	0,0257
MAL13P1.93	hypothetical protein, conserved	-1,520	-1,310	-1,300	-1,315	0.00386	-1,361	0,1060
PFL2330w	hypothetical protein, conserved	-1,110	-1,160	-1,214	-1,171	0.00387	-1,164	0,0431
PF08_0071	Fe-superoxide dismutase	1,079	1,099	1,058	1,107	0.00391	1,086	0,0219
PFI0780w	hypothetical protein, conserved	1,365	1,204	1,354	1,419	0.00392	1,336	0,0922
PFE1540w	hypothetical protein, conserved	1,536	1,322	1,281	1,407	0.00393	1,387	0,1127
PFF1525c	hypothetical protein, conserved in P. falciparum	1,260	1,381	1,432	1,241	0.00395	1,328	0,0929
PF11_0329	hypothetical protein	1,441	1,263	1,284	1,246	0.00409	1,308	0,0901
MAL13P1.413	membrane associated histidine-rich protein, MAHRP-1	-1,166	-1,237	-1,281	-1,336	0.00435	-1,255	0,0721
PFF0905w	hypothetical protein, conserved	1,079	1,106	1,152	1,131	0.00441	1,117	0,0316
PFD0755c	adenylate kinase 1	1,245	1,134	1,183	1,256	0.00442	1,204	0,0569
PFI0125c	protein kinase, FIKK family	1,082	1,077	1,089	1,045	0.00445	1,073	0,0194
PFE0045c	kinase, putative	-1,414	-1,283	-1,289	-1,202	0.00447	-1,297	0,0873
PF13_0179	isoleucine--tRNA ligase, putative	-1,298	-1,191	-1,277	-1,161	0.00448	-1,232	0,0658
PFE0715w	asparagine -- tRNA ligase, putative	1,264	1,201	1,225	1,128	0.00450	1,205	0,0571
PFD0890w	hypothetical protein, conserved	1,566	2,031	1,648	1,501	0.00457	1,687	0,2374
PFE0810c	40S ribosomal subunit protein S14, putative	1,327	1,318	1,168	1,353	0.00473	1,292	0,0838
PF14_0355	hypothetical protein	1,239	1,257	1,220	1,124	0.00475	1,210	0,0593
PF14_0516	serine/threonine-protein kinase	-1,135	-1,070	-1,099	-1,128	0.00476	-1,108	0,0297
PFL2400w	hypothetical protein, conserved	1,079	1,101	1,104	1,152	0.00482	1,109	0,0305
PFE0805w	cation-transporting ATPase 1	-1,320	-1,224	-1,463	-1,436	0.00501	-1,361	0,1100

