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Plasmodium falciparum: programmed cell death in the erythrocytic stages

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PLASMODIUM FALCIPARUM: PROGRAMMED CELL DEATH IN THE ERYTHROCYTIC STAGES

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

This thesis is presented as a series of publications and unpublished data.

Johannesburg, 2015

DECLARATION

I, Dewaldt Engelbrecht, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University. Co-author contributions to each publication are listed under "Author contributions".

_____

Dewaldt Engelbrecht

30th day of April, 2015

Ethics clearance was obtained from the University of the Witwatersrand for culturing malaria parasites in the blood of human volunteers (clearance number: M13-05-69).

To my mother,

Laetitia Engelbrecht

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ABSTRACT

Plasmodium falciparum is responsible for the majority of global malaria deaths. During the pathogenic blood stages of infection, a rapid increase in parasitaemia threatens the survival of the host before transmission of slow-maturing sexual parasites to the mosquito vector to continue the life cycle. Programmed cell death (PCD) may provide the parasite with the means to control its burden on the host and thereby ensure its own survival. PCD in *P. falciparum* remains a poorly understood and controversial topic. A gathering body of evidence suggests *P. falciparum* is capable of PCD, but there are conflicting results regarding the phenotype.

This study represents a comprehensive phenotypical characterisation of cell death in intra-erythrocytic *P. falciparum* after various physiologically relevant stress stimuli, including high parasitaemia, heat stress simulating febrile paroxysms, and exposure to natural sunlight. The latter is a novel stimulus for PCD studies in *P. falciparum*. Biochemical markers of cell death, including DNA fragmentation, mitochondrial dysregulation and phosphatidylserine externalisation on parasitized erythrocytes, were used to provide a holistic description of cell death. Data showed that the combination of cell death markers varied with different stress stimuli and with the developmental stage of the parasite. An apoptosis-like phenotype, characterised by mitochondrial depolarisation, DNA fragmentation and phosphatidylserine externalisation, was suggested after stress from high parasitaemia. Heat stress affected ring stage parasites more severely than previous data suggested and induced an apoptosis-like phenotype. In contrast, late stage parasites showed markers of an autophagic-like cell death, including slight DNA fragmentation, phosphatidylserine externalisation and cytoplasmic vacuolisation. Sunlight exposure

induced markers of PCD that included DNA fragmentation preceding mitochondrial hyperpolarisation, but the phenotype was not clear.

The paradigm of PCD in *P. falciparum* is a dynamic and ever-evolving one that will continue to challenge our thinking and understanding of how the world's deadliest parasitic killer can induce its own death to limit damage on the host. Evidence indicates that *P. falciparum* undergoes PCD and that the phenotype(s) may be unique. PCD is an important feature of *P. falciparum* biology and the elucidation of parasite PCD pathway(s) that differ from host mechanisms may yield novel drug targets.

PUBLICATIONS PRESENTED IN THIS THESIS

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Engelbrecht D, Durand PM and Coetzer TL (2012). On programmed cell death in *Plasmodium falciparum*: *status quo*. J Trop Med 2012: Article ID 646534, 15 pages.

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Engelbrecht D and Coetzer TL (2013). Turning up the heat: heat stress induces markers of programmed cell death in *Plasmodium falciparum in vitro*. *Cell Death Dis* 4: e971.

Engelbrecht D and Coetzer TL. Mob mentality: programmed cell death in *Plasmodium falciparum* at high population density. *Manuscript submitted to Microbial Cell, under review.*

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Engelbrecht D and Coetzer TL. **Sunlight kills some, saves others: growth inhibition and markers of programmed cell death in *Plasmodium falciparum* after sunlight exposure**. University of the Witwatersrand Molecular Biosciences Research Thrust Annual Research Day, Johannesburg, South Africa, December 2014.

PREFACE

To the killer

Invisible killer, you scourge of the earth

You strike at our young and those giving birth

You descend with the dark, come in the night

From you winged courier but a single bite

You poison the life force, the blood

Your army of killers advance like a flood

Is it anger or hatred that drives you to kill?

Did you choose this from your own free will?

From insect to man, the outlook is grim

But like us, you are at Nature's whim

Your curse is that your life brings disease

You must find the balance, sacrifice to appease

And here I watch you from the shadows

I follow your footsteps leading to the gallows

It was not accident, nor execution

It was suicide dictated by evolution

Is it part of you, is it set in stone;

To survive, you must also kill your own?

Jan 2015

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NOMENCLATURE

Apaf-1	Apoptotic protease activating factor 1
Atg	autophagy-related
ATM	ataxia telangiectasia mutated
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3 interacting-domain death agonist
CARD	caspase recruitment domain
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CR	cell regulator
DBD	DNA-binding domain
$\Delta\psi_m$	mitochondrial transmembrane potential
DIABLO	Direct IAP-Binding protein with Low PI
$DiOC_6(3)$	3,3'-dihexyloxacarbocyanine iodide
DISC	death-inducing signalling complex
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dUTP	deoxyuridine triphosphate
FADD	Fas-Associated protein with Death Domain
FIRE	Functional Inference using the Rates of Evolution
FITC	fluorescein isothiocyanate
HE	hydroethidine
HMM	Hidden Markov model

IAP	inhibitor of apoptosis protein
IC_{50}	half maximal inhibitory concentration
NCCD	Nomenclature Committee on Cell Death
nRBC	non-parasitised red blood cells
MAFFT	Multiple Alignment using Fast Fourier Transform
MDM2	murine double-minute 2
MCM	malaria culture medium
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCD	programmed cell death
PI	propidium iodide
pRBC	parasitised red blood cells
PS	phosphatidylserine
RBC	red blood cell
RIP	receptor-interacting protein
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
Smac	Second mitochondria-derived activator of caspases
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SPB	Sorenson's phosphate buffer
SWIB	swinged wings locus complex B
tBID	truncated BH3 interacting-domain death agonist
TdT	terminal deoxynucleotidyltransferase
TO	thiazole orange
TUNEL	TdT-mediated dUTP Nick End Labelling

1 INTRODUCTION

Malaria remains the most deadly parasitic killer globally, sowing economic and social devastation. The most recent data show that malaria claimed an estimated 584 000 lives in 2013, with 198 million cases and 3.2 billion people – nearly half the global population - at risk of being infected and developing disease. The poorest communities are hit hardest, with low and lower-income countries suffering most, particularly in the African region (Figure 1), where 90% of all malaria deaths occur. This parasitic killer also strikes at the most vulnerable members of society, with children under 5 years accounting for 78% of all deaths. Malaria control is currently one of the top priorities on the global health agenda (*World Malaria Report*, 2014).

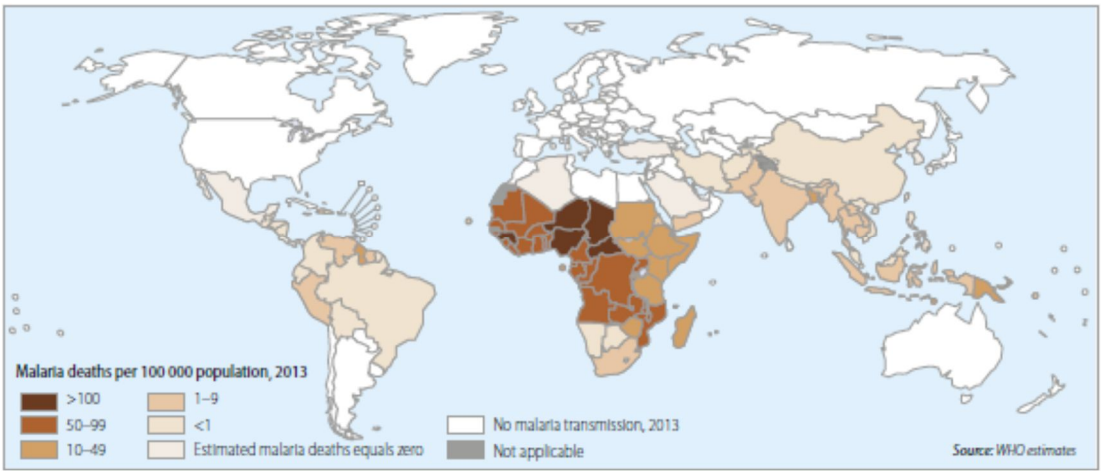

Figure 1: Malaria deaths per 100 000 in 2013 (*World Malaria Report*, 2014)

Human malaria is caused by five species of parasitic protozoa belonging to the *Plasmodium* genus, namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, the latter of which is a malaria species normally restricted to monkeys but has caused human infections. Of these species, *P. falciparum* and *P. vivax* are the greatest threats. *P. vivax* enjoys a greater global distribution than *P. falciparum* due to its resilience to lower

temperatures in the mosquito vector that allow it to survive at higher altitudes and in colder climates (Garnham, 1988). However, *P. vivax* infections in the African region are scarce, due to the absence of the Duffy gene in many African populations, the protein product of which is essential for *P. vivax* invasion (Miller *et al*., 1976). *P. falciparum* is the most prolific of the malaria killers, causing the overwhelming majority of malaria deaths. The impact of *P. falciparum* is worsened by the development of extensive drug resistance. Five countries in the region between Myanmar and Cambodia have reported resistance to artemisinin, currently the best and first line of defence against malaria (Dondorp *et al*., 2009; O'Brien *et al*., 2011; *World Malaria Report*, 2014). Artemisinin resistance has recently been shown to result from mutations in the propeller domain of the kelch protein K13 (Ashley *et al*., 2014) and *P. falciparum* along the Cambodia-Thailand border has become resistant to most available antimalarial medicines (*World Malaria Report*, 2014). Therefore, an urgent need exists for the identification of new drug targets to relieve the malaria scourge plaguing the global population.

1.1 The Life of a Killer: *P. falciparum* Life Cycle

The life cycle of *P. falciparum* is complex and diverse, consisting of both asexual and sexual stages, which thrive in widely differing and hostile conditions between the human host and *Anopheles* mosquito vector (Figure 2). The cycle begins when an infected mosquito takes a blood meal, injecting sporozoite stages of the parasite into the subcutaneous tissue or occasionally directly into the bloodstream of the human. Sporozoites travel to the liver and traverse several hepatocytes before invading and developing (Miller *et al*., 2002; Mota *et al*., 2002). Once safely enclosed in the hepatocyte, each sporozoite develops into tens of thousands of merozoites, each capable of invading a

red blood cell (RBC) upon release from the liver and starting the erythrocytic cycle of the parasite. Once invaded, merozoites form the early ring stage parasites. Ring stages develop into trophozoites, which then advance to schizonts, with each mature schizont producing around 20 new merozoites, each in turn capable of invading a new RBC. The asexual erythrocytic life cycle is responsible for the pathogenesis of malaria and repeats approximately every 48 hours (Miller *et al*., 2002).

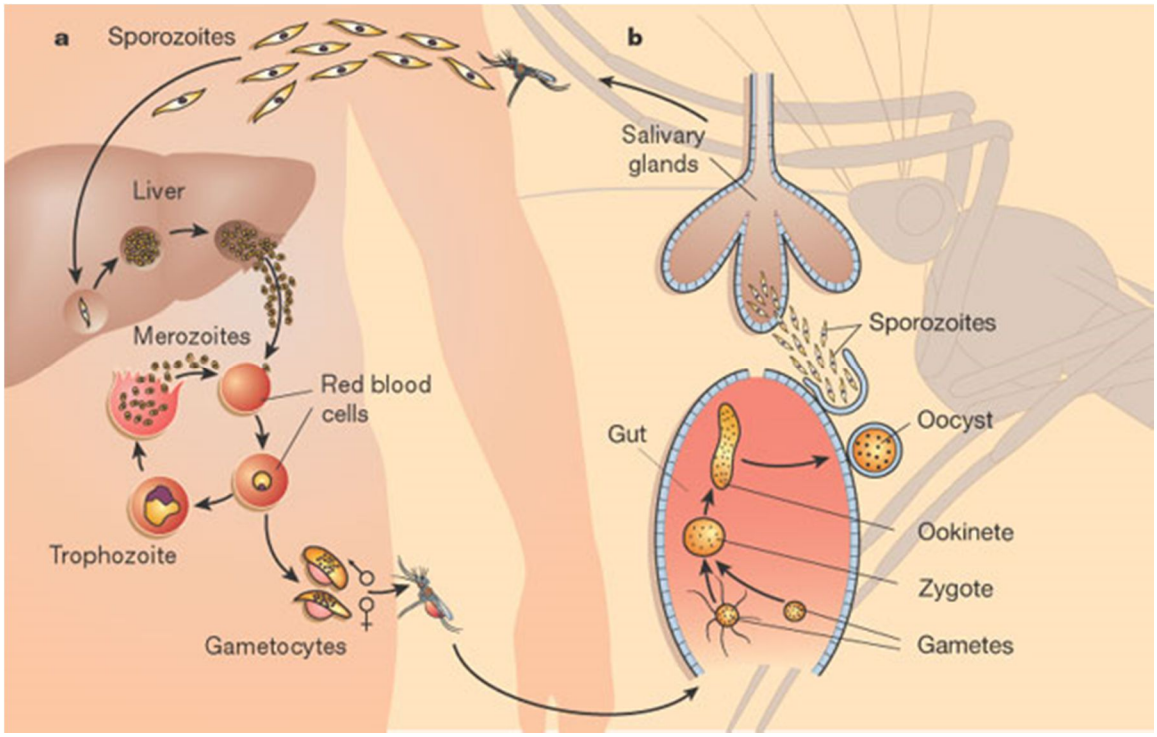

Figure 2: Parasite life cycle of *P. falciparum* (Wirth, 2002; reproduced unchanged under licence from the Nature Publishing Group).

A small portion of asexual parasites, estimated between 1 in 10 and 1 in 156, abandon the asexual cycle to form the sexual gametocytes, which are essential to transmit the infection back to the *Anopheles* vector (Miller *et al*., 2002; Talman *et al*., 2004). The formation of gametocytes, a process termed gametocytogenesis, has been likened to the puberty of *P. falciparum* (Talman *et al*., 2004). The commitment of parasites to either sexual or asexual development occurs right from the start, at merozoite formation; furthermore, from any one

schizont, all the emerging merozoites are committed to producing either male or female gametocytes. In *P. falciparum*, gametocyte maturation is a relatively slow process, with gametocytes visible in peripheral blood approximately 7-15 days after the first surge of asexual parasites (Talman *et al*., 2004).

