Applying systems biology methods to identify putative drug targets in the metabolism of the malaria pathogen *Plasmodium falciparum*

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Abstract

Despite enormous efforts to combat malaria, the disease still afflicts up to half a billion people each year, of which more than one million die. Currently no effective vaccine is within sight, and resistances to antimalarial drugs are wide-spread. Thus, new medicines against malaria are urgently needed.

In order to aid the process of drug target detection, the present work carries out a computational analysis of the metabolism of *Plasmodium falciparum*, the deadliest malaria pathogen. A comprehensive compartmentalized metabolic network is assembled, which is able to reproduce metabolic processes known from the literature to occur in the parasite. On the basis of this network metabolic fluxes are predicted for the individual life cycle stages of *P. falciparum*. In this context, a flux balance approach is developed to obtain metabolic flux distributions that are consistent with gene expression profiles observed during the respective stages. The predictions are found to be in good accordance with experimentally determined metabolite exchanges between parasite and infected erythrocyte. Knockout simulations, which are conducted with a similar approach, reveal indispensable metabolic reactions within the parasite. These putative drug targets cover almost 90% of a set of experimentally confirmed essential enzymes if the assumption is made that nutrient uptake from the host cell is limited. A comparison demonstrates that the applied flux balance approach yields target predictions with higher specificity than the topology based choke-point analysis. The previously predicted stage-specific flux distributions allow to filter the obtained set of drug target candidates with respect to malaria prophylaxis, therapy or both, providing a promising starting point for further drug development.

Keywords:
malaria, metabolic network, drug target, flux balance analysis

Zusammenfassung

Trotz weltweiter Bemühungen, die Tropenkrankheit Malaria zurückzudrängen, erkranken jährlich bis zu einer halben Milliarde Menschen an Malaria mit der Folge von über einer Million Todesopfern. Da zur Zeit eine wirksame Impfung nicht in Sicht ist und sich Resistenzen gegen gängige Medikamente ausbreiten, werden dringend neue Antimalariamittel benötigt.

Um die Suche nach neuen Angriffsorten für Medikamente zu unterstützen, untersucht die vorliegende Arbeit mit einem rechnergestützten Ansatz den Stoffwechsel von *Plasmodium falciparum*, dem tödlichsten Malaria-Erreger. Basierend auf einem aus dem aktuellen Forschungsstand rekonstruierten metabolischen Netzwerk des Parasiten werden metabolische Flüsse für die einzelnen Stadien des Lebenszyklus von *P. falciparum* berechnet. Dabei wird ein im Rahmen dieser Arbeit entwickelter Fluss-Bilanz-Analyse-Ansatz verwendet, der ausgehend von in den jeweiligen Entwicklungsstadien gemessenen Genexpressionsprofilen entsprechende Flussverteilungen ableitet. Für das so ermittelte stadienspezifische Flussgeschehen ergibt sich eine gute Übereinstimmung mit bekannten Austauschprozessen von Stoffen zwischen Parasit und infiziertem Erythrozyt. Knockout Simulationen, die mit Hilfe einer ähnlichen Vorhersagemethode durchgeführte werden, decken essentielle metabolische Reaktionen im Netzwerk auf. Fast 90% eines Sets von experimentell bestimmten essentiellen Enzymen wird wiedergefunden, wenn die Annahme getroffen wird, dass Nährstoffe nur begrenzt aus der Wirtszelle aufgenommen werden können. Die als essentiell vorhergesagten Enzyme stellen mögliche Angriffsorte für Medikamente dar. Anhand der Flussverteilungen, die für die einzelnen Entwicklungsstadien berechnet wurden, können diese potenziellen Targets nach Relevanz für Malaria Prophylaxe und Therapie sortiert werden, je nachdem, in welchem Stadium die Enzyme als aktiv vorhergesagt wurden. Dies bietet einen vielversprechenden Startpunkt für die Entwicklung von neuen Antimalariamitteln.

Schlagwörter:
Malaria, Metabolisches Netzwerk, Wirkstoff Angriffsort, Fluss-Bilanz-Analyse

Dedication

To my parents and Eugen Doce.

Contents

Chapter 1

Introduction

Malaria represents one of the major health issues worldwide. According to estimations of the World Health Organization (WHO), there were around 250 million clinical cases and almost one million disease related deaths in 2006, of which more than half were children under the age of five [545]. About 50% of the world population lives in endemic areas, situated mostly in Africa, Asia, and South-America (see Fig. 1.1). Especially in Africa, where about 90% of the malaria cases occur, the disease has significant negative impact on the economic development [428].

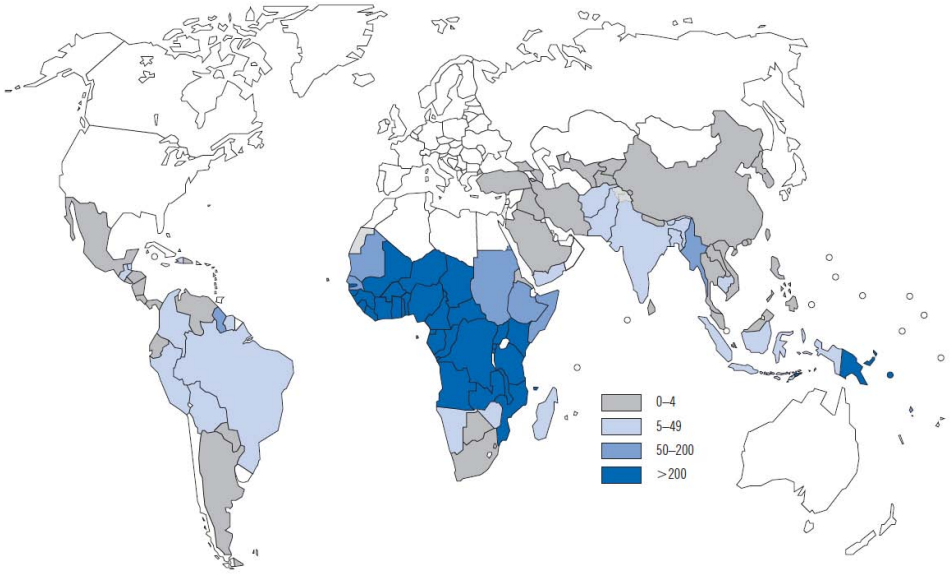


Figure 1.1: **Malaria endemic countries.** Estimated incidences of malaria in 2006 per 1,000 population (source: [545]).

The disease, which is characterized by periodical fever attacks, is caused by protozoan parasites of the genus *Plasmodium*. Among the five *Plasmodium* species that are able to infect humans, *P. falciparum* is responsible for 90% of the disease related deaths. Pathogens are typically transfered by tropical mosquitoes to the human host where they first multiply within the liver and subsequently periodically within erythrocytes.

Malaria exists presumably for more than 50,000 years [232]. It was once widespread, not only in today's endemic areas, but also in most of Europe and North-America. A worldwide program to eradicate malaria with the aid of the insecticide DDT (dichlorodiphenyltrichloro-

ethane) and available antimalarials was initiated in the 1950s by the WHO. The extinction of the disease was successful in Europe, North-America, the Soviet Union and Australia, but not in underdeveloped countries in tropical areas. Nowadays, only about one thousand malaria cases occur in Germany every year, of which all are imported from other countries. Experts see only little chances for the return of malaria to Germany, since a good health care system exists and a critical amount of infected people is missing. However, mosquito species able to transmit the disease exist in Germany and due to global warming temperatures that are needed for pathogen development might not be uncommon in the future [578].

Several non-profit initiatives, including the WHO and its Roll Back Malaria partnership, the Bill and Melinda Gates Foundation, the Global Fund to Fight AIDS, Tuberculosis and Malaria as well as the Affordable Medicines Facility for Malaria, have recently reintroduced the agenda of complete eradication of malaria in all countries [416]. In order to achieve this goal of extinction of all *Plasmodium* species causing malaria in humans, effective drugs and vaccines are needed to treat and prevent malaria.

The malaria parasite is able to outwit the host's immune system by hiding within host cells and avoiding clearance through the spleen with the help of highly variable proteins that are transported to the surface of infected erythrocytes, allowing adherence to walls of blood vessels. Therefore, vaccine development is challenging with the result that up to now no approved vaccine is available to facilitate long-term malaria prophylaxis. The global initiative Malaria Vaccine Technology Roadmap seeks to develop a malaria vaccine by 2025 that has a protective efficacy of more than 80% and lasts longer than four years [213].

Currently used antimalarial drugs correspond to five major drug classes (quinolines, antifolates, artemisinin derivatives, antibiotica and the ubiquinone analog atovaquone). Due to the strong selection pressure associated with the administration of antimalarials, drug resistances have been developed in many areas [190, 14]. Recently, reduced susceptibility even to artemisinin-based combination therapies, the recommended first-line treatments of falciparum malaria, has been reported in western Cambodia [122]. Mechanisms of resistance are still uncertain for most of the currently used drugs, making the process of drug development even more complicated.

As resistances to currently used antimalarials are spreading and a vaccine providing full and long lasting protection against clinical disease is not expected to be available soon, new effective antimalarial drugs are needed. This demand has been neglected in the last years by the pharmaceutical industry, due to the limited buying power of the population of disease-endemic countries, making cost coverage of research and development investments uncertain. To overcome this shortage in discovery of novel antimalarials, which can be sold at affordable prices, non-profit collaborations have been established between academia and pharmaceutical industry such as the Medicines for Malaria Venture [358].

A very crucial step in drug discovery is the identification of appropriate biological drug targets, as improper target selection has been found to be the main cause for the high failure rate of drug development [71]. Most of the currently used antimalarial drugs act on enzymes and thus interfere with the metabolism of the parasite [560, 220, 91]. This trend has also been found for marketed small-molecule drug targets in man of which almost 50% are enzymes [208]. Therefore, investigating the metabolic network of the malaria pathogen seems to be a promising approach to identify novel drug targets.

The majority of antimalarials interferes with the asexual blood stages of the parasite and are thus suitable for treatment of disease symptoms. In order to successfully eradicate

malaria more drugs will be needed which additionally hamper transmission of parasites by mosquitoes and kill liver stages to prevent the onset of the disease. Thorough analysis of the pathogen's individual life cycle stages will therefore be a prerequisite for the development of such drugs.

Advances in high-throughput technologies such as high-throughput genome sequencing, microarrays, mass-spectrometry, yeast two-hybrid assays and chromatin immunoprecipitation (ChIP)-chip assays have facilitated organism specific large-scale "omics" studies. It is now possible to analyze cells on a genome wide scale with respect to genomic sequences (genomics), gene expression levels (transcriptomics), expressed proteins and posttranscriptional protein modifications (proteomics), the set of present small-molecule metabolites (metabolomics) and physical interactions between proteins or proteins and DNA (interactomics). In order to evaluate this wealth of available large-scale data the young research field systems biology has evolved which has the goal to understand the complex and dynamic processes within a cell. In contrast to classical molecular biology, not only the separate system components are considered, but also the interactions between them. Based on experimental data, mathematical models describing the biological system are developed. Computers can therefore be used to simulate the behavior of the system of interest and to predict effects of system perturbations, e.g., the consequences of administering a drug that inhibits a certain enzyme. In an iterative process, model derived predictions are evaluated with wet lab experiments, and in case experiments and prediction disagree, the model is refined to further improve prediction quality (see Fig. 1.2). A systems biology approach has the advantage that system wide effects of local changes can be estimated, thus revealing how to control or optimize parts of the system. This is beneficial especially for medical applications where drug targets are sought whose inhibition have large effects on the system, as well as for biotechnological applications where the yield of certain processes has to be optimized.

Comprehensive biochemical networks, including metabolic networks [360], transcriptional regulatory networks [198, 193] and signaling networks [376], have been reconstructed and transformed into mathematical models on the basis of experimental high-throughput data. Especially metabolic networks have become available for multiple organisms from all three major domains of the tree of life [12, 84, 125, 298, 174, 138, 359, 139, 218, 443]. These reconstructions have been the subject of different applications like metabolic engineering, drug target discovery and network property analysis (see review [360] for more details). In general, good accordance between predictions and experimental data could be observed in these studies. For example, gene knockout simulations in yeast showed that predictions with 95% sensitivity and 86% specificity are possible [478], suggesting that systems biology approaches represent a valuable strategy.

Multiple studies using high-throughput technologies have been conducted to examine malaria pathogens. Genome sequences of the species *P. falciparum, P.vivax* and *P.knowlesi* are now available [161, 75, 370] and allow comparative analysis of these organisms. Furthermore, the presence of gene transcripts and proteins has been evaluated for several developmental stages of the parasites [59, 419, 427, 501, 147, 275, 276, 185, 112, 501]. Lately, even metabolite levels have been measured during different time points of the blood stage [503, 366]. Several groups have started to reconstruct the metabolic network of *P. falciparum* [170, 561, 241, 309], and the network topology has been analyzed with respect to putative drug targets [561, 136]. However, a consistent compartmentalized computational

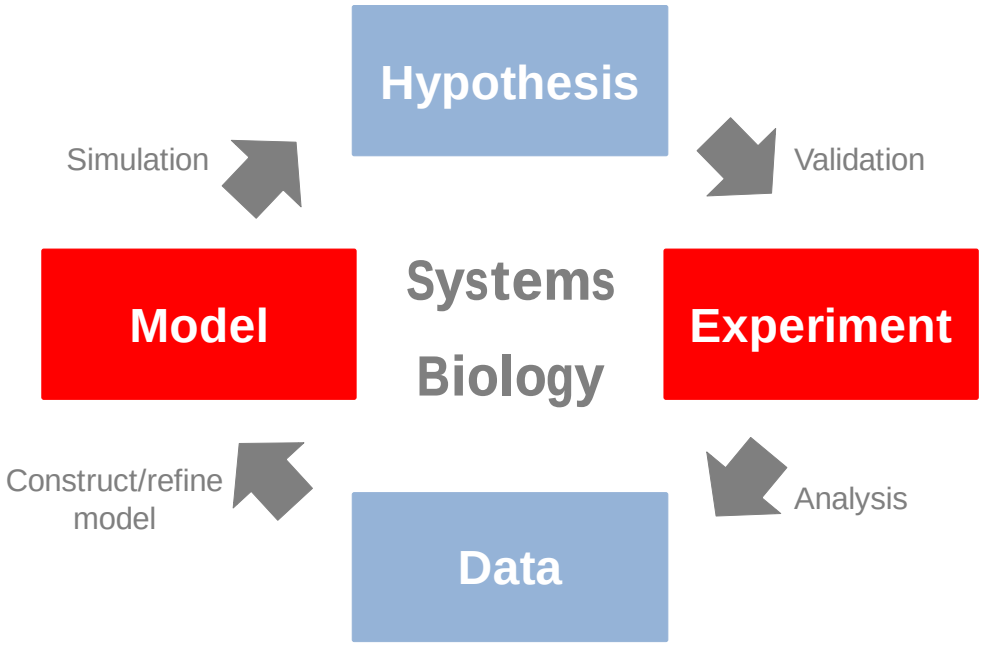


Figure 1.2: **Iterative cycle of systems biology.** Systems biology aims at understanding complex biological systems by combining experiments and computational modeling. In an iterative cycle a model of the system of interest is constructed and refined based on experimental data, while experiments are designed to investigate hypotheses suggested by simulations. By this means a model is developed that is eventually able to correctly predict the behavior of the system, thus increasing biological knowledge.

model that can be used to make life cycle stage-specific predictions does not exist yet. Such a model could contribute to elucidate the metabolism of malaria pathogens during the different life cycle stages and hence help to identify suitable drug targets, expediting the drug discovery process.

1.1 Objectives of this work

The goal of the present work is to study the metabolism of *Plasmodium falciparum*, the deadliest human malaria pathogen, with the help of systems biology methods, thus contributing to the fight against malaria. In this context, the first multi-compartment metabolic network of *P. falciparum* is assembled, which comprises data from publicly available sources. On the basis of this comprehensive network, metabolic flux distributions consistent with observed gene expression profiles are predicted, using a flux balance approach. By this means, metabolic fluxes can be inferred for all life cycle stages of the parasite for which gene expression levels have been measured. As especially the liver stage of *P. falciparum* is difficult to study and experimentally determined flux rates will not be available soon on genome scale for any life cycle stage, this computational approach is intended to augment available experimental data. In addition, the metabolic network of *P. falciparum* is analyzed with respect to essential enzymes which presumably represent good drug targets. Among these drug target

candidates those suitable for malaria prophylaxis or treatment can be determined with the help of predicted life cycle stage-specific metabolic fluxes, as such enzymes have to be active during the liver stage or the blood stage, respectively. Additional criteria like homology to human enzymes and functional similarity to therapeutic targets in other organisms help to further filter the set of putative targets.

1.2 Outline of the working program

In the following, an introduction to the topic malaria is given (Chapter 2), explaining the life cycle of the pathogen as well as disease symptoms and ways to diagnose and treat the disease. Furthermore, known antimalarial drug targets are summarized and the process underlying the discovery of new drugs is described. Chapter 3 reviews available experimental large scale data for *P. falciparum*, in particular gene expression data, which can be used to model the metabolism of the parasite. Databases relevant for the modeling process and related computational approaches are outlined in Chapter 4. This chapter includes methods allowing the prediction of metabolic fluxes on the basis of gene expression data and proposed approaches to identify putative drug targets in metabolic networks. The assembly of the compartmentalized metabolic network of *P. falciparum* as well as the human erythrocyte, one of the parasite's host cells, is described in Chapter 5. Subsequently, the approach applied to these networks in order to infer life cycle stage-specific metabolism is explained and predicted flux distributions are evaluated with respect to experimentally observed metabolite exchanges between host and parasite and in addition with proteomics and metabolomics data (Chapter 6). Finally, putative drug targets in the metabolism of *P. falciparum* detected by an *in silico* knockout study are evaluated and ranked (Chapter 7).

Chapter 2

Biological background

2.1 The disease malaria

2.1.1 The pathogen

Originally, people thought that malaria (ital.: mala aria = bad air) is caused by the air coming from swamps due to disease occurrence in these areas. Only since the discovery by Charles Louis Alphonse Laveran in 1880 of moving crescent-shaped bodies in a blood drop from a sick soldier it is known that protozoan parasites of the genus *Plasmodium* cause the disease [277]. Five species are pathogenic to humans: *Plasmodium falciparum*, which is responsible for 90% of the disease related deaths, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* as well as the primate malaria parasite *Plasmodium knowlesi*.

Plasmodium parasites are unicellular eukaryotes that not only use humans as hosts, but also rodents, birds, monkeys and reptiles. Beside a nucleus the parasites possess a mitochondrion [517], a chloroplast-like organelle without photosynthetic activity called apicoplast [403], an endoplasmic reticulum (ER) [516] and a golgi apparatus [487]. In addition, a food vacuole [120] and secretory organelles (rhoptries, micronemes and dense granules) [245] develop during certain time points of the parasite's life cycle, contributing to the different morphological states. The genome of *P. falciparum* consists of 14 nuclear chromosomes and has a size of $\sim$ 23 Mb. In addition, a small linear genome exists in the mitochondrion ($\sim$ 6 kb) and a small circular genome in the apicoplast ($\sim$ 35 kb). A first draft of the genome sequence was published in 2002 [161], revealing an unusually high percentage of A/T content (80.6%). Bioinformatics approaches identified more than 5,300 open reading frames, of which up to now only half are functionally annotated.

2.1.2 The life cycle of *Plasmodium falciparum*

P. falciparum has a rather complex life cycle, since it involves different hosts and tissues [510] as depicted in Fig. 2.1. Typically, a cycle starts with an infected female anopheline mosquito taking a blood-meal and thereby injecting parasites into the human blood stream. These sporozoites travel then to the liver where they invade hepatocytes and undergo asexual replication known as exo-erythrocytic schizogony, which involves multiple rounds of nuclear division without cytokinesis. In contrast to *P. falciparum*, *Plasmodium vivax* and *Plasmodium ovale* may rest within hepatocytes (hypnozoite stage) before they proceed with development months or even years later. The progeny of one parasite can consist of several thousand new parasites. These so-called merozoites leave the liver in hepatocyte-derived vesicles (merosomes) that protect the parasites from the host immune system. Merozoites

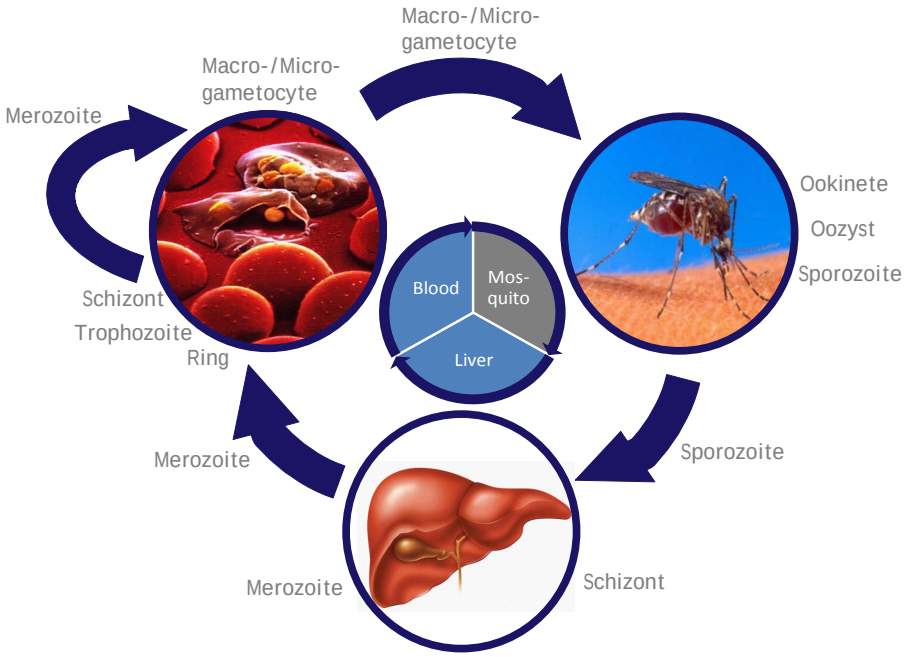


Figure 2.1: **Life cycle of *P. falciparum*.** The different parasite forms during the life cycle are annotated to the respective stages. Graphics are modified from [150, 362, 99].

released into the circulation are able to infect erythrocytes which serve the parasites as another nutrient resource. The intraerythrocytic developmental cycle (IDC) comprises several stages. Due to its characteristic morphology, the early stage is referred to as the ring form which is followed by the trophozoite stage and the formation of schizonts. The release of up to 32 new merozoites after asexual reproduction in erythrocytes triggers the symptoms of malaria, since parasite specific particles are set free that activate the human immune system. In some cases parasites differentiate during the blood stage into female (macrogametocytes) and male (microgametocytes) gametocytes, which have no further activity within the human host. If a mosquito takes a blood meal, gametocytes get into the midgut of the mosquito where the environmental conditions induce the fertilization of macrogametes by microgametes. The resultant ookinete builds a cyst on the outside of the stomach wall (oocyst), producing sporozoites (sporogony) that travel to the salivary gland of the mosquito after oocyst rupture. Here the sporozoites wait to be reinjected into the human blood stream to start a new cycle.

2.1.3 Clinical symptoms

Malaria symptoms [177, 510] occur, depending on the the *Plasmodium* species, 7–15 days (*P. falciparum*), 12–18 days (*P. vivax, P. ovale*) or 18–40 days (*P. malariae*) subsequent to a mosquito bite. Longer periods of incubation are possible due to ineffective use of prophylactic drugs or the formation of dormant liver forms (hypnozoites), leading to latency periods up to 40 years. Disease symptoms include flu-like symptoms such as fever, headache, shivering, joint pain and vomiting which makes it sometimes difficult to diagnose malaria.

In addition anemia, hemoglobinuria, convulsions, splenomegaly, hepatomegaly through to kidney failure, circulatory collapse and coma may occur. Malaria is characterized by a cyclical onset of symptoms, every second day for *P. vivax* and *P. ovale* infections (**malaria tertiana**) and every third day for *P. malariae* (**malaria quartana**). *P. falciparum* causes either fever, recurring in periods shorter than two days, or less intensive continuous fever (**malaria tropica**). Malaria episodes typically start with an intense feeling of cold and vigorous shivering (**cold stage**), followed by fever with body temperatures of 40 °C (**hot stage**). After three to four hours temperature declines to normal accompanied by strong sweating (**sweating stage**). Symptoms are triggered by the rupture of infected erythrocytes which release not only new parasites, but also toxins, inducing the secretion of cytokines by cells of the immune system. Cytokines are responsible for the fever as well as a decreasing blood sugar level and suppression of erythrocyte production in the bone marrow. Together with parasite induced lysis of erythrocytes and removal of infected cells by the spleen this may result in severe anemia. *P. falciparum* is the only human malaria pathogen that is able to produce proteins which are exported to the erythrocyte cell surface and have the ability to adhere to endothelial cells of blood vessels. As a consequence blood circulation is impaired in affected tissues, causing a shortage of oxygen and nutrients. Malaria may result in life-threatening coma (cerebral malaria) or severe adverse outcomes for pregnant women and their babies (placental malaria) if the central nervous system is involved or the placenta.

2.1.4 Clinical diagnostics

The most common technique to diagnose malaria are microscopic examinations of blood films, either using thin films or thick films derived from a larger volume of blood [177, 510]. While analyses of thick films are more sensitive and are therefore used in routine diagnosis, thin films allow species identification. In areas, where microscopic examinations are not possible, immunological tests are applied that allow the detection of parasitic antigens within minutes. However, these test are relatively expensive, are less sensitive than thick blood films and are not always able to distinguish the different *Plasmodium* species. The most sensitive method to malaria is polymerase chain reaction (PCR), which detects parasitic nucleic acids. But since PCR requires a specialized laboratory besides being expensive, it is not often applied. In case none of the above mentioned diagnostic tests are available, malaria may even be diagnosed upon symptoms such as periodic temperature patterns, nail bed pallor and splenomegaly.

2.1.5 Treatment

Drugs currently used to treat malaria include five classes of compounds: **quinolines** (quinine, chloroquine, amodiaquine, primaquine, mefloquine, piperaquine), **antifolates** (sulfadoxine, pyrimethamine, proguanil, chlorproguanil, dapsone), **artemisinin** derivatives (artemisinin, artesunate, artemether, OZ277), **antibiotica** (fosmidomycin, tetracyclines, clindamycin, azithromycin) and the ubiquinone analog **atovaquone** [332, 91]. The first widely used antimalarial, quinine, was extracted in 1820 from the bark of the Andean Cinchona tree which had been used in Peru to cure malaria and then brought to Europe by Jesuits. Later, it was replaced by the more effective synthetic quinoline compound chloroquine which be-

came the medication of choice due to its low production costs and few side effects. However, in the last years resistances to this drug have emerged in many areas of the world [14]. The antifolate fansidar, which consists of the active compounds sulfadoxine and pyrimethamine, became an alternative drug to chloroquine until recently it started to fail as well [14]. The world health organization currently advises to treat malaria on the basis of combination therapies, meaning the simultaneous use of two or more drugs with unrelated targets in the parasite to avoid fast progression of resistance development [368]. Artemisinin-based combination therapy (ACT) are recommended as first-line treatment for uncomplicated malaria in areas experiencing resistance to older medications. Artemisinin is obtained from the plant *Artemisia annua*, which has been used in traditional Chinese medicine for more than 2,000 years. In areas where resistances have not occurred yet, non-artemisinin based combinations (non-ACTs), including fansidar with chloroquine or amodiaquine are advised.

2.1.6 Prevention

Efforts to prevent the spread of malaria include the control of mosquitos, the protection of people from mosquito bites and the usage of prophylactic drugs. In order to reduce mosquito populations, their habitats have been eliminated by draining and poisoning of wetlands. Attempts of direct mosquito eradication have been made as well. In the 1950s the insecticide DDT was applied in a worldwide campaign, which resulted in the elimination of malaria among others in North-America and Europe. The discovery that DDT has negative effects on the environment, especially on birds, and that mosquitos had developed resistances discredited DDT. Today, insecticides are still used on the interior walls of homes (Indoor residual spraying), where mosquitos were found to rest after a blood meal. Less common strategies in mosquito control are radiation and genetic modification of mosquitos to obtain species that are either steril and do not produce offspring upon mating or are resistant to malaria and therefore do not transmit the disease anymore. Prevention of disease transmission is the goal of bed nets which have been shown to provide protection from mosquito bites, with improving results when treated with insecticides. This approach takes advantage of the fact that *Anopheles* mosquitos feed at night.

Another branch of malaria prevention is chemoprophylaxis which aims at stopping the disease before the onset of symptoms. Travelers in endemic areas have the option to take prophylactic drugs such as mefloquine (Lariam), doxycycline and the combination of atovaquone and proguanil hydrochloride (Malarone). Due to high costs and drug side effects, this is not applicable for residents of malarial regions.

Natural immunity to malaria pathogens does only occur after repeated infection with multiple *Plasmodium* strains. The acquired immunity, often referred to as malarial tolerance, is however not a sterile protection, but keeps parasitemia densities low and people are free of symptoms for most of the time (clinical immunity). Up to now, there are no clinically approved malaria vaccines available. Multiple vaccine candidates are currently under development and testing, though [507]. Most candidates are based on the circumsporozoite protein (CSP) which is present on the surface of sporozoites and was found to be bound by antibodies of the immune system, preventing the sporozoite from invading hepatocytes. The most promising candidate, a CSP derived vaccine, is RTS,S/AS02A, which will soon enter Phase III clinical trials [64].

2.1.7 Malaria drug targets

Most of the currently used antimalarials either target the metabolism of the pathogen or the detoxification of heme which is produced during hemoglobin degradation of the blood stage. In addition, most drugs are effective during the blood stage, the stage where malaria symptoms occur. Due to the rapidly growing resistances to currently used drugs, new antimalarials are urgently needed. New drugs can be developed either by designing new molecules inhibiting established targets or by identifying novel putative targets. Table 2.1 reviews both known targets and putative novel targets for the rational design of new antimalarial drugs [560, 220, 91].