PF14_0118	hypothetical protein	-1,852	-1,710	-1,690	-1,355	0.00512	-1,652	0,2107
PF14_0566	hypothetical protein	1,213	1,149	1,179	1,103	0.00515	1,161	0,0466
PFE0210c	hypothetical protein, conserved	1,237	1,308	1,199	1,149	0.00523	1,223	0,0670
PF14_0071	hypothetical protein	-1,070	-1,057	-1,039	-1,079	0.00531	-1,061	0,0172
PFD0220c	hypothetical protein, conserved	-1,140	-1,299	-1,254	-1,286	0.00532	-1,245	0,0724
PF13_0229	IRP-like protein	-1,150	-1,101	-1,159	-1,213	0.00539	-1,156	0,0459
PFB0100c	knob associated histidine-rich protein	-1,235	-1,357	-1,268	-1,473	0.00540	-1,333	0,1063
PF10_0298	26S proteasome subunit, putative	1,284	1,331	1,235	1,153	0.00545	1,251	0,0760
PFI0635c	hypothetical protein, conserved	1,199	1,250	1,240	1,119	0.00547	1,202	0,0598
PF14_0587	hypothetical protein	-1,256	-1,134	-1,142	-1,173	0.00610	-1,176	0,0556
PF11_0253	hypothetical protein	-1,080	-1,170	-1,132	-1,168	0.00637	-1,137	0,0421
PFL1300c	hypothetical protein, conserved	-1,145	-1,089	-1,129	-1,073	0.00649	-1,109	0,0335
PFF0060w	Maurers Cleft 2 transmembrane domain protein 6, PfMC-2TM_6	1,175	1,369	1,393	1,278	0.00651	1,304	0,0992
MAL8P1.104	CAF1 family ribonuclease, putative	-1,078	-1,113	-1,085	-1,052	0.00662	-1,082	0,0252
MAL13P1.174	MSP7-like protein	1,074	1,121	1,107	1,161	0.00664	1,116	0,0361
PF10_0242	hypothetical protein	-1,176	-1,153	-1,148	-1,077	0.00665	-1,138	0,0429
PFE1020w	u6 snRNA-associated sm-like protein Lsm2, putative	1,117	1,157	1,069	1,127	0.00668	1,117	0,0365
MAL13P1.360	hypothetical protein, conserved	-1,137	-1,100	-1,156	-1,075	0.00670	-1,117	0,0365
PFB0215c	3-5 exonuclease, putative	-1,187	-1,203	-1,356	-1,347	0.00672	-1,273	0,0906
PF10_0239	hypothetical protein	-1,298	-1,201	-1,316	-1,466	0.00673	-1,320	0,1094
PF11_0025	Maurers Cleft 2 transmembrane domain protein 11.2	1,318	1,274	1,563	1,317	0.00676	1,368	0,1316
PF11_0399	hypothetical protein	-1,107	-1,132	-1,066	-1,146	0.00678	-1,113	0,0351
PF08_0041	ribosome biogenesis protein nep1 homologue, putative	-1,208	-1,213	-1,117	-1,121	0.00678	-1,165	0,0527
PFD0790c	DNA replication licensing factor, putative	-1,572	-1,232	-1,544	-1,474	0.00691	-1,455	0,1545
PF11_0105	hypothetical protein	-1,161	-1,094	-1,143	-1,081	0.00711	-1,120	0,0384
PFE0965c	vacuolar ATP synthetase, putative	1,368	1,487	1,298	1,206	0.00715	1,340	0,1183
PFL1775c	hypothetical protein, conserved	1,074	1,063	1,076	1,123	0.00717	1,084	0,0268
PF08_0081	hypothetical protein, conserved	-1,151	-1,193	-1,128	-1,268	0.00718	-1,185	0,0615
PF13_0134	hypothetical protein, conserved	2,098	1,566	1,513	1,543	0.00718	1,680	0,2796
PF13_0257	glutamate--tRNA ligase	-1,150	-1,160	-1,074	-1,173	0.00720	-1,139	0,0443
MAL7P1.76	hypothetical protein, conserved	-1,187	-1,258	-1,141	-1,311	0.00736	-1,224	0,0755
MAL7P1.24	hypothetical protein, conserved	1,162	1,266	1,278	1,139	0.00738	1,211	0,0708
MAL13P1.302	SUMO ligase, putative	-1,109	-1,187	-1,099	-1,106	0.00740	-1,125	0,0414
PFE1100w	hypothetical protein, conserved	1,285	1,599	1,545	1,770	0.00755	1,550	0,2010
PF14_0453	hypothetical protein	1,393	1,398	1,173	1,430	0.00756	1,348	0,1183
PFF0575c	hypothetical protein, conserved	-1,348	-1,279	-1,140	-1,260	0.00756	-1,257	0,0868