Once a mosquito takes a blood meal from an infected human host, ingested male gametocytes rapidly exflagellate in response to a rise in pH and a drop in temperature and microgametes are formed before macrogametes emerge from the RBC. Fertilisation and zygote formation occur within an hour after which formation of mature ookinetes in the mosquito midgut may require as much as 30 hours (Beier, 1998). Ookinetes traverse the mosquito's midgut wall and implant beneath the basal epithelium to from oocysts (Beier, 1998). Each oocyst releases thousands of sporozoites that migrate through the mosquito haemolymph to the salivary glands, from where they are injected into the human host at the next blood meal, starting the cycle again (Beier, 1998; Sinden *et al*., 1996).

Clinically, malaria is characterised by acute cyclical febrile episodes that are triggered by the rupturing of mature schizont-infected erythrocytes releasing merozoites (Fernando, 2001; Miller *et al*., 1994). In falciparum malaria, fever typically recurs every 48 hours (Longmore *et al*., 2010; Miller *et al*., 2002), coinciding with the parasite's erythrocytic life cycle (Miller *et al*., 2002) and may peak as high as 41°C for 2–6 hours (Longmore *et al*., 2010). However, multiple asynchronous infections may lack periodicity (Fernando, 2001; Longmore *et al*., 2010) and febrile episodes may therefore occur more frequently and last longer. Falciparum malaria may further present with a number of complications. Cerebral malaria is the most serious complication and likely results in part from the sequestration of parasites in cerebral capillaries, as well as altered membrane permeability of those

capillaries. Other complications include acute renal failure, pulmonary oedema, lactic acidosis, hypoglycaemia and anaemia (Fernando, 2001).

1.2 Developmental Bottlenecks in *P. falciparum*

The complex and diverse life cycle of *P. falciparum* poses a number of transitional challenges and developmental bottlenecks for the parasite that must be overcome if its survival is to be ensured. A mosquito will ingest anything between 1 and 10^5 gametocytes from a bloodmeal. As few as 12 may form macrogametes and only two may eventually develop into oocysts, although hundreds are possible (Ghosh *et al*., 2000). Subsequently , even two oocysts may produce as many as 16 000 sporozoites, of which only 10-20 are injected into the human host at a bloodmeal (Sinden, 1999). PCD likely acts as the gatekeeper of these developmental stages. Apoptosis has been demonstrated in mosquito stages of the parasite (Ali *et al*., 2010; Al-Olayan *et al*., 2002; Arambage *et al*., 2009) and may prevent the parasite burden from overwhelming the vector and reduce competition for nutrients available for oocyst development (Hurd and Carter, 2004).

Of particular interest to this study is the maintenance of a sub-lethal erythrocytic infection to prevent overwhelming the human host, despite soaring parasitaemia resulting from every 48 hour asexual cycle, in order to allow sufficient time for gametocytes to mature and be transmitted. In the blood stages, a number of mechanisms of self-limitation have been proposed, including: (i) increasing the rate of conversion to gametocytes, thereby relocating resources from reproduction to transmission; (ii) limiting the number of merozoites released per mature schizont, (iii) altering the invasion capabilities of merozoites; (iv) regulating population synchronicity and development time; or (v) altering

the rate of cell death (Deponte and Becker, 2004; Reece *et al*., 2009). Programmed cell death (PCD) may offer the most effective mechanism of self-limitation by controlling parasite cell death independent of the host immune system (Deponte and Becker, 2004) and thereby limiting its burden on the host.

1.3 "Nothing in His Life Became Him Like the Leaving It" - Macbeth Act 1, Scene 4

For centuries, the samurai warrior class of Japan held in great esteem the tradition of *sepukku* - ritualistic suicide by disembowelment. This custom required that a samurai warrior who has failed in, or lost his purpose, must end his own life as an act of valour and commitment to the feudal system that he serves. Two iterations of this act are of particular interest in this context: (i) *oibara*, which holds that a samurai who has lost his feudal lord is obliged to also take his own life; and (ii) *munenbara*, which is sepukku committed under instruction or forced conditions (Fusé, 1980). The former is analogous to the intrinsic pathway of apoptosis, whereby the loss of some signalling factor or command results in the suicide of the cell (death by omission), while the latter reminds strongly of the extrinsic pathway of apoptosis, which is actively commanded by outside factors (death by commission). Much like apoptosis, *sepukku* was highly ritualised, with its execution following an ordered series of steps, the end result of which was the individual's demise. Most importantly, *sepukku* was (and continues to be) considered as an act of honour, as it places the good of the society as a whole above the life and existence of the individual. Herein we find the essence also of PCD: the altruistic suicide of a member of the population that contributes to the growth, survival and homeostasis of the population as a whole.

1.4 When Death Became Suicide: Recognising That Not All Deaths are Equal

The scientific community's identification and understanding of PCD has itself ancient origins, despite the terminology being fairly recent. Aristotle possibly touched on it circa 350 BCE, and Galen certainly did around 150-200 AD when the regression of the ductus arteriosus after birth was described. This was followed by the first description of the regression of the ductus venosus by Andreas Vesalius in 1564. The development of microscopy and the cell theory brought with it an explosion of works on developmental cell death, today understood to be a manifestation of apoptosis, in the 19th century. Among these were the first description of tadpole metamorphosis by Alfredo Dugès in 1835 and the first discovery of developmental cell death by Karl Vogt in 1842 (Clarke and Clarke, 1996). Walter Flemming first elucidated a number of morphological features often ascribed to apoptosis and argued that cell death resulted not just from mechanical disruption, but from chemical changes within the cell. Much of what he called chromatolytic cell death today corresponds to apoptosis (Clarke and Clarke, 1996; Galluzzi *et al*., 2015). Lockshin and Williams formally coined the term programmed cell death in 1964 in a series of studies on silkmoths (Lockshin and Williams, 1964). The term apoptosis was first used in 1972 (Kerr *et al*., 1972).

1.5 The Ritual of Programmed Cell Death: Apoptosis as a Model

PCD may present as various phenotypes, including apoptosis and autophagic-like cell death, as well as other "hybrid" phenotypes, such as necroptosis or paraptosis which are avoided in this study (Kroemer *et al*., 2009). Necrosis, which is a poorly defined phenotype in *P. falciparum*, was considered a phenotype of non-PCD cell death in this study.

Mammalian apoptosis represents the holotype of PCD and as such is used as a model below to illustrate the highly organised and regulated execution of cell death in PCD.

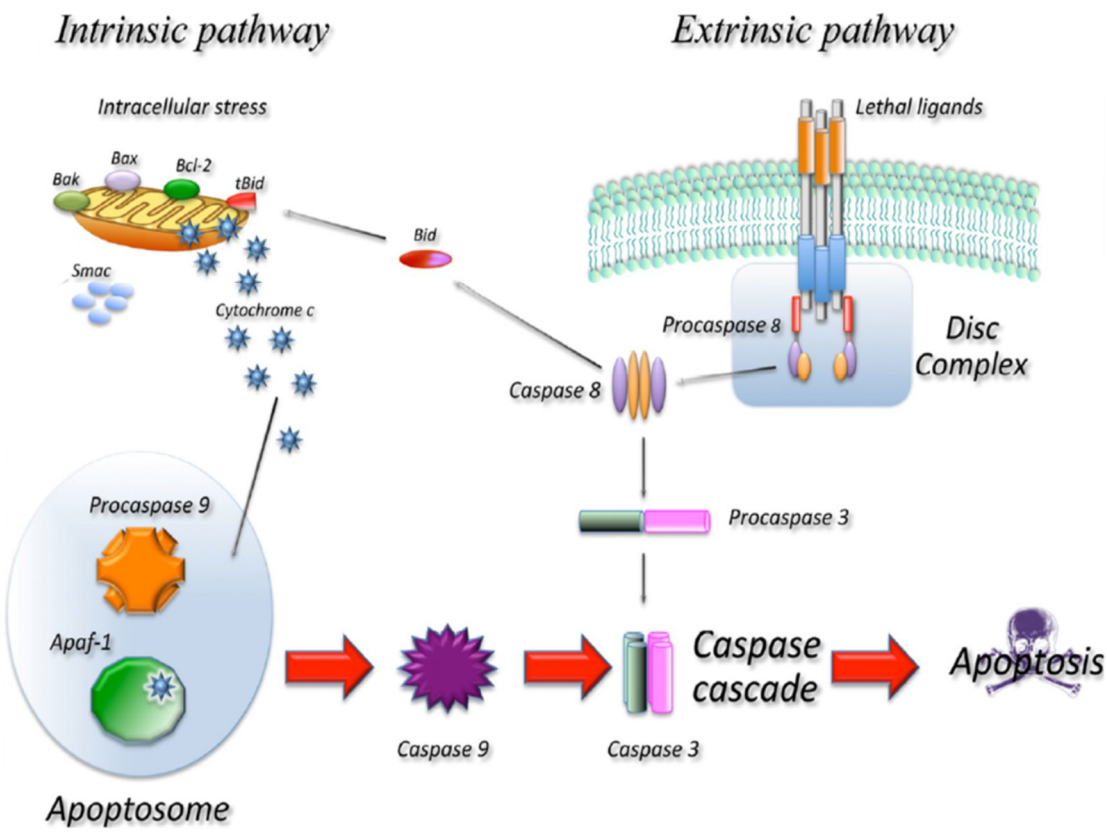

Figure 3: Schematic summary of molecular pathways leading to the activation of apoptosis (adapted from Favaloro *et al*., 2012). Abbreviations: Apaf-1 - Apoptotic protease activating factor 1; Bak - Bcl-2 homologous antagonist killer; Bax - Bcl-2-associated X protein; Bcl-2 - B-cell lymphoma 2; Bid - BH3 interacting-domain death agonist; Disc - Death-inducing signalling complex; Smac - Second mitochondria-derived activator of caspases; tBid – truncated Bid.

Two possible pathways may initiate apoptosis in mammalian cells (Figure 3). The extrinsic pathway involves the clustering of transmembrane receptor proteins upon the binding of their corresponding extracellular ligands. On the cytosolic side, receptors recruit adaptor proteins such as receptor-interacting protein (RIP) and Fas-Associated protein with Death Domain (FADD). The latter associates with procaspase-8, forming a Disc that leads to the

autolytic cleavage of procaspase-8 into caspase-8, which in turn activates caspase-3 and triggers the execution phase of apoptosis (Elmore, 2007; Wyllie, 2010).

On the other hand, the intrinsic pathway revolves around apoptosis initiation by the mitochondrion in response to non-receptor-mediated stimuli such as oxidative stress, hyperthermia, toxins or the absence of certain growth factors (Elmore, 2007). Stress stimuli induce the opening of a mitochondrial permeability transition pore, mediated either by mitochondrial swelling or the oligomerisation of Bcl-2 protein family members such as Bax, Bak and Bid (Elmore, 2007; Ly *et al*., 2003; Wyllie, 2010). The opening of a mitochondrial permeability transition pore coincides with loss of the mitochondrial transmembrane potential $\Delta\psi m$ and results in the release of pro-apoptotic proteins, including cytochrome c, Smac and a serine protease from the mitochondrial intermembrane space into the cytosol (Ly *et al*., 2003; Elmore, 2007). Cytochrome *c* complexes with Apaf-1, leading to formation of the apoptosome and activation of initiator caspase-9. Activated caspase-9 in turn activates effector caspase-3 (Elmore, 2007; Ly *et al*., 2003; Wyllie, 2010).

Both the intrinsic and extrinsic pathways of initiation converge on the activation of caspase-3 which signals the execution phase. During this phase, an extensive caspase cascade results in the activation of various proteases and cytoplasmic nucleases. Activated proteases bring about widespread synchronous proteolytic cleavage in many cell compartments, including cleavage of the plasma membrane and cytoskeletal proteins. Cleavage of the inhibitor of caspase-activated DNase allows the DNase's unfolding and localisation to the nucleus, where it is responsible for DNA cleavage (Elmore, 2007; Ly *et al*., 2003; Wyllie, 2010). The loss of plasma membrane asymmetry leads to externalisation

of phosphatidylserine (PS), amongst other phospholipids, that is normally restricted to the inner plasma membrane (van Engeland *et al*., 1998).

The above model of apoptosis allows for the monitoring of a number of biochemical markers of cell death. However, these markers are often not exclusive to apoptosis or PCD in general and some overlap between cell death phenotypes may exist in terms of biochemical criteria (Kroemer *et al*., 2009). Thus, the value and meaning of biochemical markers of cell death are considered below.

1.5.1 The mitochondrion and cell death: hitman and victim?

Loss of the mitochondrial transmembrane potential ($\Delta\psi m$) may be the "point-of-no-return" in apoptosis (Ly *et al*., 2003). Mitochondrial permeabilisation resulting in depolarisation of the $\Delta\psi m$ is an early event in apoptosis that precedes events such as chromatin condensation, DNA laddering or PS externalisation. However, decreased $\Delta\psi m$ and mitochondrial membrane permeabilisation may also occur during early necrosis (Kroemer *et al*., 1998, 2009; Skulachev, 2006).

1.5.2 PS externalisation: a wolf in sheep's clothing

This externalisation results from the activation of phospholipid scramblase and the inhibition of aminophospholipid translocase, both calcium-dependant enzymes (Balasubramanian and Schroit, 2003; Balasubramanian *et al*., 2007). PS externalisation is an early indicator of the execution phase of apoptosis, occurring prior to DNA fragmentation, but downstream of mitochondrial permeabilisation and early caspase activation. PS externalisation in apoptosis serves as a marker, tagging a cell for phagocytosis (van Engeland *et al*., 1998).