Table 2.1: **Established and suggested targets for antimalarial drugs.**

Pathway	Protein	EC no.	Ref.
Energy metabolism	aldolase	4.1.2.13	[541, 223]
	hexose transporter		[229, 434]
	lactate dehydrogenase	1.1.1.27	[509, 74]
Mitochondrial electron transport	NADH dehydrogenase	1.6.5.3	[267]
	succinate dehydrogenase	1.3.99.1	[492]
	cytochrome c reductase	1.10.2.2	[144]
Folate metabolism	dihydrofolate reductase	1.5.1.3	[347, 142, 113]
	dihydropteroate synthase	2.5.1.15	[347, 508, 316]
Heme biosynthesis	δ-aminolevulinate synthase	2.3.1.37	[520, 493]
	δ-aminolevulinate dehydratase	4.2.1.24	[55]
Protein biosynthesis	ribosomes		[546]
	threonine-tRNA ligase	6.1.1.3	[425]
	alanine-tRNA ligase	6.1.1.7	[102]
Protein modification	peptide deformylase	3.5.1.88	[60]
	protein-S-isoprenylcysteine-O-methyltransferase	2.1.1.100	[34, 548]
	farnesyl-diphosphate farnesyltransferase	2.5.1.58	[80]
	protein farnesyltransferase		[80, 547]
GPI biosynthesis	N-acetyl glucosaminylphosphatidylinositol deacetylase	3.5.1.89	[477]
Isoprenoid biosynthesis	1-deoxy-D-xylulose-5-phosphate reductoisomerase	1.1.1.267	[230]
	2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase	4.6.1.12	[109]
Ubiquinone biosynthesis	3-Demethylubiquinone-9,3-O-methyltransferase	2.1.1.64	[308]
Polyamine metabolism	ornithine decarboxylase	4.1.1.17	[20, 50, 42]
	S-adenosylmethionine decarboxylase	4.1.1.50	[556]

Continued on next page

Table 2.1 – continued from previous page

Pathway	Protein	EC no.	Ref.
	spermidine synthase	2.5.1.16	[183]
Methionine metabolism	adenosylhomocysteinase	3.3.1.1	[66, 468, 253, 326]
Phosphatidyl-choline synthesis	choline kinase	2.7.1.32	[92]
	choline carrier		[47]
	phosphoethanolamine methyl-transferase	2.1.1.103	[385, 553]
Type II fatty acid biosynthesis	3-oxoacyl-ACP synthase	2.3.1.41	[536, 396]
	3-hydroxyacyl-ACP dehydratase	4.2.1.-	[458]
	acetyl-CoA-carboxylase	6.4.1.2	[536]
	enoyl-ACP-reductase	1.3.1.9	[191, 494, 481]
Sphingolipid metabolism	serine-palmitoyl transferase	2.3.1.50	[166]
	sphingosine-N-acyltransferase	2.3.1.24	[166]
	ceramide-cholinephospho-transferase	2.7.8.3	[166]
	ceramide-glycosyltransferase	2.4.1.80	[166]
Lipases	phospholipase B	3.1.1.5	[577]
	phospholipase C	3.1.4.3	
	sphingomyelinase	3.1.4.12	[384, 186]
	surface phospholipase		[46]
Purine salvage	adenosine deaminase	3.5.4.4	[511, 165]
	GMP synthetase	6.3.5.2	[314]
	hypoxanthine guanine phosphori-bosyltransferase	2.4.2.8	[115, 438, 285]
	IMP dehydrogenase	1.1.1.205	[543]
	purine nucleos. phosphorylase	2.4.2.1	[248]
	adenylosuccinate synthetase	6.3.4.4	[129]
Pyrimidine metabolism	carbamoyl phosphate synthetase	6.3.5.5	[148]
	carbonic anhydrase	4.2.1.1	[411]
	dihydroorotase	3.5.2.3	[455]
	dihydroorotate oxidase	1.3.3.1	[266, 318]
	orotidine-5'-phosphate decarboxy-lase	4.1.1.23	[451, 454, 268]
	thymidylate synthase	2.1.1.45	[225, 354]
	ribonucleotide reductase	1.17.4.1	[33, 296, 79]
Shikimate biosynthesis	5-enolpyruvyl-shikimate-3-phosphate synthase	2.5.1.19	[415]
	amino-deoxychorism. synthase	6.3.5.8	[169]
	chorismate synthase	4.2.3.5	[318]
DNA transcription and replication	histone deacetylase		[303]
	topoisomerase I	5.99.1.2	[53]
	topoisomerase II	5.99.1.3	[356, 159, 85]

Continued on next page

Table 2.1 – continued from previous page

Pathway	Protein	EC no.	Ref.
	telomerase		[484, 7]
Hemoglobin digestion	dipeptidyl aminopeptidase 1	3.4.14.1	[258]
	falcipains (cysteine proteases)		[469, 24, 423]
	plasmepsins (aspartic acid proteases)	3.4.23.38/ 3.4.23.39	[357, 422]
	histo-aspartic protease		[30]
	leucine aminopeptidase	3.4.11.1	[352]
	M1-family aminopeptidase		[146]
Heme detox.			[490, 407]
Antioxidant defense	γ-glutamylcysteine synthetase	6.3.2.2	[390, 295, 323]
	glutathione reductase	1.8.1.7	[48, 574]
	glyoxalase I	4.4.1.5	[505]
	superoxide dismutase	1.15.1.1	[480]
	thioredoxin reductase	1.8.1.9	[264, 295]
	glutathione transferase	2.5.1.18	[187, 288, 383]
Prot. kinases	cyclin-dep. protein kinases		[259, 542]
Miscellaneous	SERA4-6		[331]
	sarco/ER Ca2+ ATPase		[173]
	subtilisin 1,2		[552, 512]

2.2 Drug discovery pipeline

In the past the discovery of drugs was often promoted by serendipity as it has been the case for the discovery of penicillin by Alexander Fleming. Nowadays, with the availability of many sequenced genomes, high-throughput technologies, combinatorial chemistry and sophisticated bioinformatics methods, new drugs are filtered in a drug discovery pipeline (see Fig. 2.2) from a large set of substances. The development of a market-ready drug is a long (10-15 years) and expensive (∼$800 million per approved drug) process, thus pharmaceutical companies try to eliminate unsuccessful substances as early as possible from the pipeline. The first step of the pipeline is the assessment of available biological data for the disease of interest and the market potential of corresponding drugs. In this course questions are addressed like the existence of an adequate animal model and whether there's a market for the drug to recover research and development expenses. Then the search for a suitable drug target begins. Improper selection during this phase has been found to be the main cause for the high failure rate of drug development [71]. 90% of current drug targets are either enzymes, G-protein coupled receptors, ion channels or transporters [208] and thus encoded by genes. The human genome as well as the genomes of human pathogens therefore represent large resources for potential targets. Genes that are further considered should meet at least two criteria: First, they need to be involved in the disease, and second, they should be druggable, meaning that their activities can be modulated through the binding of therapeutic molecules. Genes fulfilling the first requirement can be identified with the help of available information (literature searches, database mining) or conducted animal studies and clinical trials. In addition, genome-scale transcriptome profiles, comparing gene

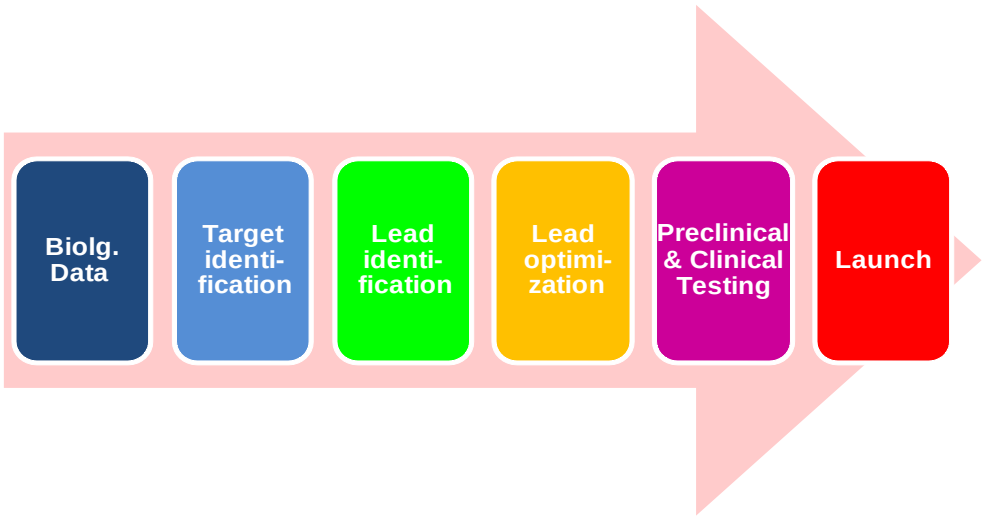


Figure 2.2: **Drug discovery pipeline.**

expression levels of healthy and diseased tissue, suggest genes that are disease relevant. Genes are assumed to fulfill the second requirement (druggability) if they belong to a gene family with members known to be able to bind small molecules (development by analogy). In general, choosing a target that is easily accessable to drugs and for which the protein structure has been resolved or predicted, simplifies subsequent steps of the drug discovery pipeline. For example, drug binding sites can be identified with bioinformatics methods if structures of the targets are available.

In case the drug target is within a pathogen, the target has to be essential for the pathogen, at least during its developmental stage in the human host. Computational analyses of respective cellular networks (metabolic or signal transduction) can give hints on such targets by unveiling those candidates that can not be circumvented by alternative paths (see section 4.4). In addition, genes homologous to the target should either be absent in the host or sufficiently distinct to avoid unwanted side effects upon drug administration. Such genes can be identified by comparative analyses of host and pathogen genomes.

Before a target can proceed in the drug discovery pipeline the importance of its role with respect to the disease needs to be validated. This can be done either with chemical or genetic methods [474], which complement each other. Chemical validation is accomplished if there is experimental evidence for disease attenuation by specific inhibition of the target with a chemical compound. This approach allows on the one hand to assess the likelihood of successful drug delivery to the target, but on the other hand positive results can not be unambiguously ascribed to the target, since the compound might bind unspecifically. Genetic approaches such as gene deletion by homologous recombination or RNA interference are thought to be more definitive with respect to testing the essentiality of a target for a disease. However, if it is not possible to obtain null-mutants this does not necessarily mean that the target is essential, since it needs to be tested in the appropriate life-cycle stage and environment. Further evidence can be obtained from conditional knockouts that are inducible during the stage of interest.

When the target is settled a lead molecule that binds to the target is sought. High-throughput screening (HTS) approaches that rely on miniaturized and automated assays allow to test large libraries of compounds within a short time. If the 3D structure of a target protein is known, the binding affinity of small molecules can alternatively be predicted by computer-aided virtual screening approaches, or a compound that fits into the binding pocket can be designed *de novo*. Those compounds (leads) that show high affinity for the target are further optimized to increase selectivity and reduce toxicity. In this context, a set of molecules that is structurally related to the lead compound is generated by means of combinatorial chemistry and subsequently tested.

After the lead optimization process is finished, pharmacokinetics and pharmacodynamics are tested in preclinical trials with animals. If these trials are successful the drug candidate is tested with a small number of healthy humans (clinical phase I). In clinical phase II the drug is administered to up to 500 hundred patients suffering from the respective disease and in clinical phase III to more than 1,000 patients. If the drug is then approved by the authorities of a country, commercialization of the drug may start and finally amortize research and development costs.

Chapter 3

Available experimental data for *P. falciparum*

The modeling process of cellular systems is always accompanied by experimental data such as kinetic constants which build a foundation for the computational model. With the advent of high-throughput techniques several types of experimental data have become available on genome-scale such as transcriptomic [59, 419, 427, 112, 501], proteomic [147, 185, 501] and protein interaction data [273]. Compared to proteomics and metabolomics data [503] more data is on-hand from measurements of gene transcript levels, providing a broad basis for computational simulations.

3.1 Genome-wide transcriptomic data

Microarrays facilitate the analysis of the transcriptome and genomic characteristics of cells in a high-throughput manner. Corresponding experiments basically consist of three steps. In the first step, the array is designed and produced. Then data are collected during the actual experiment which includes tissue preparation, mRNA isolation, mRNA labeling, hybridization and scanning. Finally, scanner images are analyzed, data points normalized and further evaluated in order to extract the underlying information. Different types of microarrays are available that vary with respect to the following aspects of the production process and application:

- array probes: **cDNA** (complementary DNA; obtained from mRNA by RT-PCR) vs. **oligo** (synthesized short DNA fragments)
- probe fixation: **spotting** (spotting robot immobilizes probes on array) vs. ***in-situ* probe synthesis** (probes are synthesized on array)
- **one color** (absolute experiment: only one sample per array) vs. **two color** experiment (comparative experiment: two samples labeled with different fluorophores are hybridized on same array)

In the last years several genome-scale experiments have been conducted, giving an insight on the transcriptome of malaria pathogens during different life cycle stages.

3.1.1 Bozdech data set

One of the first genome-scale transcriptome studies of *P. falciparum* was published by Bozdech *et al.* in 2003 [59]. Custom designed DNA microarrays with oligonucleotides respresenting predicted open reading frames (ORFs) were used to monitor gene expression during the intraerythrocytic developmental cycle (IDC) on an one hour timescale resolution.

The individual mRNA samples were hybridized in a comparative experiment against a reference pool comprising all extracted samples. The study revealed that the majority of genes was expressed during the IDC, leaving only a small percentage of genes specific to other developmental stages. Furthermore, about three quarters of those genes that were transcribed during the IDC were found to be activated only once during the 48 hours cycle. Evaluation of functionally annotated genes showed that the presence of gene transcripts correlates well with the biological function of the respective proteins.

3.1.2 Le Roch data set

An approach for gene function annotation with the help of expression profiling [419] was presented by Le Roch and colleagues. In this context they measured gene expression in *P. falciparum* during the mosquito stage (salivary gland sporozoite), the sexual stage (gametocytes present in human blood) and seven time points of the erythrocytic asexual stage (early and late ring stage, early and late trophozoite, early and late schizont, and merozoite). Gene transcript levels were analyzed in an one-color approach with custom designed high-density oligonucleotide arrays covering predicted coding and non-coding sequences. The study demonstrated that genes with similar functions exhibit similar expression profiles and thus microarray experiments can be used to further annotate functionally uncharacterized genes.

3.1.3 Sacci data set

Gene expression during the liver stage of malaria pathogen development was analyzed on genome-scale for the first time by Sacci *et al.* in 2005 [427]. Due to difficulties in these analyses for the human parasite, they constructed a liver stage cDNA library for the rodent parasite *P. yoelii*. Liver stage schizonts were extracted by laser capture microdissection. Subsequently, mRNA was isolated, transcribed into cDNA and amplified. BLAST searches with the sequenced cDNAs identified genes expressed during late liver stage.

3.1.4 Tarun data set

Recently, another transcriptome study for *P. yoelii* has been published by Tarun and colleagues [501], covering three time points during the liver stage (24 h, 40 h, and 50 h post-infection) which they compared to two time points during the mosquito stage (10 days (midgut) and 15 days (salivary gland) after blood meal) and two time points during the blood stage (schizont and mixed blood stages). For efficient isolation of infected hepatocytes, green fluorescent protein-tagged parasites and fluorescence activated cell sorting were utilized. Parasitic liver stages were found to be highly metabolically active with up-regulated mitochondrial activity.

3.1.5 Daily data set

Both above mentioned gene expression data sets covering the blood stage of *P. falciparum* are obtained from blood cultures and thus might not fully reflect *in vivo* gene expression. In contrast, the genome-scale transcriptome study presented by Daily and coworkers assesses 43 samples derived from the blood of *P. falciparum* infected patients which contain mostly

ring stages of the parasite [112]. These samples were found to correspond to three groups: starvation response accompanied by metabolism of alternative carbon sources (cluster 1), active growth based on glycolytic metabolism (cluster 2) and environmental stress response (cluster 3).

3.2 Further available high-throughput data

Most of the large-scale studies conducted for *P. falciparum* examined the transcriptome of the parasite [59, 419, 112, 576] thanks to the relatively simple microarray technology that allows to measure mRNA levels of thousands of genes simultaneously. Fewer time points of the parasite's life cycle have been analyzed with respect to present proteins. For *P. falciparum* protein profiles of the blood stage [147, 275] as well as the mosquito stage [147, 275, 276] have been determined. Up to now, the liver stage proteome has only been analyzed for the rodent malaria pathogen *P. yoelii*. Mass spectrometry studies investigating the proteome of the parasite recovered less protein species than were suggested to be present by transcriptome analyses [501]. This might be due to varying levels of sensitivity inherent to the detection methods.

Physical interactions between proteins have been assayed with a yeast two-hybrid system that revealed almost 3,000 unique interactions. A lot less is known about the metabolome of *P. falciparum*. About 50 metabolites have been identified by NMR spectroscopy in mature trophozoites [503] and about 90 in a liquid chromatography-tandem mass spectrometry study that analyzed seven time points of the intraerythrocytic developmental cycle [366].

Chapter 4

Related work

4.1 Relevant databases

4.1.1 Malaria Parasite Metabolic Pathways

A fundamental resource for information about the metabolism of *Plasmodium falciparum* is the Malaria Parasite Metabolic Pathways (MPMP) website (`http://sites.huji.ac.il/malaria/`) designed by H. Ginsburg [170], whose concern was to present available information in a clear and educative way. The website is structured in more than 100 maps, not only describing classical metabolic pathways and metabolite transport, but also biological processes such as cell-cell interaction, replication, transcription and translation. Metabolic pathway maps were manually derived from the KEGG database and adapted according to the present knowledge of parasite biochemistry and annotations of the *P. falciparum* genome. Pathways were considered to occur in *P. falciparum* if either most enzymes are suggested by genome annotation or biochemical evidences imply their functionality. Thus, certain enzymes may not be associated with any gene. Each enzyme entry is linked to supplementary information from other databases, including PlasmoDB, GeneDB, BRENDA and ExPASy ENZYME. In addition, enzymes are graphically annotated with information on inhibitors as well as a 48h clock that displays gene transcription during the blood stage.

4.1.2 PlasmoCyc

PlasmoCyc (`http://plasmocyc.stanford.edu/`) is a metabolic pathway database derived from the genome annotations of *Plasmodium falciparum*. A first draft of PlasmoCyc was generated computationally with the PathoLogic software [372], which requires a reference pathway database as input in addition to the genome annotation. Based on annotated gene product qualifiers and EC numbers PathoLogic infers biochemical reactions that occur within an organism. The more reactions of a pathway contained in the reference pathway database are predicted, the more likely is the presence of the respective pathway in the organism. The resulting network was complemented with pathways from the Malaria Parasite Metabolic Pathways website as well as reactions extracted from scientific literature. The online interface of the PlasmoCyc database visualizes the metabolic network in the form of pathway maps. Enzymatic reactions are linked to more detailed representations with chemical structures of involved metabolites as well as information on the protein subunits of enzyme complexes. PlasmoCyc is integrated into the BioCyc website which provides a collection of organism-specific pathway databases.

4.1.3 KEGG

The Kyoto Encyclopedia of Genes and Genomes, or simply KEGG, is a knowledge base developed and maintained by the Kanehisa lab in Kyoto (`http://www.genome.jp/kegg/`). KEGG consists of four main databases (PATHWAY, BRITE, GENES and LIGAND) that are divided into several subdatabases, containing data among others on enzymes, reactions, diseases and drugs [239, 241, 240]. KEGG PATHWAY provides manually drawn pathway maps, representing metabolic pathways and other cellular processes. On the basis of these maps and the subdatabase KEGG orthology (KO), which catalogues orthologous genes, organism specific metabolism can be inferred. Orthologous genes are determined in a semi-automated procedure involving pairwise genome comparison and the integration of gene clusters from the COG database [502] and protein families classified by experts. KEGG provides tools to browse and search functional hierarchies, to analyze transcriptomic and metabolome data in the context of pathway maps and to draw chemical compound structures that can be used to search the KEGG databases.

4.1.4 BRENDA

BRENDA (BRaunschweig ENzyme DAtabase, `http://www.brenda-enzy- mes.org`) provides a comprehensive collection of biochemical and molecular information on more than 4,000 different metabolic enzymes classified according to the Enzyme Commission list of enzymes [82]. The manually curated database entries contain enzyme related information from primary literature such as enzyme nomenclature, enzymeligand interactions, functional parameters, organism related information, information on enzyme structure, molecular properties and links to other databases. Two additional databases, AMENDA (Automatic Mining of ENzyme DAta) and FRENDA (Full Reference ENzyme DAta), have been developed that contain enzyme data extracted from PubMed abstracts by text-mining procedures. These databases cover more organism-specific enzyme information than BRENDA, but are less precise due to missing manual validation.

4.1.5 Reactome

The Reactome online platform `http://www.reactome.org` [524, 525, 309] represents a comprehensive resource for human metabolic pathways all curated by expert biological researchers and peer-reviewed. In addition, tools are provided for pathway visualization and analysis of large-scale datasets such as transcription profiles. The curated human data is used to infer pathways and reactions in non-human species with sequenced and annotated genomes like *Plasmodium falciparum*. In this context similarity clusters are compiled on the basis of reciprocal best similarity between human genes and those of an organism of interest. If matching enzymes of a particular pathway are found in the organism, the pathway is assumed to be present and missing enzymes are postulated.

4.1.6 Transport Classification Database

The Transporter Classification Database (TCDB) is a curated database cataloging membrane transport proteins according to an IUBMB approved classification system [429, 430].

Similar to the Enzyme Commission (EC) system for classification of enzymes, TCDB provides a classification of membrane transport proteins and in addition information about protein sequence, structure and function as well as evolutionary information about transport systems from multiple organisms including *P. falciparum*. The database is hosted at `http://www.tcdb.org/` and currently encompasses about 5,000 representative transporters and putative transporters in more than 500 families which were compiled from primary literature. Several tools are offered that allow to classify newly identified membrane proteins and to annotate genome sequences.

4.1.7 Gene Ontology

The goal of the Gene Ontology (GO) initiative is to provide a controlled vocabulary that standardizes attributes assigned to genes and gene products to describe their roles in any organism [19]. Attributes are related to three main categories: cellular component, biological process and molecular function. Each of these categories is structured as a hierarchical directed acyclic graph where terms are linked by either of the two relationships "is-a" or "part-of". Terms can have several parent terms and several child terms. Multiple databases including PlasmoDB, the Plasmodium genome resource [22], have already annotated their data with GO terms and provide references to support the annotations. This data is available at `http://www.geneontology.org` together with tools to access and process the data.

4.2 Flux balance analysis

Flux balance analysis (FBA) is a commonly used method to investigate the metabolic capabilities of cellular systems, since it allows to estimate unknown fluxes in metabolic networks with respect to cellular constraints [130, 394, 138, 280, 125, 139, 218, 298, 365, 12, 84, 137, 359, 398, 337, 360, 404] (see Fig. 4.1 for illustration). Among others, FBA has been used for metabolic engineering studies [387, 281], drug target prediction [233, 135, 279] as well as growth prediction on different media [320, 522]. In contrast to kinetic modeling, no detailed rate equations are required, predestining the method for large metabolic networks which lack full kinetic characterization of all enzymes. Prerequisite is rather the stoichiometric matrix S, which describes the number of metabolite molecules consumed and produced by each reaction of the metabolic network, as well as knowledge about the cellular objective. The basis of FBA is the flux balance principle which assumes the system to be at quasi steady-state, as biochemical reactions are typically much faster than changes in the phenotype of a cell during growth or differentiation. Thus, the sum of fluxes producing a metabolite equals the sum of fluxes consuming the metabolite:

$$Sv = 0$$

where v is the metabolic flux vector, containing internal metabolite fluxes as well as exchange fluxes. The solution space of the resultant linear equation system is a high dimensional flux cone, containing all flux configurations that are feasible at steady-state. Constraints derived from thermodynamics and cellular environment conditions further restrict the solution space. Organisms are thought to be optimized in the course of evolution. Therefore, a biologically meaningful objective function, which is a linear function of fluxes, is applied

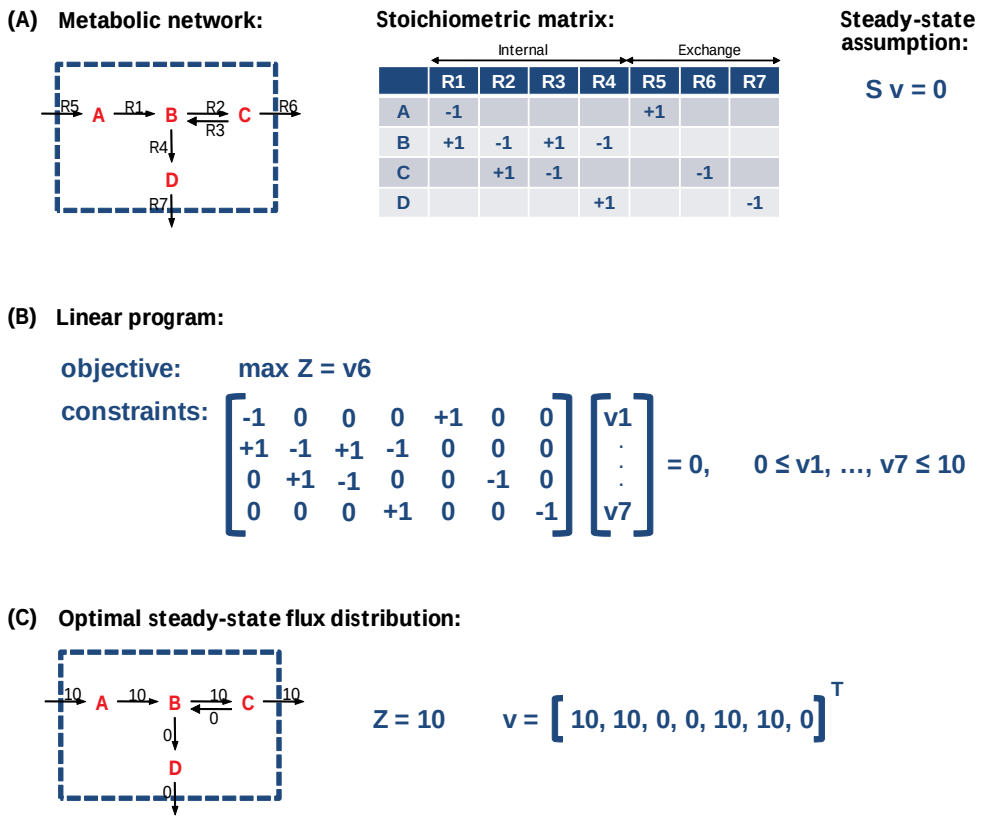


Figure 4.1: **Illustration of flux balance analysis.** Given the stoichiometric matrix S of a network and the steady-state assumption (A), a set of algebraic constraints on metabolic fluxes v can be formulated. Additional constraints defining lower and upper bounds on each flux can be inferred for example from thermodynamics. Usually the number of variables (unknown metabolic fluxes) is larger than the number of equations (equals number of metabolites) for the resulting linear equation system (B). Therefore, optimization is applied to identify a flux distribution among all possible solutions, which is optimal with respect to a chosen criterion. Here, flux v_6, which corresponds to the secretion of a certain target metabolite, is maximized. Given the set of constraints, a maximum flux value of 10 can be obtained for v_6 if v_1, v_2 and v_5 are assigned to equal 10 (C).

to the set of possible solutions to identify a flux distribution that is optimal with respect to the cellular objective. Maximization of biomass is one such target function that is assumed to be suitable especially for microorganisms [523]. For eukaryotic cells such as hepatocytes, for which reproduction is not the primary goal, cellular maintenance at minimal efforts has been proposed as an alternative objective function [203]. Altogether this yields the following FBA formalism:

$$\begin{aligned} \min / \max_{v} \quad & c^T v && \text{(objective function)} \\ \text{s. t.:} \quad & Sv = 0 && \text{(steady-state assumption)} \\ & v_{\min,i} < v_i < v_{\max,i} && \text{(constraints)} \end{aligned}$$

where c is the vector of linear combination coefficients and v_{min} and v_{max} are the lower and upper bounds on fluxes.

4.2.1 MinModes

Determining metabolic fluxes that are linked to certain cellular functions is often a difficult task, especially in large metabolic networks. To avoid tedious experiments several structural analysis concepts have been developed, including elementary modes and extremal pathways [442, 449]. These methods explore for a given metabolic network all routes (modes) converting substrates into output metabolites. The main disadvantage of these methods is the large number of modes arising from combinatorial multiplicity when network size increases. The concept of MinModes (see Fig. 4.2) is an approach based on flux balance analysis to decompose flux distributions into a manageable number of functionally interpretable modes [201]. A MinMode is defined as a minimal steady-state flux distribution that allows for the production of a exactly one target metabolite, corresponding to a certain functional requirement of the cell. The underlying assumption is that flux distributions are basically superpositions of multiple MinModes with different relative intensities, which are reflected in the coefficients of the linear combination. Thus, comparing the coefficients of two flux distributions decomposed into MinModes unveils directly changes in cellular functions.

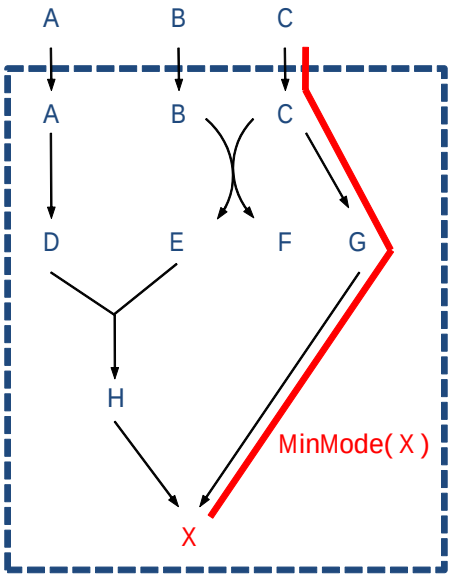


Figure 4.2: **MinMode illustration.** A MinMode is defined as a steady-state flux distribution which is optimal with respect to the flux minimization principle (sum of non-zero fluxes is minimal) and yields a single unit of the metabolite of interest. In the illustrated example network the MinMode for metabolite X is indicated by the red line.

4.3 Integrating gene expression data into flux calculations

Genome sequence analysis only reveals which metabolic reactions could possibly occur within an organism, but not which reactions are actually active during a certain time point, not to mention the quantitative contributions of each reaction. Objective functions representing cellular goals are supposed to drive flux balance analysis to the identification of true flux distributions. However, considering that currently no organism is fully characterized, suggests that proposed objective functions do not fully cover the true cellular objectives. Furthermore, organisms might pursue different goals during different stages of their lifespan and cells of multi-cellular organisms might even fulfill different tasks depending on the tissue. A hint on the cellular status can be provided by gene expression experiments which monitor the presence of gene transcripts during a certain time point and allow inferences on the subsequent presence of corresponding enzymes. Integration of gene expression data into flux balance calculations therefore has the potential to improve the modeling of cellular metabolism.

4.3.1 GIMME algorithm

A straight forward way to integrate transcriptomic data into FBA was proposed by Åkesson *et al.* [4]. The basic assumption is that if a gene is not expressed, the corresponding enzyme is absent and thus the respective reaction is not catalyzed. In FBA calculations this is implemented as additional constraints that keep fluxes through reactions to zero if enzymes are not present, thereby reducing the feasible solution space. Improved predictions of the metabolic behavior of yeast cells in batch cultures were obtained with this approach. Due to the simplicity of this approach, no flux distributions can be predicted if required metabolic functionalities can not be achieved without enzymes suggested by gene expression data to be absent.

An approach solving this problem was published by Becker and Palsson [38] who named their method GIMME (Gene Inactivity Moderated by Metabolism and Expression, see Fig. 4.3). Similar to the Åkesson approach, reactions with expression values below a certain threshold are removed from the network, and an optimal flux distribution is sought that meets the required metabolic functionalities. In case the problem is infeasible, optimization is used to find the most consistent set of reactions to obtain a valid flux distribution:

$$
\begin{aligned}
&\min \sum c_i \mid v_i \mid \\
\text{s. t.:}\ &Sv = 0 \\
&a_i < v_i < b_i \\
&c_i = \begin{cases} x_{cutoff} - x_i & \text{if } x_{cutoff} > x_i \\ 0 & \text{otherwise} \end{cases}
\end{aligned}
$$

where x_i is the corresponding gene expression value and x_{cutoff} a threshold above which enzyme presence is assumed. The degree of inconsistency between predicted fluxes and gene expression data is given by the value of the objective function.

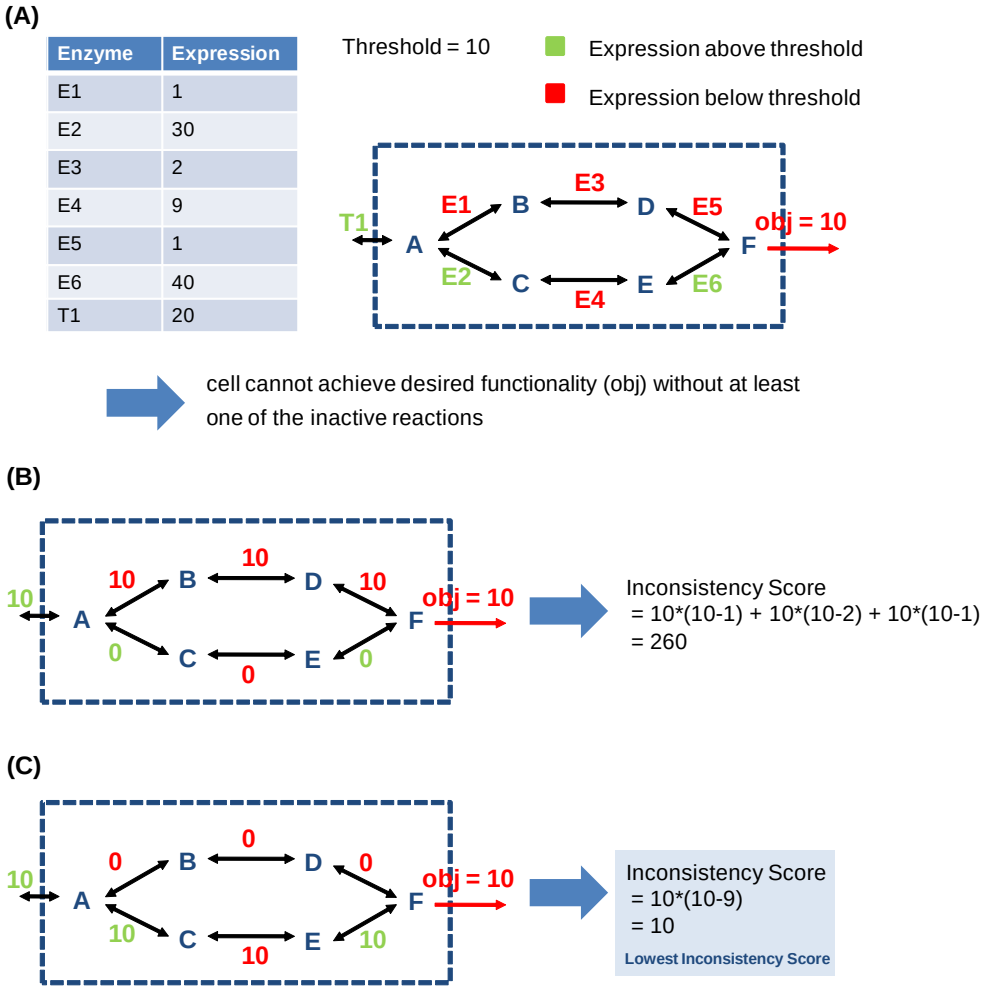


Figure 4.3: **GIMME algorithm illustration.** For the depicted metabolic network available gene expression data is listed in the blue table (A). Applying a threshold of 10 to the expression levels reveals that only enzymes E2, E6 and transporter T1 are active. Thus, the network can not fulfill the objective of producing 10 units of metabolite F if only those reactions are allowed to carry non-zero fluxes, where the level of corresponding gene transcripts is higher than the chosen threshold. This objective can only be accomplished if either enzymes E1, E3 and E5 are assumed to be active as well (B) or enzyme E4 (C). The latter assumption yields a lower Inconsistency Score, as the expression status of only one enzyme has to be changed, which in addition is expressed at a level close to the threshold. Therefore, the GIMME algorithm returns the flux distribution depicted in (C).