MAL13P1.274	serine/threonine protein phosphatase pfPp5	1,160	1,141	1,160	1,070	0.00757	1,133	0,0429
PFF0480w	hypothetical protein, conserved	-1,093	-1,162	-1,140	-1,079	0.00771	-1,119	0,0391
PF07_0079	60S ribosomal protein L11a, putative	1,169	1,164	1,074	1,170	0.00780	1,144	0,0470
PFA0435w	hypothetical protein, conserved	1,305	1,234	1,125	1,207	0.00788	1,218	0,0745
PF10_0015	acyl CoA binding protein, putative	1,076	1,039	1,042	1,047	0.00794	1,051	0,0167
PF10_0183	hypothetical protein	-1,189	-1,274	-1,157	-1,344	0.00795	-1,241	0,0844
PFC0400w	60S Acidic ribosomal protein P2	1,162	1,150	1,095	1,225	0.00797	1,158	0,0534
PFE0445c	Soluble N-ethylmaleimide-sensitive factor Attachment Protein	1,240	1,178	1,186	1,097	0.00806	1,175	0,0593
PF10_0038	ribosomal protein S20e, putative	1,382	1,394	1,246	1,621	0.00818	1,411	0,1555
PF11_0250	high mobility group-like protein NHP2, putative	1,011	1,015	1,024	1,019	0.00820	1,017	0,0055
PF13_0140	dihydrofolate synthase/folylpolyglutamate synthase	-1,082	-1,082	-1,049	-1,043	0.00822	-1,064	0,0211
PFC10_API0024	tRNA-Gly	-2,288	-1,695	-1,484	-1,702	0.00832	-1,792	0,3456
PF14_0390	hypothetical protein	1,161	1,277	1,232	1,389	0.00839	1,265	0,0955
PF14_0044	hypothetical protein	-1,108	-1,245	-1,157	-1,231	0.00845	-1,186	0,0642
PFF1265w	oxidoreductase, short-chain dehydrogenase family, putative	-1,073	-1,051	-1,110	-1,108	0.00848	-1,085	0,0285
PFL2310w	hypothetical protein, conserved	-1,215	-1,121	-1,099	-1,141	0.00848	-1,144	0,0501
PF13_0215	hypothetical protein, conserved	-1,086	-1,120	-1,063	-1,060	0.00850	-1,082	0,0277
PFL2110c	hypothetical protein, conserved	1,278	1,427	1,181	1,418	0.00857	1,326	0,1182
PFA0730c	hypothetical protein	1,328	1,221	1,435	1,197	0.00866	1,295	0,1093
PFF1505w	hypothetical protein, conserved	-1,318	-1,185	-1,160	-1,163	0.00869	-1,206	0,0753
PF11_0168	hypothetical protein	-1,258	-1,098	-1,215	-1,196	0.00904	-1,192	0,0674
PF10_0178	hypothetical protein	-1,257	-1,397	-1,195	-1,186	0.00914	-1,259	0,0976
PFD1175w	Plasmodium falciparum trophozoite antigen r45-like protein	-1,172	-1,093	-1,085	-1,158	0.00916	-1,127	0,0445
PF14_0585	ribosomal protein S28e, putative	1,155	1,184	1,113	1,267	0.00927	1,179	0,0651
PFI0155c	PfRab7, GTPase	1,326	1,167	1,216	1,149	0.00929	1,214	0,0796
MAL8P1.124	hypothetical protein, conserved	-1,296	-1,488	-1,231	-1,232	0.00929	-1,312	0,1214
PF13_0148	hypothetical protein, conserved	-1,445	-1,254	-1,484	-1,711	0.00929	-1,473	0,1874
PFC0285c	T-complex protein beta subunit, putative	-1,179	-1,124	-1,080	-1,187	0.00937	-1,142	0,0503
PFA0410w	hypothetical protein, conserved	-1,034	-1,078	-1,083	-1,061	0.00941	-1,064	0,0220
PF14_0277	coatamer protein, beta subunit, putative	1,046	1,108	1,112	1,117	0.00953	1,096	0,0331
PF11_0226	petidase, M16 family	1,177	1,133	1,287	1,301	0.00960	1,224	0,0825
PF14_0615	ATP synthase (C/AC39) subunit, putative	1,084	1,132	1,070	1,064	0.00963	1,088	0,0311
PF08_0040	hypothetical protein, conserved	-1,248	-1,206	-1,143	-1,105	0.00966	-1,176	0,0638
PFL2065c	mitochondrial import inner membrane translocase subunit	1,298	1,319	1,361	1,130	0.00977	1,277	0,1015
PFE0300c	60S ribosomal subunit protein L24, putative	1,050	1,069	1,056	1,027	0.00985	1,050	0,0175
PFL1700c	V-type K+ independent h+ translocating inorganic pyrophosphatase	1,304	1,153	1,140	1,263	0.00997	1,215	0,0809