1.5.3 DNA fragmentation: the final act of cell suicide

The endonucleolytic degradation of nuclear DNA into nucleosome-sized fragments of ~200 bp is characteristic of apoptosis (Arends *et al*., 1990; Jonker *et al*., 1993; Wyllie, 1980). However, DNA fragmentation is not exclusive to apoptosis, and can also occur during autophagy and non-PCD phenotypes of cell death, such as necrosis (Charriaut-Marlangue and Ben-Ari, 1995; de Torres *et al*., 1997; Grasl-Kraupp *et al*., 1995; Vermes *et al*., 2000)

1.6 Down the Rabbit Hole: Making Sense of Death in an Ever-Changing World

In recent times, the landscape of cell death is changing and evolving more rapidly than ever. Even within the modest timespan of this project, paradigms have shifted. At the beginning of this project, the Nomenclature Committee on Cell Death (NCCD), a veritable authority on cell death that strives towards unified criteria, recognised apoptosis and autophagic-like cell death as phenotypes of PCD. It was already clear that PCD can be caspase-independent (Kroemer *et al*., 2009). Necrosis was widely used as a non-PCD phenotype of cell death, although it was recognised that necrosis can occur via a regulated and active process (Galluzzi and Kroemer, 2008; Hitomi *et al*., 2008) and should not merely be concluded in the absence of apoptotic or autophagic markers. The NCCD argued against clear-cut distinctions of cell death phenotypes, as many modes of cell death display mixed features, but advised against the use of hybrid terms such as “aponecrosis” or “necroapoptosis”. Importantly, the NCCD called for the replacement of morphological criteria with biochemical markers of cell death (Kroemer *et al*., 2009). This last recommendation in particular laid the foundation for this project, which aimed to use

biochemical markers of cell death as far as possible. Others called attention to the seemingly trivial, but critically important, conceptual difference between PCD and a cell death program (Nedelcu *et al*., 2011). The latter better describes a single-celled system such as *P. falciparum*, where the cell is the entire system and its own constitutive elements contribute to its demise. It was recommended that the broad term of PCD be replaced with "active cell death", removing the question as to whether cell death is programmed to contribute to a higher level organism or simply follows an active cell death program (Nedelcu *et al*., 2011).

Recent updates by the NCCD (Galluzzi *et al*., 2015) classify cell death into one of two categories: (i) accidental cell death, which results from physical, chemical or mechanical trauma and is insensitive to pharmacological or genetic intervention; or (ii) regulated cell death which is controlled by genetically encoded molecular machinery. Regulated cell death may occur either in response to disturbances in the microenvironment or as a process of development, immune response or maintenance of homeostasis (Galluzzi *et al*., 2015). In *P. falciparum*, disturbances in the microenvironment are most likely responsible for the induction of cell death as described in this study, although maintenance of homeostasis might also be considered. Apoptosis is recognised as a caspase-dependant variant of regulated cell death (Galluzzi *et al*., 2015).

1.7 Suicide in the Single-Celled World: Evidence of PCD in *P. falciparum*

PCD has been demonstrated in unicellular organisms, including parasitic protozoa (Lüder *et al*., 2010; van Zandbergen *et al*., 2010). Apart from the ability to orchestrate their own death, parasites can facilitate their development and survival by inducing PCD in host cells

(Bienvenu *et al*., 2010). These host-pathogen interactions are complex, and the role of PCD in balancing pathogenic and survival mechanisms remains poorly understood. In *Plasmodium*, the appearance of "crisis form" morphology, first described in *P. brasilianum* in 1944 (Taliaferro and Taliaferro, 1944), has been widely observed in *P. falciparum* and correlated with retardation of growth and development, loss of synchronicity, and decline in parasite numbers (Carlin *et al*., 1985; James *et al*., 1985; Jensen *et al*., 1982; Ockenhouse *et al*., 1984). This morphological phenomenon was later linked to PCD (Picot *et al*., 1997). Many studies have since cited the appearance of "crisis forms" as evidence of PCD (Deponte and Becker, 2004; López *et al*., 2010; Meslin *et al*., 2007; Nyakeriga *et al*., 2006; Oakley *et al*., 2007; Picot *et al*., 1997). However, the definition of "crisis forms" is not entirely clear, often being simply described as degenerate or abnormal parasites, making it a parameter that is difficult to objectively observe and quantify. In addition, we and others have also observed the appearance of such degenerate parasites in untreated *in vitro* cultures (Deponte and Becker, 2004; Picot *et al*., 1997).

Furthermore, a preference has already been expressed for biochemical criteria of cell death (Kroemer *et al*., 2009). Several PCD markers of an apoptosis-like phenotype have been documented in the ookinetes and zygotes of *P. berghei*, both *in vivo* in the *Anopheles* mosquito as well as in *in vitro* cultures, without external experimental stimuli (Ali *et al*., 2010; Al-Olayan *et al*., 2002; Hurd and Carter, 2004; Matthews *et al*., 2012) although Le Chat and colleagues found very little evidence to support this view (Le Chat *et al*., 2007). It was shown that ookinetes of *P. falciparum* exhibited evidence of an apoptosis-like cell death in the midgut of the mosquito (Arambage *et al*., 2009). Apoptosis was also documented in the liver stages of *P. berghei* (Eickel *et al*., 2013). In the pathogenic asexual human blood stages of *P. falciparum*, the biochemical evidence for PCD and especially the

phenotype of cell death remains highly controversial. Some studies support the occurrence of PCD as apoptosis (Ch'ng *et al*., 2010; Cheema *et al*., 2014; Kumar *et al*., 2008; Meslin *et al*., 2007; Mutai and Waitumbi, 2010; Oakley *et al*., 2007; Picot *et al*., 1997; Sharma *et al*., 2014), while others suggest that the phenotype more closely resembles autophagic cell death (Eickel *et al*., 2013; Totino *et al*., 2008) or necrosis (Porter *et al*., 2008). Some overlap of apoptosis and autophagy has also been noted (López *et al*., 2010), while other authors simply describe the cell-death phenotype as non-apoptotic (Nyakeriga *et al*., 2006; Pankova-Kholmyansky *et al*., 2003).

1.8 "Well, We Were Born to Die" – Romeo and Juliet Act 3, Scene 4

Phenotypic characterisation represents a crucial first step in elucidating PCD pathways in *P. falciparum* and their effect on host-pathogen interactions. A better understanding of both the proximate ("how") and the ultimate ("why") reasons of such a mechanism will impact on our knowledge of PCD in unicellular parasites and may provide clues for prospective drug targets.

1.9 Study Outline

In the following chapters, the study is presented as a series of published articles or submitted manuscripts, followed by a brief conclusion uniting the data. The biochemical assays for cell death are outlined in the appendix.

2 RESEARCH AIMS

The study aims to characterise the phenotype(s) of cell death in *P. falciparum* under physiologically-relevant stress stimuli, including:

(i) High parasitaemia

(ii) Heat stress similar to febrile episodes during malaria

(iii) Exposure to natural sunlight.

The cell death phenotype will be described using biochemical markers of cell death, including:

(i) DNA fragmentation

(ii) Changes in mitochondrial transmembrane polarisation

(iii) PS exposure by parasitised red blood cells (pRBC)

3 INTRODUCTION TO PUBLICATION 1

Engelbrecht D, Durand PM and Coetzer TL

On programmed cell death in *Plasmodium falciparum*: *status quo*

Journal of Tropical Medicine (2012), 2012, Article ID 646534

Journal impact factor: not yet established

Author contributions: DE was responsible for all literature research and drafting of the manuscript. DE and TLC contributed to the conception of the manuscript. TLC and PMD contributed to the editing. PMD provided genomic evidence of a PCD pathway in *Plasmodium*. All authors read and approved the final version of the manuscript.

Citations of this publication, excluding self-citation by DE:

Ch'ng J-H, Renia L, Nosten F, Tan KSW (2012). Can we teach an old drug new tricks? *Trends Parasitol* 28(**6**): 220–224.

Durand PM, Choudhury R, Rashidi A, Michod RE (2014). Programmed death in a unicellular organism has species-specific fitness effects. *Biol Lett* 10: 20131088.

Eickel N, Kaiser G, Prado M, Burda P-C, Roelli M, Stanway RR, *et al*. (2013). Features of autophagic cell death in *Plasmodium* liver-stage parasites. *Autophagy* 9(**4**): 568–580.

Matthews H, Ali M, Carter V, Underhill A, Hunt J, Szor H, *et al*. (2012). Variation in apoptosis mechanisms employed by malaria parasites: the roles of inducers, dose dependence and parasite stages. *Malar J* 11: 297.

Orellana M V, Pang WL, Durand PM, Whitehead K, Baliga NS (2013). A role for programmed cell death in the microbial loop. *PLoS One* 8(**5**): e62595.

Ortiz LMG (2012). Chronicles of a silent death : Apoptosis. *Res cell Biol* 1: 1–7.

Proto WR, Coombs GH, Mottram JC (2013). Cell death in parasitic protozoa: regulated or incidental? *Nat Rev Micro* 11: 58–66.

Sinai AP, Roepe PD (2012). Autophagy in Apicomplexa: a life sustaining death mechanism? *Trends Parasitol* 28(**9**): 358–364.

Taylor-Brown E, Hurd H (2013). The first suicides: a legacy inherited by parasitic protozoans from prokaryote ancestors. *Parasit Vectors* 6: 108.

Summary

This publication provides a review of the state of knowledge on PCD in *P. falciparum*. Previous publications are summarised according to phenotype conclusions, parasite strains used and cell death markers monitored. Possible reasons for discrepancies in results and conclusions from previous studies are explored. Consideration is given to various phenotype definitions that were applied throughout the course of this study. Some genomic evidence for PCD in *P. falciparum* is provided. Pertinent and unanswered questions are posed that were used to direct the manner of thinking while moving forward in this study.

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Review Article

On Programmed Cell Death in *Plasmodium falciparum: Status Quo*

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Conflicting arguments and results exist regarding the occurrence and phenotype of programmed cell death (PCD) in the malaria parasite *Plasmodium falciparum*. Inconsistencies relate mainly to the number and type of PCD markers assessed and the different methodologies used in the studies. In this paper, we provide a comprehensive overview of the current state of knowledge and empirical evidence for PCD in the intraerythrocytic stages of *P. falciparum*. We consider possible reasons for discrepancies in the data and offer suggestions towards more standardised investigation methods in this field. Furthermore, we present genomic evidence for PCD machinery in *P. falciparum*. We discuss the potential adaptive or nonadaptive role of PCD in the parasite life cycle and its possible exploitation in the development of novel drug targets. Lastly, we pose pertinent unanswered questions concerning the PCD phenomenon in *P. falciparum* to provide future direction.

1. Introduction

Programmed cell death (PCD) forms an integral physiological part of multicellular organisms, where it plays an essential role in normal development and maintenance of integrity and homeostasis. In addition, it forms part of the defense response to combat infectious pathogens as well as being involved in the pathogenesis of certain human diseases (reviewed in [1–3]). This self-sacrificial cell-death phenomenon has also been demonstrated in unicellular organisms, including parasitic protozoa (reviewed in [4, 5]). Apart from the ability to orchestrate their own death, parasites can facilitate their development and survival by inducing PCD in host cells (reviewed in [6]). These host-pathogen interactions are complex, and the role of PCD in balancing pathogenic and survival mechanisms remains poorly understood.

The definitions of PCD and its various phenotypes are considered in Table 2 of Appendix A. Observations of PCD have their foundations in the middle-late nineteenth century as an awareness of physiological cell death [7, 8] although the term was first coined in 1964 by Lockshin and Williams [9]. Apoptosis, now recognised as a prominent phenotype of PCD, was described in 1972 by Kerr and coworkers [10]. More than 20 years later, apoptosis was demonstrated in a unicellular trypanosome [11], and in 1997, it was described in two species of malaria parasites, *Plasmodium falciparum* [12] and *P. yoelii* [13]. Different phenotypes of PCD have been shown in evolutionary diverse unicellular eukaryote lineages [14, 15] as well as in prokaryotes [16]. However, a growing body of conflicting evidence regarding PCD in *Plasmodium* has followed. We present a critical review of current knowledge of this phenomenon, focusing on the asexual intraerythrocytic stage of *P. falciparum*, and offer possible explanations for discrepancies in the data. We also highlight some of the unanswered questions in this field, including the possible adaptive value of PCD, and allude to the possible exploitation of this process in the identification of novel drug targets.

2. Evidence of PCD in *Plasmodium falciparum*

The appearance of "crisis form" morphology, first described in *P. brasilianum* in 1944 [17], has been widely observed in *P. falciparum* and correlated with retardation of growth and development, loss of synchronicity, and decline in parasite numbers [18–22]. This morphological phenomenon was linked to PCD by Picot et al. [12]. Many studies have since cited the appearance of "crisis forms" as evidence of PCD [12, 23–27]. However, the definition of "crisis forms" is not entirely clear, often being simply described as degenerate or abnormal parasites, making it a parameter that is difficult to objectively observe and quantify. In addition, we and others have also observed the appearance of such degenerate parasites in untreated *in vitro* cultures ([12, 23], Engelbrecht et al., unpublished). Striving towards a unified description of cell death phenotypes, it has been suggested that morphological descriptions be replaced by functional and/or biochemical criteria [28].

Several PCD markers of an apoptosis-like phenotype have been documented in the ookinetes and zygotes of *P. berghei,* both *in vivo* in the *Anopheles* mosquito as well as in *in vitro* cultures, without external experimental stimuli [29–31] although Le Chat and colleagues found very little evidence to support this view [32]. Recently, it was shown that ookinetes of *P. falciparum* exhibited evidence of an apoptosis-like cell death in the midgut of the mosquito [33].

In the pathogenic asexual human blood stages of *P. falciparum,* the biochemical evidence for PCD and especially the phenotype of cell death remains highly controversial. Some studies support the occurrence of PCD as apoptosis [12, 26, 27, 34–36], while others suggest that the phenotype more closely resembles autophagic cell death [37] or necrosis [38]. Some overlap of apoptosis and autophagy has also been noted [24], while other authors simply describe the cell-death phenotype as nonapoptotic [25, 39]. At this stage, it cannot be conclusively confirmed whether any PCD phenotype is typical and whether its manifestation is essential and/or beneficial to the parasite. Resolution of these issues is an essential first step in elucidating the underlying PCD pathways in *P. falciparum* and their effect on host-pathogen interactions. A better understanding of both the proximate ("how") and the ultimate ("why") reasons of such a mechanism will impact on our knowledge of PCD in unicellular parasites and may provide clues for prospective drug targets.

A summary of studies on PCD markers in *P. falciparum* is presented in Table 1. On face value, the conflicting data offered by these studies seem daunting when attempting to reach a conclusion on PCD in *P. falciparum*. Consideration is thus given to possible explanations for the discrepancies that may lessen the controversy, and thereby either answer pertinent questions or raise new issues about the cell-death mechanisms of *P. falciparum*. This consideration is addressed in two parts. First, by considering confounding variables of the system, such as differences in the strain, stimulus, or life stage studied. Second, methodological pitfalls that may distort the interpretation of results are examined. By evaluating conflicting evidence as individual pieces of the same puzzle, rather than different pieces for the same open space, a larger and more descriptive picture forms.