4.3.2 Shlomi approach

Shlomi *et al.* [465] presented a more sophisticated approach to predict tissue-specific metabolic behavior on the basis of gene expression data and flux balance analysis. In contrast to common FBA methods, no assumptions about cellular objectives are made. According to the authors, hints on tissue-specific metabolic goals can rather be inferred from predicted

metabolite uptake and secretion. The concept of the Shlomi approach is to maximize the conformity of metabolic fluxes with the expression status of corresponding genes, but not to insist on total agreement like the Åkesson approach in the case of unexpressed genes (see Fig. 4.4). This takes into account post-transcriptional regulation, which arranges that on the one hand certain gene transcripts are not translated into enzymatic proteins, while on the other hand, certain gene transcripts present at almost undetectable levels are translated with high efficiency. The corresponding mixed integer linear programming problem is formulated as follows:

$$\max_{v,y_0,y_{1p},y_{1n}} \sum_{i \in R_{NE}} y_{0,i} + \sum_{i \in R_E} (y_{1p,i} + y_{1n,i}) \tag{4.1}$$

$$\text{s. t.:} \quad Sv = 0 \tag{4.2}$$

$$v_{\min} \leq v \leq v_{\max} \tag{4.3}$$

$$v_i + y_{1p,i}(v_{min,i} - \epsilon) \geq v_{min,i}, \ \forall i \in R_E \tag{4.4}$$

$$v_i + y_{1n,i}(v_{max,i} + \epsilon) \leq v_{max,i}, \ \forall i \in R_E \tag{4.5}$$

$$v_{min,i}(1 - y_{0,i}) \leq v_i \leq v_{max,i}(1 - y_{0,i}), \ \forall i \in R_{NE} \tag{4.6}$$

$$v \in R^m \tag{4.7}$$

$$y_{0,i}, y_{1p,i}, y_{1n,i} \in [0, 1] \tag{4.8}$$

where v is the flux vector and S the stoichiometric matrix describing the topology of the metabolic network. The steady-state assumption is ensured by (4.6). Thermodynamic constraints restricting flux directions are imposed on the solution space by (4.7). Two boolean variables ($y_{1p,i}$, $y_{1n,i}$) are introduced for reactions corresponding to expressed genes (R_E). Inequalities (4.8) and (4.9) enforce fluxes in forward or backward direction to be larger than threshold $\epsilon = 1$ if the respective boolean variables are set to 1. Similar, inequality (4.10) guarantees that reactions associated with unexpressed genes (R_{NE}) do not carry any flux if the boolean variables $y_{0,i}$ are equal to 1. The sum of these binary variables composes the objective function that is sought to be maximized, in order to obtain flux distributions that are consistent with corresponding expression profiles.

This approach was applied to predict tissue-specific metabolism for ten human tissues including liver, brain and heart. Comparisons with available data sources for tissue specificity of genes, reactions and metabolites showed significant correlations between predictions and these data sets.

4.3.3 E-Flux algorithm

Very recently Colijn *et al.* proposed a novel method called E-Flux to infer the metabolic status of a cell from gene expression profiles [96]. Like the approaches reviewed in the previous sections, E-Flux extends the flux balance analysis concept. The basic idea is to introduce lower and upper flux bounds for all reactions of a metabolic network as a function of measured gene expression (see Fig. 4.5). Upper flux bounds are set to the expression values of corresponding genes and are normalized with respect to the highest bound. If a reaction is catalyzed by an enzyme complex, the minimum expression value of all complex components is chosen as an upper flux bound. In case of isozymes catalyzing the same reaction, the upper flux bound is set to the sum of all corresponding expression values.

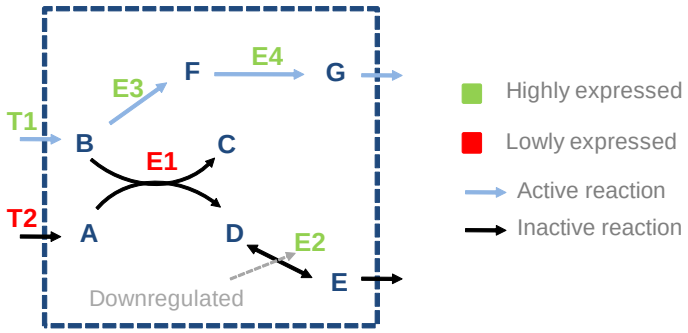


Figure 4.4: **Illustration of the approach by Shlomi *et al.* to calculate metabolic fluxes consistent with gene expression data.** The example metabolic network consists of four enzymes (E1-E4) and two transporters (T1, T2) whose corresponding genes are all highly expressed except for those corresponding to E1 and T2. The predicted steady-state flux distribution is indicated by light blue arrows. An alternative steady-state flux distribution which assigns a non-zero flux to enzyme E2 would yield a lower objective value, as the expression status of two enzymes/transporters (E1, T2) have to be changed instead of one (E2). Therefore, gene transcripts corresponding to enzyme E2 are assumed to be post-transcriptionally downregulated. Illustration adapted from [465].

Lower flux bounds are for all reversible reactions equal to the upper bounds multiplied by -1 and for all irreversible reactions equal to 0. Thus, metabolic fluxes are constrained to values between -1 and 1. If a reaction is associated with genes that are only sparsely expressed, tight constraints are assigned to the fluxes, while flux constraints are looser, if corresponding genes are highly expressed.

The E-Flux method was applied to the metabolism of the tuberculosis pathogen *Mycobacterium tuberculosis*, using as an objective function biomass maximization and mycolic acid production, respectively. Several hundred gene expression experiments, comparing treated cells with untreated cells, were considered to infer metabolic fluxes in treated cell lines and control cell lines, respectively. The ratio of the maximal biomass production rate obtained for treated cells and for untreated cells was then calculated to assess the impact of the treatment on mycolic acid production. By this means several known inhibitors of mycolic acid or fatty acid synthesis were correctly identified among the compounds used to treat the bacterium and several yet unknown inhibitors and enhancers were predicted.

4.4 Network based drug target analysis

Identification of suitable drug targets represents the first step in the drug discovery pipeline (see section 2.2). In general, a large number of cellular components come into consideration for any particular disease. Experimental screening of all candidates is time consuming and expensive. Therefore, computational methods that are able to prioritize a subset of these candidates can help to reduce the number of targets that are experimentally tested and thus reduce the costs, as computational methods are far less expensive than wet lab experiments.

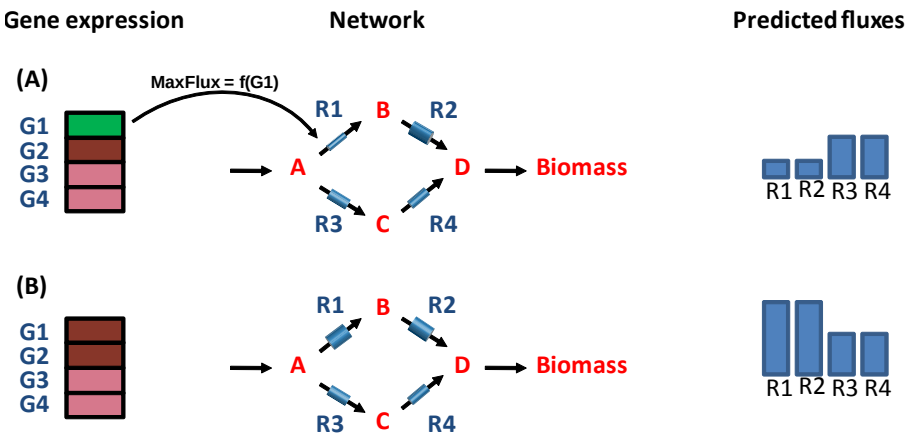


Figure 4.5: **Illustration of the E-Flux method.** Constraints imposed by the E-Flux method on possible metabolic fluxes through the example network are illustrated by pipes surrounding reaction arrows. The higher expression levels of corresponding genes are, the higher are the upper limits on fluxes and thus the larger are the diameters of the pipes. (A) and (B) show the flux rates obtained with the E-Flux algorithm for two different gene expression states (green: low expression, red: high expression). When gene G1 is lowly expressed (A), reaction R1 is restrained to carry a small flux. Although gene G2 is highly expressed, the flux through reaction R2 cannot be higher than that through R1 due to the steady-state condition. However, if G1 has a similarly high expression level as G2 (B) R1 and R2 are both assigned to high flux values, which in contrast to (A) exceed the fluxes through the moderately active reactions R3 and R4. Illustration adapted from [96].

4.4.1 Metabolic control analysis

As outlined in section 2.2 components of a biological network are generally considered to be good drug targets if they are essential for disease progression. Complete inhibition of any of these components would result in disease attenuation. However, complete inhibition of enzymes is difficult, since most drugs are designed as reversibly binding competitive substrate analogs and thus can be supplanted by the substrate which accumulates upon enzyme inhibition [101]. Therefore, drugs are more effective if they act on targets that have a large influence on pathways even when inhibited only partially. Such targets can be identified with the help of metabolic control analysis (MCA) [235, 195, 141], a mathematical framework that was originally developed for metabolic networks, but has been extended to other biological networks [210, 421]. So called **flux control coefficients** C_i^J and **concentration control coefficients** C_i^X (see Fig. 4.6) quantify the impact of a network component i (e.g., an enzyme) on the network flux J or component concentration X (e.g., metabolite concentration), respectively, as follows:

$$C_i^J = \frac{\frac{\partial \ln J}{\partial \ln p_i}}{\frac{\partial \ln v_i}{\partial \ln p_i}}, \quad C_i^X = \frac{\frac{\partial \ln X}{\partial \ln p_i}}{\frac{\partial \ln v_i}{\partial \ln p_i}}$$

with p_i being a parameter such as enzyme concentration that is infinitesimally changed and v_i being the enzyme rate. Changes of the parameter may shift the system from one steady-state to another. Control coefficients basically relate the resulting relative changes of steady-state fluxes (concentrations) to the relative activity changes of an enzyme with all other enzyme activities being constant. The larger a control coefficient, the stronger is the impact of an enzyme on the respective flux or concentration. A flux control coefficient of 1 implies that changing the enzyme's activity changes the steady-state flux in the same proportion. The enzyme is thus a true rate-limiting enzyme. In ideal networks the sum of all flux control coefficients equals 1 while all concentration coefficients sum up to 0 (summation theorem). Control coefficients are system properties and therefore change with the status of a system.

MCA has been applied in drug discovery [76, 211] to suggest drug targets among others for the treatment of cancer [97] and African sleeping sickness caused by the parasite *Trypanosoma brucei* [26]. Glycolytic enzymes are assumed to be good drug targets in *T. brucei*, since this pathway is the parasite's only source for ATP. Bakker *et al.* proposed to choose targets that have high flux control coefficients in the parasite, but low flux control coefficients in the host (differential control analysis), thus increasing selective inhibition. Four glycolytic enzymes (aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase) were found to fulfill this criterion. Drugs specifically designed to inhibit these enzymes in the parasite are therefore assumed to have a large impact on parasite development, but few side effects on homologous host enzymes, due to lower binding affinities for host enzymes and lower influence of these enzymes on the glycolytic pathway in the host.

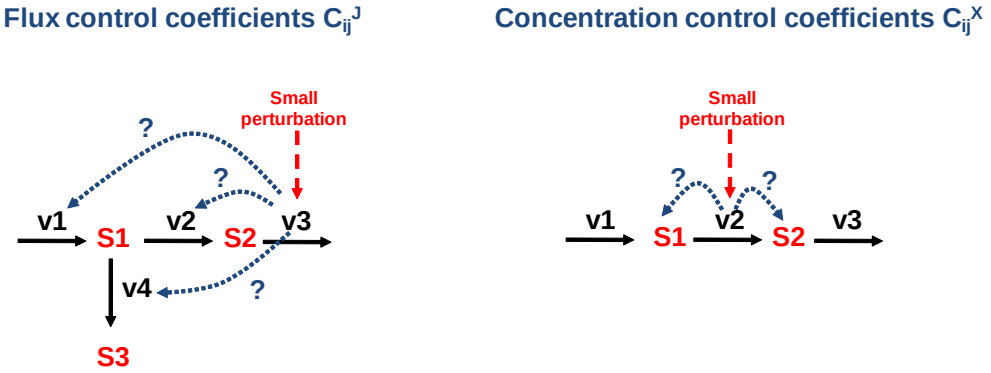


Figure 4.6: **Flux control coefficients and concentration coefficients in metabolic control analysis.** The coefficients indicate the effect of changes in the activity of a specific enzyme (dotted blue arrows) on steady-state fluxes or concentrations, respectively, within a metabolic network.

The major downside of MCA is the requirement of a kinetic model for the system of interest. Since kinetic data is not yet available for genome-scale networks, control coefficients can only be calculated for well studied pathways. Furthermore, control coefficients are linear approximations, thus overestimating larger variations in enzyme activities [447].

4.4.2 Choke-point analysis

Choke-point analysis is a simple topology based approach to identify presumably essential enzymes even in genome-scale metabolic networks. **Choke-points** are enzymes that either uniquely consume or produce a certain metabolite (see Fig. 4.7). Inhibition of such an enzyme likely results either in accumulation of metabolites, which is potentially toxic for the cell, or in depletion of possibly essential metabolites, making the enzyme indispensable. The essentiality of enzymes has been assessed with this method for several organisms [561, 401, 472, 136], revealing that choke-point analysis is able to detect validated drug targets. Rahman *et al.* combined choke-point analysis with the concept of **load-points**, which are network hot spots with a high number of k-shortest paths passing through, but a low number of attached nearest neighbors [401]. Ranking choke-points according to their load is assumed to prioritize biochemically important enzymes, since a high load indicates that an enzyme connects a large number of metabolites via a shortest path. Another improvement of choke-point analysis was proposed by Fatumo *et al.* who suggested an algorithm that analyses whether all compounds downstream of a knocked-out reaction can be produced by other pathways with an equal number of enzymatic steps as it would be possible with the complete metabolic network [136]. Combination of this algorithm and choke-point analysis resulted in a higher accuracy and precision for predicted drug targets.

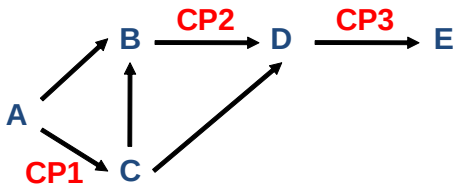


Figure 4.7: **Illustration of choke-points.** The example metabolic network contains three choke-points: CP1, because it produces a unique product; CP2, because it consumes a unique substrate and CP3 because it both consumes a unique substrate and produces a unique product. Illustration adapted from [561].

Choke-point analysis is a simple and fast method to predict drug targets, requiring only the network topology as input, but no further experimental data. However, the method tends to yield many false positives [561] and thus low accuracy, especially when the network has a large periphery, which might be the case in incomplete networks.

4.4.3 Minimal cut sets

Another network topology based approach to detect components critical for metabolic functioning is the concept of minimal cut sets (MCS) [256, 255], which is implemented in the CellNetAnalyzer software available at `http://www.mpi-magdeburg.mpg.de/projects/cna/cna.html`. A MCS is a irreducible set of network components whose removal will lead to a failure in certain network functions such as the production of target metabolites (see Fig. 4.8). Given a user defined objective function all MCSs of a metabolic network can be computed on the basis of elementary flux modes (EM), which are minimal metabolic routes through a network operating at steady-state [448, 449]. Any feasible steady-state flux dis-

tribution can be represented by a linear combination of elementary flux modes. For each EM that involves the objective reaction, a MCS contains at least one reaction active in this EM. Thus, removing all reactions of a MCS from the metabolic network of interest ensures that no feasible and balanced flux distribution through the network exists that involves the objective reaction, as all EMs containing this reaction have vanished. The proposed algorithm calculates MCSs by iteratively enlarging a set of reactions by adding a reaction contained in an EM that involves the objective reaction, but does not contain any reaction of the current set. When a set of reactions coveres all EMs corresponding to the objective function and is not a superset of other sets with the same characteristic, the set is considered to be a MCS.

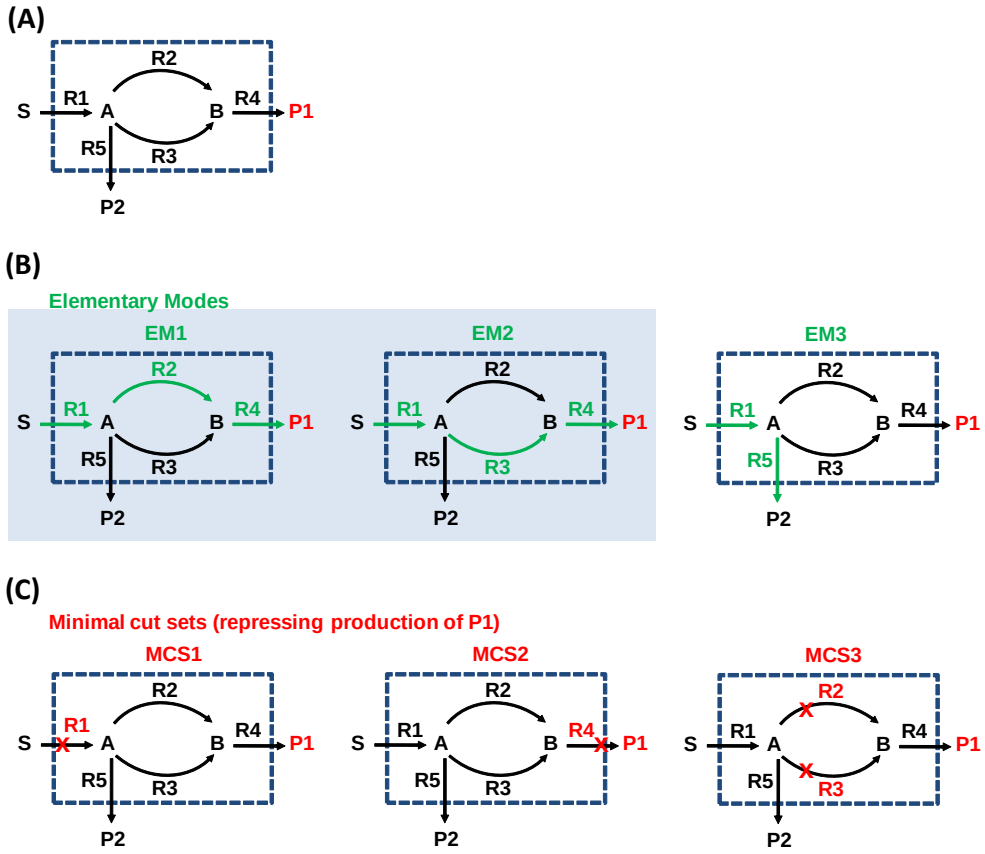


Figure 4.8: **Illustration of minimal cut sets concept.** The metabolic network depicted in (A) contains three elementary modes (B) of which two include the objective function R4 (highlighted by blue box). Based on these elementary modes the minimal cut sets which repress the synthesis of P1, the product of the objective function, can be computed (C). Illustration adapted from [255].

4.4.4 Gene knockout simulations based on flux balance analysis

Flux balance analysis methods allow to simulate the outcome of gene knockouts, when information is available on nutrients that can be imported and target metabolites that

are produced by a cell [131, 377]. In this context a reaction of interest is restricted to carry no flux, while the network is asked to produce the target metabolites. This basically corresponds to the calculation of MinModes for each essential metabolite without using the reaction under examination. If the simulation is successful and a flux distribution satisfying the constraints can be found, the tested reaction is assumed to be not essential, since the network utilizes other reactions to fulfill the task. Otherwise, the enzyme catalyzing this reaction can be considered as a putative drug target, as its inhibition would prevent the synthesis of essential metabolites and thus cell maintenance or growth.

Chapter 5

Metabolic Network Reconstruction

5.1 Introduction

Living organisms apply a large set of biochemical reactions to fulfill a variety of cellular tasks such as energy provision, maintenance of the cellular state, provision of precursors for cellular reproduction and synthesis of signaling molecules. Most of the reactions within these **metabolic networks** are catalyzed by genome encoded enzymes, since on the one hand reactions may exhibit slow reaction kinetics, and on the other hand organisms are able to regulate their metabolism by varying enzyme activities. Fig. 5.1 depicts a typical metabolic network. Metabolites imported into the cell are either degraded by catabolic reactions to yield energy and essential building block precursors, or they are utilized by anabolic reactions to produce larger molecules such as biomass components. End products fulfill their task within the cell or are excreted for further purposes. Metabolic networks can be subdivided into **metabolic pathways** which consist of a series of reactions that participate in the synthesis or degradation of the same metabolite. In the past, metabolism was analyzed on this level of complexity, leaving out the crosstalk between pathways caused by shared metabolites such as branch point metabolites and co-factors.

The reconstruction of genome-scale metabolic networks is guided by different data sources [373]. Biochemical experiments provide the strongest evidence for the presence of enzymes in an organism and thus corresponding biochemical reactions. Functionally annotated genes that are homologous to the genomic sequence of interest give further clues on the structure of a metabolic network. In addition, physiological knowledge on the capability of an organism to produce certain metabolites supports the assumption that respective biosynthesis pathways are present. And finally, *in silico* simulations of known cellular behaviour may identify missing reactions in case simulations fail due to gaps in the underlying metabolic network. The confidence levels of these sources differ, being the highest for the direct biochemical proof of enzyme functions and the lowest for reactions inferred by computational simulations. Therefore, as long as not all reactions are experimentally verified and organisms are fully characterized, reconstructed metabolic networks are still hypothetical and subject to constant revision and extension.

Since the development of sequencing technologies, genomes of almost one thousand organisms have been sequenced, and the sequencing of several thousand genomes is in progress [272]. This deluge of sequenced genomes and the subsequent homology based gene annotations allowed for the reconstruction of genome scale metabolic networks even for those organisms for which direct biochemical information is scarce. Large scale metabolic networks are now available for organisms of all three major domains of the tree of life [12, 84, 125, 298, 174, 138, 359, 398, 139, 218, 443].

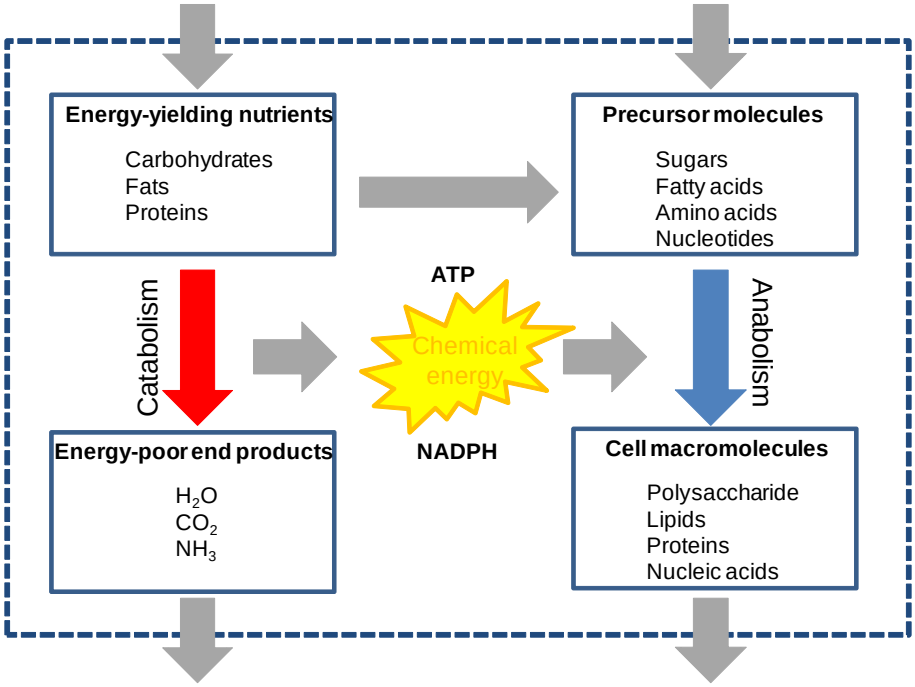


Figure 5.1: **Metabolic network scheme.** Adapted from [162].

5.2 Metabolic network of *P. falciparum*

The basis for any genome scale metabolic network of *P. falciparum* constitute the functional annotations of the genome sequence [161], which are catalogued in the PlasmoDB database [22] (`http://www.plasmodb.org/plasmo/home.jsp`). Based on these annotations, as well as literature knowledge, several groups have started to infer metabolic pathways occurring in the parasite [170, 561, 241, 309]. However, none of these initiatives made the effort to construct a functional, compartmentalized and consistent metabolic model.

The presented thesis aims at compiling a compartmentalized, comprehensive metabolic network which is able to fulfill cellular functions known from the literature to occur in *P. falciparum*. Therefore, data from multiple online resources have been integrated in the assembly process. Biochemical reactions were gathered from the Malaria Parasite Metabolic Pathways (MPMP) website (release of November 2007), the KEGG database (release of February 2009), the BioCyc database (release of August 6th 2007 for *P. falciparum* 3D7), the Reactome database (release of November 2007) and the BRENDA database (release of January 2007).

KEGG reactions were considered as present in *P. falciparum* if genes of the parasite were assigned via the KEGG Orthology (KO), a classification of orthologous and paralogous groups of genes determined based on high sequence similarities to genes of known function. Hagai Ginsburg kindly provided a list of genes and EC numbers corresponding to metabolic pathways listed on the MPMP website. All KEGG reactions associated with these EC numbers were checked for their presence in any of the pathways depicted at the MPMP website and, where appropriate, added to the metabolic network. Similarly, all KEGG

reactions associated with EC numbers that are annotated as present in *P. falciparum* by the BRENDA database were included in the network. Furthermore, all reactions from BioCyc that are listed in the PlasmoCyc subdatabase as well as all reactions from Reactome that are inferred by sequence homology to occur in *P. falciparum* were added to the network. As BioCyc reactions are mostly computationally inferred, reactions were removed that are associated to pathways that are assumed to not be present in *P. falciparum* [171], except for the chorismate biosynthesis pathway.

Since each resource uses individual identifiers for reactions and metabolites, it was necessary to map the reactions onto each other. The KEGG identifiers were chosen as an overall means of identification. *P. falciparum* reactions suggested by BioCyc and Reactome were mapped to KEGG reactions via annotated genes or EC numbers. In case that way the corresponding KEGG reaction was not found, involved metabolites were mapped to KEGG metabolites either with the help of annotated ChEBI or PubChem identifiers, or, if such cross-references were not available, the PubChem database was queried for matching synonyms. The KEGG metabolite identifiers were then assembled to find the corresponding KEGG reaction. For several reactions no matching KEGG reaction could be found by this means. In such cases, the reaction equation translated to respective KEGG metabolites was assigned with a new unique identifier starting with the letter 'Q' followed by five digits in the style of the KEGG identifiers.

Each reaction of the resulting metabolic network (see Fig. 5.2 and Table 5.1) was assigned to one of the following compartments: cytosol, nucleus, mitochondrion, apicoplast, endoplasmic reticulum, or food vacuole. The default compartment for all reactions is the cytosol. This assignment was overruled if a gene catalyzing a certain reaction is annotated with another compartment by the Gene Ontology database. In addition, compartments were reassigned when reactions are assigned by the MPMP website to a different compartment or when corresponding genes contain signal sequences for the apicoplast or mitochondrion. Information on transporters transferring metabolites between the different compartments as well as between the host and the parasite, were mostly obtained from the MPMP website, and in addition, from the Transport Classification database, BioCyc, and the Reactome database.

Reactions were assumed to be irreversible if high Gibbs free energies were predicted or observed [311, 312, 221]. In addition, pairs of synthesis and decay reactions were defined as irreversible in case the synthesis step involved energy-rich metabolites such as ATP or CoA conjugate but the decay step not, e.g.,`Acyl-CoA + 1-Acyl-sn-glycero-3-phosphocholine <=> CoA + Phosphatidylcholine` and `Phosphatidylcholine + H2O <=> 1-Acyl-sn-glycero-3-phosphocholine + Fatty acid .`

Organism	**Reactions**	**Transporters**	**Metabolites**	**Genes**	**Compartments**
Plasmodium falciparum	998	377	1,622	579	6
Human erythrocyte	349	88	566		1

Table 5.1: **Overview of assembled metabolic networks for *P. falciparum* and the human erythrocyte.**

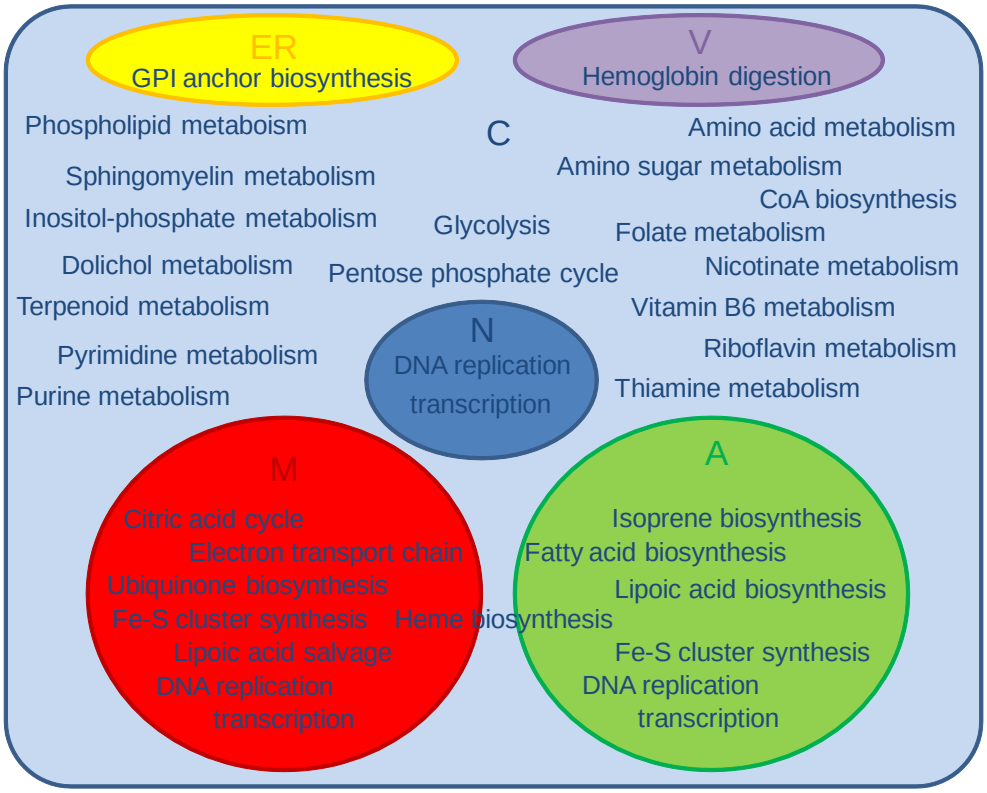


Figure 5.2: **Overview of metabolic pathways present in *P. falciparum*.**

5.3 Metabolic network of the human erythrocyte host cell

The best studied system among the host cells of *P. falciparum* is the human erythrocyte, due to its reduced metabolic capacity and relatively simple availability. As predictions are thought to improve when the parasite is integrated into its natural environment, the metabolic network of this cell type was additionally compiled. For the human erythrocyte multiple metabolic networks have been published that comprise the cell's core metabolism, including glycolysis, pentose phosphate pathway, glutathione metabolism and adenine nucleotide metabolism (see [351, 217] and contained references). However, these networks lack metabolic pathways providing cofactors such as thiamin diphosphate and pyridoxal phosphate that are required for full cellular functioning. Therefore, the metabolic network of the human erythrocyte was assembled from available online resources to obtain a network that is as complete as possible. As a main source served the enzyme database BRENDA [82], from which all EC numbers were extracted that are assigned to enzymes of human erythrocytes. Furthermore, the human red blood cell database [379], which catalogues the proteome of human red blood cells, was queried for enzymes to complement those obtained from BRENDA. Subsequently, the EC numbers were used to extract reactions from the KEGG database. Transport processes listed at the Malaria Parasite Metabolic Pathways website were added to the network and amended with transporters extracted from primary literature [222, 413, 529, 414, 433, 467, 257, 366, 43, 94, 260, 328, 61, 573, 15]. An

overview of the network can be found in Table 5.1. The best studied system among the host cells of *P. falciparum* is the human erythrocyte, due to its reduced metabolic capacity and relatively simple availability. As predictions are thought to improve when the parasite is integrated into its natural environment, the metabolic network of this cell type was additionally compiled. For the human erythrocyte multiple metabolic networks have been published that comprise the cell's core metabolism, including glycolysis, pentose phosphate pathway, glutathione metabolism and adenine nucleotide metabolism (see [351, 217] and contained references). However, these networks lack metabolic pathways providing cofactors such as thiamin diphosphate and pyridoxal phosphate that are required for full cellular functioning. Therefore, the metabolic network of the human erythrocyte was assembled from available online resources to obtain a network that is as complete as possible. As a main source served the enzyme database BRENDA [82], from which all EC numbers were extracted that are assigned to enzymes of human erythrocytes. Furthermore, the human red blood cell database [379], which catalogues the proteome of human red blood cells, was queried for enzymes to complement those obtained from BRENDA. Subsequently, the EC numbers were used to extract reactions from the KEGG database. Transport processes listed at the Malaria Parasite Metabolic Pathways website were added to the network and amended with transporters extracted from primary literature [222, 413, 529, 414, 433, 467]. An overview of the network can be found in Table 5.1.