Influence des conditions environnementales sur le métabolisme de Plasmodium falciparum

Résumé

P. falciparum est le principal responsable des formes graves du paludisme. Le parasite évolue entre deux hôtes (homme et moustique) qui lui imposent différents environnements; et tout particulièrement, des modifications des pressions partielles d'O_2 nécessitant des capacités d'adaptation surprenantes pour un parasite microaérophile. Chez l'hôte vertébré, les phénomènes de cytoadhésion, ralentissant la progression du parasite notamment au niveau des poumons, augmentent la durée d'exposition aux conditions hyperoxiques.

La dynamique de la réponse parasitaire à l'hyperoxie a été étudiée par une approche combinée de transcriptomique et de protéomique. Certains mécanismes de défense contre les espèces réactives d'oxygène ont été appréciés, dont une éventuelle fonction oxydase alternative.

L'exposition du parasite à 21% d'O_2 induit un retard de croissance au niveau de la schizogonie. Le stress oxydatif induit par l'hyperoxie entraîne des perturbations métaboliques comme une inhibition de la glycolyse en faveur de la respiration et un ralentissement du métabolisme de la vacuole digestive. Cette action combinée sur le métabolisme mitochondrial et vacuolaire permet au parasite de s'adapter à un environnement hyperoxydant, en régulant la production d'espèces réactives d'oxygène. Nos travaux ont montré qu'un inhibiteur de la fonction oxydase alternative, l'acide salicylhydroxamique ou SHAM, avec un effet mineur sur la croissance parasitaire en microaérophilie, avait un effet létal sur les parasites en hyperoxie.

Une meilleure compréhension de la biologie parasitaire pourrait contribuer au développement de nouveaux traitements antipaludiques associés à une thérapie hyperbarique.

Mots Clés: *Plasmodium falciparum, mitochondrie, hyperoxie, SHAM, puce à ADN, DIGE.*

Impact of environmental conditions on Plasmodium falciparum metabolism

Abstract

P. falciparum is the main species responsible for severe case of malaria. The parasite evolves between two hosts (human and mosquito), imposing to it different environments; especially changes in the O_2 pressure, demanding astonishing adaptation skills for a microaerophilic parasite. In the vertebrate host, the phenomena of cytoadhesion, which slow down the spread of the parasite among others in the lungs, increase the timing of exposure to hyperoxic conditions.

The parasitic response dynamic to hyperoxia has been analysed by a combined transcriptomic and proteomic approach. Some of the defense mechanisms against reactive oxygen species have been evaluated, among which a potential alternative oxidase function.

The exposure of the parasite to 21%O_2 atmosphere leads to a growth delay at schizogony level. The oxidative stress resulting from the hyperoxia conducts to metabolic alterations, as an inhibition of the glycolysis in favour of respiration and as a slowdown of the metabolism of the digestive vacuole. This combined action on the mitochondrial and vacuolar metabolisms allows the parasite to adapt itself to hyperoxic environment, by regulating reactive oxygen species. Our works have shown that an inhibitor of the alternative oxidase function, the salicylhydroxamic acid or SHAM, with a minor effect on the parasite growth in microaerophily, had letal effect on parasites in hyperoxia.

A better understanding of the parasitic biology could contribute to the development of new antimalarial treatments, associated with a hyperbaric oxygen therapy.

Keywords: *Plasmodium falciparum, mitochondria, hyperoxia, SHAM, microarray, DIGE.*