3. Conflict due to Confounding Variables: Is There Method in the Madness?

3.1. P. Falciparum Strains. Numerous strains of *P. falciparum* have been used to study PCD, including chloroquine-resistant strains such as 7G8, FCR3, Lili, K1, PSS1, and Dd2, as well as strains that are sensitive to the drug, for example, 3D7 and F32 (Table 1). This complicates interpretation of the results when chloroquine (8 out of 12 studies) and other drugs are used to induce cell death, especially since it has been speculated that the development of chloroquine drug resistance may be correlated with a decreased susceptibility to undergo PCD [26]. Recently, reduced sensitivity to artemisinin has been described [40], but gene expression studies have not linked this phenotype to changes in PCD [41]. Different strains seem to differ in their susceptibility to undergo PCD and manifest different phenotypes. The 3D7 strain appears to be most susceptible to PCD with an apoptotic phenotype resulting from exposure of parasite cultures to chloroquine [12, 26, 34] etoposide [26], or increased temperatures [27]. Apoptosis has also been reported in the Dd2 strain under high *in vitro* parasite densities [36]. Other strains manifest entirely different cell death phenotypes, such as the PSS1 strain that showed evidence of autophagic cell death, as indicated by cytoplasmic vacuolisation without chromatin condensation or DNA fragmentation and caspase involvement [37]. The CSC-1 strain lacked markers of apoptosis and instead showed swelling and food vacuole lysis, resulting in secondary necrosis after stimulation with drugs or febrile temperatures [38]. Drug-induced cell death of the F32 strain did not exhibit any of the typical markers of apoptosis although the occurrence of PCD was not ruled out [25]. Conflicting results exist for the 7G8 strain, where chloroquine either induced apoptosis [26, 34] or caused cell death showing features of both apoptosis and autophagy [24]. Studies utilising multiple *P. falciparum* strains subjected to the same stimuli within the same laboratory provide the best evidence to show interstrain differences in PCD phenotypes and susceptibilities [12, 26, 34].

3.2. Cell-Death Stimulus. Widely differing stimuli (Table 1), concentrations, and exposure times have been used to study PCD in *P. falciparum,* which makes direct comparisons problematic. Antimalaria drugs are the most commonly used experimental trigger, but results have not been consistent. Treatment of cultures for 6 and 24 hours with 40 nM chloroquine (corresponding to the IC_{50} value of the drug) revealed DNA fragmentation suggestive of apoptosis [12]. However, another study using the same strain and stimulus showed almost no response at a comparable chloroquine concentration of 30 nM, with a significant effect only evident above 30 μM chloroquine treatment for 8 or 10 hours [34]. Apart from this dose-dependent effect of a single drug, the mode of action of a drug also impacts significantly on the type of cell death. Nyakeriga and coworkers treated the F32

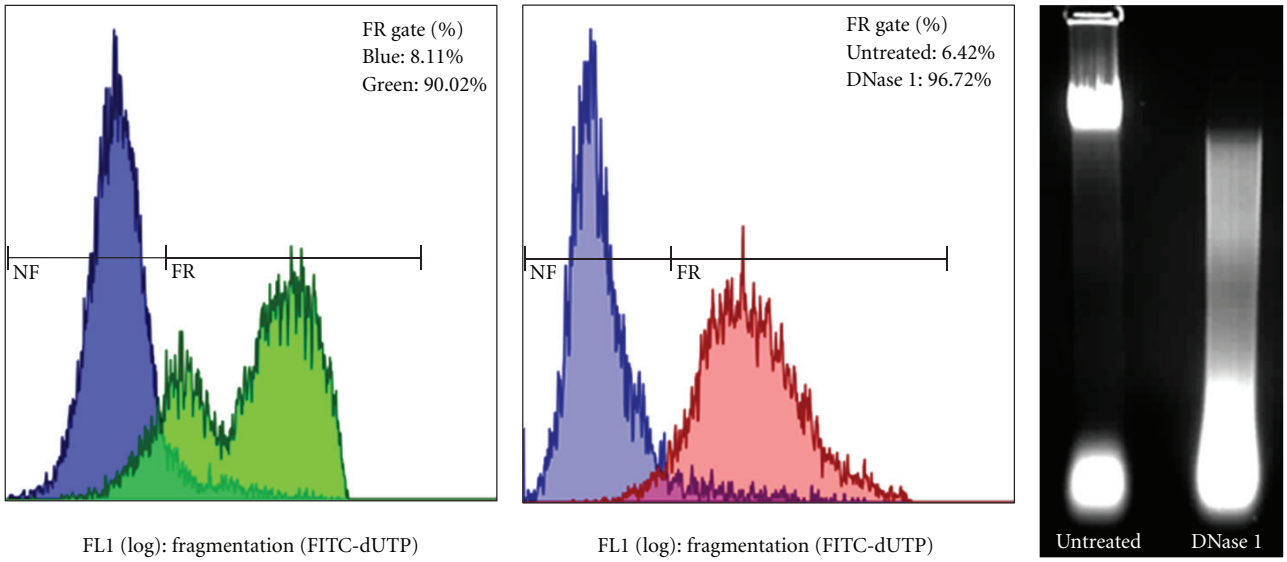

Figure 1: DNA fragmentation as illustrated by the TUNEL assay and agarose gel electrophoresis. Plot regions are denoted as nonfragmented (NF) and fragmented (FR). (a) TUNEL results showing parasites at 5.0% parasitaemia (blue) and parasites at parasitaemia of 7.2%, which failed to progress beyond the ring stage (green), after a decline from a high parasitaemia of >11%. (b) TUNEL results of untreated parasites (blue) and parasites treated with DNase 1 (red). (c) Agarose gel electrophoresis of genomic DNA from intact untreated (left) and DNase 1-treated (right) parasites showing a smear of DNA fragments.

P. falciparum strain with several drugs with different modes of action and demonstrated that the effect on numerous PCD markers varied significantly [25].

A question arises as to whether antimalaria drugs are an appropriate stimulus, since the reaction of the parasite to the drug may be different in the *in vitro* system compared to the *in vivo* disease. In PCD experiments, the dose of the drug is adjusted so that the death rate is less than 100%, which correlates with the concept of PCD, where some parasites die to benefit the rest of the population although it may also reflect the fact that not all parasites have taken up the drug. However, *in vivo*, when a patient with malaria is treated, the drugs rapidly and effectively kill all parasites, implying that at least in some parasites, death is an uncontrolled event as opposed to a preprogrammed mechanism. Physiologically relevant triggers may therefore better reflect the pathways that operate *in vivo*.

During the intraerythrocytic cycle of development, *P. falciparum* is exposed to temperatures up to 41°C during periodic bouts of fever in the human host, which occur in response to erythrocyte rupture and release of new merozoites. Incubation of parasites at febrile temperatures has been used as a natural stimulus to study PCD but with conflicting results. One study showed DNA fragmentation after 2 hours at 41°C [27], whereas a similar study demonstrated no effect after incubation at 40°C for as long as 16 hours [38], albeit with different strains (3D7 and CSC-1, resp.).

The self-limiting effect of increasing population densities has also been investigated as a stressor for triggering PCD [36]. The Dd2 strain showed growth stagnation at <6% parasitaemia in highly synchronous cultures, with apoptotic markers including mild DNA fragmentation as measured by the TUNEL assay [36]. This contrasts with our studies on the 3D7 strain, which reached peak parasitaemia levels of >11% and following decline, exhibited very high levels of DNA fragmentation, also measured by the TUNEL assay (Figure 1(a)). These parasites remained in the ring stage of the life cycle and failed to progress to trophozoites and schizonts.

3.3. Life Cycle Stages and Culture Conditions. *P. falciparum* has a complex life cycle and is subjected to very different environmental conditions as it shuttles between its human and mosquito hosts. Evidence relating to PCD of the ookinete stage in the mosquito midgut was briefly mentioned earlier. Subsequent to invasion of human erythrocytes, the parasite develops through sequential stages of proliferation, which respond differently to external stimuli that may trigger PCD. This hampers the generation of consistent results, since researchers have used either asynchronous cultures or else different stages of synchronous cultures (rings, trophozoites, or schizonts). In addition, there is wide variation in initial parasitaemia and haematocrit as well as the composition of the culture medium (e.g., the use of human plasma or serum or different concentrations of Albumax).

Another variable in *in vitro* culture experiments relates to the presence of residual white blood cells (leukocytes) despite wash steps to purify erythrocytes. These cells may undergo PCD in response to the stimulus applied to induce parasite death, and since they contain DNA, this may interfere with

studies of DNA fragmentation. This dilemma was illustrated by Porter et al. [38], who demonstrated oligonucleosomal DNA laddering by agarose gel electrophoresis in parasitised erythrocyte cultures treated with chloroquine, similar to that reported by Picot et al. [12]. However, the laddering pattern was absent once cultures had been depleted of leukocytes by affinity chromatography. Moreover, treatment of whole blood with chloroquine produced a similar laddering pattern [38]. Cognisance should therefore be taken of possible false positive results when interpreting DNA laddering or fragmentation experiments.

Finally, each stage of development is associated with a different repertoire of mRNA and proteins, which also confounds the issue and may offer another explanation of discrepancies in results, since the appropriate executors of PCD may not be present in the experimental stage under investigation.

4. Conflict due to Methodological Choice or Design: Madness in the Methods?

Apart from discrepancies in data due to differences in the studied system, as described above, it is also possible that problems may be encountered in the methods used to study the system. Morphological markers are unreliable although the cellular and molecular methods that have been recommended to replace them [28] are currently also not perfect and suffer from diverse drawbacks [42]. Technological approaches to detect markers of PCD in protozoan parasites are borrowed from studies in metazoans, and commercial kits have typically been optimised for mammalian systems. They may therefore not be appropriate for studies in *Plasmodium*. Some of the most common markers of PCD, especially apoptosis, that have been used in *P. falciparum* are DNA fragmentation, alterations in the mitochondrial and plasma membranes and the involvement of proteases. Some of the methodological pitfalls associated with these techniques are considered below.

4.1. DNA Fragmentation: Agarose Gel Electrophoresis versus TUNEL Assay. Oligonucleosomal DNA fragmentation is considered one of the hallmarks of the late stages of apoptosis, resulting from internucleosomal DNA cleavage, which produces a characteristic ladder of DNA fragments (see Table 2 in Appendix A) [2, 28]. Two methods of detection are used in *P. falciparum* studies: (1) agarose gel electrophoresis involving the electrophoretic resolution and visualisation of isolated DNA by ethidium bromide or SYBR green staining, or more commonly (2) the TUNEL (Terminal deoxynucleotidyltransferase-mediated dUTP Nick End Labelling) assay, which relies on enzyme (TdT)-mediated integration of a fluorochrome-conjugated base (dUTP) to free 3′-OH ends of fragmented DNA strands in whole cells, detected by flow cytometry, fluorescence microscopy, or light microscopy with appropriate filters [2, 43]. Table 1 provides an overview of the DNA fragmentation results obtained in various *P. falciparum* PCD studies. These results are occasionally in conflict with other detected markers of PCD in the same study. This may be due to the choice or execution of the detection method, as considered below.

The initial suggestion of PCD in *P. falciparum* was based on oligonucleosomal DNA fragmentation demonstrated by a ladder of DNA fragments resolved on agarose gels. The low sensitivity of conventional ethidium bromide staining necessitated radiolabelling of free DNA ends prior to electrophoresis and Southern blotting [12]. Our own data attest to the problem of low sensitivity: despite significant growth inhibition and the prominent appearance of crisis forms in chloroquine- and heat-treated 3D7 parasites, we have been unable to demonstrate the expected oligonucleosomal laddering pattern on agarose gels (1-2 μg DNA loaded) (Engelbrecht et al., unpublished data). This contrasts with the findings of Ch'ng and colleagues who demonstrated DNA fragmentation by the TUNEL assay at comparable concentrations of chloroquine, using the same strain [34].

The TUNEL assay analysed by flow cytometry appears to be accepted as the standard for detecting DNA fragmentation in *P. falciparum*. However, the major drawback of this method is that it cannot distinguish the type of DNA fragmentation, and therefore, oligonucleosomal DNA fragments, as well as random fragments, will yield positive results. No definite conclusion on the type of PCD can thus be reached, since fragmented DNA with liberated 3′-OH ends may be generated in cells undergoing apoptosis, necrosis, or autophagy [43–46]. Our own results confirmed this shortcoming: *in situ* DNA digestion in *P. falciparum* with DNase 1, which is utilised in several studies as a positive control [25–27, 36, 37], showed ~90% TUNEL positive parasites (Figure 1(b)) although this treatment manifested as a homogenous smear of DNA (that could be associated with necrosis) on agarose gels stained with ethidium bromide (Figure 1(c)). A careful choice of controls for both apoptosis and necrosis is thus essential for correct interpretation of DNA fragmentation results. Perhaps exploitation of the ability of flow cytometry to measure several parameters simultaneously in individual cells may also provide the opportunity to include additional markers to discriminate between DNA fragmentation in apoptotic and necrotic cells.

Furthermore, analysis of the TUNEL assay by fluorescence microscopy [26, 27] is only semiquantitative and may not provide an accurate representation of the portion of the parasite population manifesting DNA fragmentation. These results should therefore be corroborated by quantitative flow cytometry. Totino et al. [37], observed <10% of TUNEL positive parasites using flow cytometry, despite significant (~40%–75%) parasite death, after treatment with staurosporine (an inducer of apoptosis), or SNAP (a nitric oxide donor) or the antimalaria drug, chloroquine. The authors thus suggested that TUNEL positive fragmentation, detected solely by fluorescence microscopy [26], may only represent a small fraction of the population [37], and since a small percentage of TUNEL positive parasites have also been observed in untreated cultures [34, 37], these results should be interpreted with caution. It should be noted, however, that Totino and coworkers assessed parasite viability with

rhodamine staining, which correlates with an early apoptotic loss of mitochondrial membrane potential, whereas DNA degradation is a later event, and this may partly explain the lack of correlation between DNA fragmentation and parasite death [37].

With each method exhibiting its own drawbacks, one may ask which one, if any, of the above should be used to detect DNA fragmentation? Agarose gel electrophoresis is simple and economical to perform and provides conclusive evidence for the type of DNA fragmentation but suffers from low sensitivity and is only semiquantitative. The low sensitivity may be overcome by substantial, time-consuming modifications, such as Southern blot analysis [12]. The TUNEL assay, in contrast, is highly sensitive, rapid, and quantitative if assessed by flow cytometry. However, it cannot discriminate between specific oligonucleosomal cleavage of DNA and random degradation, which is essential in determining the type of cell death. It would thus be prudent to verify the type of DNA fragmentation with agarose gels for those samples that produce positive TUNEL results.