5.4 Consistency checks

The compiled metabolic networks of *P. falciparum* and the human erythrocyte were tested for consistency to obtain functional metabolic models. In a first step, highly specific metabolites such as α-D-Glucose were replaced by more general metabolites (D-Glucose), using a manually compiled list of substitutions. Moreover, reactions containing generic terms like 'fatty acid' were split into a set of reactions, each using a respectively more specific metabolite (tetradecanoic acid, hexadecanoic acid, etc.). Next, generic reactions (`A + n B` $\rightarrow$ `n C + D`) were removed and reactions describing the synthesis or degradation of macromolecules (`A + B` $\rightarrow$ `A`) were modified in such a way that one metabolite does not occur on both sides of a reaction equation. Finally, reaction duplets that were already present in the original data or were generated by the consistency procedure were deleted.

Up to this point, the metabolic network is basically just a set of accumulated reactions. To transform the network into a fully operative model, flux balance simulations were conducted with regard to the producibility of important biomass precursors such as lipids, nucleotides, amino acids, carbohydrates, and cofactors (see Table 5.2). In this context MinModes [201] were calculated for each of these metabolites, which are basically minimal steady-state flux distributions that allow for the production of a certain metabolite. In case no MinMode could be calculated, i.e., no path exists that converts external nutrients into the metabolite of interest, precursors of the metabolite (as defined by metabolic maps of KEGG and MPMP) were consecutively checked with respect to their producibility until one was found that is producible, thus revealing missing reactions and transporters. Endproducts and intermediates of pathways known from the literature to occur in *P. falciparum*, e.g., glycolysis, pentose phosphate cycle, hemoglobin degradation and glutathione conjugate export, were examined in the same way. This process revealed that mostly intracellular transporters

were missing in the network. Reactions that had to be added include tRNA ligases in the mitochondrion and apicoplast, DNA and protein synthesis reactions, and the biosynthesis of unsaturated fatty acids.

Table 5.2: **Essential metabolites for parasite and erythrocyte.** Cellular compartments: [n] = nucleus, [a] = apicoplast, [m] = mitochondrion, [er] = endoplasmic reticulum, [v] = food vacuole. The first nine metabolites represent pseudo-metabolites, which are needed to test respective reactions.

Metabolite	**Parasitic stages where assumed to be essential**
active-cytochrome-c-reductase[m]	all stages
active-glutathione-reductase	all stages
active-glutathione-transferase	all stages
active-glyoxalase-II	all stages
active-SAH-hydrolase	all stages
active-superoxide-dismutase	all stages
active-superoxide-dismutase[m]	all stages
active-superoxide-dismutase[v]	all stages
active-thioredoxin-reductase	all stages
ATP	all stages
cAMP	all stages
Cardiolipin	all stages
Cholesterol	all stages
CoA	all stages
Diacylglycerol	all stages
DNA[a]	all stages except: merozoite, mosquito midgut sporozoite, mosquito salivary gland sporozoite, gametocyte
DNA[m]	all stages except: merozoite, mosquito midgut sporozoite, mosquito salivary gland sporozoite, gametocyte
DNA[n]	all stages except: merozoite, mosquito midgut sporozoite, mosquito salivary gland sporozoite, gametocyte
eIF5A-precursor-hypusine	all stages
FAD	all stages
Fe-S protein[a]	all stages
Fe-S protein	all stages
Glycoprotein	all stages
GPI-protein[er]	all stages
Heme[m]	all stages
Hemozoin[v]	only blood stages

Continued on next page

Table 5.2 – continued from previous page

Metabolite	Parasitic stages where assumed to be essential
Hexadecanoic acid	all stages
H+-pumped[m]	all stages
Isopentenyl diphosphate	all stages
mRNA[a]	all stages
mRNA[m]	all stages
mRNA[n]	all stages
NAD+	all stages
NADP+	all stages
Octadecanoic acid	all stages
Phosphatidate	all stages
Phosphatidylcholine	all stages
Phosphatidyl-D-myo-inositol	all stages
Phosphatidylethanolamine	all stages
Phosphatidylserine	all stages
Protein[a]	all stages
Protein[c]	all stages
Protein[m]	all stages
Protein-C-terminal-S-farnesyl-L-cysteine-methyl-ester	all stages
Protein-N6-(lipoyl)lysine[a]	only late liver stages
Protein-N6-(lipoyl)lysine[m]	all stages
Pyridoxal phosphate	all stages
S-adenosyl-L-methionine	all stages
S-geranylgeranyl-protein	all stages
Sphingomyelin	all stages
Tetrahydrofolate	all stages
Thiamin diphosphate	all stages
Triacylglycerol	all stages
Ubiquinone[m]	all stages
(9Z)-Hexadecenoic acid	all stages
(9Z)-Octadecenoic acid	all stages
(9Z,12Z)-Octadecadienoic acid	all stages

5.5 Discussion

Genome-scale metabolic reconstructions are typically derived from genome annotations which are coupled with data from comprehensive databases about enzymes and metabolic pathways such as EXPASY and KEGG. Primary literature is then used to complement the initial reconstruction. This process of validating each individual reaction of a reconstructed metabolic network is very time consuming and may easily demand several man-years. Therefore, the presented metabolic network of *P. falciparum* was instead compiled from preprocessed data generated by several scientific groups based on primary literature

and computational inferences. Consistency checks and flux balance simulations identified gaps in pathways and facilitated the development of the first functional compartmentalized metabolic network of *P. falciparum*.

Most metabolic networks previously reconstructed do not consider the environment of an organism in terms of other organisms. The first multispecies metabolic network was published by Stolyar and colleagues [486] which represents syntrophic interactions among the anaerobic microorganisms *Desulfovibrio vulgaris* and *Methanococcus maripaludis*. Conducted simulations accurately predicted metabolic fluxes and the relative abundance of the two organisms. So far, no efforts have been made to computationally analyze the metabolic interactions between *P. falciparum* and its host cells. Hence, the compiled metabolic networks of *P. falciparum* and the human erythrocyte present a good opportunity to explore the exchange of metabolites during the blood stage of parasite development.

Reconstructed metabolic networks have been proven useful for multiple applications, including interpretation of high-throughput data, guidance of metabolic engineering, hypothesis testing and analysis of network properties (see [361] and references therein). In general, the quality of results derived from such reconstructions depends heavily on the information used to attain them. For example, about 50% of the predicted genes in *P. falciparum* are not annotated yet, suggesting that the compiled metabolic network is not complete. Furthermore, as pointed out in [171], most of the dababases (BioCyc, KEGG, Reactome) infer metabolic pathways computationally which results in predictions where actually only one or two enzymes of the pathway are associated with genomic sequences and therefore very likely do not occur in the parasite. To cope with this problem, those pathways were removed that are suggested by [171] to be not present in *P. falciparum*. Moreover, it is likely that in the future genes will be discovered in the genome of the parasite that encode for missing enzymes thereby filling current annotation gaps of the metabolic network. But still, as not every single reaction has been validated in the network compilation process, it is likely that fractions of the metabolic network are not correct which may influence simulation outcomes. However, compared to currently available reconstructions of the *P. falciparum* metabolism, the network presented here is not only the most comprehensive one, but has also been proven to be functional, hence providing a basis for further analysis.

Chapter 6

Predicting life cycle specific metabolism

6.1 Introduction

Reconstructions of metabolic networks as presented in the previous chapter provide the basis for predictions on the metabolic behavior of a cell. Not only local, but also system-wide effects of cellular perturbations like varying environments or inhibition of certain enzymes can be assessed with the help of such large-scale networks [361].

The metabolism of *P. falciparum* has been analyzed in a network based approach by Yeh *et al.* [561] and Fatumo *et al.* [136]. Yeh and colleagues developed PlasmoCyc, a sub-database of BioCyc, which contains *P. falciparum* related metabolic reactions annotated with genomic sequences. Putative drug targets were identified in the resulting metabolic network by choke-point analysis. Metabolic data from PlasmoCyc was also analyzed by Fatumo *et al.* with respect to essential enzymes. The applied breadth first search approach inspects whether metabolites downstream of a blocked reaction are producible via alternative routes and thus the reaction is dispensable. Combined with choke-point analysis this approach improves the precision of the prediction results compared to choke-point analysis alone. However, drug targets proposed by these approaches are presumably of limited quality, since they are derived from metabolic networks that are not further processed using additional information such as cellular compartments and transport processes. Moreover, they did not consider the different life cycle stages of the parasite, during which the metabolism might vary due to different cellular environments.

As outlined in section 4.2 flux balance analysis (FBA) is a commonly used computational method to gain insight into the metabolic capabilities of a cell. Several extensions of this methodology have been proposed that integrate high-throughput data in order to obtain more realistic metabolic flux predictions for a particular cellular condition [4, 38, 465, 96, 417]. These approaches add constraints to the basic FBA formalism that reflect gene or protein expression, which, in contrast to metabolite levels, are available on genome-scale for multiple organisms. For example, reactions were constrained to a flux value of zero if expression of the corresponding transcript or enzyme could not be detected [4, 417, 96]. Less strict approaches do not exclude such reactions, but rather seek flux distributions which are most consistent with expression data [465, 38].

This work intends to deduce stage-specific metabolism for all stages of the life cycle of *P. falciparum* with the help of flux balance analysis and gene expression profiles. For this purpose the approach developed by Shlomi *et al.* [465] was adapted and applied to the assembled metabolic network under consideration of gene expression profiles measured during different life cycle stages.

6.2 Applied gene expression profiles

Large-scale gene expression profiles are available for the blood stage of *P. falciparum* as well as the mosquito stage. However, the liver stage in the human host is experimentally difficult to assess and therefore gene expression data covering this stage in *P. yoelii*, a close relative of *P. falciparum* causing malaria in rodents, were additionally considered in this study. Gene expression is assumed to be sufficiently similar in these two *Plasmodium* species, justifying the utilization of *P. yoelii* data to infer metabolic fluxes in *P. falciparum*. Gene expression data has been gathered from five publications (see Table 1): (a) a genome-scale transcriptome analysis of *P. falciparum* covering the intraerythrocytic developmental cycle (IDC) by Bozdech *et al.* [59] (see section 3.1.1), (b) a genome-scale transcriptome analysis by Le Roch *et al.* [419] examining the blood stage and the mosquito stage of *P. falciparum* (see section 3.1.2), (c) a cDNA library obtained from liver stage expression analysis of the rodent malaria pathogen *P. yoelii* by Sacci *et al.* [427] (see section 3.1.3), (d) a data set from Tarun *et al.* [501], which includes samples extracted from *P. yoelii* during the liver stage, the mosquito stage and the blood stage (see section 3.1.4) and (e) a genome-scale transcriptome analysis by Daily *et al.* of 43 samples derived from the blood of *P. falciparum* infected patients which contain mostly ring stages of the parasite [112] (see section 3.1.5). In total, 110 gene expression samples were analyzed.

In a first step, the accordance of these data sets was checked with regard to corresponding stages. For this purpose the expression status was determined for each gene of every expression sample. For the Le Roch data set the same parameter cutoffs were applied as mentioned in [419] (thermocycling treatments data): expression level $E > 10$ and likelihood of gene absence (calculated by the match-only integral distribution algorithm) $logP < -0.5$. Genes covered by the Bozdech and Tarun data sets were considered to be expressed if the corresponding signal-to-noise ratio (s2n = $(mean(\text{FG}) - mean(\text{BG}))$ / $std(\text{BG})$, with FG being the pixel signal intensity resulting from specific hybridization and BG being the background intensity caused by non-specific binding), is greater or equal to a threshold. Signal-to-noise ratios were extracted from the raw data, averaged over all replicates and normalized using quantile normalization [54]. Applying a too low or too high threshold results in almost all genes being expressed or silenced, respectively. For the Bozdech data set the threshold t was chosen in such a way that the average Euclidean distance between the binary expression values

$$expr\,(g \mid t) = \begin{cases} 0 & \text{if gene } g \text{ is silent given threshold } t \\ 1 & \text{if gene } g \text{ is expressed given threshold } t \end{cases}$$

is maximal for non-consecutive pairs of samples and minimal for consecutive pairs:

$$\arg\max_{t} \underbrace{\frac{\sum_{i=1}^{n-2}\sum_{j=i+2}^{n}\sqrt{\sum_{k=1}^{m}\left[expr\left(g_{i,k} \mid t\right) - expr\left(g_{j,k} \mid t\right)\right]^2}}{\sum_{i=1}^{n-2}\sum_{j=i+2}^{n} 1}}_{\text{avg. Euclidean distance between non-consec. pairs}} - \underbrace{\frac{\sum_{i=1}^{n-1}\sqrt{\sum_{k=1}^{m}\left[expr\left(g_{i,k} \mid t\right) - expr\left(g_{i+1,k} \mid t\right)\right]^2}}{n-1}}_{\text{avg. Euclidean distance between consec. pairs}}$$

with n being the number of samples per set, m being the number of genes per sample and $g_{i,k}$ being the k^{th} gene in the i^{th} sample.

A threshold of 17 turned out to be optimal. This approach could not be applied to the Tarun set, since the time span between extractions of samples is much larger. Instead, the signal-to-noise ratio threshold was set to 25 which yielded largest similarity between Tarun samples and corresponding Le Roch samples. As the Sacci data set is a cDNA library that captures mRNA present at a certain time point, all genes of the library were accounted as expressed. For the Daily samples a gene expression signal cutoff of 139 yielded largest similarity between samples of cluster 2 (active growth) and ring stage expression samples of the Le Roch set and was therefore used to determine expression in Daily samples.

Similarities and differences between the expression patterns of the individual life cycle stages were then assessed with a simple and intuitive distance measure, the normalized Hamming distance:

$$dist(i,j) = \frac{\mid \mathrm{E}_i \cap \mathrm{S}_j \mid + \mid \mathrm{E}_j \cap \mathrm{S}_i \mid}{\mid \mathrm{G}_i \cap \mathrm{G}_j \mid}$$

with E_i, S_i and G_i being the set of expressed, silenced or all analyzed genes, respectively, in sample i. The numerator equals the sum of genes with different expression status, while the denominator equals the total number of common genes in two samples. The resulting Hamming distance matrix for all gene expression sample pairs is shown in Fig. 6.1.

The distance matrix reveals that samples of the same data set covering consecutive time points tend to be more similar compared to those covering non-consecutive time points, especially samples of the Bozdech or the Le Roch data set. Furthermore, similar gene expression patterns can be observed for samples of different data sets that correspond to the same life cycle stage, e. g. Le Roch ring stage samples and Bozdech samples extracted during hour 1 to 20 after erythrocyte infection. In addition, the distance matrix agrees well with the observation by Daily *et al.* that the *in vivo* ring stage expression samples can be ascribed to three clusters [112]: starvation response accompanied by metabolism of alternative carbon sources (cluster 1), active growth based on glycolytic metabolism (cluster 2) and environmental stress response (cluster 3). The distance matrix confirms that samples of the same clusters are more similar in terms of expressed enzymes. Only a fraction of cluster 3 samples exhibits similarity to cluster 1 samples. Moreover, it shows that samples of cluster 2 are similar to those samples of the Bozdech and Le Roch data sets which

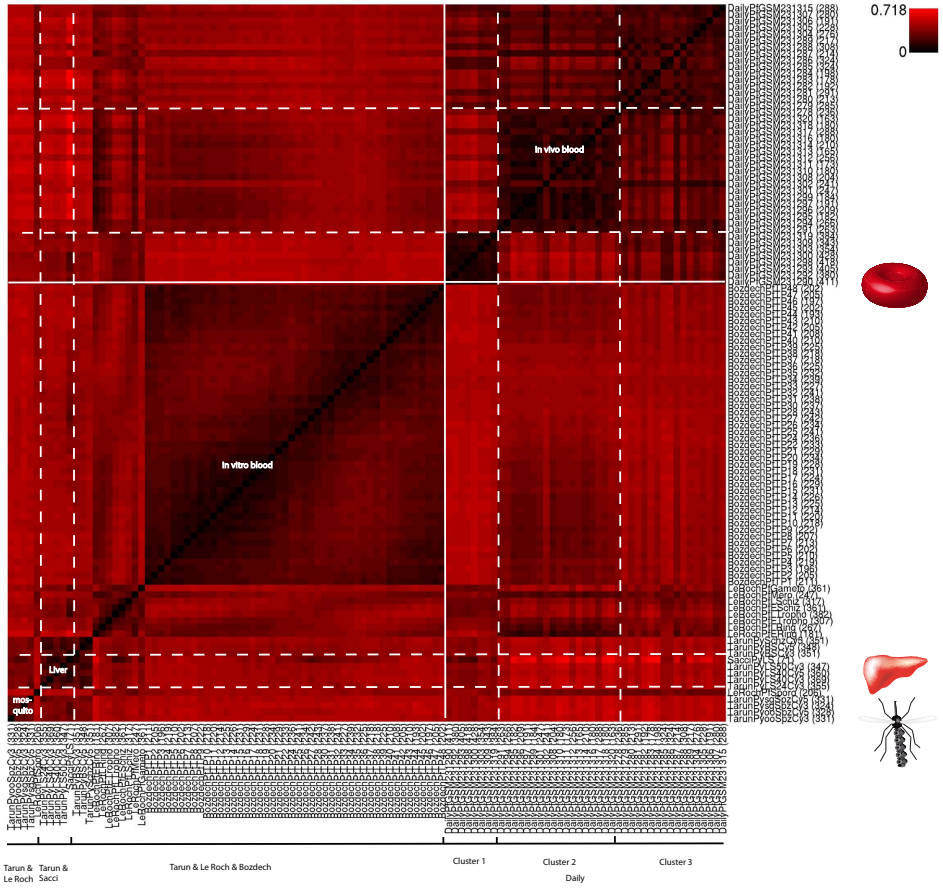


Figure 6.1: **Normalized Hamming distance matrix for gene expression samples.** In order to compare the gene expression profiles of the different time points, normalized Hamming distances have been calculated as described in the text for each pair of gene expression samples. The darker the color of a matrix entry, the lower is the corresponding Hamming distance. Sample labels are composed of the sample abbreviation (see Table 1) and the number of expressed genes.

correspond to early or very late blood stages. In general, Hamming distances are larger when samples belong to different data sets. This can be ascribed to one or more of the following issues (i) different methods have been used to analyze gene expression (microarray analysis vs. cDNA library), (ii) experiments are conducted with different organisms (*P. falciparum* vs. *P. yoelii*) and (iii) for the individual data sets different criteria have been applied to classify genes as absent or present.

6.3 Gene expression data mapped onto metabolic pathways

Mapping gene expression data onto metabolic networks may uncover active pathways for each stage and metabolic differences between the individual life cycle stages. For this

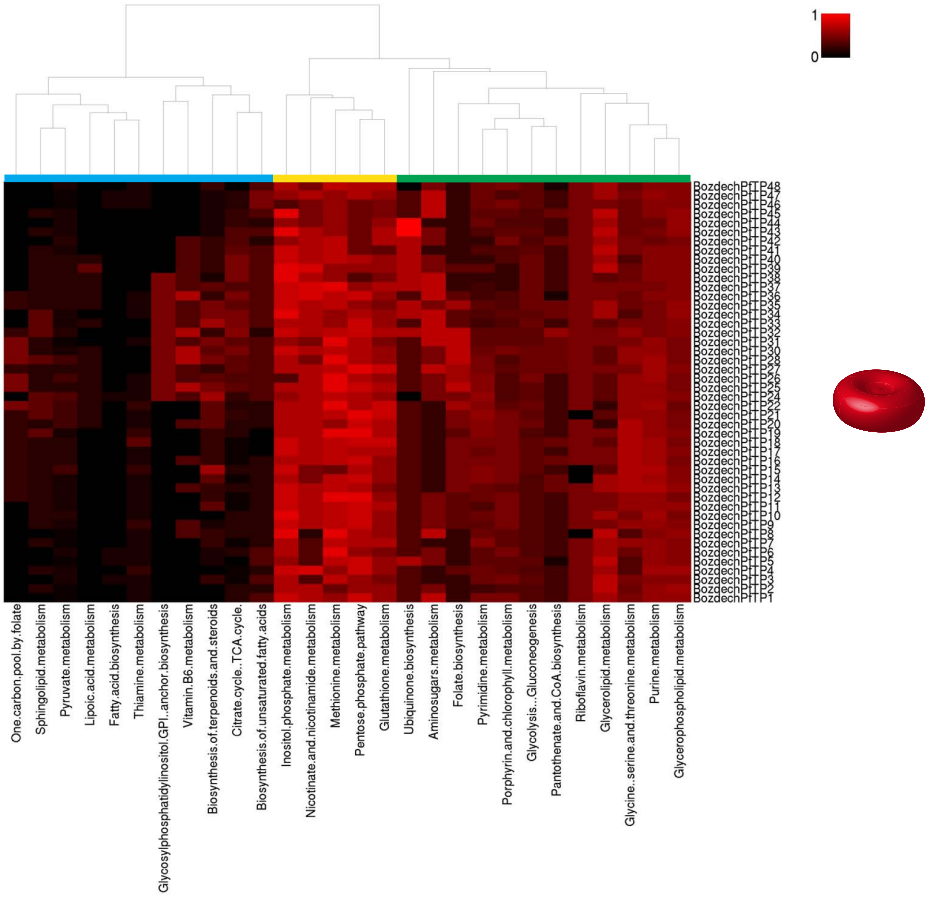


Figure 6.2: **Bozdech gene expression samples mapped onto metabolic pathways.** Mapping gene expression data onto metabolic networks may uncover active pathways for each stage as well as metabolic differences between the individual life cycle stages. For this purpose, the ratio of expressed genes per KEGG pathway (# expressed genes / # of pathway associated genes with available expression data) was calculated for each Bozdech gene expression sample. The darker the color of a matrix entry, the lower is the ratio. Clusters of pathways with similar patterns of expressed genes during the individual life cycle time points were calculated with the built-in function hclust ('average' method) of the statistics software R (colored bars).

purpose, the ratio of expressed genes per pathway (as defined by KEGG) was calculated for each gene expression sample (see Fig. 6.2 - 6.5) and pathway clusters were determined.

Three pathway clusters can be identified for the Bozdech and the Le Roch data sets, which roughly correspond to each other (see green, blue, and yellow bar in Fig. 6.2 and Fig. 6.3). The green clusters, which have 10 pathways in common (e. g. glycolysis, glycerophospholipid metabolism and porphyrin metabolism), contain pathways where only a fraction of pathway associated genes are expressed during the IDC with a maximum of expressed genes during late trophozoite and early schizont stage. Pathways associated with the blue clusters (common pathways: fatty acid biosynthesis, lipoic acid metabolism, vitamin B6 metabolism and one carbon pool by folate metabolism) show gene expression almost exclu-

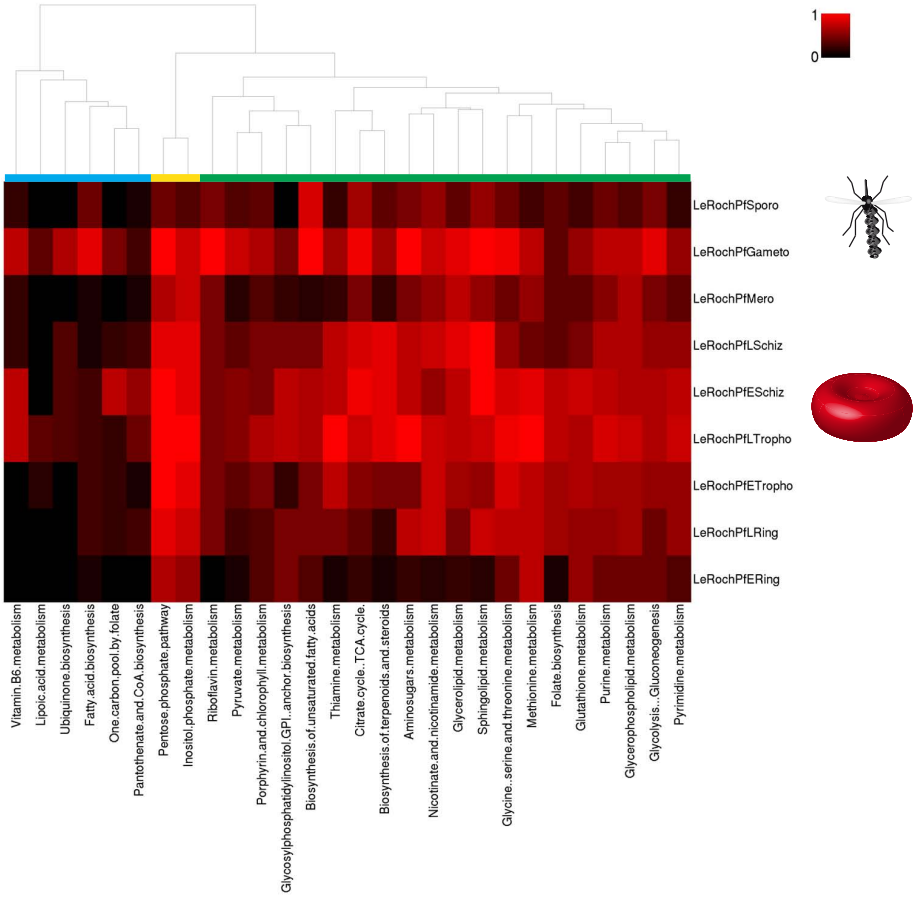


Figure 6.3: **Le Roch gene expression samples mapped onto metabolic pathways.** See caption of Fig. 6.2.

sively during late trophozoite and early schizont stages of the IDC, while those of the yellow clusters (common pathways: pentose phosphate pathway, inositol phosphate metabolism) exhibit many expressed genes during all phases of the intraerythrocytic developmental cycle. Notably, during the gametocyte stage (Le Roch data set) a high number of genes are expressed in all pathways and in contrast only few genes during the invasive stages (Le Roch data: sporozoite, merozoite). Pathway clusters as described above for the blood stage are not as apparent for the Tarun data, since this stage has not been analyzed as detailed. Nevertheless, certain pathways group together as seen for the Bozdech and Le Roch data sets (see colored bars in 6.4). Daily and coworkers found that their ring stage expression samples can be divided into three clusters: starvation response accompanied by metabolism of alternative carbon sources (cluster 1), active growth based on glycolytic metabolism (cluster 2) and environmental stress response (cluster 3). These clusters can be found as well when expression samples are mapped to metabolic pathways as shown in 6.5. In samples of cluster 1 almost all enzymes are expressed in each of the considered pathways, suggesting that required metabolites cannot be obtained in sufficient amounts from the host and thus the parasite has to activate all possible pathways in order to obtain these metabolites. In

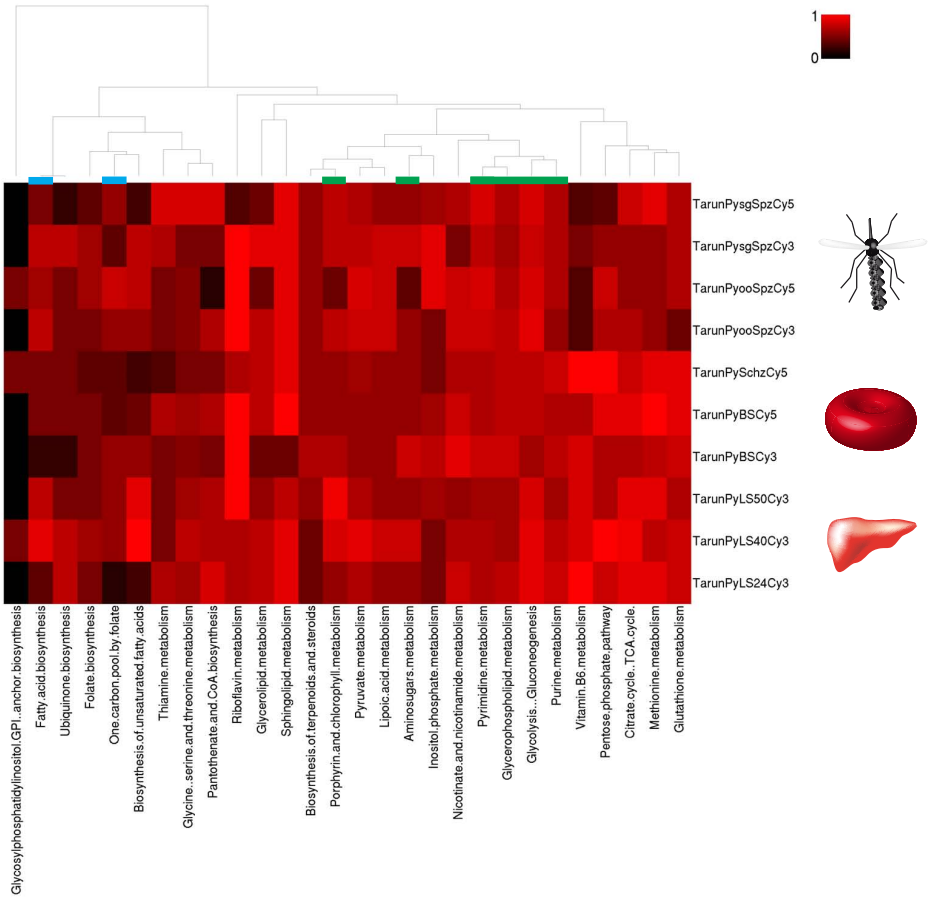


Figure 6.4: **Tarun gene expression samples mapped onto metabolic pathways.**
See caption of Fig. 6.2.

contrast, less enzymes are expressed in samples corresponding to cluster 2. Here, enzymes of sugar metabolism related pathways like glycolysis, pentose phosphate pathway and glycerophospholipid metabolism (blue bar) are mostly activated. Similar expression patterns can be observed for cluster 3 samples. However, more enzymes are expressed in certain pathways such as fatty acid synthesis, thiamine metabolism and ubiquinone biosynthesis (pink bar).