4.2. Loss of Mitochondrial Transmembrane Potential ($\Delta\psi_m$). Mitochondria play a key role in PCD, and a loss of membrane potential ($\Delta\psi_m$) usually precedes complete permeabilisation of the mitochondrial membrane [28, 47], which triggers downstream events in the PCD cascade. In apoptosis, loss of $\Delta\psi_m$ is an early event occurring prior to other apoptotic manifestations such as chromatin condensation, DNA laddering, or phosphatidylserine (PS) externalisation [47]. However, decreased $\Delta\psi_m$ and mitochondrial membrane permeabilisation may also occur during early necrosis [28, 47, 48] prior to the appearance of vacuolisation and cytoplasmic swelling [47].

The mitochondrial membrane potential is normally evaluated with lipophilic cationic probes [47], such as $DiOC_6$ [24, 25, 38], JC-1 [26, 34, 35], TMRE (tetramethylrhodamine ethyl ester) [36], or rhodamine 123 [37]. The fluorescent probes are detected either by spectrofluorimetry, flow cytometry, or fluorescence microscopy. As summarised in Table 1, a decrease in $\Delta\psi_m$ was observed after cell death had been induced in various strains with drugs or heat or bilirubin, but due to the nonspecificity of this parameter, it was not always correlated with apoptosis. Conflicting results were obtained by Nyakeriga and colleagues, who could not detect any loss of $\Delta\psi_m$ after treatment of the chloroquine-sensitive F32 strain with chloroquine or etoposide, a topoisomerase II inhibitor [25]. As expected, atovaquone, which targets the mitochondria, decreased the $\Delta\psi_m$ [25]. The choice of mitochondrial probe may have a significant influence on results, as probes differ in both their specific and nonspecific binding targets and are often mitochondrial inhibitors themselves [49, 50]. Various probes have also shown different responses depending on the stimulus used to induce mitochondrial dysregulation, albeit in a human cell line [50]. It is also crucial to ensure proper staining of the parasites and to include appropriate experimental controls. In addition to an untreated parasite culture and a nonparasitised erythrocyte control to monitor nonspecific binding, it is important to include a positive control, such as a mitochondrial uncoupling agent (CCCP), to ensure the validity of the results. Furthermore, the mitochondrial localisation of the dye should be verified with fluorescence microscopy. In view of the ambiguous nature of this marker, it cannot be used in isolation to determine the phenotype of PCD.

4.3. Phosphatidylserine (PS) Externalisation. Viable erythrocytes normally maintain an asymmetrical transbilayer distribution of phospholipids, with the anionic aminophospholipid phosphatidylserine (PS) localised almost exclusively on the inner leaflet of the plasma membrane [51]. In a number of conditions, such as sickle cell anaemia and thalassaemia as well as senescent erythrocytes, the asymmetry of the plasma membrane is lost, leading to externalisation of PS to the outer leaflet [51].

During apoptosis, complete mitochondrial membrane permeabilisation in response to a PCD stimulus results in the release of cytochrome c and calcium, which triggers the translocation of PS [28, 52]. This early PS externalization is a widely used marker of apoptosis in mammalian cells and has also been used in unicellular lineages [42]. In mammalian cells, the externalised PS is thought to serve as a signal to phagocytes to engulf and digest apoptotic cellular remnants, thereby preventing an immune response although the phenomenon has yet to be fully explained or exclusively linked to apoptosis [2, 53]. This flip-flop of PS is commonly detected with fluorochrome-labelled Annexin V, which binds to PS with high affinity although it may also bind to other anionic phospholipids [53]. The assay usually includes a membrane-impermeable vital dye such as propidium iodide to exclude demised cells that have become permeable.

In *Plasmodium*, PS externalization has been detected during apoptosis of the extracellular ookinetes and zygotes of *P. berghei* [29, 30, 33]. However, detection of PS exposure in the intracellular erythrocytic stages is complicated by the presence of several membranes. Apart from the erythrocyte membrane, the parasite has a plasma membrane and is surrounded by a parasitophorous vacuolar membrane (PVM). The host cell membrane may be removed by extensive affinity purification steps and selective lysis of the PVM may be achieved with increasing concentrations of saponin or sorbitol [23, 54] although the purity of the parasite plasma membrane would have to be verified. The functional relevance of potential PS translocation to the outer surface of one of the parasite membranes is not clear, since the parasite is still within the intracellular milieu of the erythrocyte. However, *P. falciparum* remodels the host erythrocyte membrane extensively during its intracellular development, and although it initially prevents PS externalization to protect the infected host cell from clearance by macrophages, PS exposure becomes apparent during the schizont stage [6, 55, 56]. It is not known whether this change is specifically induced by a parasite undergoing PCD, but phagocytosis of the dead parasite will limit the immune response and production of inflammatory cytokines, which represents a survival advantage for the parasite. An investigation of the exposure of PS on the outer erythrocyte membrane surface

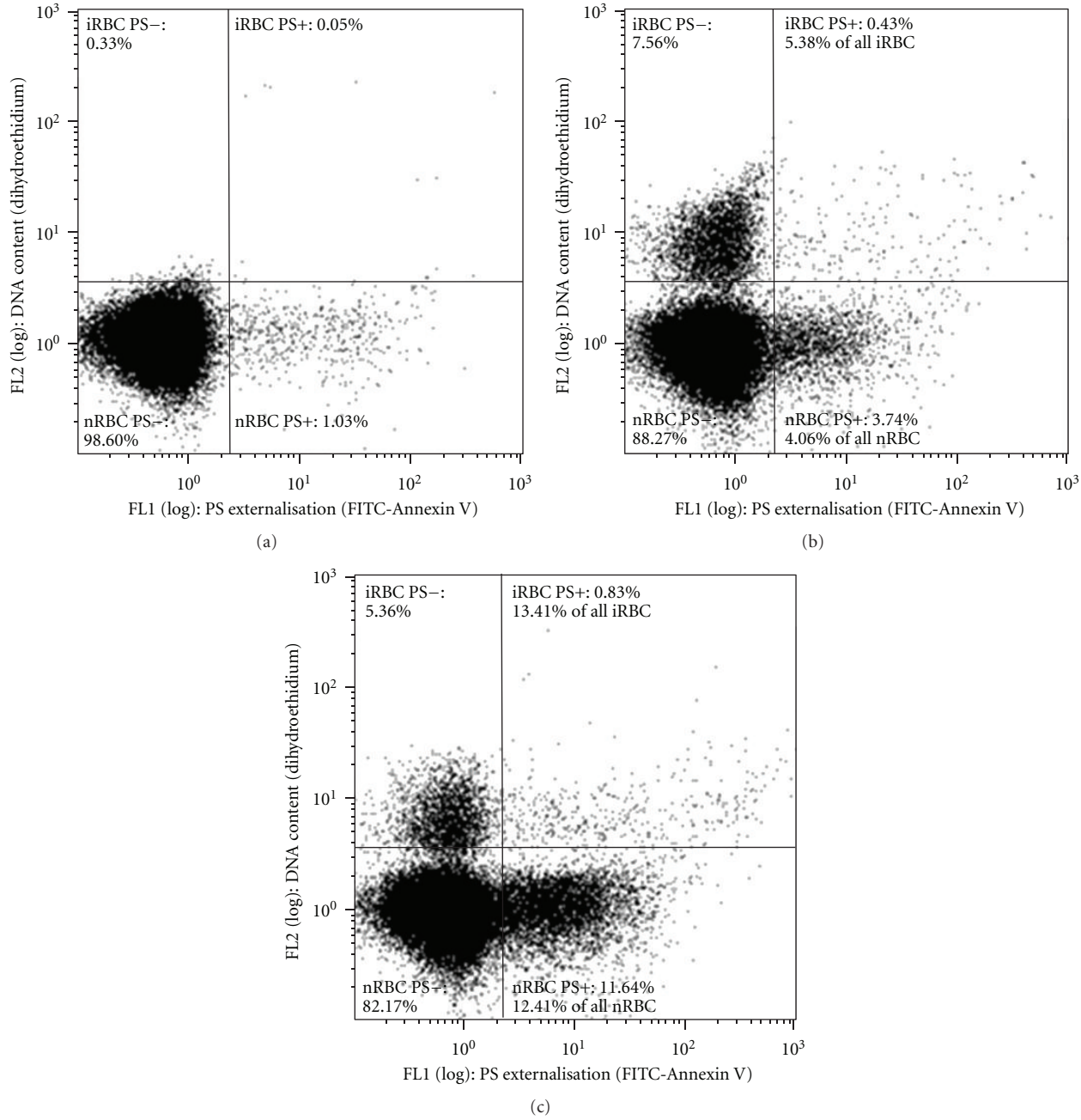

Figure 2: Phosphatidylserine externalisation (PS+) measured by FITC-Annexin V binding in both infected erythrocytes (iRBC) and their neighbouring noninfected erythrocytes (nRBC). iRBC were identified by the uptake of the cell-permeable DNA-binding dye dihydroethidium. An increase in PS externalisation can be seen by comparing an uninfected erythrocyte culture (a), to a parasite culture (b), both maintained at 37°C, and a heat-stressed parasite culture maintained at 40°C for 48 hours (c).

after stimulating PCD in *P. falciparum* and a comparison to PS levels in untreated infected cells (Figure 2) should be correlated with other markers of PCD in the parasite.

4.4. Cysteine Protease Involvement. Apoptosis is a genetically regulated catabolic process, which is executed by a proteolytic cascade of cysteine proteases, known as caspases [28]. Thus far, no true caspases have been identified in *Plasmodium* [23] although plant-like metacaspases have been found [57]. Nevertheless, the activation of caspase-like enzymes during PCD has often been used as a marker for apoptosis as summarised in Table 1.

Detection of caspase-like activity in *P. falciparum* relies on the use of fluorochrome-conjugated substrates or inhibitors which emit a fluorescent signal after proteolytic cleavage of the peptide substrate (such as VAD or DEVD) or irreversible binding of the inhibitor to the enzyme. In addition, substrate analogues linked to fluoromethyl ketone

have also been used as inhibitors. The emitted fluorescence is quantified by flow cytometry or spectrofluorimetry and/or visualization by fluorescence microscopy.

Increased caspase-like activity was demonstrated in response to chloroquine [34], staurosporine [34], and bilirubin [35]. However, other groups found no evidence of cysteine protease involvement after treatment with various drugs [37, 38] or heat [38]. During chloroquine-induced PCD, Ch'ng and coworkers used an array of inhibitors and concluded that clan CA cysteine proteases such as cathepsins and calpains mediated parasite death [34]. This is in contrast to studies implicating clan CD proteases (metacaspases) in response to chloroquine treatment [26] or caspase 3-like enzymes following bilirubin exposure [35].

These conflicting results highlight several important caveats. (1) Commercial kits and reagents have primarily been developed for mammalian systems, and *Plasmodium* enzymes may exhibit different substrate specificity. (2) Broad spectrum caspase inhibitors also inhibit other cysteine proteases [58], and off-target effects may also complicate the interpretation of results [59]. (3) The enzymes implicated by substrate and/or inhibitor assays in *P. falciparum* have not been characterised and conclusively linked to parasite apoptosis. (4) Caspase-independent apoptosis has also been described [60–62], and this may occur when proteases have been inhibited. Thus, the inhibitors may not prevent cell demise but simply shift the phenotype of cell death to one that is not dependent on the affected protease [63].

5. Is There a Cure for the Madness?

The bulk of the evidence presented in this paper favors the occurrence of some form of PCD in *P. falciparum*; however, the major debate centres around the phenotype. The current classification of distinct cell death phenotypes [28] is important, since a uniform nomenclature provides clarity to the field; however, it may also exacerbate the problem, since it is becoming evident that parasite death may in some instances display features and markers of more than one phenotype or possibly early and late characteristics of a single phenotype. This may relate to different subsets of parasites in the study population responding in a slightly different way to the death stimulus or initiating PCD at slightly different time intervals if cultures are not tightly synchronised, since some stages may be more susceptible to a specific trigger. The NCCD has recognised this dilemma and proposes that the move away from morphological features to biochemical characteristics may alleviate the situation [28].

To facilitate interstudy comparison and consistency, an integrated strategy whereby researchers evaluate as many appropriate markers as possible using standardised methods and culture conditions in a single study may be beneficial to resolve the current inconsistencies in the data. In addition, a more rigorous experimental approach will increase the confidence in the data. Appropriate controls are therefore essential, and an adequate number of technical and biological replicates should be assessed to ensure a reliable and consistent outcome.

6. Genomic Evidence of PCD in *Plasmodium*

Plasmodium lacks true caspases [23, 64, 65] but genomic evidence for metacaspases (see Table 3 in Appendix B) has been found in *P. falciparum* [23, 26, 32, 57, 66, 67], *P. berghei* [32], and *P. vivax* [32, 66] as well as *P. knowlesi and P. yoelii* [66].

These metacaspases belong to the C14 family of clan CD cysteine proteases. PfMC1 (PF13_0289) has been partly characterised using computational methods [26] and contains an N-terminal caspase recruitment domain (CARD) and a C-terminal catalytic domain including the histidine-cysteine dyad essential for activity. *P. berghei* parasites have been genetically manipulated to produce a knockout clone lacking the orthologous PbMC1 (PB001074) gene in an attempt to evaluate the role of the enzyme in apoptosis of sexual stage parasites [32]. A comparison of wild-type and knockout parasites has produced conflicting results. The gene either seems to be functionally redundant, since it had no effect on apoptotic markers [32] (unpublished data cited in [42]), or it appeared to modulate ookinete numbers [68].

Transcription data revealed that PbMC1 is actively expressed in all mosquito stages of *P. berghei* and in female gametocytes but not in the asexual erythrocytic stages of development. PfMC1 is transcribed in the blood stages of *P. falciparum*. However, transcription does not guarantee active expression of the protein, and thus, the involvement of metacaspases in cell death processes is still unresolved.

A *P. falciparum* gene (PF10450c) coding for a putative apoptosis-related protein showed increased mRNA expression in parasites exposed to bilirubin, and this correlated with several other markers of apoptosis [35].