In general, the majority of metabolic pathway genes is expressed during starvation (Daily data) or late trophozoite and early schizont stage where reproduction of biomass is at its peak [366]. Pathways showing similar patterns of activation and suppression are presumably co-regulated. Especially in the case of purine and pyrimidine biosynthesis this is plausible, since both nucleotide types are needed in large amounts at the same time during DNA replication and mRNA synthesis. Likewise the expression of genes associated with fatty acid biosynthesis, pyruvate metabolism, and lipoic acid metabolism seems to be coupled, since only few genes are expressed during blood stages but significantly more during liver stages. Most of the reactions assigned to these three KEGG pathways are assumed to take place in the apicoplast and are linked to each other. When fatty acid demand is fully

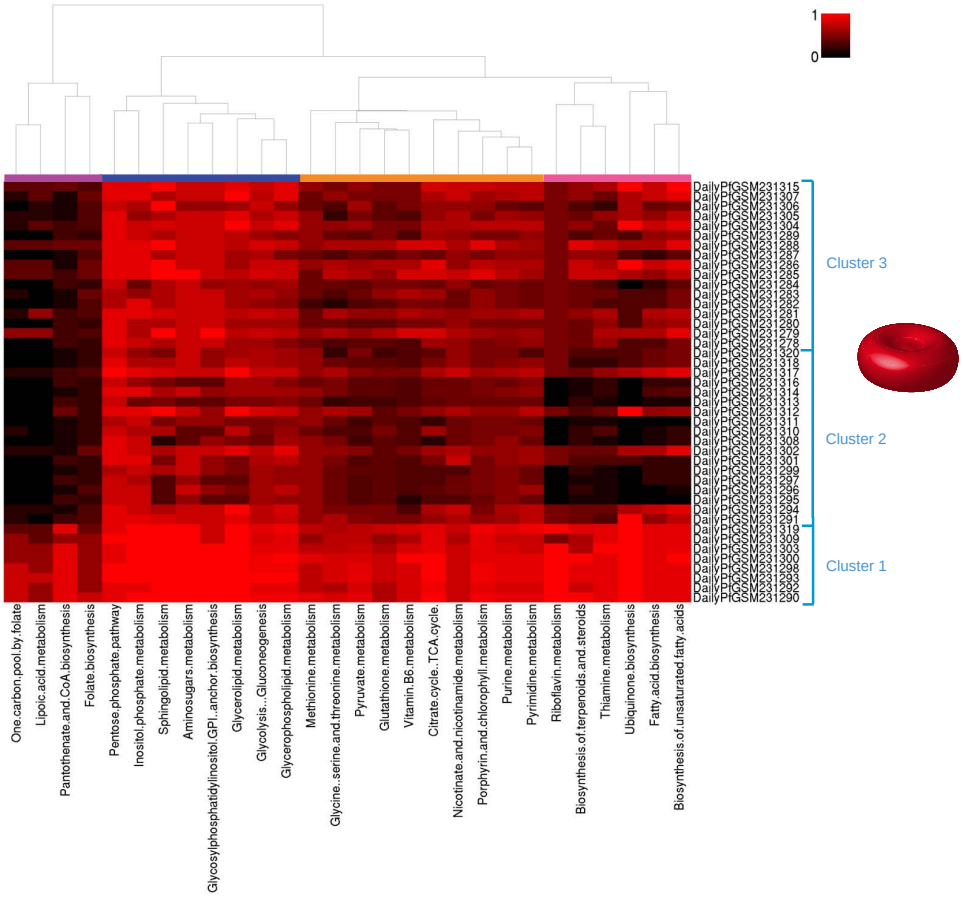


Figure 6.5: **Daily gene expression samples mapped onto metabolic pathways.** See caption of Fig. 6.2.

satisfied by import, which is suggested for the blood stage by findings of Vaughan *et al.* [526], neither the precursor acetyl-CoA (pyruvate pathway) nor lipoic acid, a cofactor of pyruvate dehydrogenase catalyzing acetyl-CoA synthesis, are needed within the apicoplast. Thus, it seems reasonable that these three pathways, which cluster together when considering the Bozdech dataset, are co-regulated.

6.4 Metabolic flux predictions for different life cycle stages

Gene expression data give an impression of the metabolic status of a cell, as has been shown in the previous section. However, gene expression data alone are not sufficient to deduce metabolic fluxes. Simply assuming a correlation between gene expression levels and metabolic fluxes is not appropriate, since it has been shown that neither mRNA levels and protein levels always correlate well [181, 214, 176, 86] nor enzyme levels and corresponding flux rates of catalyzed reactions [134, 504]. Furthermore, expression data is not always available for all genes of interest, or in some cases, there are no genes assigned to metabolic reactions at all, if they are rather inferred from metabolic context than based on direct biochemical evidence. In addition, from gene expression data alone no information can be gained with regard to which nutrients are imported into the cell and which metabolites are secreted. Flux balance approaches provide a means to predict flux distributions despite such obstacles. Flux values can be predicted even for those reactions where no gene expression data is available, as fluxes are not independent due to the assumed steady-state condition, which requires the sum of fluxes producing a metabolite to equal the sum of fluxes consuming it. A reasonable objective function describing the cellular goals is essential to obtain meaningful flux distributions with FBA. In the case of *P. falciparum* the overall goal is reproduction, and thus biomass production. At the same time the parasite has an interest to keep its host cell alive for the period of replication which sets an upper limit on the replication rate. Depending on how well the organism of interest is already studied, the objective function might neglect certain metabolically important issues, e.g., a metabolite essential for reproduction that has not been noticed yet. On this account, an approach is applied here that combines FBA and gene expression information to calculate flux distributions for the different parasitic life cycle stages. The integration of expression data ensures that metabolic aspects not captured by the objective function are nevertheless represented in the calculated flux distributions.

Flux distributions were predicted for the different parasitic life cycle stages based on the method proposed by Shlomi *et al.* [465], which maximizes the number of reactions whose activity is consistent with the expression state of corresponding genes. The original method by Shlomi has several limitations, though: synthesis of metabolites needed for growth is not guaranteed; reactions of low confidence are treated the same as those with annotated genes; in order to maximize the objective function, all reactions catalyzed by the same gene product are sought to be active simultaneously in case the gene is expressed; furthermore, no limits are set on available nutrients. Therefore, this approach was modified in several ways (see Fig. 6.6): (a) Constraints were added to ensure the production of important biomass precursors such as phospholipids, which are needed for parasite reproduction (see Table 5.2). These target metabolites might vary depending on the stage covered by the given gene expression sample. DNA synthesis is important for stages aiming at reproduction, like trophozoites. In contrast, for invasive forms of the parasite (sporozoite, merozoite, etc.) this is not an issue and therefore not considered in flux calculations corresponding to these stages. Furthermore, blood stages need to incorporate heme from hemoglobin degradation into hemozoin molecules in order to prevent intoxication and cell lysis. This makes hemozoin a target metabolite for these stages, but not for liver or mosquito stages where hemoglobin is not present. Targeted deletions of critical fatty acid synthesis enzymes in *P. yoelii* and *P. falciparum* suggest that fatty acid synthesis in the apicoplast is only essential during late

liver stage [526]. This implies that pyruvate dehydrogenase (PDH), which provides fatty acid synthase with acetyl-CoA, is not essential during any other stage and thus lipoate, a cofactor of PDH, is as well dispensable in the apicoplast. Since PDH is the only enzyme in the apicoplast that requires lipoate, lipoate biosynthesis is demanded in this organelle only during late liver stage. (b) For all reactions whose existence is not supported by genome annotations, boolean variables ($y_{2,i}$) and constraints were introduced that keep these reactions inactive if the respective variable is set to 1. This is useful as especially data from the BioCyc database contain reactions without any associated gene that are nevertheless declared to be present in *P. falciparum* to fill gaps in pathways. (c) In contrast to the original Shlomi approach, the modified method does not seek all reactions catalyzed by the same enzyme to be simultaneously active if the respective gene is expressed. As the objective function of the approach proposed by Shlomi *et al.* maximizes the sum of boolean variables that represent reaction activity, higher objective function values are obtained if non-zero fluxes are assigned to all reactions corresponding to expressed genes. However, this might not always be appropriate. Therefore, for each expressed gene a boolean variable ($y_{3,i}$) and constraints were added, ensuring that at least one of the reactions associated to an expressed gene has to carry a non-zero flux in case the respective variable is set to 1. Replacing variables y_{1p} and y_{1n} (see section 4.3.2 for more details of the Shlomi approach) in the objective function with this variable type avoids that all reactions catalyzed by the same expressed enzyme are sought to be active at the same time to further optimize the objective function value. (d) With respect to the restrained access of the parasite to nutrients of the host, the network is forced to fulfill its tasks with a minimum amount of externally supplied metabolites by subtracting the absolute flux values through the parasite's plasma membrane transporters from the objective function value. The corresponding mixed integer linear program reads as follows:

$$\max_{v^+,v^-,y_0,y_2,y_3} \sum_{i\in R_{NE}} \alpha y_{0,i} + \sum_{i\in R_{NG}} \beta y_{2,i} + \sum_{i\in G_E} \gamma y_{3,i} - \sum_{i\in T} \delta v_i^+ \tag{6.1}$$

$$\text{s. t.:}\quad Sv = 0 \tag{6.2}$$

$$v_{\min,i} \le v \le v_{\max,i} \tag{6.3}$$

$$v_i \ge 1, \quad \text{for all target fluxes} \tag{6.4}$$

$$v_i + y_{1p,i}(v_{\min,i} - \epsilon) \ge v_{\min,i},\ \forall i \in R_E \tag{6.5}$$

$$v_i + y_{1n,i}(v_{\max,i} + \epsilon) \le v_{\max,i},\ \forall i \in R_E \tag{6.6}$$

$$v_{\min,i}\,(1 - y_0) \le v_i \le v_{\max,i}\,(1 - y_0)\,,\ \forall i \in R_{NE} \tag{6.7}$$

$$v_{\min,i}\,(1 - y_2) \le v_i \le v_{\max,i}\,(1 - y_2)\,,\ \forall i \in R_{NG} \tag{6.8}$$

$$y_{3,i} - y_{1p,r(i)} \ge 0,\ \forall i \in G_E \tag{6.9}$$

$$y_{3,i} - y_{1n,r(i)} \ge 0,\ \forall i \in G_E \tag{6.10}$$

$$y_{3,i} \le \sum y_{1p,r(i)} + \sum y_{1n,r(i)},\ \forall i \in G_E \tag{6.11}$$

$$v = v^+ - v^- \tag{6.12}$$

$$y_0, y_{1p}, y_{1n}, y_2, y_3 \in [0, 1] \tag{6.13}$$

$$v^+, v^- \ge 0 \tag{6.14}$$

Within the solution space, which is defined by (6.2)-(6.14), a flux distribution is sought that maximizes the objective function given in (6.1). α denotes the reward for setting the fluxes of reactions to zero that correspond to unexpressed genes (R_{NE}), β represents the reward for assigning a zero flux to such reactions that are not associated with any gene (R_{NG}), γ denotes the reward for having a non-zero flux through any reaction assigned to an expressed gene (G_E), while δ is the penalty for forward fluxes v^+ through parasitic plasma membrane transporters (T) importing external metabolites. The variables were set as follows: $\alpha = 2$, $\beta = 0.1$, $\gamma = 1$, and $\delta = 0.5$. Inequalities (6.5)-(6.7) are the same as in [465] with $\epsilon = 1$ and (6.8) is analogous to (6.7) to obtain a zero flux through reactions without associated genes. The subsequent inequalities (6.9)-(6.11) ensure that if a binary variable of type y_3 is set to 1, at least one of the corresponding variables of type y_1 have to equal 1 as well. Matching variables y_1 and y_3 are identified via function $r(i)$ that maps a gene onto a set of reactions that are catalyzed by the gene product. Biomass production is ensured by (6.4), which requires effluxes of at least one molecule of the respective target metabolites through system boundaries.

The modified approach was applied to each of the gene expression samples extracted from different time points of parasite development (see Table 1) to predict respective metabolic fluxes during these time points. In order to compare the resulting flux distributions for the individual time points, normalized Hamming distances were computed for all flux distribution pairs (see Fig. 6.7). In general, the calculated flux distributions are more similar to each other (max. Hamming distance: 0.188) than the corresponding gene expression samples (max. Hamming distance: 0.718). However, similarity patterns as observed for the gene expression data are also detectable for the flux profiles. For example, flux profiles corresponding to consecutive time points during the blood stage are more similar than those of non-consecutive time points (see Bozdech data; trophozoite stage vs. schizont) and flux profiles corresponding to *in vivo* expression profiles of the same cluster tend to be more similar than those of different clusters.

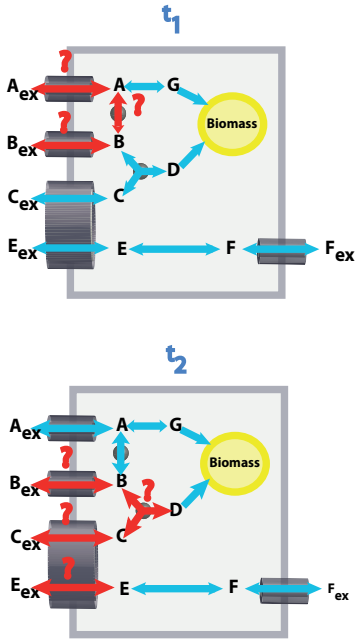
As experimental data on intra-parasitic metabolic fluxes are not available, the plausibility of calculated flux distributions was checked by comparing predicted exchange fluxes between host and parasite with available experimental observations.

6.5 Metabolite exchange with host

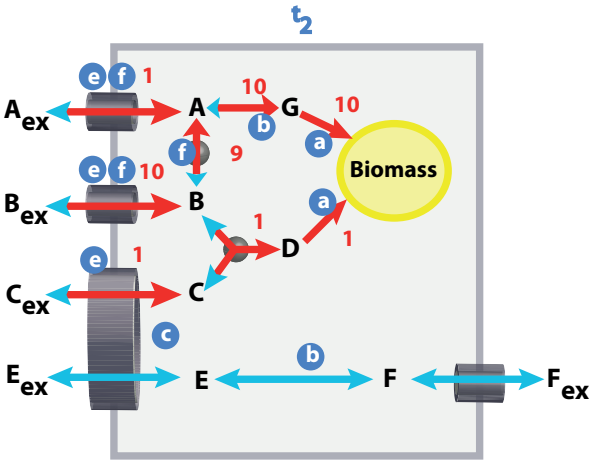
During its life cycle, *P. falciparum* is exposed to different environments and has to deal with different nutrient supply. It has been shown for *Trypanosoma brucei* that the parasite is able to remodel its metabolism depending on the available carbon sources [104]. It is very likely that *P. falciparum* utilizes likewise varying parts of its enzyme equipment as a function of available nutrients, which results in varying sets of metabolites transported through the parasite's plasma membrane during the individual developmental stages.

Reactions / transporters	Gene	Gene expression t_1	t_2
$A_{ex} \leftrightarrow A$	G_1	present	
$B_{ex} \leftrightarrow B$	G_2	present	present
$C_{ex} \leftrightarrow C$	G_3		present
$E_{ex} \leftrightarrow E$	G_3		present
$F_{ex} \leftrightarrow F$	G_4		
$A \leftrightarrow B$	G_5	present	
$B+C \leftrightarrow D$	G_6		present
$E \leftrightarrow F$			
$A \leftrightarrow G$			

A



B



- **a Biomass composition:**
  G = 10
  D = 1
- **b Penalize reactions without assigned genes**
- **c Do not require all reactions catalyzed by same gene to be active at same time**
- **d Minimize amount of utilized nutrients**
- **e Environmental condition:**
  $c[B_{ex}] >> c[A_{ex}] = c[C_{ex}]$
- **f Consider gene expression of previous time points**

Figure 6.6: **Illustration of presented flux balance approach to predict life cycle specific metabolism.** Given the gene expression data (blue table) flux distributions within the present example metabolic network (red arrows) can be inferred for time points t_1 and t_2 as depicted in (A). However, neither flux direction nor flux strength can be deduced from gene expression alone. The set of all possible flux distributions that are conform with the gene expression data can be reduced by knowledge about target fluxes such as biomass production (a). Reactions that are not supported by genome annotation might represent errors in the network assembly. Therefore it is desirable to prevent the usage of such reactions in calculated flux distributions (b). An enzyme or a transporter that is able to process different metabolites does not necessarily convert all substrates at same rates. If one reaction product is not converted further by subsequent enzymes, it accumulates and as a consequence the net production rate is close to zero, even if the gene is expressed and substrate is available (c). The flux solution space can be narrowed down further, when assuming that biomass production is achieved with a minimal amount of nutrients (d), which are of varying availability (e). Gene products can be present within a cell, even when the gene transcript is not detectable, as proteins appear later than the corresponding mRNA and protein degradation might be delayed compared to mRNA degradation. Considering proteins to be present whose transcript was detectable during a previous time point (f) presumably reflects the actual cellular status better than taking only the current transcription snapshot into account. The flux distribution calculated by the flux balance approach, which incorporates all these issues, is shown in (B).

Fig. 6.8 gives an overview of imported (red) and secreted (green) metabolites during the individual life cycle stages, as predicted with the above described FBA method when considering only the metabolic network of the parasite, without any further information about the parasite's environment. Glucose, the main energy source for *P. falciparum* during the IDC, is converted via glycolytic enzymes to lactate, which is then excreted [463]. This phenomenon is recovered by the simulations for multiple time points. Strikingly, for many stages amino acids are predicted to be exported, which does not seem beneficial for protein biosynthesis. In fact, the parasite digests up to three quarters of the host hemoglobin during the IDC to provide amino acids for protein synthesis, to gain space in the host cell for further growth [265] and to maintain the osmotic stability of the host cell [284]. Excess amino acids not incorporated into the parasite's proteome are exported out of the infected host cell [569] and is therefore in accordance with the predictions. Experiments have shown that *P. falciparum* relies on exogenous supply of isoleucine, the only amino acid that is not present in hemoglobin [289]. For all time points covered by gene expression samples isoleucine uptake has been correctly predicted. Moreover, in concordance with experiments by Kirk and Saliba [251], coenzyme A precursor pantothenate is imported in all the simulations. According to the predictions, the parasite satisfies its demand for NAD+ precursors mostly with the import of nicotinamide, but also with nicotinate import, which is conform with experimental findings [366] that suggest *P. falciparum* to be dependent on exogenous supply of these metabolites. The parasite possesses an antioxidant defense system, which involves glutathione to eliminate peroxides and toxic substances. Resulting glutathione disulfate and glutathione conjugates are subsequently either regenerated or removed from the cell [344]. All predicted flux distribution correctly suggest the export of glutathione conjugates.

However, there are predicted transport processes that are not conform with literature knowledge. For example, phosphate is mostly predicted to be exported, while it is reported

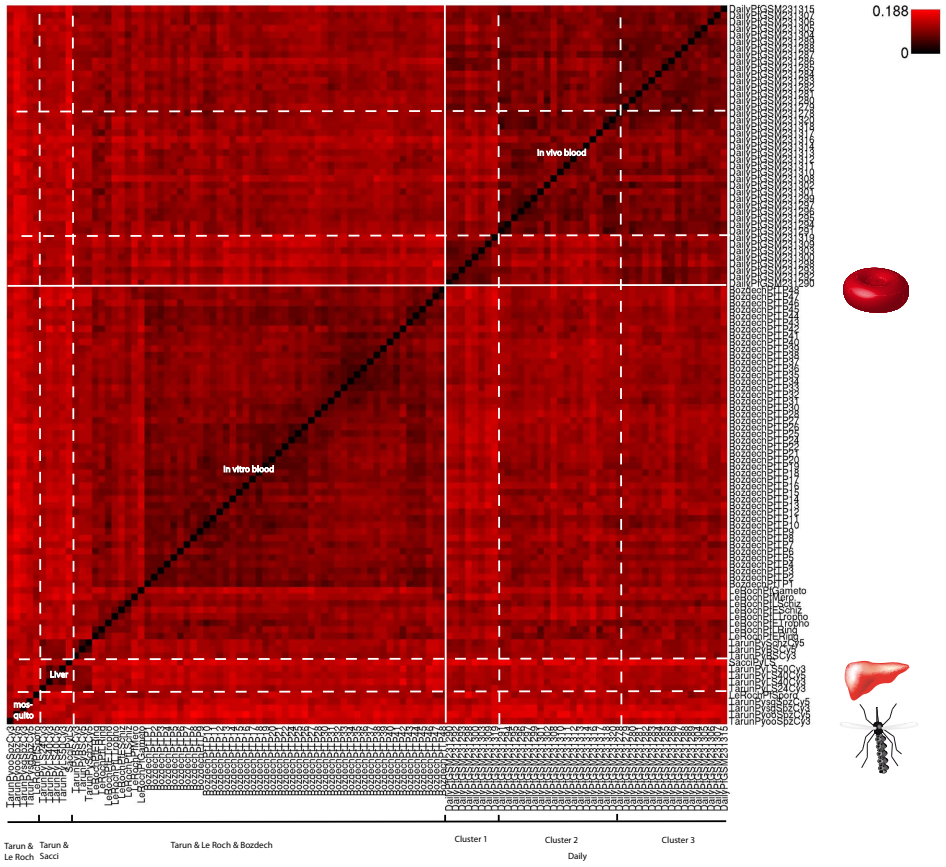


Figure 6.7: **Normalized Hamming distance matrix for calculated flux distributions.** Flux distributions have been predicted with the developed flux balance approach for each time point of the parasite's life cycle for which a gene expression profile exists. Simulations were conducted considering only the metabolic network of the parasite without any further constraints reflecting the parasite's environment and without considering the expression status of genes during preceding time points. In order to compare the individual flux distributions normalized Hamming distances (see text for formula) have been determined for all pairs of flux distributions. The darker the color of a matrix entry, the lower is the corresponding Hamming distance.

to be taken up [435]. Likewise, ATP is imported rather than exported to support the host as proposed by Kanaani and Ginsburg [237] and hemoglobin is predicted to be taken up during liver stages where no hemoglobin is present. Furthermore, purine precursors are imported mostly in the form of inosine, guanosine, and adenosine, while hypoxanthine is exported, despite being available in higher concentrations in the blood plasma than the other purine precursors. These discrepancies indicate that further constraints reflecting the parasites environment are needed in order to obtain more reliable flux predictions. In addition, uptake of host proteins during other stages than the blood stage and subsequent degradation of these proteins in the food vacuole seems to be conceivable, as respective

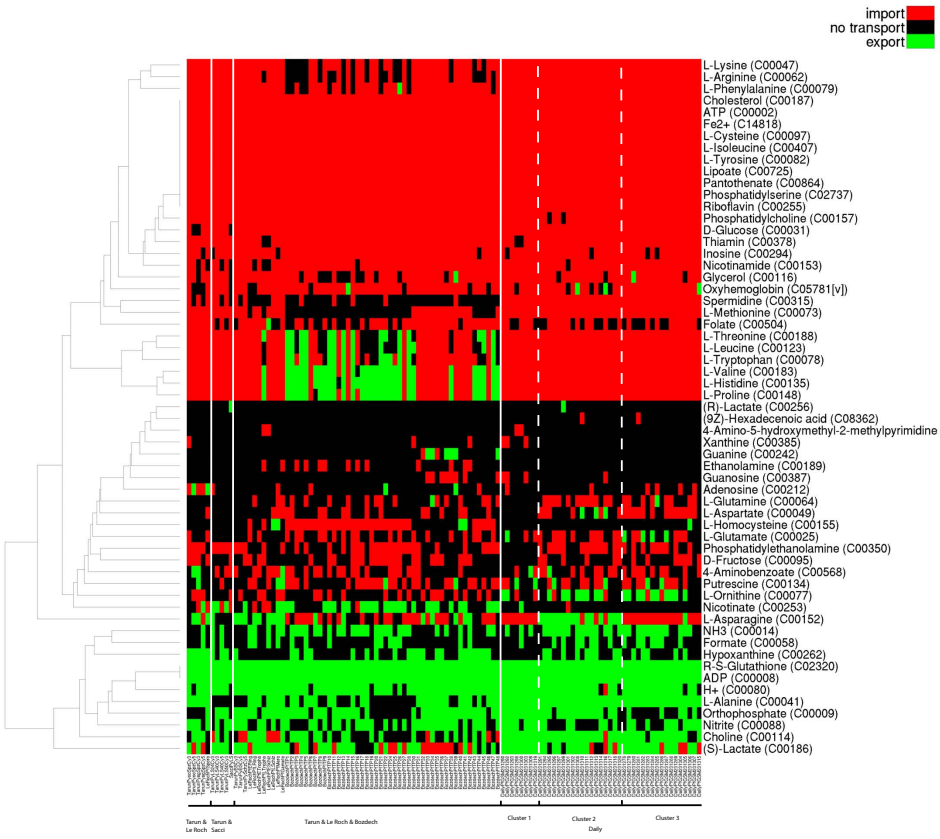


Figure 6.8: **Predicted host parasite metabolite exchanges.** Flux distributions have been predicted with the flux balance approach for each time point of the parasite's life cycle for which a gene expression profile exists. Simulations were conducted considering only the metabolic network of the parasite without any further constraints reflecting the parasite's environment and without considering the expression status of genes during preceding time points. Resulting metabolite exchanges between host and parasite are depicted in this figure. Red matrix entries represent metabolites that are predicted to be imported into the parasite while green matrix entries represent metabolites secreted into the host compartment.

peptidases have been detected throughout the developmental cycle.

6.6 Improved metabolic flux predictions for the blood stage

Most of the knowledge about the malaria pathogen *P. falciparum* is related to the blood stage, due to simple experimental accessibility and the fact that among the host cells the erythrocyte is the best studied system. As predictions for the blood stage are assumed to improve when this knowledge is integrated, the parasite's metabolic network was combined with that of the erythrocyte, and the combined network was forced to produce in

addition metabolites important for erythrocyte metabolism. Furthermore, constraints reflecting knowledge about the blood stage were incorporated into the calculations. For example, the parasite was forced to consume at least a certain amount of hemoglobin and glucose, since both metabolites are known to be consumed by the parasite in large amounts [133, 251]. Uptake limits were set for several metabolites, including purine precursors, sugars, cofactors and phospholipid headgroup precursors, which are proportional to the respective blood plasma concentrations as listed in the Human Metabolome Database (http://www.hmdb.ca/). In addition, constraints were added that ensure trapping of purine precursors as well as choline and ethanolamine within the parasite due to phosphorylation [252, 282]. Observations by Mehta *et al.* [322] indicate that *P. falciparum* is able to repress glycolysis of erythrocytes by inhibiting their glycolytic enzymes phosphofructokinase and pyruvate kinase. In concordance with these observations both host enzymes were inhibited in flux calculations that correspond to stages subsequent to the ring stage of the IDC.

For a certain fraction of *P. falciparum* genes, the transcriptome was found to correlate better with the proteome of the following than the current stage [420]. For this reason, considering only the gene expression snapshot of a single time point does not give a complete picture on the actual present proteome. Including the gene expression status of previous time points into the calculations should therefore further improve flux predictions. The algorithm was modified in such a way that genes were considered to be expressed, if a transcript is either present during the time point of interest or during a time span of approximately 12 hours prior to this time point (concerns 12 preceding samples of Bozdech data set and 2 preceding samples of Le Roch data set). Since the time intervals between samples covering the liver or the mosquito stage are larger, this modification was only applied to flux predictions related to the blood stage.

Metabolites predicted to be exchanged between host and parasite when using the improved algorithm (combination of host and parasite network, additional constraints reflecting knowledge about the blood stage as well as consideration of gene expression status of preceding time points; see Fig. 6.9 for Hamming distance matrix between calculated flux distributions) are in considerably better accordance with experimental findings (see Table 6.1 and Fig. 6.10) than before. In contrast to the previous predictions, purine precursors and choline are only imported and not secreted, while amino acids (except for isoleucine) are only exported. For the flux calculations hemoglobin is assumed to be digested at higher rates during trophozoite and schizont stages of the IDC, which releases iron that can be utilized by the parasite [168, 292, 132]. When the host's glycolytic enzymes phosphofructokinase and pyruvate kinase are inhibited (trophozoite and schizont stages), ATP can no longer be obtained from the host. Thus, the parasite needs to produce more ATP on its own, which requires the import of additional phosphate. Moreover, in order to ensure host survival for a certain time period, the parasite provides the host with ATP, which is in accordance with findings by Kanaani and Ginsburg [237].

In general, flux profiles derived from *in vitro* (Bozdech and Le Roch data set) and *in vivo* gene expression samples (Daily data set) are fairly similar in terms of exchanged metabolites between host and parasite, especially those corresponding to the active growth like cluster (cluster 2). One main difference is that more purine precursor types are predicted to be imported for the *in vivo* samples. The main differences between *in vivo* clusters is the absence of predicted fatty acid and choline import for samples of the starvation like cluster (cluster 1). This could be attributed to environmental conditions that result in a lack

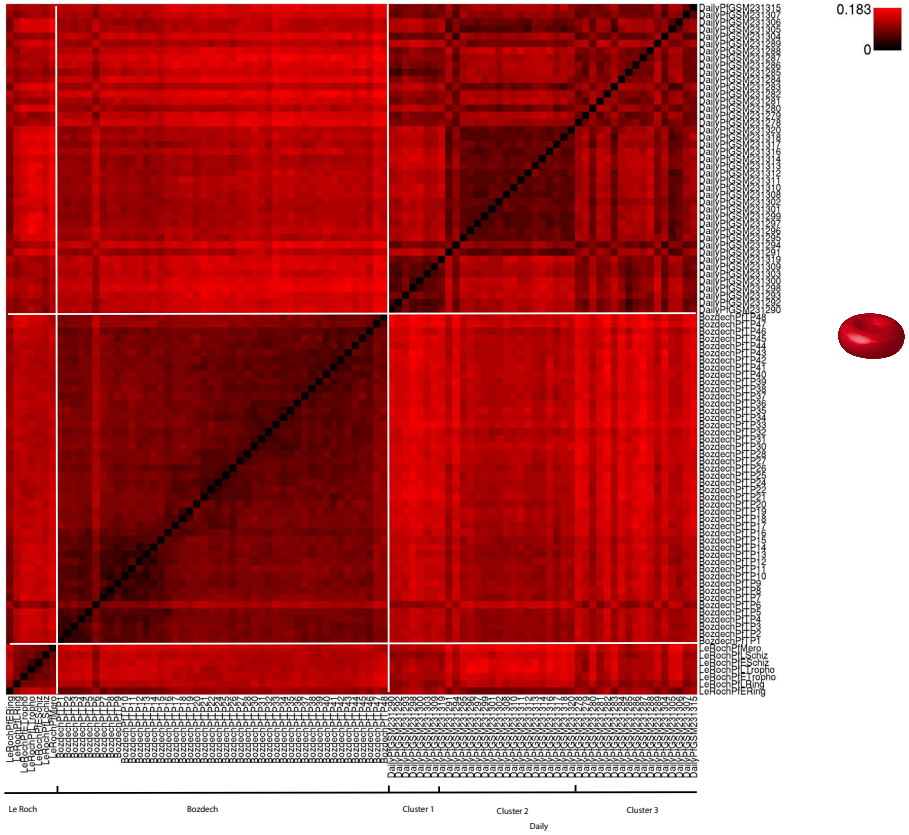


Figure 6.9: **Normalized Hamming distance matrix for calculated flux distributions using improved algorithm.** Flux distributions have been predicted with the improved flux balance approach for each gene expression sample derived from the blood stage. Simulations were conducted on the basis of the combined metabolic network of parasite and host and additional constraints reflecting knowledge about the blood stage. Furthermore, the expression status of genes during preceding time points was considered for the flux calculations. In order to compare the individual flux distributions normalized Hamming distances (see text for formula) have been determined for all pairs of flux distributions. The darker the color of a matrix entry, the lower is the corresponding Hamming distance.

of nutrients required for parasite growth. Cell membrane production is then either not possible or not the main focus and thus the import of membrane precursors like fatty acids and choline is not required.