Genomic evidence [66] for the existence of elements of a PCD pathway in *P. falciparum* is presented in Table 3 of Appendix B. These genes encode proteins that are involved in all stages of PCD, including induction, regulation, and execution, and although it remains to be proven experimentally, these findings suggest that a classical PCD pathway exists in *Plasmodium*. However, the possibility that these proteins/domains have unrelated pleiotropic functions cannot be excluded. The identification of amino acid sequences with structural similarity to p53 DNA-binding domains in *P. falciparum* is a particularly exciting finding. The low sequence similarity between *Plasmodium* and known p53 DBDs and the evidence for p53-like activity in plants and green algae [69, 70] raise the intriguing possibility that p53-dependant apoptosis is extremely ancient or arose more than once in eukaryote evolution.

7. What Is the Role of PCD in *P. falciparum*?

Despite conflicting evidence presented in the previous sections, it seems clear that markers of PCD can be detected in *P. falciparum* in response to numerous stimuli. This raises the question of the relevance of this phenomenon in a protozoan parasite. Although the adaptive advantage of PCD is more easily understood in multicellular organisms, where the sacrifice of some cells contributes to the development and maintenance of the higher-level organisms [14], the idea

of suicide is difficult to reconcile with unicellular parasites (reviewed in [1, 14]). It is generally argued that PCD in *P. falciparum* may have developed by group-level selection, providing a survival benefit to kin in a population of closely related individuals [23]. In *P. falciparum*, PCD may provide a number of benefits, and one of the most widely suggested is the self-limitation of the parasite's burden on the host, to facilitate transmission. However, it should not be assumed that the existence of a PCD mechanism in *P. falciparum* is an adaptive trait even if its execution provides a survival benefit to kin, population or species.

Empirical evidence for adaptive programmed death in unicellular lineages has only been provided in two organisms so far. In the yeast, *S. cerevisiae*, a genetically encoded altruistic aging programme has been demonstrated [71, 72], and in the green alga *C. reinhardtii*, the substances released during PCD were shown to benefit others, suggesting the phenomenon is a group level adaptation in this organism [73]. However, PCD may have evolved and been maintained as a nonadaptive or pleiotropic trait in other organisms and still be genetically regulated [14]. Furthermore, the lessons learnt from other unicellular organisms, including protozoan parasites, such as *Trypanosomes* or *Toxoplasma*, may not always apply to *P. falciparum,* especially to the highly virulent intraerythrocytic stages, which are under very different environmental constraints and pressure.

8. Can We Exploit PCD as a Novel Drug Target?

If PCD is a group-level parasite survival mechanism, it would have to be downregulated in a therapeutic context. In addition, PCD in *Plasmodium* would have to be adaptive in its nature in order for its manipulation by drug therapy to come at a significant survival cost to parasite populations.

To minimise harmful side effects in patients, a protein in the PCD pathway that is targeted should be sufficiently different from the human counterpart. Metacaspases have been implicated as PCD effector molecules in *P. falciparum,* and these enzymes are only found in protozoa. However, treatment of mice with a pan-caspase inhibitor, z-VAD, did not protect them from the lethal effects of experimentally induced cerebral malaria. A similar antiapoptotic therapeutic approach using transgenic mice that overexpressed Bcl-2 also failed [74].

An interesting aspect that has not been dealt with in this paper is that *Plasmodium* induces apoptosis in numerous human host cell types, such as endothelial, neuronal, and retinal cells [6]. In contrast, infected hepatocytes and erythrocytes are protected from apoptosis by the parasite to ensure its own survival [55]. An antiapoptotic therapy that uses erythropoietin as a neuroprotective adjuvant to prevent neuronal apoptosis provides a new treatment option. It has been successful in experimental murine cerebral malaria [75], and a recent human study showed that African children with high levels of erythropoietin were protected against neurological sequelae of cerebral malaria [76].

Since malaria is an acute and aggressive infection that requires immediate treatment with drugs that rapidly and effectively kill all parasites, the concept of treating patients with agents that promote PCD may not have the required effect unless they are used in the context of an adjuvant. However, future elucidation of PCD pathways may yield potential target proteins that are multifunctional, which will broaden the scope of the drug and interfere with more than one aspect of the parasite's biology.

9. Perspective

With the controversy surrounding PCD phenotypes in *P. falciparum* being further complicated by methodological difficulties in the detection of several PCD markers and the often ambiguous nature of the markers themselves, a more innovative approach may be required to successfully characterise and exploit possible PCD pathways in *P. falciparum*.

Current thinking on PCD in *P. falciparum* is based on a multicellular PCD paradigm, which may not be appropriate for the unicellular malaria parasite, since it may differ in its ultimate and proximate reasons in reaching the same cellular endpoint. However, with our current lack of knowledge of PCD in *P. falciparum,* the metazoan template is a useful frame of reference and provides a starting point to identify homologous PCD genes and proteins in *Plasmodium* species. The development of new and more powerful computational algorithms to investigate the highly unusual *P. falciparum* genome and proteome will help direct future investigations. The identification of key genes and biochemical characterisation of the recombinant *P. falciparum* proteins will facilitate development of parasite-specific reagents, which will provide new tools and improve the specificity and sensitivity of marker assays. Genomic evidence of PCD machinery also paves the way for parasite gene manipulation and the generation of selective knockout parasites, which can then be used to assess the functional role of the gene in PCD.

Numerous challenges still exist for researchers in this field, and a few of these unanswered questions are delineated in Appendix C. The current phase of research is mainly descriptive, whereby markers of PCD are assessed and documented following *in vitro* exposure of *P. falciparum* cultures to adverse conditions. This provides indirect evidence of key participants in PCD, such as cysteine proteases although there is still no clear molecular link between the markers and the actual biochemical events in the parasite and the final phenotype. This should form the basis for the next exciting phase of study and the challenge for researchers will be to elucidate and clarify the phenotypic expression of PCD and to identify and characterise the pathway(s) that underlie this fundamental biological process in *P. falciparum*.

Appendices

A. Characteristics of Programmed Cell Death (PCD) Phenotypes (Table 2)

PCD may be defined as any cell death process that results from the activation of an intrinsic cell death programme; that is, it is a genetically regulated sequential process. In

Table 1: Summary of PCD studies in *P. falciparum*. Symbols indicate the following: Δ—change observed (increase or decrease indicated by ↑ or ↓, resp.); ×—not done. SNAP—S-nitroso-N-acetyl-penicillamine.

Phenotype	Stimulus	Strain	Morphological changes	DNA fragmentation	Mitochondrial membrane potential	Protease involvement	Ref
Apoptosis	Antimalaria drugs	HB3 K1	Δ Microscopy	Δ ↑ TUNEL	×	×	[23]
	Bilirubin	NF-54	Δ Fluorescence microscopy	×	Δ ↓ JC-1 Spectrofluorimetry and fluorescence microscopy	Δ ↑ Caspase assay, spectrofluorimetry, DEVD substrate, and DEVD inhibitor	[35]
			×	Δ ↑ Laddering, Agarose gel and Southern blotting	×	×	[12]
		3D7	Δ Microscopy	Δ ↑ TUNEL by fluorescence microscopy	Δ ↓ JC-1 Fluorescence microscopy	Δ VAD inhibitor	[26]
	Chloroquine		×	Δ ↑ TUNEL by flow cytometry and fluorescence microscopy	Δ ↓ JC-1 Flow cytometry and fluorescence microscopy	Δ ↑ Caspase assay, flow cytometry and fluorescence microscopy, VAD substrate, VAD, FA, FF, LLL, CA-074, and E64d inhibitors	[34]
		7G8	Δ Microscopy	Δ ↑ TUNEL by fluorescence microscopy	Δ ↓ JC-1 fluorescence microscopy	NO CHANGE VAD inhibitor	[26]
		K1	×	×	Δ ↓ JC-1 flow cytometry	×	[34]
	Etoposide	3D7 7G8		Δ ↑ TUNEL by fluorescence microscopy		Δ VAD inhibitor NO CHANGE VAD inhibitor	[26]
	Chloroquine Oxidants	HB3 K1		Δ ↑ TUNEL			
	Etoposide Starvation	HB3 K1	Δ Microscopy	×	×	×	[23]
	Heat	3D7		Δ ↑ TUNEL by fluorescence microscopy			[27]
	Increased parasite density	Dd2		Δ ↑ TUNEL by flow cytometry and fluorescence microscopy	Δ ↓ TMRE flow cytometry	Δ ↑ protease mRNA and protein	[36]

Table 1: Continued.

Phenotype	Stimulus	Strain	Morphological changes	DNA fragmentation	Mitochondrial membrane potential	Protease involvement	Ref
Apoptosis/ Autophagy	Chloroquine *S. nudum* extracts	7G8	Δ Microscopy, electron microscopy	Δ ↑ TUNEL by flow cytometry	Δ ↓ $DiOC_6$ Flow cytometry	×	[24]
Autophagy	Chloroquine SNAP Staurosporine	PSS1	Δ Electron microscopy	NO CHANGE TUNEL by flow cytometry	Δ ↓ Rhodamine 123 Flow cytometry	NO CHANGE VAD inhibitor	[37]
Necrosis	Chloroquine Staurosporine Heat	CSC-1	Δ Microscopy, electron microscopy	NO CHANGE Agarose gel with SYBR Green staining	Δ ↓ $DiOC_6$ Flow cytometry	Δ ↓ Caspase assay, Flow cytometry and fluorescence microscopy, VAD, LETD, LEHD, and AEVD inhibitors	[38]
Undefined	Chloroquine	Lili	×	NO CHANGE Agarose gel and Southern blotting	×		[12]
	Etoposide	F32	NO CHANGE Microscopy	NO CHANGE TUNEL by flow cytometry	NO CHANGE $DiOC_6$ flow cytometry	×	[25]
	Atovaquone				Δ ↓ $DiOC_6$ flow cytometry		
	SNAP		Δ Microscopy	Δ ↑ Smear, Agarose gel with ethidium bromide staining			
	Artemisinin Ceramide Mefloquine	FCR3	NO CHANGE Electron microscopy	NO CHANGE TUNEL and agarose gels	×	NO CHANGE Caspase 3 assay	[39]
	Staurosporine	3D7	×	Δ ↑ TUNEL by flow cytometry and fluorescence microscopy	Δ ↓ JC-1 Flow cytometry	Δ ↑ Caspase assay, flow cytometry, and VAD substrate NO CHANGE VAD inhibitor	[34]

Table 2

Cell death phenotype	Morphology	Common biochemical markers
Apoptosis	(i) Decreased cellular volume (pyknosis) (ii) Rounding-up of cell	(i) Loss of mitochondrial membrane potential ($\Delta\Psi_m$)
	(iii) Intact plasma membrane blebbing	(ii) Cysteine protease activation
	(iv) Chromatin condensation	(iii) Phosphatidylserine externalization
	(v) Nuclear fragmentation	(iv) Oligonucleosomal DNA fragmentation
	(vi) Apoptotic body formation	(v) Exclusion of cell-impermeable vital dyes in early stages
	(vii) Minor changes in cytoplasmic organelles	(vi) Increase in reactive oxidants
Autophagy	(i) Cytoplasmic vacuolization	(i) Starvation response
	(ii) Autophagic vesicles with double membranes	(ii) Induction of membrane rearrangement genes (e.g. ATG1 and ATG8) to form autophagosomes
	(iii) No chromatin condensation	(iii) Caspase 8 involvement
Necrosis	(i) Increased cellular volume (oncosis)	(i) Nonspecific DNA degradation
	(ii) Loss of integrity and rupture of plasma membrane	(ii) Inclusion of cell-impermeable vital dyes
	(iii) Swelling of cytoplasmic organelles	(iii) Loss of $\Delta\Psi_m$

recognition of the recommendations of the Nomenclature Committee on Cell Death (NCCD) in striving towards uniform nomenclature [28], apoptosis, and autophagy are considered phenotypes of PCD in *Plasmodium*. **Apoptosis** is associated with highly characteristic cellular and biochemical changes in response to detrimental external or internal stimuli, resulting in cell death [2]. **Autophagy** represents the sequestration of cellular material into autophagosomes for subsequent degradation and typically promotes survival, but under extreme or prolonged adverse conditions, the cell may die [77]. Necrosis, once viewed as an entirely uncontrolled manner of cell death, may also be a genetically regulated and energy-dependent form of cell death [78]. However, necrosis is a poorly defined phenotype in *Plasmodium* and in the absence of evidence for a regulated sequence of events it is considered a non-PCD form of cell death in *Plasmodium*. Each cell death phenotype is characterised by a distinctive morphology; however, the NCCD has recommended that morphological cell death definitions be replaced with more objective functional or biochemical criteria. The main morphological features of the different cell death phenotypes and some of the common markers for each are listed in the table below. It should be noted that phenotypes may not be exclusive and overlap may therefore occur between the markers. Any marker in isolation should thus not be considered proof of a particular PCD phenotype.

B. Genomic Elements of a PCD Pathway in Four *Plasmodium* Genomes (Table 3)

Evidence has recently accumulated demonstrating the manifestation of one or more markers of PCD in the asexual blood stages of *P. falciparum* in response to various experimental stimuli to induce parasite death. In the sexual developmental stages within the mosquito host, features of apoptosis have also been observed in *P. berghei* and *P. falciparum*. However, the presence of these markers does not necessarily imply the existence of a complete pathway.

There is very little information concerning the genes and proteins involved in PCD in *Plasmodium*. Some of the reasons for this lack of data relate to the fact that the *P. falciparum* genome is a notoriously difficult genome to investigate. It is evolutionary distant from other taxa due to fast-evolving genes and is phylogenetically ancient and complex [79]; there are frequent low-complexity regions and other inserts in genes, and the nucleotide and codon biases render the methods for orthologue identification less sensitive and specific. Conventional homology methods have therefore yielded limited results, which is evident from the finding that ~60% of predicted proteins in the *P. falciparum* genome have no significant homology to known proteins [80].