There are still some metabolites whose predicted transfer direction through the parasite's plasma membrane does not meet literature knowledge. For example, it has been found that arginine contained in the medium is imported and depleted by the parasite, resulting in ornithine which is then excreted [366]. In contrast, the simulations suggest arginine export. Since the experimental observations were made at excessively high arginine concentrations, further experiments are needed to examine arginine transport under physiological condi-

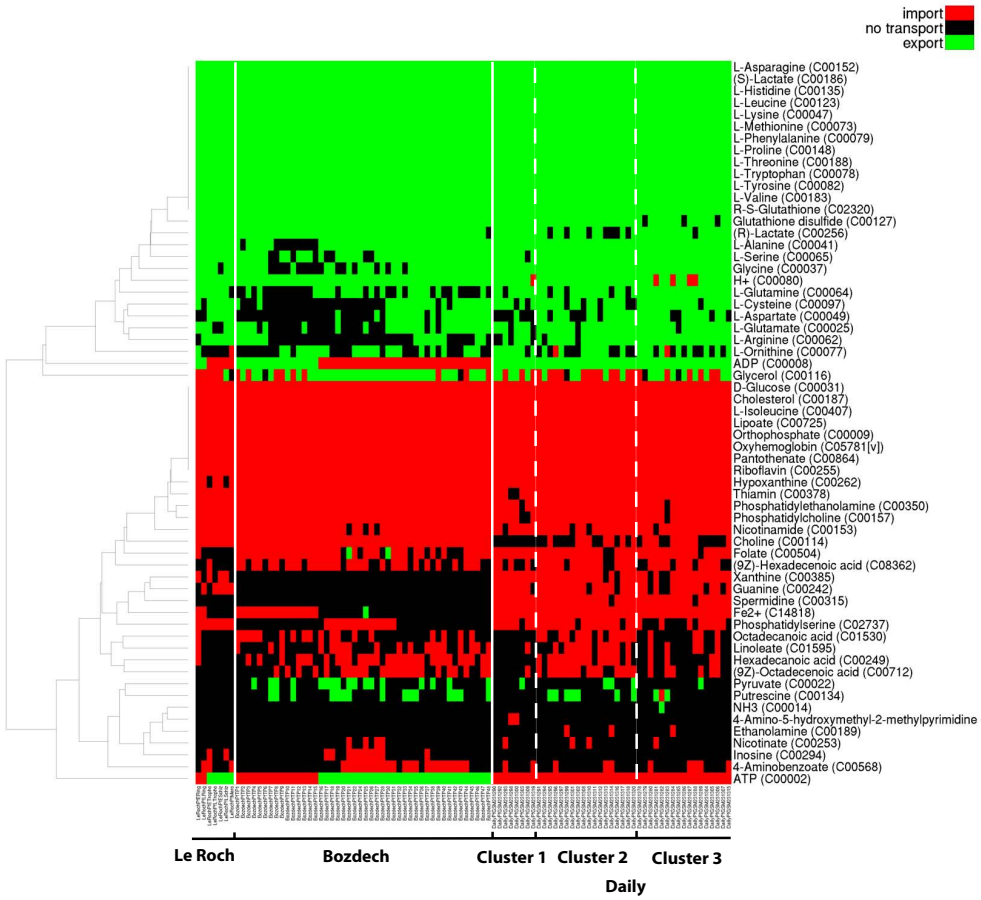


Figure 6.10: **Predicted host parasite metabolite exchanges using improved algorithm.** Flux distributions have been predicted with the improved flux balance approach (see Fig. 6.6) for each time point of the intraerythrocytic developmental cycle for which a gene expression profile exists. Simulations were conducted on the basis of the combined metabolic network of parasite and host and additional constraints reflecting knowledge about the blood stage. Furthermore, the expression status of genes during preceding time points was considered for the flux calculations. Resulting metabolite exchanges between host and parasite are depicted in this figure. Red matrix entries represent metabolites that are predicted to be imported into the parasite, while green matrix entries represent metabolites secreted into the host compartment.

tions. The predictions are more in line with experiments by Liu et al. [289] which indicate that hemoglobin degradation satisfies the parasite's demand for all amino acids during the blood stage except for isoleucine and thus isoleucine is the only amino acid required to be taken up. Other predicted metabolite transports that contradict literature knowledge are those of formate, hydrogencarbonate and ethanolamine. Almost no transport is predicted for ethanolamine, because the transport process is not associated with any gene and therefore our algorithm avoids to assign non-zero fluxes. However, this is not the case for formate and hydrogencarbonate, but rather missing reactions and transport processes in the

erythrocyte that are needed to dispose of these metabolites. Thus, predictions can still be further improved when more knowledge is available on the particular developmental stages as well as the host cell.

Counting for each metabolic reaction and transport process in how many stage-specific flux distributions it is predicted to be active when using the improved algorithm (see Fig. 6.11), reveals that nearly 350 reactions carry a non-zero flux in all 96 flux profiles that are derived from gene expression samples extracted during the IDC. In comparison to these globally active reactions, others that are present during fewer time points can be considered as more stage-specific.

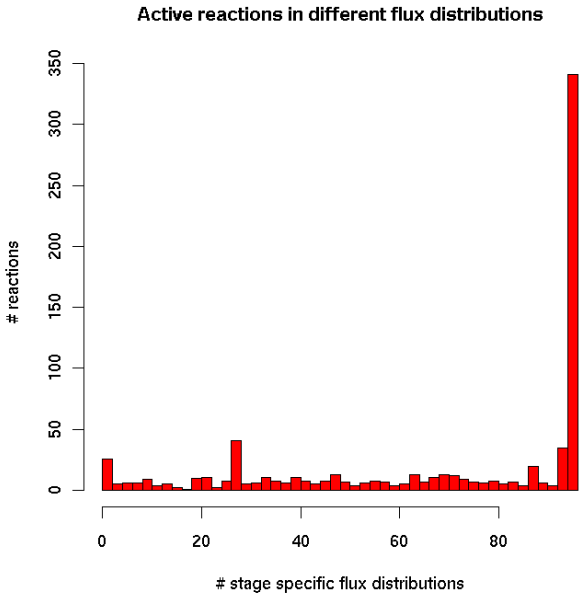


Figure 6.11: **Reaction distribution among stage-specific fluxes.** For each reaction of the parasite's metabolic network it was counted in how many of the flux distributions calculated with the improved approach the reaction carries a non-zero flux. The resulting histogram is shown here. The x-axis gives the number of flux distributions which assign a non-zero flux to a reaction and the y-axis indicates the number of reactions that are present in a certain number of flux distributions. In other words, the left most bar of the histogram represents the number of reactions that exclusively occur in a single flux distribution and are therefore very stage-specific, while the right most bar represents the number of reactions that are present in all 96 flux distributions related to the blood stage.

Table 6.1: **Metabolite exchanges between parasite and host: prediction vs. experiment.**

Metabolite	Predicted	Experiment	Comment	References
Glucose	uptake	uptake		[170, 306, 463]
Lactate	secretion	secretion		[170, 306, 463]
Hemoglobin	uptake	uptake		[170, 463]
Amino acids	secretion	secretion		[170, 306, 463]
Isoleucine	uptake	uptake		[170, 306], [463]
Nucleosides / nucleobases	uptake	uptake		[170, 306, 378]
Choline	uptake	uptake		[170, 306, 3, 282]
Ethanolamine	no exchange	uptake	No gene assigned to ethanolamine transporter	[170, 3]
Phosphatidylcholine	uptake	uptake		[170, 184]
Phosphatidylethanolamine	uptake	uptake		[170, 184]
Phosphatidylserine	uptake	uptake		[170]
Phosphate	uptake	uptake		[170, 306, 435]
Pantothenate	uptake	uptake		[170, 306, 251]
Nicotinamide / nicotinate	uptake	uptake		[170, 366]
Folate	mostly uptake	uptake		[170, 306]
4-aminobenzoic acid	uptake	uptake		[170, 306]
ADP	uptake / secretion	uptake	secretion in early blood stage when ATP can still be salvaged from host	[170, 306, 237]
ATP	uptake / secretion	secretion	uptake in early blood stage when host glycolysis is not repressed yet	[170, 306, 237]
Glycerol	uptake / secretion	uptake / secretion		[170, 306]
Glutathione conjugate	secretion	secretion		[170, 306, 344]

Continued on next page

Table 6.1 – continued from previous page

Metabolite	Predicted	Experiment	Comment	References
Ornithine	no exchange / secretion	secretion		[366]
Cholesterol	uptake	uptake		[170]
Formate	no exchange	secretion	Erythrocyte lacks reactions and transport processes to dispose of metabolite	[170, 306]
Fe2+	no exchange / uptake	uptake		[170, 306]
H+	mostly secretion	secretion		[170, 306]
HC03-	no exchange	secretion	Erythrocyte lacks reactions and transport processes to dispose of metabolite	[170, 306]

6.7 Calculated fluxes mapped onto metabolic pathways

In order to explore the predicted flux distributions derived from the improved algorithm on the level of metabolic pathways, a second mapping was done. This time, instead of counting expressed genes per KEGG pathway, reactions carrying non-zero fluxes were counted, to assess whether a pathway is active or not and whether there are changes during the IDC (see Fig. 6.12, 6.13 and Fig. 6.14). Due to the integration of gene expression states of preceding time points into the calculations, more enzymes are considered to be present than are suggested by the corresponding gene expression sample. In general, for consecutive time points the number of active reactions per pathway does not vary as much as the number of expressed genes per pathway (compare to Fig. 6.2 and Fig. 6.3). Therefore, fewer metabolic changes are predicted between the individual time points than are suggested by the gene expression data. Nevertheless, time dependent activity variation can be observed for certain pathways. For example, fewer reactions corresponding to folate biosynthesis are predicted to be active during the ring stage than during subsequent stages. In contrast, for the citric acid cycle as well as sphingolipid metabolism an increase in active reactions is predicted during schizont and early ring stage.

Predictions derived from *in vivo* gene expression samples show mainly similar pathway activity levels. Samples of the starvation-like cluster (cluster 1) vary from the others only with respect to lipid metabolism related pathways, which have a higher fraction of active reactions (especially the fatty acid biosynthesis pathway), and energy metabolism. Less reactions of the glycolytic pathway are active while more reactions of the citric acid cycle are active compared to cluster 2. The same holds true for some samples of the stress response-like cluster (cluster 3).

However, for some pathways the flux distributions vary substantially from the reported expression profiles. For instance, according to the flux predictions, synthesis of terpenoids (part of steroid synthesis pathway) takes place during the complete IDC, while gene expression data suggest this pathway to be only active during ring and trophozoite stages. This is due to the chosen set of target metabolites required to be produced during the individual stages (see Table 5.2), which includes isopentenyl diphosphate. This might not be appropriate for all time points.

6.8 Validation with proteomics and metabolomics data

For further validation the predicted stage-specific flux distributions were compared to available proteomics data. Five data sets were considered in this process, including three data sets extracted from *P. falciparum* during blood and mosquito stages [147, 275, 276], one data set that covers these stages in the rodent malaria pathogen *P. berghei* [185] and one data set obtained from the liver stage of *P. yoelii*, another rodent malaria pathogen [501]. The individual life cycle stages that were analyzed in these studies are listed in Table 2 and Table 3. For each observed protein sample the percentage of enzymes that is predicted to be active in the stage-specific flux distributions was calculated and plotted in Fig. 6.15.

Tendentially, if only few enzymes were detected during a specific stage, like the salivary gland sporozoite stage in the study by Hall *et al.* (HallPbSporo), a higher percentage of these enzymes are recovered by the predictions. Accordance between proteome data sets

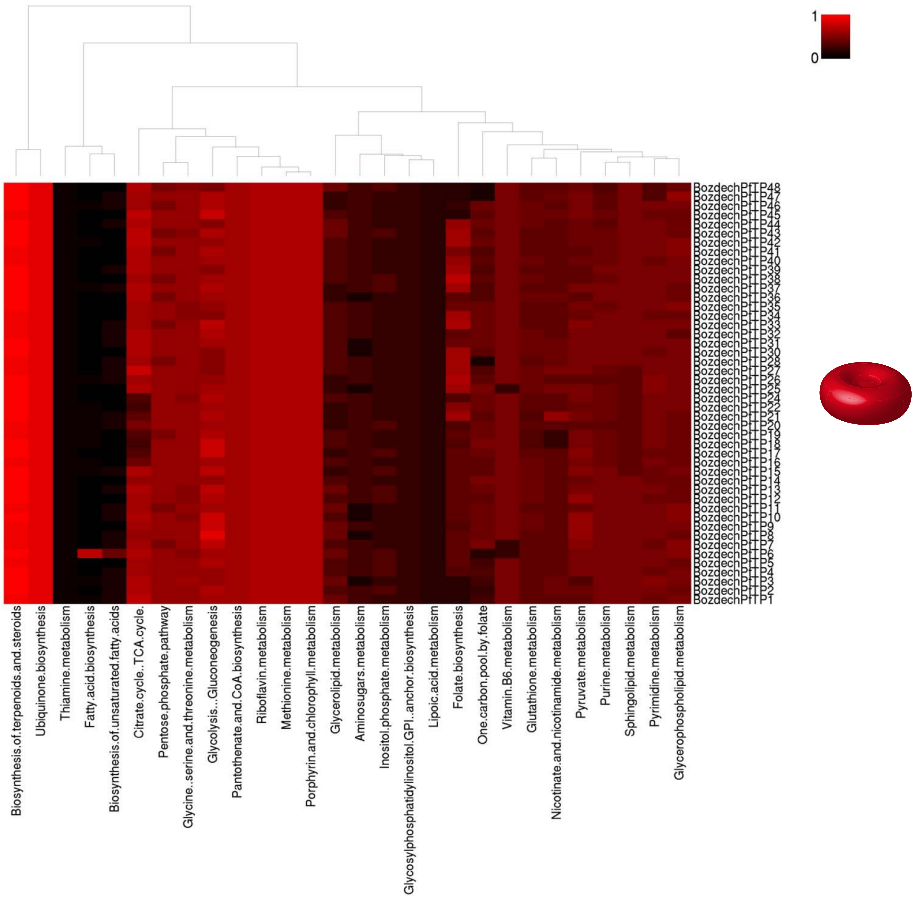


Figure 6.12: **Predicted metabolic fluxes consistent with Bozdech gene expression data mapped onto metabolic pathways.** Flux distributions have been predicted with the improved flux balance approach for each time point of the intraerythrocytic developmental cycle for which a gene expression profile exists. Simulations were conducted on the basis of the combined metabolic network of parasite and host and additional constraints reflecting knowledge about the blood stage. Furthermore, the expression status of genes during preceding time points was considered for the flux calculations. In order to explore the predicted flux distributions on the level of metabolic pathways, the flux profiles were mapped onto KEGG pathways and active reactions were counted, revealing active pathways and changes during the IDC. The darker the color of a matrix entry the fewer reactions of the corresponding pathway are active.

and predicted flux distributions does not only depend on the size of the proteome data set, though. For example, the gametocytes sample (LasonderPfGametocyte) extracted from blood cultures correlates better with flux distributions predicted for blood stages than the smaller sporozoite derived sample (FlorensPfSporo). In general, flux distributions predicted for mosquito stages show less agreement with proteome samples than flux distributions predicted for other stages. Nevertheless, flux distributions associated with salivary gland sporozoites (TarunPyooSpz, TarunPysgSpz) have large overlaps with proteome samples of

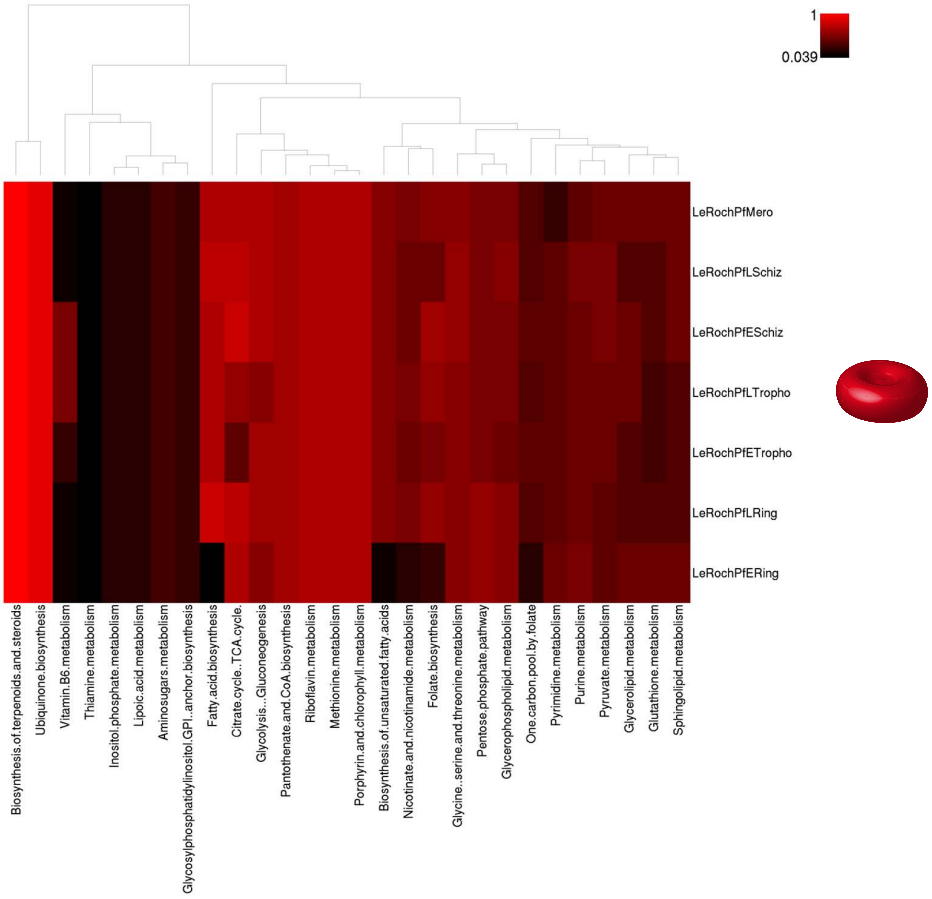


Figure 6.13: **Predicted metabolic fluxes consistent with Le Roch gene expression data mapped onto metabolic pathways.** Same as Fig. 6.12, but fluxes were calculated using Le Roch gene expression data.

the same stage (LasonderPfSgSporo, HallPbSporo, HallPbOocyst). Likewise, proteome samples extracted during the liver stages show best correlation with corresponding predicted flux profiles. Concerning the blood stage of the parasite's life cycle, a higher percentage of enzymes detected during the trophozoite and schizont stage (LasonderPfTrophoSchz, FlorensPfTropho) are predicted to be active during the schizont stage when considering those flux distributions derived from Le Roch gene expression samples. For blood stage related flux distributions derived from Bozdech gene expression samples the trophozoite proteome samples correlate slightly better with trophozoite stage related flux distributions. Regarding the *in vivo* expression sample derived predictions more agreement with blood stage proteome is found for samples of cluster 1 and 3.

Additionally, predicted flux distributions were analyzed with respect to metabolites that are produced or consumed by active reactions and thus should be present during the respective stage. These metabolites were compared to experimentally determined sets of metabolites detected during seven time points of the intraerythrocytic developmental cycle (Olszewski data set) [366] or extracted with different methods from mature trophozoites

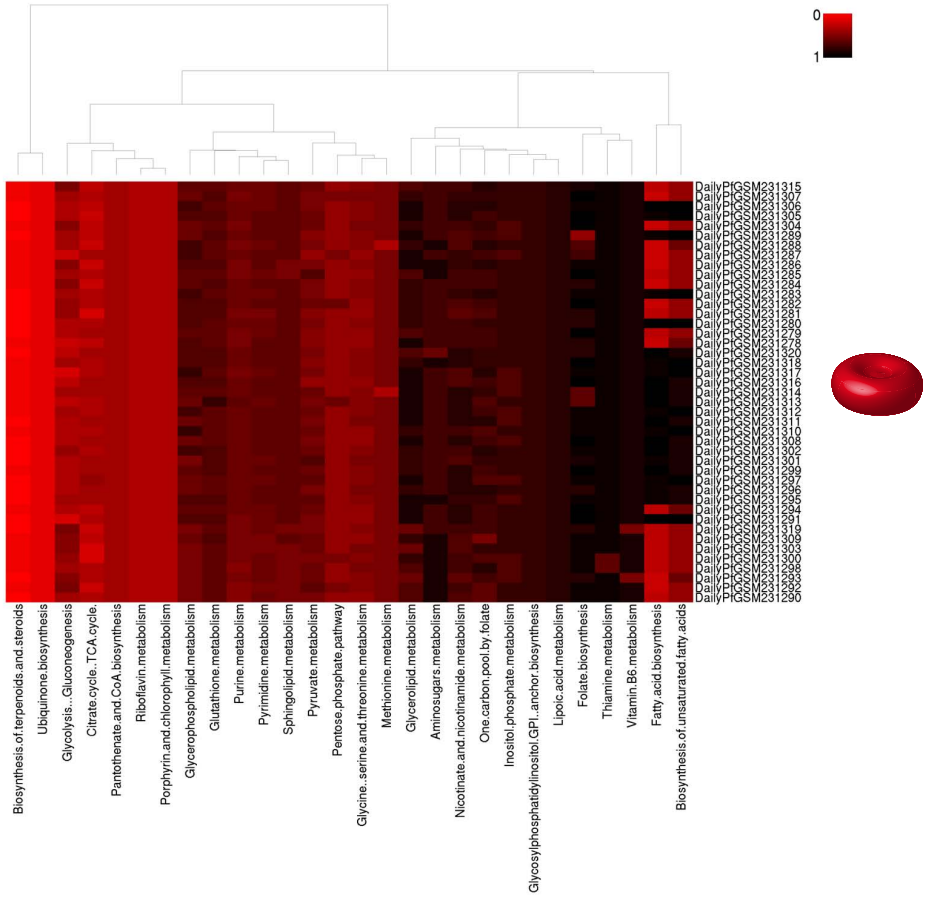


Figure 6.14: **Predicted metabolic fluxes consistent with Daily gene expression data mapped onto metabolic pathways.** Same as Fig. 6.12, but fluxes were calculated using Daily gene expression data.

(Teng data set) [503], respectively. The percentage of experimentally detected metabolites covered by predictions was computed for each of the eleven sets (see Fig. 6.16). Since the Teng metabolomics samples are quite small and contain mostly metabolites of the core metabolism, they are almost all present in all life cycle stage-specific flux predictions. In contrast, less metabolites of the Olszewski samples are recovered by the predictions. Nevertheless, more than 80% of the metabolites contained in these sets (which are more than double the size of the Teng metabolite sets) are involved in reactions that are predicted to be active during the blood stage. All seven Olszewski samples consist of the same metabolites (only concentrations vary during the individual time points), suggesting that these metabolites are present during the whole IDC. Discrepancies between predictions and experimental results are not necessarily due to limited prediction quality. For example, it is conceivable that metabolites diffuse into the cell and are experimentally detected, but no enzyme is expressed that is able to convert the metabolite. Furthermore, the metabolomics samples contain less than one hundred metabolites, suggesting that a large fraction of the actually present metabolites were not experimentally analyzed.

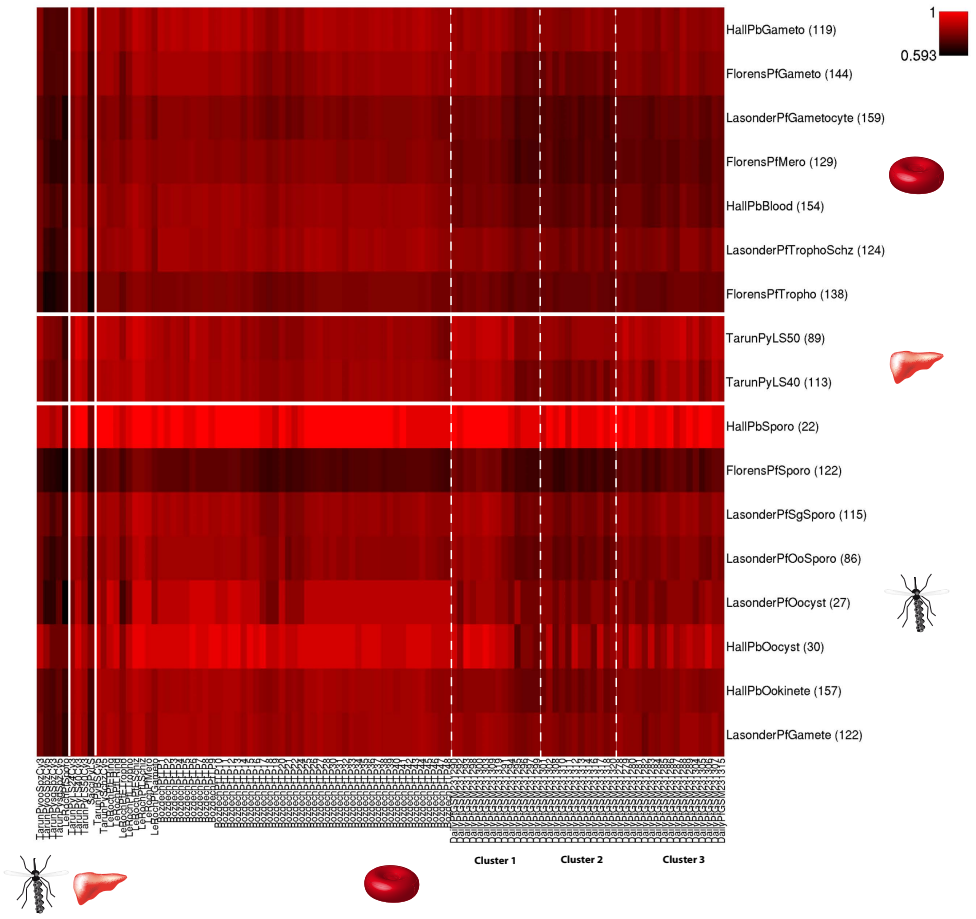


Figure 6.15: **Overlap of experimentally determined proteins with stage-specific sets of enzymes predicted to be active.** The applied proteome samples (y-axis) are specified in Table 2. Numbers in parenthesis indicate the number of genes that correspond to the metabolic network and are covered by a proteomics sample (y-axis) or the number of genes associated with active reactions (x-axis), respectively. Matrix entries depict the percentage of experimentally determined metabolic enzymes recovered by the stage-specific flux predictions. Improved predictions were considered for blood stage flux distributions.

6.9 Determination of fluxes with higher reliability

For each stage of the parasite's developmental cycle, so-called consensus reactions were determined that are predicted to be active for all gene expression samples covering the same stage. These reactions are more likely to actually occur during a certain stage, since they are derived from different data samples. In order to identify consensus reactions, all calculated flux profiles were grouped into 13 sets (LS24, LS40, LS50, eRing, lRing, eTropho, lTropho, eSchiz, lSchiz, Mero, Gameto, ooSporo, sgSporo; see Table 1) with respect to represented stages, and those reactions were determined that carry a non-zero flux in all flux profiles of the same set. For all stages of the intraerythrocytic developmental cycle those flux profiles were considered that were calculated with the improved algorithm.

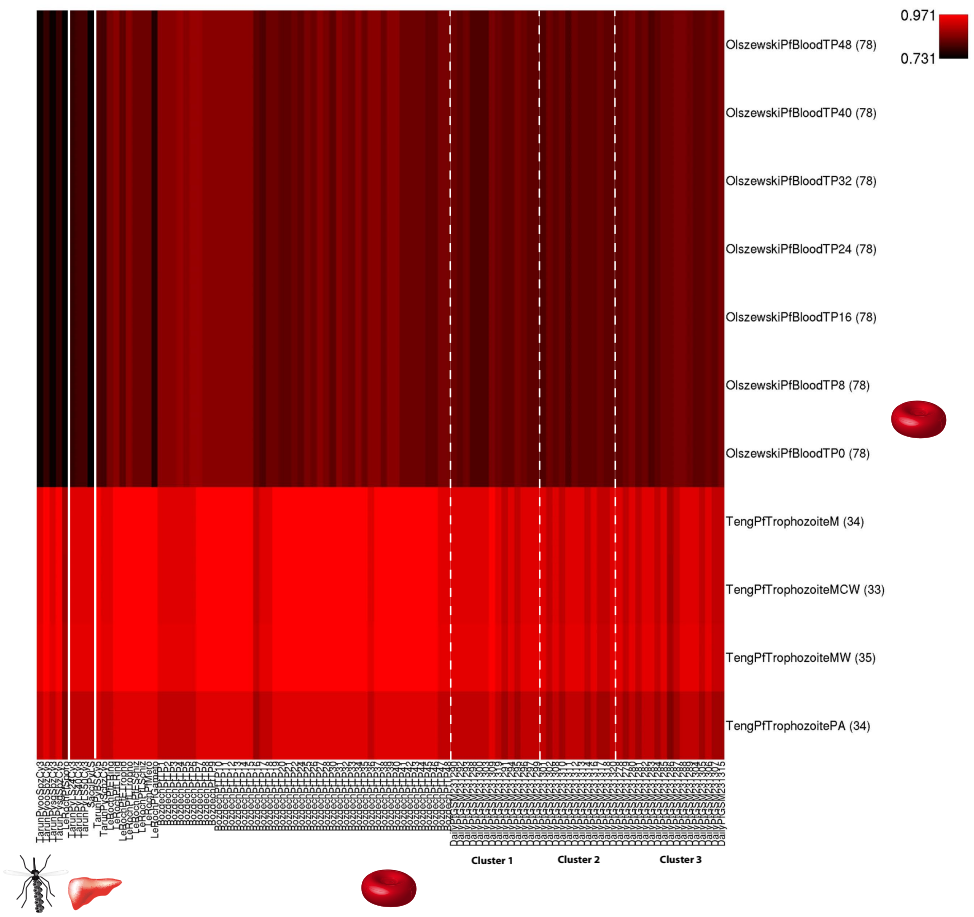


Figure 6.16: **Overlap of experimentally determined metabolites with stage-specific sets of metabolites predicted to be present.** The applied metabolomics samples (y-axis) are specified in Table 3. Numbers in parenthesis indicate the number of metabolites that correspond to the metabolic network and are covered by a metabolomics sample (y-axis) or the number of metabolites involved in a predicted flux distribution (x-axis), respectively. Matrix entries depict the percentage of experimentally determined metabolites recovered by the stage-specific flux predictions. Improved predictions were considered for blood stage flux distributions.

For each blood stage and each metabolic pathway the fraction of consensus reactions per total number of pathway reactions was computed to uncover the distribution of consensus reactions among pathways (see Fig. 6.17). To get an impression of how many consensus reactions are shared between the different stages, this fraction was also computed for those consensus reactions that two sets have in common. In several pathways the same reactions are active during the whole IDC (e. g. ubiquinone biosynthesis and terpenoid biosynthesis). These are mostly biosynthesis pathways where alternative routes are rare and the endproducts are considered to be essential. Therefore, the network is forced to produce them, which results in a large overlap between the predicted fluxes through these pathways for all time

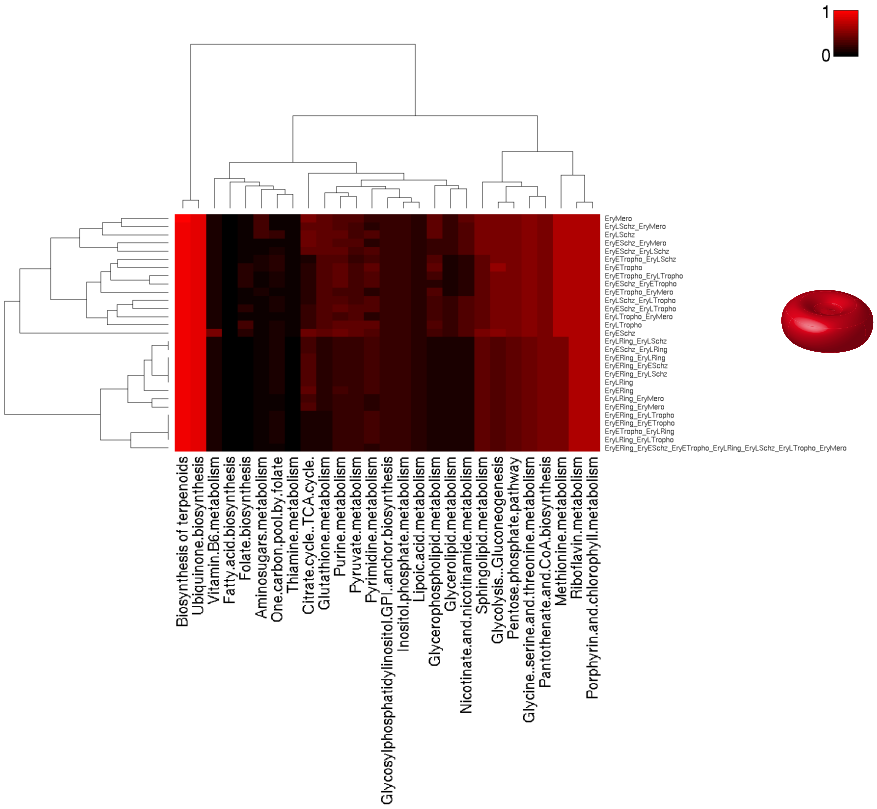


Figure 6.17: **Overview of pathway specific consensus reactions for different time points of intraerythrocytic cycle.** Consensus reactions (reactions that are predicted to be active for all gene expression samples, covering the same stage) were determined for all stages of the IDC. These reactions are more likely to actually occur during a certain stage, since they are derived from different data samples. In order to identify consensus reactions, all calculated flux profiles corresponding to the blood stage were grouped into seven sets (see Table 1) with respect to represented stages and those reactions were determined that carry a non-zero flux in all flux profiles of the same set. For each blood stage and each metabolic pathway the fraction of consensus reactions per total number of pathway reactions was computed to uncover the distribution of consensus reactions among pathways. To get an impression of how many consensus reactions are shared between the different stages, this fraction was also computed for those consensus reactions that two sets have in common. The darker the color of a matrix entry the lower is the percentage of consensus reactions.

points. By contrast there are pathways where more accordance can be observed for trophozoite and schizont stages (e.g. glutathione metabolism, pentose phosphate pathway). This is mainly due to the fact that consensus reactions for the early and late ring stage are derived from many more samples than those for other stages. Especially the heterogeneous *in vivo* expression samples contribute to a low number of consensus reactions for these stages.

The citric acid cycle is one of the few pathways where a certain level of agreement exists between ring and schizont stages.

6.10 Discussion

A relatively simple approach to obtain global insight into the metabolism of a cell under certain conditions is to analyze gene expression and to infer active metabolic pathways based on the presence of corresponding enzyme mRNA transcripts (e.g. [56, 567, 234, 437]). Multiple tools have been developed in the last years to aid this process [93, 374, 334, 286]. However, more detailed information such as flux rates and flux directions cannot be gained from gene expression data alone. Additional metabolomics and tracer experiments are required to obtain this information. A computer based alternative is flux balance analysis, which derives metabolic flux rates by solving an optimization problem that reflects the flux balance principle and an assumed objective for the metabolism of an organism of interest. In contrast to kinetic modeling, no detailed rate equations are required, thus making this method appropriate for large-scale metabolic networks lacking full kinetic characterization. In this work an approach that combines gene expression data with flux balance analysis has been applied to *P. falciparum* to infer life cycle stage-specific metabolism. This strategy was assumed to complement the weaknesses of each approach, allowing to predict metabolic fluxes even for those parts of the metabolic network which are not associated with gene expression data or which might not be covered by the applied objective function due to incomplete knowledge of cellular objectives. As has been shown, flux distributions derived from this approach differ for certain pathways from what is expected when only taking into account gene expression profiles mapped onto metabolic pathways. For example, the terpenoid biosynthesis pathway is predicted to be active during the whole IDC, while few genes of this pathway are expressed during merozoite and ring stage. These discrepancies are due to the chosen target metabolites whose production requires enzymes of this pathway to be active. To evaluate whether these predictions are inadequate or mRNA levels are simply not fully conclusive, experimental data of present enzymes and pathway intermediates are needed. Comparisons with experimentally observed enzyme and metabolite profiles revealed good accordance for most of the stage-specific flux distributions. However, due to the small number of examined stages as well as the relatively small number of measured enzymes and metabolites, detailed evaluation of predicted flux distributions is not possible. Differences in predicted fluxes and observed enzyme profiles might additionally be caused by the applied signal-to-noise cutoff for the classification of gene expression. Especially for the Tarun data set, where a high cutoff was chosen and thus more genes are considered to be not expressed, this could play a role. Another issue could be the fact that only one solution was considered here, but FBA might yield multiple solutions, which are equally optimal with respect to the applied objective function. It is possible that alternative optimal solutions agree better with experimental data. Therefore, prediction improvements could be obtained when flux ranges are considered that reflect alternative solutions rather than fixed flux values. Such ranges can be determined with the help of a concept called flux variability analysis [300]. Integration of available information about present enzymes and metabolites will further improve FBA predictions.

In general, better predictions could be obtained if more details were known about the

biochemical composition of the parasite during the different developmental stages, allowing to use for each stage an adapted set of target metabolites. Furthermore, incorporation of additional information such as available nutrients, capacities of transport proteins and host cell metabolism would further improve predictions as has been demonstrated for stages of the intraerythrocytic developmental cycle. Until such information is available for all stages of parasite development, flux distributions derived from flux balance analysis and gene expression data are a good starting point to get an insight on the parasite's metabolism on genome-scale.

Chapter 7

Identification of putative drug targets

7.1 Introduction

Typically, the first step in the drug discovery process is the identification and validation of a suitable drug target. Subsequently, a potential drug is determined by either high-throughput screening against a library of drug-like compounds or rational drug design. In order to prevent waste of resources, it is important that the target candidate is well chosen. Several criteria exists that help to identify potent drug targets. The two most important properties are: (1) the target is druggable, i.e., the target possesses a binding site for small molecules that might modulate its activity and (2) the target is essential for disease progression.

Druggable proteins have been analyzed with respect to common structural features, revealing that these characteristics are good predictors for potential targets [575, 353, 89]. In case structural information is not available, genomic sequence properties shared among drug targets have been shown to be an alternative basis for druggability predictions [25].

Proteins critical for processes involved in disease progression can be detected by network based approaches that consider the whole system and not only local properties. If a pathway has been found to be essential for disease progression and kinetic information is available for all involved enzymes, metabolic control analysis can be used to determine those enzymes that have the largest influence on the pathway and thus presumably represent efficient targets [76, 211]. However, very often such information is not available. A relative simple alternative method is the choke-point analysis, which identifies reactions that are for particular metabolites the only source or sink in the metabolic network of interest [561, 188, 472, 473]. Inhibition of such reactions results in accumulation or depletion of these metabolites with presumably negative effects on the cellular system. Many known drug targets in the metabolism of pathogens have been shown to be choke-points [561, 472]. A major disadvantage of this method is the high number of predicted targets. Other proposed network based drug target detection approaches have in common that a certain knowledge about the cellular objective is required, e.g., target metabolites essential for the development of pathogenic cells. An example hereof is a concept called minimal cut sets, which determines a minimal set of blocked reactions that are required for the inactivation of certain network functions [256, 255, 189]. Computation time for large metabolic networks is quite high, though, as the approach requires to compute all feasible and balanced flux distributions for the network of interest (elementary flux modes) whose number grows exponentially with network size. An approach that is characterized by relatively low computation times is flux balance analysis. This concept has been applied to simulate knockouts in several organisms with good agreement between phenotype predictions and

experimental observations [135, 279, 233, 377, 131].

7.2 Detection of essential reactions via flux balance analysis

The main goal of this work is the identification of novel putative drug targets in *Plasmodium falciparum*. For this purpose flux balance simulations were conducted, in order to analyze the compiled metabolic network with regard to essential reactions required for the production of important cellular metabolites. Enzymes catalyzing such reactions represent potential drug targets, since their inhibition would result in a shortage of required metabolites and thereby in impaired development. The linear program underlying the knockout simulations conducted in this study reads as follows:

$$\min_{v} \sum \mid v_i \mid \tag{7.1}$$

$$\text{s. t.: } Sv = 0 \tag{7.2}$$

$$v_{\min,i} < v_i < v_{\max,i} \tag{7.3}$$

$$v_i > 1 \quad \text{for target metabolite efflux} \tag{7.4}$$

$$v_i = 0 \quad \text{for knocked-out enzymes} \tag{7.5}$$

Here, S is the stoichiometric matrix of the cellular system, v_i are internal fluxes as well as exchange fluxes, and v_{min} and v_{max} represent lower and upper bounds on fluxes. The solution space that contains all feasible flux distributions is defined by linear equations derived from the steady-state assumption (7.2) as well as thermodynamics and knowledge about enzyme and transporter capacities (7.3). Production of metabolites assumed to be essential during any time point of parasite development (see Table 5.2) is ensured by forcing an efflux of these metabolites through the system boundary (7.4). In addition, fluxes through a reaction of interest are fixed to zero, mimicking the knockout of the corresponding gene. For each reaction of the metabolic network of *P. falciparum* such a linear program was formulated. In case the solution space of the problem was empty, the knocked-out reaction was considered to be indispensable. Hence, the corresponding enzyme represents a putative drug target, since no path exists converting external nutrients into all target metabolites, which presumably results in impaired parsite development. In contrast to the FBA calculations conducted in the previous chapter, no gene expression data was considered for the identification of essential reactions. Furthermore, no assumptions were made about the cellular environment and nutrient uptake was not restricted.

As not all metabolites are equally important during all time points of parasite development, e.g., DNA building blocks are not required to be synthesized during the merozoite stage, a reaction detected by this means might not be globally essential, but rather during those time points when the particular metabolite requiring the activity of the reaction is indispensable. On the other hand, certain reactions that are not detected as essential might be incorrectly classified if nutrients are in reality not available in sufficient amounts during all time points, which is in contradiction to the simulation assumption. In this case essential metabolites cannot be derived from imported precursors, but rather need to be completely synthesized, making additional reactions indispensable. To sum up, all reactions identified

as essential by this approach are essential during at least one developmental stage, but not all reactions that are essential during any stage are necessarily recovered.

7.3 Essential reactions in the metabolism of *P. falciparum*

Flux balance knockout simulations identified 307 reactions that are essential for the parasite. To evaluate these results a gold standard set was compiled containing 57 enzymes that were shown to have antimalarial effects upon inhibition or silencing (see Table 7.1). 35 of these gold standard enzymes correspond to reactions that were predicted to be indispensable. Blocking simultaneously all reactions catalyzed by the same gene product recovered two further targets (dihydrofolate reductase and hypoxanthine phosphoribosyltransferase) contained in the gold standard set. Among the 20 remaining enzymes 13 were detected as essential, if additionally transporters were constrained that import biomass precursors bypassing the respective enzyme. For example, inhibition of the spermidine transporter makes the spermidine synthase indispensable. These enzymes are therefore either not metabolically essential during all environmental conditions, but only during those stages where particular nutrients are not present in sufficient amounts, or the respective parasitic transporters are not efficient enough. Furthermore, GMP synthase was detected if the import of guanine and guanosine was constrained and all reactions catalyzed by the enzyme were blocked. Ornithine decarboxylase was not found by this means, as a an alternative path exists to synthesize putrescine, which involves carbamoylputrescine amidase. Blocking this enzyme in addition to the spermidine and putrescine transporters makes ornithine decarboxylase indispensable. Carbamoylputrescine amidase is very likely not present in *P. falciparum*, since it was added in the BioCyc database to fill gaps in the putrescine biosynthesis pathway of which only one enzyme is associated with a gene. Succinate dehydrogenase and NADH dehydrogenase, which are not even detected when nutrient import is limited, are presumably false positives. For both enzymes of the mitochondrial electron transport chain antimalarial activity was demonstrated through inhibition with substrate analogs of ubiquinone [267, 492]. As ubiquinone, the substrate of both enzymes, can be provided with electrons by several enzymes (succinate dehydrogenase, NADH dehydrogenase, dihydroorotate dehydrogenase, FAD-dependent glycerol-3-phosphate dehydrogenase, malate-quinone oxidoreductase), it is not quite obvious why inhibition of only one enzyme should result in impaired parasite development. One explanation could be that the ubiquinone analogs do not only inhibit one enzyme, but also others that use ubiquinone as a substrate. This is supported by the findings of Painter *et al.* [371] who discovered that an active mitochondrial electron transport chain might only be required for the disposal of electrons derived from dihydroorotate dehydrogenase, an essential enzyme for pyrimidine biosynthesis. They showed that parasites expressing the corresponding yeast enzyme, which does not require ubiquinone as an electron acceptor, are insensitive to atovaquone, an inhibitor of the electron transport chain Complex III. This suggests that the antimalarial effect of ubiquinone analogs was rather due to either additional inhibition of the downstream Complex III or simultaneous inhibition of all enzymes providing ubiquinone with electrons (including dihydroorotate dehydrogenase), instead of the sole inhibition of succinate dehydrogenase or NADH dehydrogenase. The simulations indicate furthermore that neither sole inhibition of choline kinase nor sole inhibition of phosphoethanolamine methyltransferase kills the parasite, be-

cause phosphatidylcholine can be derived from choline as well as from ethanolamine [385]. The question remains whether these pathways are indeed able to fully compensate the loss of the alternative pathway, as both have been suggested to affect parasite growth [92, 553]. However, the findings by Choubey *et al.* [92] can also be explained by unspecific inhibitor binding (both experiments utilized miltefosin analogs), resulting in the inhibition of both choline kinase and phosphoethanolamine methyltransferase [385]. Finally, the gold standard enzyme fructose-bisphosphate aldolase was not detected by the knockout simulations. Essentiality of this glycolytic enzyme was not confirmed by the simulations, since ATP can be generated from glycerol-3-phosphate (derived from imported glycerol) and glyceraldehyde-3-phosphate (produced in pentose phosphate pathway), which enter glycolysis downstream of the enzyme. These metabolites might yield lower amounts of ATP, though, due to the lower concentration of glycerol in the blood compared to glucose. Buscaglia *et al.* [68] demonstrated that aldolase bridges the actin-myosin motor, which is involved in gliding motility and host cell invasion, and an invasin protein that connects to host cell receptors. Metabolite (and drug) binding to aldolase prevents interactions between aldolase and the invasin protein, which has negative effects on parasite motility. Therefore, the observed reduction of parasitemia upon aldolase inhibition might rather be caused by impaired host cell invasion than metabolic effects.

Table 7.1: **Gold standard set of essential enzymes.**CP: choke-point; FBA: found by FBA

EC no.	Reaction	CP	FBA	Comment	Reference
1.1.1.205	IMP dehydrogenase	Yes	No	Precursor import (adenosine, hypoxanthine, inosine)	[543]
1.1.1.267	1-deoxy-D-xylulose-5-phosphate reductoisomerase	Yes	Yes		[230]
1.3.1.9	enoyl-ACP-reductase	Yes	Yes		[191, 494, 481]
1.3.3.1	dihydroorotate oxidase	No	Yes		[27, 52, 194, 318, 266]
1.3.99.1	succinate dehydrogenase	No	No	Presumably unspecific off-target effects	[492]
1.5.1.3	dihydrofolate reductase	No	No	Necessary to block all reactions catalyzed by enzyme	[62, 347, 142, 113]
1.6.5.3	NADH dehydrogenase (ubiquinone)	No	No	Presumably unspecific off-target effects	[267]
1.8.1.7	glutathione reductase	Yes	Yes		[48, 574]
1.8.1.9	thioredoxin reductase	Yes	Yes		[264, 295]
1.10.2.2	cytochrome c reductase	Yes	Yes		[144]
1.15.1.1	superoxide dismutase	Yes	Yes		[480]
1.17.4.1	ribonucleoside-diphosphate reductase	No	Yes		[79, 33, 296]
2.1.1.100	protein-S-isoprenylcysteine-O-methyltransferase	Yes	Yes		[34, 548]
2.1.1.103	phosphoethanolamine methyltransferase	Yes	No	Presumably unspecific off-target effects	[385, 553]
2.1.1.45	thymidylate synthase	No	Yes		[225, 354]
2.1.1.64	3-Demethylubiquinone-9,3-O-methyltransferase	Yes	Yes		[308]
2.3.1.24	sphingosine-N-acyltransferase	Yes	Yes		[166]
2.3.1.37	delta-aminolevulinate synthase	Yes	Yes		[520, 493]

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Table 7.1 – continued from previous page

EC no.	Reaction	CP	FBA	Comment	Reference
2.3.1.41	3-Oxoacyl-[acyl-carrier protein] synthase	Yes	Yes		[536, 396]
2.3.1.50	serine-palmitoyl transferase	No	Yes		[166]
2.4.2.1	purine-nucleoside phosphorylase	Yes	No	Precursor import (hypoxanthine, xanthine)	[248]
2.4.2.8	hypoxanthine phosphoribosyltransferase	Yes	No	Necessary to block all reactions catalyzed by enzyme	[115, 438, 285]
2.5.1.15	dihydropteroate synthase	Yes	No	Precursor import (folate)	[316, 508, 347]
2.5.1.16	spermidine synthase	Yes	No	Precursor import (spermidine)	[183]
2.5.1.18	glutathione transferase	Yes	Yes		[157, 187, 288, 383]
2.5.1.19	3-Phosphoshikimate-1-carboxyvinyltransferase	Yes	No	Precursor import (4-Aminobenzoate, folate)	[415]
2.5.1.58	farnesyl-diphosphate-farnesyltransferase	No	Yes		[80]
2.7.1.32	choline kinase	No	No	Presumably unspecific off-target effects	[92]
2.7.8.3	ceramide-cholinephosphotransferase	Yes	Yes		[166]
3.3.1.1	S-adenosyl-l-homocysteine hydrolase	Yes	Yes		[326, 253, 468, 66]
3.4.11.1	leucine aminopeptidase	Yes	No	Precursor import (amino acids)	[352]
3.4.14.1	dipeptidyl aminopeptidase 1	No	No	Precursor import (amino acids)	[258]
3.4.23.38	plasmepsins (aspartic acid proteases)	No	No	Precursor import (amino acids)	[357, 422]
3.4.23.39	plasmepsins (aspartic acid proteases)	No	No	Precursor import (amino acids)	[357, 422]
3.5.1.89	N-acetyl-glucosaminylphosphatidylinositol deacetylase	No	Yes		[477]

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Table 7.1 – continued from previous page

EC no.	Reaction	CP	FBA	Comment	Reference
3.5.2.3	dihydroorotase	Yes	Yes		[455, 266]
3.5.4.4	adenosine deaminase	Yes	No	Precursor import (hypoxanthine, inosine, xanthine)	[165, 511]
4.1.1.17	ornithine decarboxylase	No	No	Precursor import (spermidine, putrescine); alternative reaction	[20, 50, 42]
4.1.1.23	orotidine-5-phosphate decarboxylase	Yes	Yes		[41, 327, 268, 454, 451]
4.1.1.50	adenosylmethionine decarboxylase	No	No	Precursor import (spermidine)	[556]
4.1.2.13	fructose-bisphosphate aldolase	Yes	No	Presumable main antimalarial effect is impaired host cell invasion	[541, 223]
4.2.1.1	carbonic anhydrase	Yes	Yes		[411]
4.2.1.24	δ-aminolevulinic acid dehydratase	No	Yes		[55]
4.2.1.58-61	3-hydroxyacyl-ACP dehydratase	Yes	Yes		[458]
4.2.3.5	chorismate synthase	Yes	No	Precursor import (4-Aminobenzoate, folate)	[318]
4.4.1.5	lactoylglutathione lyase	Yes	Yes		[505]
4.6.1.12	2-C-methyl-D-erythritol-2,4-cyclodiphosphate-synthase	No	Yes		[109]
5.99.1.2	topoisomerase I	Yes	Yes		[53]
5.99.1.3	topoisomerase II	Yes	Yes		[159, 85, 356]
6.1.1.3	threonine-tRNA ligase	Yes	Yes		[425]
6.1.1.7	alanine-tRNA ligase	Yes	Yes		[102]
6.3.2.2	γ-glutamylcysteine synthetase	Yes	Yes		[390, 295, 323]
6.3.4.4	adenylosuccinate synthetase	Yes	Yes		[129]

Continued on next page

Table 7.1 – continued from previous page

EC no.	Reaction	CP	FBA	Comment	Reference
6.3.5.2	GMP synthetase	No	No	Necessary to block all reactions catalyzed by enzyme; precursor import (guanine/guanosine)	[314]
6.3.5.5	carbamoyl-phosphate synthase	No	Yes		[148]
6.3.5.8	amino-deoxychorismate synthase	No	No	Precursor import (4-Aminobenzoate, folate)	[536]

7.4 Performance comparison with choke-point analysis

Previously, two methods have been presented to predict drug targets in *P. falciparum* [561, 136]. Both approaches apply the concept of choke-points, which are reactions that are the only source or sink for at least one metabolite. To compare the performance of the FBA method and that of choke-point analysis, all choke-points were identified in the compiled metabolic network and assessed with the gold standard set of drug targets (see Table 7.1). 679 choke-point reactions were detected in the network, including reactions that are catalyzed by 37 enzymes of the gold standard. Thus, choke-point analysis recovers more true targets than the above described flux balance approach. However, due to the large number of reactions predicted as essential by the choke-point analysis, the flux balance approach results in higher values for specificity, accuracy and precision and therefore a better enrichment of true targets (see Fig. 7.1). This means that a choke-point analysis might end up detecting more true targets, but the flux balance approach will result in a set of predicted targets with a lower percentage of false positives, which reduces the waste of experimental resources needed to validate the predictions.

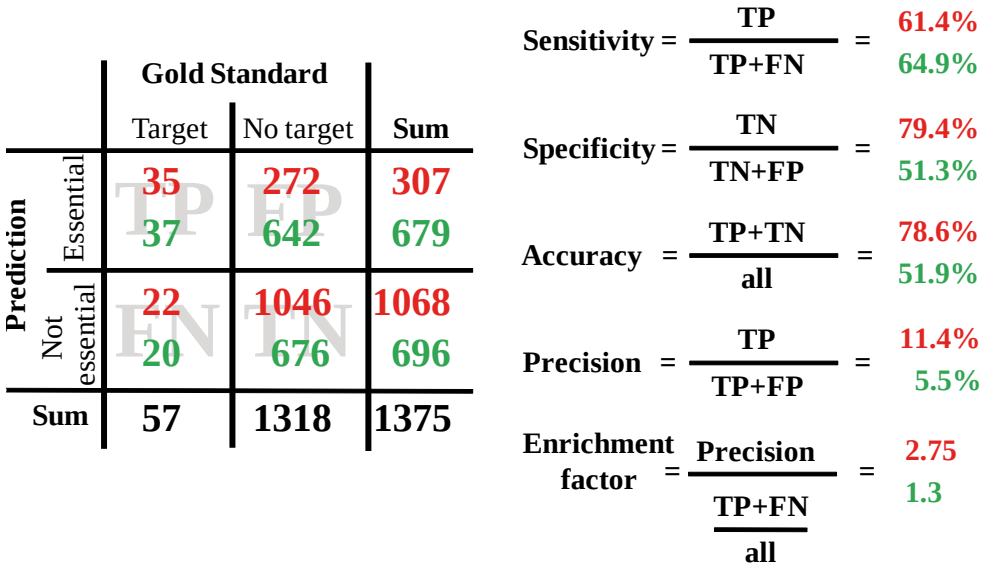

Figure 7.1: **Evaluation of predicted drug targets.** FBA based knockout simulations were conducted to uncover reactions within the parasite's metabolic network that are essential for the production of development relevant metabolites (see Table 5.2). The predicted set of indispensable reactions, which presumably represent good drug targets, was evaluated on the basis of a gold standard set that contains 57 experimentally verified essential enzymes. True positives (TP), false positives (FP), false negatives (FN), and true negatives (TN) were determined in order to calculate based on these numbers sensitivity, specificity, accuracy and precision of the method as well as the corresponding enrichment factor (red numbers). To compare the performance of the FBA method to the previously proposed choke-point analysis, additionally all choke-points within the parasite's metabolic network were identified and the same statistics were calculated (green numbers).

7.5 Ranking of predicted drug targets

Some of the targets predicted by the flux balance approach that are not covered by the gold standard might nevertheless be valid targets. To address this question, the SuperTarget database [179] was queried with the respective EC numbers to check whether some of these reactions are catalyzed by enzymes that were previously targeted in the course of medical treatments or were shown to have effects on the viability of an organism or pathogenic cell. Of 155 reactions that are assigned to genes and are predicted to be essential but are not covered by the gold standard enzymes, 39 correspond to enzymes that are listed in the SuperTarget database as drug targets.

To avoid unwanted side effects within the human host, it is beneficial to target parasitic enzymes that are not homologous to human enzymes. Therefore a BLAST search was completed on human protein sequences (non-redundant protein sequences) using the predicted parasitic protein sequences from PlasmoDB as queries. Analogous to [561] an E-value significance threshold of < 0.075 was applied to determine homologous sequences. Of the 198 reactions that were predicted to be essential and are associated with genes, 168 are catalyzed by enzymes that are homologous to human enzymes. The gene products catalyzing the remaining 30 reactions represent presumably good drug target candidates. Targets with human homologs may still be interesting targets as long as binding sites for potential inhibitors are sufficiently distinct.

As discussed above, reactions predicted to be essential are not necessarily essential during all time points. Therefore, the stage-specific metabolic fluxes calculated in the previous chapter were considered to get an idea during which stages reactions are essential. In this context, the consensus reactions calculated for each life cycle stage (reactions that carry a non-zero flux in all flux profiles corresponding to the same life cycle stage) were mapped onto the set of essential reactions (see Table 4). This mapping gives a hint on targets that are present during all time points as opposed to those that are only active during certain stages. Depending on the time point when a reaction of the latter type is essential, drugs designed to inhibit the respective enzyme can either be applied as prophylactic drugs to kill the liver stages of the parasite and prevent disease symptoms or to treat the disease by killing blood stages of the parasite, respectively.

Predicted drug target reactions that do not correspond to the gold standard set, but are associated with genes, were ranked according to a score that represents the sum of weighting factors assigned to the following features (see Table 4): weighting factor 2 if corresponding genes are not homologous to human genes, weighting factor 1 if the enzymatic function is targeted in any organism (i.e., EC number is listed in the SuperTarget database) and weighting factor 1 if the reaction is active during all parasitic life cycle stages. The latter weighting factor prioritizes exemplarily those targets that are suitable for prophylaxis and disease treatment. Depending on which developmental stage of the parasite one is interested in to inhibit this weighting factor can be adapted. The 30 top ranking targets are listed in Table 7.2. Strikingly, among the eight top ranking enzymes/transporters four enzymes and one transporter are assigned to the apicoplast, which harbors prokaryotic biochemical pathways and was therefore proposed as an interesting drug target [403, 546]. Three of the top ranking enzymes are involved in the biosynthesis of isopentenyl diphosphate (IPP). Since two enzymes of the IPP biosynthesis pathway (1-deoxy-D-xylulose-5-phosphate reductoisomerase, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate-synthase) have already been suggested as poten-

tial targets for antimalarial therapy [230, 77, 109], it is likely that other enzymes of this linear pathway have also this potency. *P. falciparum* possesses a pathway to synthesize pyrimidine nucleotides *de novo*. Multiple reactions of this pathway are predicted to be essential and for some of them (carbonic anhydrase, carbamoyl-phosphate synthase, dihydroorotase, dihydroorotate oxidase, orotidine-5'-phosphate decarboxylase) inhibition has already been shown to have negative effects on growth [411, 148, 451, 454, 268, 318, 27, 52, 194, 455, 41, 327], suggesting that the parasite cannot fully compensate the loss of pyrimidine biosynthesis by import. Thus, other enzymes that catalyze intermediate reactions might be likewise essential as proposed by the predictions. The heme (porphyrin) biosynthesis pathway has previously been proposed as a target for antimalarial drugs [493, 55, 520], as heme is needed as a prosthetic group in proteins such as cytochromes. Heme, which is freed in large amounts during hemoglobin digestion, is presumably unavailable to the parasite outside of the food vacuole [493, 517], making *de novo* synthesis essential. This is in accordance with the predictions that classified several enzymes involved in heme biosynthesis to be indispensable. Another pathway that contains reactions predicted to be essential is that of Coenzyme A (CoA) biosynthesis. It has been demonstrated that the parasite relies on the uptake of pantothenate, a precursor of CoA, and that it is sensitive to pantothenate analogs [482]. Therefore, inhibition of downstream enzymes within this linear pathway might hamper parasite growth as well. Multiple top ranking enzymes are involved in the process of protein biosynthesis (tRNA ligases, deoxyhypusine synthase). The eukaryotic translation initiation factor eiF5A, whose activation involves deoxyhypusine synthase, has already been suggested to be the target of a drug with antimalarial effect [236]. Despite the obvious global effect upon inhibition of the translational machinery, relatively few is known about this process in *P. falciparum*. Therefore, an EU funded consortium called MEPHITIS has started very recently to explore the protein synthesis machinery in *Plasmodium* with the goal to design drugs against malaria. Adenylate cyclase obtained among others the highest score by the scoring function. Experimental findings by Ono *et al.* [367] indicate that knockouts of adenylate cyclase result in a decreased level of cAMP, leading to reduced apical regulated exocytosis and thus impaired infection of host cells. The essentiality of this enzyme is therefore related rather to signaling than to metabolism, but nevertheless represents an effective target. These evidences for the plausibility of the results together with the proven high specificity and accuracy rates for known targets indicate that the predicted set of essential reactions presents a promising starting point for further experimental investigations.

Table 7.2: **Top ranking predicted essential reactions.** T: listed as target in SuperTarget database; S: score

Enzyme name	Compartment	Pathway	EC no.	T	S
adenylate cyclase	apicoplast	Purine metabolism	4.6.1.1	x	4
fumarase	cytosol	Citrate cycle	4.2.1.2	x	4
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	apicoplast	Biosynthesis of isopentenyl diphosphate	2.7.7.60	x	4
4-hydroxy-3-methylbut-2-enyl-diphosphate synthase	apicoplast	Biosynthesis of isopentenyl diphosphate	1.17.4.3		3
CDP-ME kinase	apicoplast	Biosynthesis of isopentenyl diphosphate	2.7.1.148		3
pantetheine-phosphate adenylyltransferase	cytosol	CoA biosynthesis	2.7.7.3		3
pyridoxal 5-phosphate synthase	cytosol	Vitamin B6 metabolism			3
amino acid transporter	transport: cytosol <-> apicoplast	Transport			3
geranyl-diphosphate synthase	cytosol	Terpenoid biosynthesis	2.5.1.1	x	2
farnesyl-diphosphate synthase	cytosol	Terpenoid biosynthesis	2.5.1.10	x	2
2-Octaprenylphenol hydroxylase	mitochon.	Ubiquinone biosynthesis	1.14.13.-	x	2
ubiquinone biosynthesis methyltransferase	mitochon.	Ubiquinone biosynthesis	2.1.1.-	x	2
uroporphyrinogen decarboxylase	apicoplast	Porphyrin metabolism	4.1.1.37	x	2
coproporphyrinogen oxidase	apicoplast	Porphyrin metabolism	1.3.3.3	x	2
uridylate kinase	apicoplast	Pyrimidine metabolism	2.7.4.14/ 2.7.4.22/ 2.7.4.4	x	2
aspartate carbamoyltransferase	cytosol	Pyrimidine metabolism	2.1.3.2	x	2
orotate phosphoribosyltransferase	cytosol	Pyrimidine metabolism	2.4.2.10	x	2

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Table 7.2 – continued from previous page

Enzyme name	Compartment	Pathway	EC no.	T	S
cysteine desulfurase	apicoplast/ mitochon.	Fe-S-protein biogenesis	2.8.1.7	x	2
tRNA ligase (Ile, Lys, Met, Trp, Tyr)	cytosol/ apicoplast/ mitochon.	Aminoacyl-tRNA biosynthesis	6.1.1.1/ 6.1.1.2/ 6.1.1.5/ 6.1.1.6/ 6.1.1.10	x	2
glutathione synthase	cytosol	Glutathione metabolism	6.3.2.3	x	2
riboflavin kinase	cytosol	Riboflavin metabolism	2.7.1.26	x	2
glucosamine 6-phosphate synthase	apicoplast	Aminosugars metabolism	2.6.1.16	x	2
mannose-6-phosphate isomerase	cytosol	Fructose and mannose metabolism	5.3.1.8	x	2
cytochrome-c oxidase	mitochon.	Oxidative phosphorylation	1.9.3.1	x	2
choline-phosphate cytidylyltransferase	cytosol	Glycerophospholipid metabolism	2.7.7.15	x	2
deoxyhypusine synthase	cytosol	Activation of eiF5A	2.5.1.46	x	2

7.6 Discussion

Experimental validation of drug targets with the help of gene knockout techniques is a time consuming issue. Flux balance analysis provides a fast means to reduce the search space, since reactions essential for parasite development can be identified on the basis of their involvement in the synthesis process of indispensable metabolites. Drug targets predicted in this work were evaluated on the basis of a set of known essential enzymes, revealing that the applied flux balance approach is able to enrich true targets in the set of predicted targets. The presented FBA approach has the advantage over other computational drug target detection methods that it performs reasonably well, as has been demonstrated by a comparison with choke-point analysis, and that it requires only short computation times. The latter plays a role especially when the metabolic network of interest is large and beyond the capabilities of methods such as minimal cut sets that need to explore all paths through a network.

Additional information including homology to host enzymes and reported negative effects on cell development in other organisms was used to rank proposed targets. Targets with high ranks were found to be involved in metabolic pathways, which contain enzymes already known to be essential for parasite development.

As has been shown for fatty acid synthase [526], which is only essential during late liver stage, the importance of enzymes might vary during different life cycle stages. Therefore, predictions of metabolic fluxes during the individual stages of parasite development may help to identify reactions that are always active opposed to those that only occur during certain stages. By this means, targets can be identified that are suitable for prophylaxis, therapy or both (depending on the applied scoring criterion), thereby aiding the drug target detection process.

Chapter 8

Conclusions and outlook

The goal of this work was to explore the capabilities of systems biology methods in the context of drug target detection within the metabolism of the malaria pathogen *P. falciparum* and distinction between targets suitable for malaria prophylaxis, therapy or both. For this purpose, a comprehensive multi-compartment metabolic network was compiled for the parasite based on data from several resources. This network was validated by flux balance simulations to obtain a consistent network that is able to fulfill metabolic processes, such as hemoglobin digestion, known from the literature to occur in *P. falciparum*. With the help of an adapted version of the method proposed by Shlomi *et al.* [465], metabolic flux distributions corresponding to the parasite's individual life cycle stages were derived from gene expression profiles measured during respective stages. These predicted flux distributions were shown to agree well with literature knowledge in terms of exchanged metabolites between parasite and host during the blood stage. However, it was necessary to integrate additional information into the calculations in order to obtain this level of accordance. The metabolic network of the parasite was combined with that of the erythrocyte and stage-specific knowledge like plasma metabolite concentrations was considered to account for the actual cellular environment of the parasite. In addition, gene expression data of previous stages were included, since enzymes might be present although corresponding gene transcripts are already degraded. Experimentally determined exchanges between host and parasite are only available for the blood stage, preventing evaluation of predictions corresponding to other stages. As predictions depend on the incorporation of further information, prediction quality is likely to be limited for those stages where it was not possible to integrate such information (liver and mosquito stages). This is also suggested by proteomics data, which coincide better with blood stage flux distributions than with those corresponding to mosquito stages. Considering available proteomics and metabolomics data, will presumably improve predictions, as such data are closer to the phenotype than gene expression data due to posttranscriptional regulation. Flux predictions for liver stages can be further improved when the metabolic network of the hepatocyte, which has been completed very recently, is integrated into the calculations.