In a recent study, six key proteins or domains were selected based on their involvement in the four main stages of the p53-dependent pathway: induction (ATM), initiation (p53), regulation (MDM2, CR6 and IAP) and execution (peptidase C14). Hidden Markov model (HMM) libraries were constructed (supplementary file which is available online at doi:10.1155/2012/646534, [66]) based on multiple sequence alignments performed with MAFFT [81] and edited with BioEdit [82]. Construction, calibration and implementation of HMMs were conducted with HMMER [83]. A detailed analysis of the four *Plasmodium* genomes was performed using these HMM libraries, as well as an array of computational approaches including standard homology methods, phylogenetics, structural models and a novel evolutionary rate-based alignment algorithm FIRE (Functional Inference using the Rates of Evolution), which was developed to identify homologous and analogous genes in organisms with unusual genomes, such as *P. falciparum*, and hence low sequence similarity [84]. Hits are listed in the table below with E-values in parentheses. Fifteen hits with negative E-values were retrieved from the HMM library

Table 3

Protein/domain with function	*P. falciparum*	*P. knowlesi*	*P. vivax*	*P. yoelii*
ATM (detection of DNA damage)	PF13_0072 (−2) PFD0690c (−2)	PKH_051590 (−2)	PVX_084530 (−5)	—
p53 DBD (initiator of PCD)	PFE1120w (−2) PFE0325w (−1)	—	—	—
MDM2/SWIB (negative regulation of p53 activity)	PF10_0167 (−1)	PKH_060130 (−1)	PVX_001730 (−2)	PY00201 (−1)
CR6 (negative regulation of cell cycle)	MAL7P1.212 (−3)	PKH_101090 (−1)	—	—
IAP (inhibitor of apoptosis)	PFE0985w (−2)	PKH_101280 (−3)	PVX_080265 (−4)	PY00703 (−1)
Peptidase C14 (execution of apoptosis)	PF13_0289 (−22) PF14_0160 (−1) PF14_0363 (−1)	PKH_111640 (−25) PKH_133100 (−2) PKH_126800 (−1)	PVX_114725 (−22) PVX_085640 (−2) PVX_118575 (−1)	— PY04718 (−2) PY00663 (−1)

Abbreviations: ATM: ataxia telangiectasia mutated; CR: cell regulator; DBD: DNA-binding domain; IAP: inhibitor of apoptosis protein; MDM2: murine double-minute 2; SWIB: swinged wings locus complex B; —: no orthologue detected.

for p53 DNA binding domains (DBD) and two of these are included in the table. These encode proteins containing predicted p53 DBD-like structural folds (antiparallel beta sheets with greek key topology) according to PlasmoDB v5.5 and positive FIRE scores. The FIRE algorithm predicts the function of a domain based upon similar evolutionary rates and produced scores of 0.71 and 0.68 for PFE1120w and PFE0325, respectively (FIRE scores greater than 0.6 are suggestive of similar functions).

C. Missing Pieces of the Puzzle

Apart from the problematic aspects regarding experimental studies on PCD in *P. falciparum*, which are highlighted in this paper and require further research, numerous additional and challenging questions remain to be answered. A few of these are outlined below.

(1) Which Life-Cycle Stage of P. falciparum Is Most Susceptible to PCD? During its complex life cycle in two hosts and diverse tissues, some stages may be more prone to PCD than others to maximise parasite survival. Ookinetes in the mosquito and rapidly growing intraerythrocytic trophozoites and schizonts appear to be more susceptible to various stimuli, in contrast to early ring stages. In particular, ring stages are apparently not affected by febrile temperatures, and this has biological relevance, since periods of fever in the host occur during this developmental stage.

Does the exposure of merozoites to host immune factors render these extracellular forms of the parasite vulnerable to PCD?

(2) What Makes Some Parasites within the Population More Susceptible to PCD? The concept of PCD being adaptive in unicellular organisms implies that only a subset of parasites is destined to commit suicide to benefit the remaining members of the population, but how are they selected? Natural infections of *P. falciparum* in endemic areas exhibit a high degree of genetic diversity due to multiple infectious mosquito bites, and since PCD should benefit genetically similar or identical kin, this may play a role in the selection process. In areas of low transmission or in *in vitro* studies, the parasites are clonal, implying that other selection criteria are utilised. These are not yet known, but they may relate to small differences in metabolic activity of individual parasites or to differences in the microenvironment.

(3) What Are the Suicide Signals Inducing PCD? Several clues are available from the pathogenesis of malaria, which imply that the signals may potentially be provided by the host, an individual parasite or a population of parasites. In malaria patients, anaemia is a major and often life-threatening consequence of infection, and to keep the host alive, the parasite may elicit PCD in response to host factors such as a decreased haemoglobin content or haematocrit and altered blood viscosity. High parasitaemia in patients and in *in vitro* cultures, or a large ookinete burden in the mosquito, intuitively constitutes a parasite-derived PCD signal to protect the host, and thereby ensure parasite survival.

Are the signals positive or negative? If trophozoites or schizonts develop abnormally or are damaged by internal or external factors, does this trigger removal of these defective parasites? Alternatively, if a merozoite fails to invade an erythrocyte, does the lack of a receptor-ligand interaction trigger a default PCD pathway?

(4) Is There Communication between Parasites within a Population? For parasite-derived signals to be effective, a quorum sensing mechanism must exist to allow parasites to be in touch with each other, but the nature of this communication system in *P. falciparum* is currently unknown. Furthermore, since PCD involves killing a subset of parasites only, how do parasites receiving the same message ensure that only some of them react?

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4 INTRODUCTION TO PUBLICATION 2

Engelbrecht D and Coetzer TL

The walking dead: Is hydroethidine a suitable viability dye for intra-erythrocytic *Plasmodium falciparum*?

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Summary

The distinction between live and dead parasites is essential in PCD studies, particularly in a haemoparasite such as *P. falciparum*, where the encapsulation of the parasite within multiple cell membranes renders the use of exclusion dyes based on cell permeability, such as propidium iodide, unfeasible for live/dead discrimination. Hydroethidine is used as a vital dye based on its conversion to cell-impermeable ethidium by actively metabolising cells. However, it was discovered that dead cells continued to stain positively with hydroethidine. This finding provides essential information for studies on *P. falciparum* PCD, including this thesis.

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Short communication

The walking dead: Is hydroethidine a suitable viability dye for intra-erythrocytic *Plasmodium falciparum*?

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ABSTRACT

Hydroethidine has been used as a viability dye in various haemoparasites, including *Plasmodium* species. We compared flow cytometric quantification by hydroethidine and thiazole orange in *P. falciparum*. Dead parasites that did not develop or replicate exhibited high levels of DNA fragmentation and abnormal microscopic morphology, but were detected as viable ring-stage parasites by hydroethidine. Hydroethidine quantification was similar to thiazole orange, a DNA-binding dye that stains live and dead parasites. Data obtained cast concerns on hydroethidine as a suitable viability dye in *P. falciparum* and highlight the necessity of proper gating in flow cytometric studies quantifying parasitaemia.

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Introduction

Quantification of intra-erythrocytic parasites by flow cytometry with DNA-binding dyes offers a simple, rapid and accurate alternative to microscopy, without the bias problem inherent in microscopic evaluations of morphological forms and parasitaemia, with much larger sample populations than are counted on Giemsa-stained smears. Dihydroethidium, or hydroethidine (HE), has been utilised in several studies for this purpose, as well as for assessing parasite viability. The suitability of HE as a viability dye in haemoparasites was first described in *Babesia bovis* [1], and later applied to several *Plasmodium* spp. [2–5]. HE is an electronically neutral fluorophore that readily passes through lipid cell membranes and is oxidised by living cells to cationic ethidium, which becomes trapped intracellularly due to its charge and binds nuclear DNA, resulting in a fluorescence shift that allows discrimination of living parasites [6,7].

In this study, we compared flow cytometric quantification of *in vitro* intra-erythrocytic *P. falciparum* using two flow cytometric dyes, namely HE and thiazole orange (TO). HE flow cytometry detected a population of parasites for a number of consecutive days that were judged to be non-viable due to their failure to develop and the presence of DNA fragmentation as measured by the Terminal deoxynucleotidyltransferase-mediated Nick End Labelling (TUNEL) assay. These findings raise concerns about the suitability of HE as a viability dye for *in vitro P. falciparum* experiments.

The 3D7 strain of *P. falciparum* was maintained asynchronously in continuous culture according to established methods [8] with some modifications [9]. Briefly, parasites were cultured in washed donor erythrocytes at 5% haematocrit in malaria culture medium composed of RPMI 1640 supplemented with 50 mg/L gentamycin, 50 mg/L hypoxanthine, 0.21% (w/v) sodium bicarbonate and 0.5% Albumax II (w/v) (Gibco, USA) to a 5 ml final culture volume. For the duration of the study, culture medium was changed daily for both control and test cultures and optimal atmosphere maintained by gassing with a gas mixture of 2% O_2, 5% CO_2 and 93% N_2. Control and test cultures were infected with parasites at the same time (Day 0) and test cultures were allowed to replicate freely for 9 days without the addition of fresh erythrocytes, while parasitised control cultures were maintained at a lower parasitaemia by diluting to ~1% parasitaemia (5 ml culture volume) with fresh erythrocytes every second day. Non-parasitised erythrocyte cultures were maintained in parallel and used as background for flow cytometric staining. Growth and development were monitored daily by Giemsa-stained thin smears and flow cytometry with HE and TO. Inclusion gates for ring- and trophozoite/schizont-stage parasitised erythrocytes were established with parasitised cultures synchronised by incubation in 10 volumes of a 5% (w/v) D-sorbitol solution for 20 min at room temperature, similar to a previous method [10] (Fig. 1). The developmental stage of synchronised parasites was confirmed by Giemsa-stained thin smears.

HE staining was performed similar to a previous method [5]. Briefly, 10 µl whole culture was diluted to 1 ml with phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 50 µM HE (5 µl 10 mM stock in DMSO), mixed and incubated at 37 °C in the dark for 20 min. Cells were counted by flow

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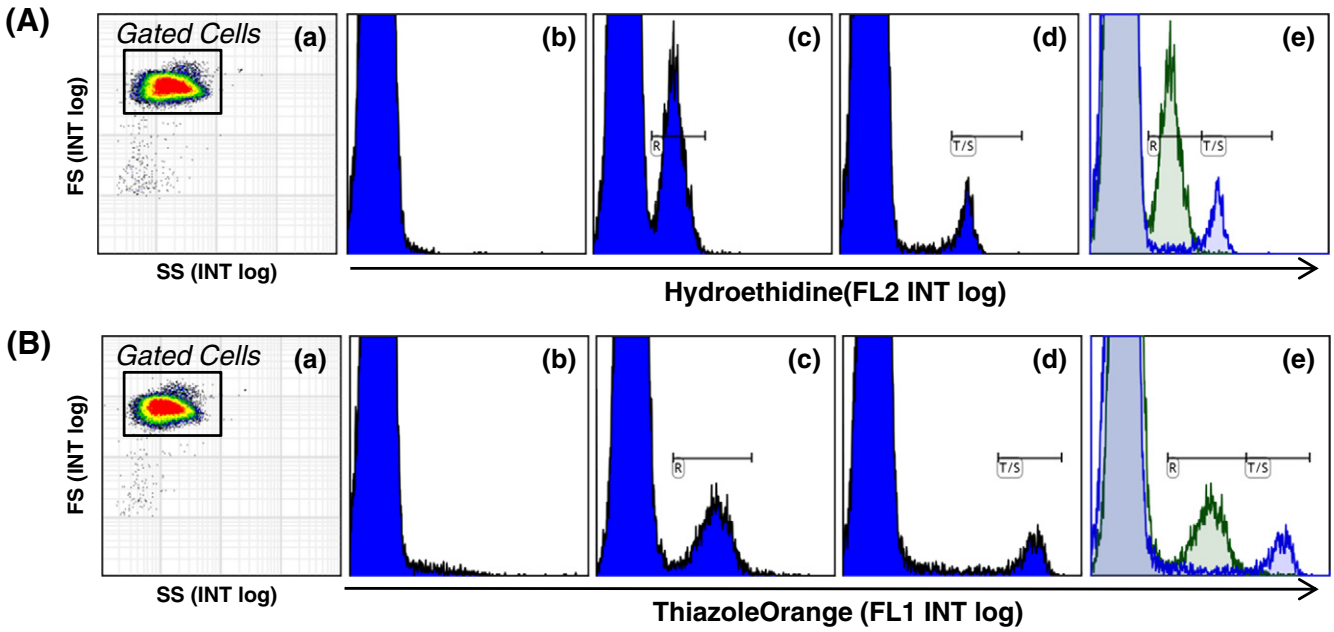

Fig. 1. Flow cytometry gating for quantification of *P. falciparum*. Gating strategy for parasitaemia quantification by hydroethidine (A) and thiazole orange (B). (a) Forward- vs. side-scatter density plots were used to gate a cell population which was evaluated for parasitaemia by single parameter histogram plots. Examples of histograms include: (b) non-infected erythrocytes; (c) synchronised ring stage-infected erythrocytes; and (d) synchronised trophozoite/schizont stage-infected erythrocytes. (e) Superimposition of histograms from synchronised parasites confirmed the separation of ring stage (R) and trophozoite/schizont stage (T/S) peaks and the validity of the gates.

cytometry using a forward- vs. side-scatter density plot to gate a cell population (Fig. 1A a). The gated population was analysed on a FL2 fluorescence integral (INT log) histogram for HE fluorescence (Fig. 1A b–e).

TO staining was performed similar to a previous method [11]. Briefly, 10 µl whole culture was diluted to 1 ml with Sorenson's phosphate buffer (SPB: 47 mM Na_2HPO4, 20 mM KH_2PO_4, pH 7.2) containing 1 µM TO (diluted from a 10 mM stock in methanol), mixed and incubated at room temperature in the dark for 20 min. Similar to HE, cells were counted on a flow cytometer using a forward- vs. side-scatter density plot to gate a cell population (Fig. 1B a), but the gated population was analysed on a FL1 fluorescence integral (FL1 INT log) histogram for TO fluorescence (Fig. 1B b–e).

DNA fragmentation was measured by the TUNEL assay, using the APO-DIRECT TUNEL kit from BD Pharmingen (USA) according to the manufacturer's recommendations, with modifications similar to a previous method [12]. Briefly, whole culture was centrifuged and the pellet was fixed in 4% formaldehyde in PBS (v/v) on ice for 60–90 min, followed by permeabilisation with a solution of 0.1% tri-sodium citrate (w/v) and 0.1% Triton X-100 (v/v) for 3 min on ice. Labelling was performed with DNA staining solution (including TdT enzyme and FITC-dUTP) for 60–90 min at 37 °C, followed by staining with propidium iodide (PI) for 30 min at room temperature, according to the manufacturer's recommendations. Samples were kept in the dark and analysed by flow cytometry. Parasite populations were gated on a FL2 time-of-flight (FL2 TOF lin) vs. FL2 integral (FL2 INT lin) fluorescence dot blot for PI fluorescence. Gated populations were then analysed on a FL1 integral (FL1 INT log) histogram for TUNEL stain (FITC) fluorescence. Staining controls were used to delineate stain-negative populations. DNase 1-treated parasites were used as a positive control.