The assembled metabolic network of *P. falciparum* has been shown to be useful with respect to identification of putative drug targets. About two thirds of experimentally validated essential enzymes could be recovered by flux balance knockout simulations. Almost all remaining true targets were detected when additionally available nutrients were constrained, suggesting that the parasite is not able to compensate inhibited pathways by import of respective nutrients although transporters exist. More information about provided nutrients as well as target metabolites is required to improve predictions, as enzyme essentiality does not only depend on network topology, but also on the cellular environment present during

a stage of interest. This is supported by the results of a comparison between choke-point analysis, a pure topology based method, and the here applied flux balance approach, which succeeds the former in terms of specificity, accuracy and precision.

For the knockout simulations a set of target metabolites was considered that includes all metabolites essential during any life cycle stage of the parasite. Therefore, not all enzymes that were found to be indispensable are essential during all stages. Overlaying the metabolic network with stage-specific flux predictions, reveals which enzymes come into question as targets during the different stages, i.e., which targets are suitable for malaria prophylaxis (essential during liver stages) or malaria treatment (essential during blood stages).

With regard to the iterative cycle underlying systems biology approaches (see Fig. 1.2), the present work is currently in the phase of hypothesis generation. A model describing the metabolism of *P. falciparum* has been constructed based on available data and simulations have been conducted that allow to make predictions on stage-specific metabolism and on effects of knockouts. These predictions now await experimental validation. Data derived from these experiments will then help to refine the model and to improve predictions until the model reproduces reality reasonably well.

The current trend is to refine predictions by combining reconstructed cellular networks with different types of high-throughput data, e.g., metabolic networks with gene expression data as presented here. Since proteomics data and to a smaller extent metabolomics data are available for *P. falciparum*, future extensions of the parasite's metabolism model could include constraints reflecting these data. In addition, observed protein-protein interactions could be used to distinguish isoenzymes from components of a multi-enzyme complex, which only fulfill their function when all components are expressed simultaneously. *P. falciparum* genes are assumed to be posttranscriptionally regulated, but increasing hints suggest that regulation at the level of transcription initiation also plays a role [59, 271, 290, 291]. Transcription factors have been identified by bioinformatics approaches [161, 72] and putative cis-regulatory elements have been detected by statistical analysis of promoter regions of co-expressed genes [564]. In addition, ChIP-on-chip (chromatin immunoprecipitation) technology is available that will help to investigate interactions between proteins and DNA, thus identifying transcription factors and respective promoter regions. These efforts will eventually allow to reconstruct the gene regulatory network of the parasite, which can then be combined with the metabolic network. By this means, the impact on metabolism and hence parasite viability upon inhibition of transcription factors can be assessed and putative drug targets identified. The more experimentally determined information can be incorporated into the model, the less assumptions have to be made. The ultimate goal is a comprehensive model that combines metabolism, gene regulation and signal transduction, making assumptions about cellular objectives redundant, as they are intrinsic to the model itself.

Applied experimental data

1 Gene expression data

Table 1: **Gene expression samples.**

Sample	Set	Source	Organism	Stage	Experiment
TarunPyLS24Cy3	LS24	Tarun	*P. yoelii*	24 h post liver infection; Cy3 labeled	2-color oligo microarray
TarunPyLS40Cy3	LS40	Tarun	*P. yoelii*	40 h post liver infection; Cy3 labeled	2-color oligo microarray
TarunPyLS40Cy5	LS40	Tarun	*P. yoelii*	40 h post liver infection; Cy5labeled	2-color oligo microarray
TarunPyLS50Cy3	LS50	Tarun	*P. yoelii*	50 h post liver infection; Cy3 labeled	2-color oligo microarray
TarunPyBSCy3	eRing, lRing, eTropho, lTropho, eSchiz, lSchiz	Tarun	*P. yoelii*	mixed erythrocytic stages; Cy3 labeled	2-color oligo microarray
TarunPyBSCy5	eRing, lRing, eTropho, lTropho, eSchiz, lSchiz	Tarun	*P. yoelii*	mixed erythrocytic stages; Cy5 labeled	2-color oligo microarray
TarunPySchzCy5	eSchiz, lSchiz	Tarun	*P. yoelii*	erythrocytic schizonts; Cy5 labeled	2-color oligo microarray
TarunPyooSpzCy3	ooSporo	Tarun	*P. yoelii*	1 d post blood meal mosquito midgut sporozoites; Cy3 labeled	2-color oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
TarunPyooSpzCy5	ooSporo	Tarun	*P. yoelii*	1 d post blood meal mosquito midgut sporozoites; Cy5 labeled	2-color oligo microarray
TarunPysgSpzCy3	sgSporo	Tarun	*P. yoelii*	15 d post blood meal mosquito salivary gland sporozoites; Cy3 labeled	2-color oligo microarray
TarunPysgSpzCy5	sgSporo	Tarun	*P. yoelii*	15 d post blood meal mosquito salivary gland sporozoites; Cy5 labeled	2-color oligo microarray
SacciPyLS40	LS40	Sacci	*P. yoelii*	40 h post liver infection	cDNA library
LeRochPfERing	eRing	LeRoch	*P. falciparum*	early ring stages	oligo microarray
LeRochPfLRing	lRing	LeRoch	*P. falciparum*	late ring stages	oligo microarray
LeRochPfETropho	eTropho	LeRoch	*P. falciparum*	early trophozoites	oligo microarray
LeRochPfLTropho	lTropho	LeRoch	*P. falciparum*	late trophozoites	oligo microarray
LeRochPfESchiz	eSchiz	LeRoch	*P. falciparum*	early erythrocytic schizonts	oligo microarray
LeRochPfLSchiz	lSchiz	LeRoch	*P. falciparum*	late erythrocytic schizonts	oligo microarray
LeRochPfMero	Mero	LeRoch	*P. falciparum*	merozoite stage	oligo microarray
LeRochPfGameto	Gameto	LeRoch	*P. falciparum*	gametocyte stage	oligo microarray
LeRochPfSporo	sgSporo	LeRoch	*P. falciparum*	mosquito salivary gland sporozoites	oligo microarray
BozdechPfTP1	eRing	Bozdech	*P. falciparum*	1 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP2	eRing	Bozdech	*P. falciparum*	2 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP3	eRing	Bozdech	*P. falciparum*	3 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP4	eRing	Bozdech	*P. falciparum*	4 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
BozdechPfTP5	eRing	Bozdech	*P. falciparum*	5 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP6	eRing	Bozdech	*P. falciparum*	6 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP7	eRing	Bozdech	*P. falciparum*	7 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP8	eRing	Bozdech	*P. falciparum*	8 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP9	lRing	Bozdech	*P. falciparum*	9 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP10	lRing	Bozdech	*P. falciparum*	10 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP11	lRing	Bozdech	*P. falciparum*	11 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP12	lRing	Bozdech	*P. falciparum*	12 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP13	lRing	Bozdech	*P. falciparum*	13 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP14	lRing	Bozdech	*P. falciparum*	14 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP15	lRing	Bozdech	*P. falciparum*	15 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP16	lRing	Bozdech	*P. falciparum*	16 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP17	eTropho	Bozdech	*P. falciparum*	17 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP18	eTropho	Bozdech	*P. falciparum*	18 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
BozdechPfTP19	eTropho	Bozdech	*P. falciparum*	19 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP20	eTropho	Bozdech	*P. falciparum*	20 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP21	eTropho	Bozdech	*P. falciparum*	21 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP22	eTropho	Bozdech	*P. falciparum*	22 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP24	lTropho	Bozdech	*P. falciparum*	24 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP25	lTropho	Bozdech	*P. falciparum*	25 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP26	lTropho	Bozdech	*P. falciparum*	26 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP27	lTropho	Bozdech	*P. falciparum*	27 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP28	lTropho	Bozdech	*P. falciparum*	28 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP30	eSchiz	Bozdech	*P. falciparum*	30 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP31	eSchiz	Bozdech	*P. falciparum*	31 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP32	eSchiz	Bozdech	*P. falciparum*	32 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP33	eSchiz	Bozdech	*P. falciparum*	33 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP34	eSchiz	Bozdech	*P. falciparum*	34 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
BozdechPfTP35	eSchiz	Bozdech	*P. falciparum*	35 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP36	eSchiz	Bozdech	*P. falciparum*	36 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP37	eSchiz	Bozdech	*P. falciparum*	37 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP38	lSchiz	Bozdech	*P. falciparum*	38 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP39	lSchiz	Bozdech	*P. falciparum*	39 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP40	lSchiz	Bozdech	*P. falciparum*	40 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP41	lSchiz	Bozdech	*P. falciparum*	41 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP42	lSchiz	Bozdech	*P. falciparum*	42 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP43	lSchiz	Bozdech	*P. falciparum*	43 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP44	lSchiz	Bozdech	*P. falciparum*	44 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP45	lSchiz	Bozdech	*P. falciparum*	45 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP46	lSchiz	Bozdech	*P. falciparum*	46 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP47	Mero	Bozdech	*P. falciparum*	47 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray
BozdechPfTP48	Mero	Bozdech	*P. falciparum*	48 h post erythrocyte infection; Cy5 labeled	2-color oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
DailyPfGSM231278	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231279	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231280	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231281	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231282	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231283	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231284	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231285	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231286	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231287	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231288	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231289	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231290	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231291	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
DailyPfGSM231292	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231293	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231294	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231295	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231296	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231297	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231298	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231299	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231300	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231301	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231302	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231303	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231304	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231305	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
DailyPfGSM231306	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231307	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231308	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231309	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray
DailyPfGSM231310	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231311	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231312	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231313	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231314	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231315	eRing, lRing	Daily	*P. falciparum*	Environmental stress cluster	oligo microarray
DailyPfGSM231316	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231317	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231318	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray
DailyPfGSM231319	eRing, lRing	Daily	*P. falciparum*	Starvation cluster	oligo microarray

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Table 1 – continued from previous page

Sample	Set	Source	Organism	Stage	Experiment
DailyPfGSM231320	eRing, lRing	Daily	*P. falciparum*	Active growth cluster	oligo microarray

2 Proteomics data

Table 2: **Proteomics data sets.**

Sample	Source	Organism	Stage
LasonderPfTrophoSchz	[275]	*P. falciparum*	trophozoites and schizonts
LasonderPfGametocyte	[275]	*P. falciparum*	gametocytes
LasonderPfGamete	[275]	*P. falciparum*	gametes
LasonderPfOocyst	[276]	*P. falciparum*	oocysts, 7-8 days after mosquito infection
LasonderPfOoSporo	[276]	*P. falciparum*	oocyst-derived sporozoites, 13-14 days after mosquito infection
LasonderPfSgSporo	[276]	*P. falciparum*	salivary gland sporozoites, 18-22 days after mosquito infection
TarunPyLS40	[501]	*P. yoelii*	40 h post liver infection
TarunPyLS50	[501]	*P. yoelii*	50 h post liver infection
FlorensPfSporo	[147]	*P. falciparum*	salivary gland sporozoites, 14 days after mosquito infection
FlorensPfMero	[147]	*P. falciparum*	merozoites
FlorensPfTropho	[147]	*P. falciparum*	trophozoites
FlorensPfGameto	[147]	*P. falciparum*	gametocytes
HallPbSporo	[185]	*P. berghei*	salivary gland sporozoites
HallPbBlood	[185]	*P. berghei*	mixed asexual blood stages
HallPbOokinete	[185]	*P. berghei*	ookinetes
HallPbOocyst	[185]	*P. berghei*	oocysts, 9-12 days after mosquito infection
HallPbGameto	[185]	*P. berghei*	gametocytes

3 Metabolomics data

Table 3: **Metabolomics data sets.**

Sample	Source	Organism	Stage
TengPfTrophozoitePA	[503]	*P. falciparum*	trophozoites, perchloric acid extraction method
TengPfTrophozoiteMW	[503]	*P. falciparum*	trophozoites, methanol/water extraction method
TengPfTrophozoiteMCW	[503]	*P. falciparum*	trophozoites, methanol/chloroform/water extraction method
TengPfTrophozoiteM	[503]	*P. falciparum*	trophozoites, methanol extraction method
OlszewskiPfBloodTP0	[366]	*P. falciparum*	0 h post erythrocyte infection
OlszewskiPfBloodTP8	[366]	*P. falciparum*	8 h post erythrocyte infection
OlszewskiPfBloodTP16	[366]	*P. falciparum*	16 h post erythrocyte infection
OlszewskiPfBloodTP24	[366]	*P. falciparum*	24 h post erythrocyte infection
OlszewskiPfBloodTP32	[366]	*P. falciparum*	32 h post erythrocyte infection
OlszewskiPfBloodTP40	[366]	*P. falciparum*	40 h post erythrocyte infection
OlszewskiPfBloodTP48	[366]	*P. falciparum*	48 h post erythrocyte infection

Complete list of predicted essential reactions with assigned gene

Table 4: **Ranked predicted essential reactions.** a = LS24, b = LS40, c = LS50, d = EryERing, e = EryLRing, f = EryETropho, g = EryLTropho, h = EryESchz, i = EryLSchz, j = EryMero, k = EryGameto, l = MosquitoSgSporo, m = MosquitoOoSporo, T = enzyme listed in SuperTarget DB, S = score

Equation	**a**	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**l**	**m**	**EC no.**	**T**	**S**
ATP <=> 3;5-Cyclic AMP + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	4.6.1.1	x	4
(S)-Malate <=> Fumarate + H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	4.2.1.2	x	4
2-C-Methyl-D-erythritol 4-phosphate + CTP <=> 4-(Cytidine 5-diphospho)-2-C-methyl-D-erythritol + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.60	x	4
2-C-Methyl-D-erythritol-2,4-cyclodiphosphate + Reduced-ferredoxin + 2 H+ -> 1-Hydroxy-2-methyl-2-butenyl-4-diphosphate + H2O + Oxidized-ferredoxin	x	x	x	x	x	x	x	x	x	x	x	x	x	1.17.4.3		3
ATP + Pantetheine 4-phosphate <=> Pyrophosphate + Dephospho-CoA	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.3		3
4-(Cytidine 5-diphospho)-2-C-methyl-D-erythritol + ATP <=> 2-Phospho-4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol + ADP	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.1.148		3
D-Glyceraldehyde 3-phosphate + D-Ribulose 5-phosphate + L-Glutamine <=> Pyridoxal phosphate + L-Glutamate	x	x	x	x	x	x	x	x	x	x	x	x	x			3
Glycine + H+ <-> Glycine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Lysine + H+ <-> L-Lysine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Aspartate + H+ <-> L-Aspartate[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Arginine + H+ <-> L-Arginine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
L-Glutamine + H+ <–> L-Glutamine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Serine + H+ <–> L-Serine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Tryptophan + H+ <–> L-Tryptophan[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Phenylalanine + H+ <–> L-Phenylalanine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Tyrosine + H+ <–> L-Tyrosine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Cysteine + H+ <–> L-Cysteine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Leucine + H+ <–> L-Leucine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Histidine + H+ <–> L-Histidine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Asparagine + H+ <–> L-Asparagine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Valine + H+ <–> L-Valine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Threonine + H+ <–> L-Threonine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
L-Isoleucine + H+ <–> L-Isoleucine[a] + H+[a]	x	x	x	x	x	x	x	x	x	x	x	x	x			3
Oxygen + 4 Cytochrome-c <=> 4 Cytochrome-c-oxidized + 2 H2O + H+-pumped	x	x	x	x	x	x	x	x	x	x	x	x	x	1.9.3.1	x	2
Protein-L-cysteine + L-Cysteine –> L-Alanine + protein-S-sulfanylcysteine	x	x	x	x	x	x	x	x	x	x	x	x	x	2.8.1.7	x	2
ATP + UMP <=> ADP + UDP	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.4.-/ 2.7.4.14/ 2.7.4.22/ 2.7.4.4	x	2

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + gamma-L-Glutamyl-L-cysteine + Glycine <=> ADP + Orthophosphate + Glutathione	x	x	x	x	x	x	x	x	x	x	x	x	x	6.3.2.3	x	2
ATP + Riboflavin <=> ADP + FMN	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.1.26	x	2
L-Glutamine + D-Fructose 6-phosphate <=> L-Glutamate + D-Glucosamine 6-phosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.6.1.16	x	2
D-Mannose 6-phosphate <=> D-Fructose 6-phosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	5.3.1.8	x	2
Carbamoyl phosphate + L-Aspartate <=> Orthophosphate + N-Carbamoyl-L-aspartate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.1.3.2	x	2
Dimethylallyl diphosphate + Isopentenyl diphosphate <=> Pyrophosphate + Geranyl diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.1/ 2.5.1.10/ 2.5.1.29	x	2
Orotidine 5'-phosphate + Pyrophosphate <=> Orotate + 5-Phospho-alpha-D-ribose 1-diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.4.2.10	x	2
CTP + Choline phosphate <=> Pyrophosphate + CDP-choline	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.15	x	2
Geranyl diphosphate + Isopentenyl diphosphate <=> Pyrophosphate + trans,trans-Farnesyl diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.1/ 2.5.1.10/ 2.5.1.29	x	2
ATP + L-Tyrosine + tRNA(Tyr) <=> AMP + Pyrophosphate + L-Tyrosyl-tRNA(Tyr)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.1	x	2
Uroporphyrinogen III <=> Coproporphyrinogen III + 4 CO2	x	x	x	x	x	x	x	x	x	x	x	x	x	4.1.1.37	x	2
Coproporphyrinogen III + Oxygen <=> Protoporphyrinogen IX + 2 CO2 + 2 H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	1.3.3.3	x	2

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + L-Isoleucine + tRNA(Ile) <=> AMP + Pyrophosphate + L-Isoleucyl-tRNA(Ile)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.5	x	2
ATP + L-Lysine + tRNA(Lys) <=> AMP + Pyrophosphate + L-Lysyl-tRNA	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.6	x	2
ATP + L-Methionine + tRNA(Met) <=> AMP + Pyrophosphate + L-Methionyl-tRNA	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.10	x	2
ATP + L-Tryptophan + tRNA(Trp) <=> AMP + Pyrophosphate + L-Tryptophanyl-tRNA(Trp)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.2	x	2
2-Octaprenyl-6-methoxyphenol + Oxygen + NADPH <=> 2-Octaprenyl-6-methoxy-1,4-benzoquinone + NADP+ + H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	1.13.12.-/ 1.14.13.-/ 1.14.3.-	x	2
2-Octaprenyl-6-methoxy-1,4-benzoquinone + S-Adenosyl-L-methionine <=> 2-Octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone + S-Adenosyl-L-homocysteine	x	x	x	x	x	x	x	x	x	x	x	x	x	2.1.1.-	x	2
eIF5A-precursor-lysine + Spermidine <=> eIF5A-precursor-deoxyhypusine + 1,3-Diaminopropane	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.46	x	2
Geranylgeranyl-diphosphate + Protein-L-cysteine -> S-geranylgeranyl-protein + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.59/ 2.5.1.60		1
(R)-S-Lactoylglutathione + H2O <=> Glutathione + (R)-Lactate + active-glyoxalase-II	x	x	x	x	x	x	x	x	x	x	x	x	x	3.1.2.6		1
ATP + (9Z)-Hexadecenoic-acid + CoA <=> AMP + (9Z)-Hexadecenoyl-CoA + Diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	6.2.1.3		1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + Octadecanoic-acid + CoA <=> AMP + Stearoyl-CoA + Diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	6.2.1.3		1
ATP + (9Z)-Octadecanoic-acid + CoA <=> AMP + Oleoyl-CoA + Diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	6.2.1.3		1
ATP + Linoleate + CoA <=> AMP + Linoleoyl-CoA + Diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	6.2.1.3		1
eIF5A-precursor-deoxyhypusine + NADH + H+ + Oxygen <=> Protein-N6-[(R)-4-amino-2-hydroxybutyl]-L-lysine + NAD+ + H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	1.14.99.29		1
2 Fe2+ + Reduced-ferredoxin + protein-S-sulfanylcysteine –> Oxidized-ferredoxin + Fe-S-complex + Protein-L-cysteine	x	x	x	x	x	x	x	x	x	x	x	x	x			1
GlcN-PI-Palm + 3 Dolichyl-phosphate-D-mannose –> ManAlpha1-2ManAlpha1-6ManAlpha1-4GlcN-PI-Palm + 3 Dolichyl-phosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.4.1.-/ 2.4.1.130		1
EtN-P6-ManAlpha1-2ManAlpha1-2ManAlpha1-6ManAlpha1-4GlcN-PI-Palm + EtN-P6-ManAlpha1-2ManAlpha1-6ManAlpha1-4GlcN-PI-Palm + Protein –> GPI-protein	x	x	x	x	x	x	x	x	x	x	x	x	x			1
Fe-S-complex + Protein –> Fe-S-protein	x	x	x	x	x	x	x	x	x	x	x	x	x			1
Fe-S-complex + Protein –> Fe-S-protein	x	x	x	x	x	x	x	x	x	x	x	x	x			1
4 Porphobilinogen + H2O <=> Hydroxymethylbilane + 4 NH3	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.61		1
ATP + Dephospho-CoA <=> ADP + CoA	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.1.24		1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + FMN <=> Pyrophosphate + FAD	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.2		1
Orthophosphate + Pyrophosphate + S-Adenosyl-L-methionine <=> ATP + L-Methionine + H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.6		1
Protoporphyrin + Fe2+ <=> Heme + 2 H+	x	x	x	x	x	x	x	x	x	x	x	x	x	4.99.1.1		1
ATP + GMP <=> ADP + GDP	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.4.8		1
Inositol 1-phosphate <=> D-Glucose 6-phosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	5.5.1.4		1
GTP + D-Mannose 1-phosphate <=> Pyrophosphate + GDP-mannose	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.13		1
UDP-N-acetyl-D-glucosamine + Dolichyl phosphate <=> UMP + N-Acetyl-D-glucosaminyldiphosphodolichol	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.8.15		1
GDP-mannose + Dolichyl phosphate <=> GDP + Dolichyl phosphate D-mannose	x	x	x	x	x	x	x	x	x	x	x	x	x	2.4.1.83		1
ATP + D-Ribose 5-phosphate <=> AMP + 5-Phospho-alpha-D-ribose 1-diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.6.1		1
N6-(1,2-Dicarboxyethyl)-AMP <=> Fumarate + AMP	x	x	x	x	x	x	x	x	x	x	x	x	x	4.3.2.2		1
Reduced ferredoxin + NADP+ <=> Oxidized ferredoxin + NADPH + H+	x	x	x	x	x	x	x	x	x	x	x	x	x	1.18.1.2/ 1.18.1.3		1
ATP + Hexadecanoic acid + CoA <=> AMP + Palmitoyl-CoA + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	6.2.1.3		1
Malonyl-CoA + Acyl-carrier protein <=> CoA + Malonyl-[acyl-carrier protein]	x	x	x				x	x			x			2.3.1.39/ 2.3.1.85/ 2.3.1.86	x	1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
CDP-diacylglycerol + myo-Inositol <=> CMP + 1-Phosphatidyl-D-myo-inositol	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.8.11		1
D-Mannose 6-phosphate <=> D-Mannose 1-phosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	5.4.2.8		1
trans,trans-Farnesyl diphosphate + Isopentenyl diphosphate <=> Pyrophosphate + Geranylgeranyl diphosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.29		1
Triacylglycerol + CoA <=> 1,2-Diacyl-sn-glycerol + Acyl-CoA	x	x	x	x	x	x	x	x	x	x	x	x	x	2.3.1.20		1
Acetyl-CoA + Enzyme N6-(dihydrolipoyl)lysine <=> CoA + [Dihydrolipoyllysine-residue acetyltransferase] S-acetyldihydrolipoyllysine	x	x	x	x	x	x	x	x	x	x	x	x	x	2.3.1.12		1
UDP-N-acetyl-D-glucosamine + 1-Phosphatidyl-D-myo-inositol <=> UDP + N-Acetyl-D-glucosaminylphosphatidylinositol	x	x	x	x	x	x	x	x	x	x	x	x	x	2.4.1.198		1
Sphinganine + NADP+ <=> 3-Dehydrosphinganine + NADPH + H+	x	x	x	x	x	x	x	x	x	x	x	x	x	1.1.1.102		1
ATP + Pantothenate <=> ADP + D-4′-Phosphopantothenate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.1.33		1
2 Protoporphyrinogen IX + 3 Oxygen <=> 2 Protoporphyrin + 6 H2O	x	x	x	x	x	x	x	x	x	x	x	x	x	1.3.3.4		1
(R)-4′-Phosphopantothenoyl-L-cysteine <=> Pantetheine 4′-phosphate + CO2	x	x	x	x	x	x	x	x	x	x	x	x	x	4.1.1.36		1
ATP + L-Arginine + tRNA(Arg) <=> AMP + Pyrophosphate + L-Arginyl-tRNA(Arg)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.19		1
ATP + L-Asparagine + tRNA(Asn) <=> AMP + Pyrophosphate + L-Asparaginyl-tRNA(Asn)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.22		1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + L-Cysteine + tRNA(Cys) <=> AMP + Pyrophosphate + L-Cysteinyl-tRNA(Cys)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.16		1
ATP + L-Glutamine + tRNA(Gln) <=> AMP + Pyrophosphate + Glutaminyl-tRNA	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.18		1
ATP + Glycine + tRNA(Gly) <=> AMP + Pyrophosphate + Glycyl-tRNA(Gly)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.14		1
ATP + L-Histidine + tRNA(His) <=> AMP + Pyrophosphate + L-Histidyl-tRNA(His)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.21		1
ATP + L-Leucine + tRNA(Leu) <=> AMP + Pyrophosphate + L-Leucyl-tRNA	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.4		1
ATP + L-Phenylalanine + tRNA(Phe) <=> AMP + Pyrophosphate + L-Phenylalanyl-tRNA(Phe)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.20		1
ATP + L-Proline + tRNA(Pro) <=> AMP + Pyrophosphate + L-Prolyl-tRNA(Pro)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.15		1
ATP + L-Serine + tRNA(Ser) <=> AMP + Pyrophosphate + L-Seryl-tRNA(Ser)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.11		1
ATP + L-Valine + tRNA(Val) <=> AMP + Pyrophosphate + L-Valyl-tRNA(Val)	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.9		1
Dolichyl diphosphooligosaccharide + Protein asparagine <=> Dolichyl diphosphate + Glycoprotein with the oligosaccharide chain attached by N-glycosyl linkage to protein L-asparagine	x	x	x	x	x	x	x	x	x	x	x	x	x	2.4.1.119		1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
(3R)-3-Hydroxybutanoyl-[acyl-carrier protein] + NADP+ <=> Acetoacetyl-[acp] + NADPH + H+		x	x				x	x						1.1.1.100/ 2.3.1.85/ 2.3.1.86	x	1
(3R)-3-Hydroxyoctanoyl-[acyl-carrier protein] + NADP+ <=> 3-Oxooctanoyl-[acp] + NADPH + H+		x	x				x	x						1.1.1.100/ 2.3.1.85/ 2.3.1.86	x	1
(R)-3-Hydroxyhexanoyl-[acp] + NADP+ <=> 3-Oxohexanoyl-[acp] + NADPH + H+		x	x				x	x						1.1.1.100/ 2.3.1.85/ 2.3.1.86	x	1
trans,trans-Farnesyl diphosphate + Isopentenyl diphosphate <=> trans,trans,cis-Geranylgeranyl diphosphate + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.-		1
tRNA(Asp) + L-Aspartate + ATP <=> L-Aspartyl-tRNA(Asp) + Pyrophosphate + AMP	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.12/ 6.1.1.23		1
tRNA(Glu) + L-Glutamate + ATP <=> L-Glutamyl-tRNA(Glu) + Pyrophosphate + AMP	x	x	x	x	x	x	x	x	x	x	x	x	x	6.1.1.17/ 6.1.1.24		1
all-trans-Hexaprenyl diphosphate + Isopentenyl diphosphate <=> all-trans-Heptaprenyl diphosphate + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.30		1
all-trans-Pentaprenyl diphosphate + Isopentenyl diphosphate <=> all-trans-Hexaprenyl diphosphate + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.33		1
all-trans-Octaprenyl diphosphate + 4-Hydroxybenzoate <=> 3-Octaprenyl-4-hydroxybenzoate + Pyrophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x	2.5.1.-/ 2.5.1.39		1
Pyruvate + D-Glyceraldehyde 3-phosphate <=> 1-Deoxy-D-xylulose 5-phosphate + CO2	x	x	x	x	x	x	x	x	x	x	x	x	x	2.2.1.7		1

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
G00144 + Palmitoyl-CoA <=> G00145 + CoA	x	x	x	x	x	x	x	x	x	x	x	x	x	2.3.-.-		1
Enzyme N6-(dihydrolipoyl)lysine + NAD+ <=> Enzyme N6-(lipoyl)lysine + NADH + H+	x	x	x	x	x	x	x	x	x	x	x	x	x	1.8.1.4		1
ATP + Lipoate <=> Pyrophosphate + Lipoyl-AMP	x	x	x	x	x	x	x	x	x	x	x	x	x	2.7.7.63		1
Lipoyl-AMP + Apoprotein <=> Protein N6-(lipoyl)lysine + AMP	x	x	x	x	x	x	x	x	x	x	x	x	x	1.2.4.2/ 2.7.7.63		1
(S)-Malate + 2-Oxoglutarate[m] –> (S)-Malate[m] + 2-Oxoglutarate	x	x	x	x	x	x	x	x	x	x	x	x	x			1
S-Adenosyl-L-methionine <–> S-Adenosyl-L-methionine[m]	x	x	x	x	x	x	x	x	x	x	x	x	x			1
Fe-S-complex[m] + ATP + H2O –> Fe-S-complex + ADP + Orthophosphate	x	x	x	x	x	x	x	x	x	x	x	x	x			1
Fe2+ –> Fe2+[m]	x	x	x	x	x	x	x	x	x	x	x	x	x			1
L-Isoleucine[out] <–> L-Isoleucine	x	x	x	x	x	x	x	x	x	x	x	x	x			1
ATP + R-S-Glutathione –> ADP + Orthophosphate + R-S-Glutathione[out]	x	x	x	x	x	x	x	x	x	x	x	x	x			1
Ferriprotoporphyrin_IX –> Hemozoin			x	x	x	x	x	x	x	x	x					0
2-Phospho-D-glycerate <=> Phosphoenolpyruvate + H2O	x	x	x	x	x	x	x	x	x	x	x			4.2.1.11		0
ATP + 3-Phospho-D-glycerate <=> ADP + 3-Phospho-D-glyceroyl phosphate	x	x	x	x	x	x	x	x	x	x	x			2.7.2.3		0
2-Phospho-D-glycerate <=> 3-Phospho-D-glycerate	x	x	x	x	x	x	x	x	x	x	x			5.4.2.1		0
ATP + dTDP <=> ADP + dTTP	x	x	x	x	x	x	x	x	x					2.7.4.6		0
ATP + dTMP <=> ADP + dTDP	x	x	x	x	x	x	x	x	x					2.7.4.12/ 2.7.4.9		0
dUTP + H2O <=> dUMP + Pyrophosphate	x		x	x	x		x	x	x		x			3.6.1.19/ 3.6.1.23		0

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Table 4 – continued from previous page

Equation	a	b	c	d	e	f	g	h	i	j	k	l	m	EC no.	T	S
ATP + dCDP <=> ADP + dCTP	x	x	x	x	x	x	x	x	x					2.7.4.6		0
ATP + Holo-[carboxylase] + HCO3- <=> ADP + Orthophosphate + Carboxybiotin-carboxyl-carrier protein	x	x	x				x	x			x			6.3.4.14		0

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Abbreviations

Abbreviation	Explanation
ACT	artemisinin-based combination therapy
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BRENDA	Braunschweig Enzyme Database
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CoA	Coenzyme A
CSP	circumsporozoite protein
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
EC	enzyme commission
EM	elementary mode
FBA	flux balance analysis
GIMME	Gene Inactivity Moderated by Metabolism and Expression
GO	Gene Ontology
HTS	high-throughput-screening
IDC	intraerythrocytic developmental cycle
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG Orthology
MCA	metabolic control analysis
MCS	minimal cut set
MinMode	minimal flux mode
MPMP	Malaria Parasite Metabolic Pathways
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
WHO	World Health Organization

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Declaration

Herewith, I declare that I prepared the present dissertation myself and without the use of illegitimate aids. I used no other but the indicated sources and accessories. Further, I insure that this dissertation has not before been submitted to any other faculty for examination.

Berlin, Carola Huthmacher