All flow cytometric analyses were performed using a Beckman Coulter Gallios flow cytometer. The instrument was cleansed daily and calibration was monitored by Beckman Coulter Flow Check Pro fluorospheres. Excitation was at 488 nM. Post-acquisition analyses were performed using Beckman Coulter Kaluza v1.1 software. Graphs of parasite growth and development were generated with GraphPad Prism v5.01.

HE flow cytometry of asynchronous *in vitro P. falciparum* (3D7) showed clearly defined populations distinguished by differential nucleic acid content, allowing discrimination between ring- and late-stage (trophozoites and schizonts) parasites (Fig. 1A c–e). During the first three days after initiating cultures (Fig. 2A a–d) there was normal parasite development with ring-stages progressing to late-stages of approximately equal magnitude the following day, and each late-stage parasite forming multiple merozoites that re-invaded to form new rings the following day, with a corresponding increase in parasitaemia. The progression of developmental stages correlated well with morphology observed on Giemsa-stained smears and with the expected 48 hour asexual erythrocytic cycle of *P. falciparum* [13]. Peak parasitaemia was achieved on day 3 and 4 (Fig. 2B a). A poorly defined parasite peak without distinction between ring- and late-stage populations on day 4 (Fig. 2A e) was correlated with the appearance of numerous darkly-stained, condensed parasites observed microscopically (Fig. 2C Day 4). Thereafter, parasites were observed as a single ring-stage peak that did not progress to late stages on subsequent days (Fig. 2A f–j). The ring peak decreased notably in magnitude by ~4% (mean parasitaemia) on day 5, measured by HE flow cytometry, before reaching a plateau and finally declining further on day 9 (Fig. 2B a). The appearance of a single ring peak correlated with the microscopic observation of numerous small, darkly-stained parasites, resembling merozoites (Fig. 2C Day 6), which were observed on all subsequent days, with practically no morphologically normal parasites remaining. TO, which stains DNA in live and dead parasites, produced similar results to HE, with only a slightly lower peak parasitaemia (~12% and ~13%, respectively) and an apparent earlier decline from peak parasitaemia (Fig. 2B b). From day 5 both TO and HE detected practically all remaining parasites as ring-stages. Substitution of HE with ethidium bromide at equivalent concentrations did not yield comparable results to HE staining, with ethidium bromide showing low fluorescence in the FL2 channel (data not shown).

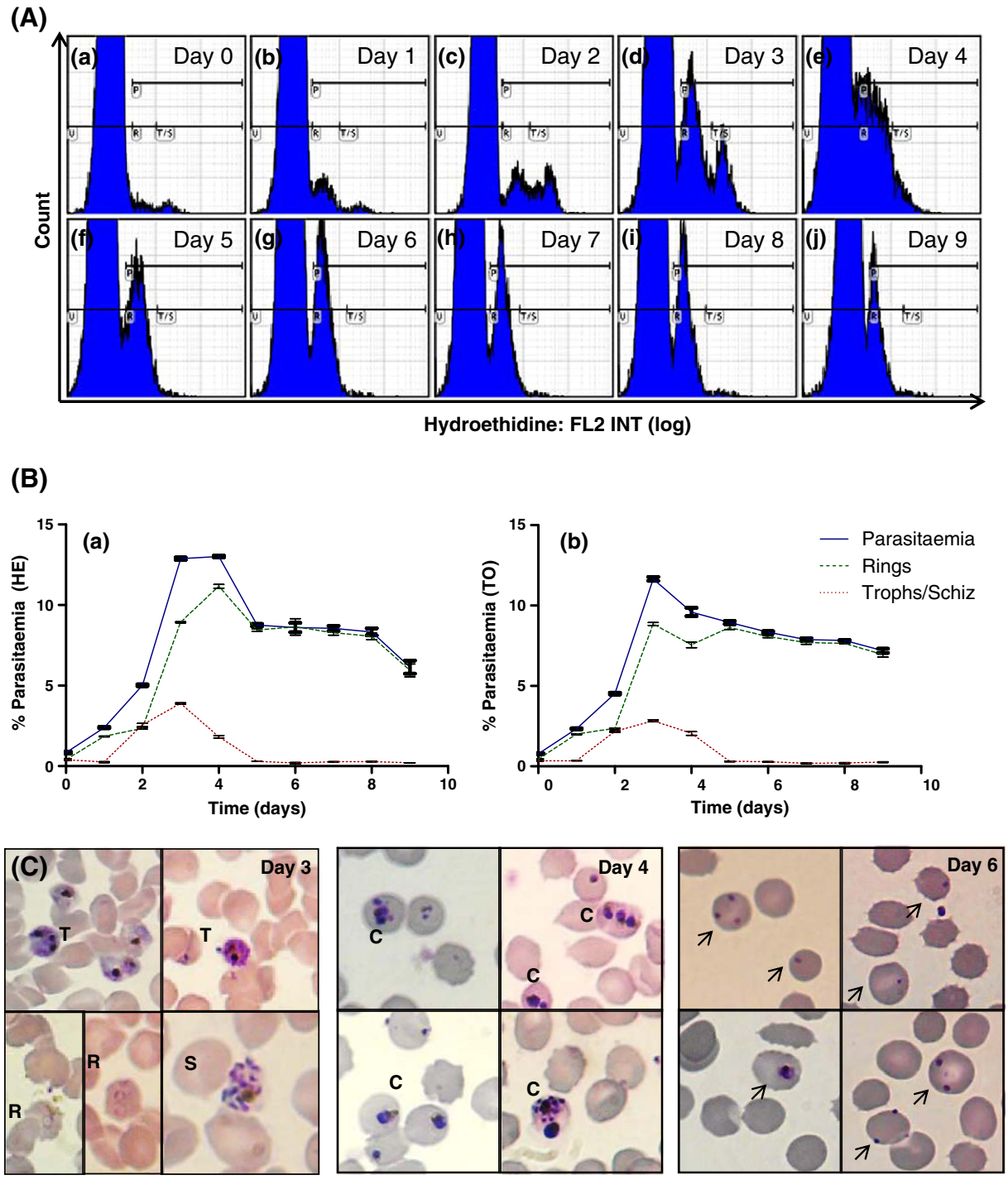

Fig. 2. Quantification of *P. falciparum*. (A) Raw flow cytometric data for HE staining with ring stage (R) and trophozoite/schizont stage (T/S) peaks are indicated. The expected parasite development and replication was seen (a–d) but was followed by a broader, non-distinct peak (e) and subsequently only a ring-stage peak was observed (f–j). (B) Comparison of HE (a) and TO (b) measurements indicated a decline following peak parasitaemia, with practically all remaining parasites detected as ring stage. Similar results were obtained for HE and TO. Values are indicated as arithmetic mean +/− S.E.M., with n = 4 (two biological replicates, two technical replicates) between Day 0 and 6, and n = 2 (two technical replicates) thereafter. (C) Microscopic observation of parasite morphology on Giemsa-stained thin smears (magnification ×1000). At the onset of peak parasitaemia on Day 3, rings (R), trophozoites (T) and schizonts (S) were observed, with darkly-stained, condensed parasites (C) observed on Day 4. Small, darkly-stained parasites, resembling merozoites, were observed on subsequent days and are indicated by arrows at Day 6.

TO has been used in *in vitro Plasmodium* studies to monitor and quantify parasitaemia by detecting parasite DNA [11], irrespective of cell viability. HE also detects parasite DNA, but only after it has been metabolised to ethidium by live parasites [1,6], implying that the parasites detected on days 5 to 9 as a single ring stage peak, were viable. This contradicts the failure of these parasites to develop or replicate and we therefore set out to confirm parasite death by measuring DNA fragmentation with the TUNEL assay on days 6 and 9. When compared to control parasites that were maintained at a lower parasitaemia so as not to induce stress, test cultures showed high levels of DNA fragmentation (~80% more than controls) (Fig. 3), indicating that parasites were in fact dead. The availability of nutrients from the medium was not a limiting factor to the growth and development of parasites observed as a single ring peak following peak

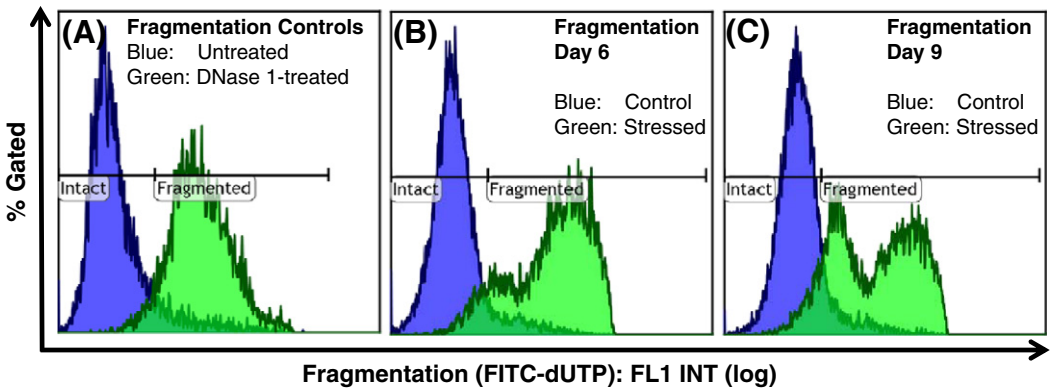

Fig. 3. Increased DNA fragmentation in parasites maintained at high parasitaemia. DNA fragmentation was measured by the TUNEL assay. Gates for parasites with Fragmented or Intact DNA were delineated with the use of untreated- and DNase 1-treated parasites (A, fragmentation controls). Individual histograms for control and stressed parasites on day 6 (B) and day 9 (C) were superimposed to show fragmentation in parasites that were observed as a single ring peak following stress from high parasitaemia (green), when compared to control parasites on the same day (blue).

parasitaemia, since culture medium was changed daily. Positive HE staining of dead cells has also been reported in other unicellular organisms [14].

Encapsulation by the host red blood cell may have trapped dead parasites intracellularly, leaving parasite DNA available for detection. Furthermore, the host cell may have provided an enzymatically active and oxidative environment for the conversion of HE to ethidium and/or related fluorescent products by one or more mechanisms, including: $NADP^+$-dependent enzymatic dehydrogenation [7]; oxidation by host cell haemoglobin [15] or reactive oxygen species [7,16,17]; the latter either derived from the host cell or resulting from parasite metabolism. Once oxidised, the dye would easily have gained access to parasite DNA if the parasite membranes had been permeabilised as a result of cell death.

During this study, our flow cytometry gating structure allowed discrimination between morphological parasite stages, which provided the key evidence that parasites were failing to develop and had not simply reached a parasitaemia plateau. Without such gating, this important distinction may be overlooked, which would result in misleading data in studies that use HE to determine parasite growth inhibition.

In conclusion, we detected a population of *P. falciparum* parasites by HE flow cytometry that were confirmed to be dead by the following observations: (i) parasites did not develop or replicate; (ii) displayed abnormal microscopic morphology; and (iii) had high levels of DNA fragmentation. Our data cast concerns on the validity of HE as a viability dye in the intra-erythrocytic stages of *P. falciparum*. Furthermore, the necessity of proper gating of various morphological stages of *P. falciparum* in flow cytometry was highlighted.

Acknowledgments

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5 INTRODUCTION TO PUBLICATION 3

Engelbrecht D and Coetzer TL

Turning up the heat: heat stress induces markers of programmed cell death in *Plasmodium falciparum in vitro*

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Summary

Malaria illness is characterised by febrile episodes. Fever bouts are generally thought to assist the host in parasite clearance, but may also benefit the parasite by inducing PCD. This publication characterises the cell death phenotypes of *in vitro P. falciparum* after heat stress similar to fever paroxysms experienced during malaria. Early stage parasites were shown to be more severely affected by heat stress than suggested by previous studies and exhibited an apoptosis-like phenotype characterised by mitochondrial depolarisation and DNA fragmentation. Late stage parasites, in contrast, showed markers of an autophagic-like form of cell death, including slight DNA fragmentation, mitochondrial hyperpolarisation, phosphatidylserine externalisation and cytoplasmic vacuolisation. Crucially, the data illustrated that the phenotypical markers of cell death in *P. falciparum* may vary with parasite developmental stage.

OPEN

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Turning up the heat: heat stress induces markers of programmed cell death in *Plasmodium falciparum in vitro*

D Engelbrecht*[,1] and TL Coetzer[1,2]

Malaria is characterised by cyclical febrile episodes that result from the rupture of mature schizont-infected erythrocytes releasing merozoites. In patients infected with *Plasmodium falciparum*, fever may reach peak temperatures as high as 41 °C. Febrile episodes typically have a deleterious effect on parasites and probably benefit the host by aiding parasite clearance; however, the parasite may also gain advantage from limiting its burden on the host and prolonging infection to ensure development and transmission of slow-maturing gametocytes. Programmed cell death (PCD) may provide the parasite with a mechanism of self-limitation, although the occurrence and phenotype of PCD in the erythrocytic stages remain controversial due to conflicting data. This study aimed to characterise the cell death phenotype of *P. falciparum* in response to *in vitro* heat stress. A variety of biochemical markers of PCD, including DNA fragmentation, mitochondrial dysregulation and phosphatidylserine externalisation, as well as morphological studies of Giemsa-stained thin smears and real-time microscopy were utilised to characterise the phenotype. Heat stress decreased *P. falciparum* growth and development *in vitro*. Late-stage parasites were more susceptible, although early stages were more affected than expected. Early-stage parasites exposed to 41 °C exhibited markers of an apoptosis-like PCD phenotype, including DNA fragmentation and mitochondrial depolarisation. Heat-stressed late-stage parasites showed no significant DNA fragmentation or mitochondrial dysregulation; however, cytoplasmic vacuolisation was suggestive of an autophagy-like form of PCD. Our results therefore showed that biochemical and morphological markers of PCD varied with intra-erythrocytic parasite development and that *P. falciparum* exhibited facets of both apoptosis- and autophagy-like phenotypes after exposure to febrile temperatures, which may reflect a unique PCD phenotype.
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Subject Category: Experimental Medicine

Malaria is characterised by acute cyclical febrile episodes that are triggered by the rupturing of mature schizont-infected erythrocytes releasing merozoites.[1] In *Plasmodium falciparum* malaria, fever typically recurs every 48 h,[2,3] coinciding with the parasite's erythrocytic life cycle,[2] and may peak as high as 41 °C for 2–6 h.[3] However, multiple asynchronous infections may lack periodicity[3] and febrile episodes may therefore occur more frequently and last longer.

Elevated temperatures of 40 °C for 6 h or more significantly inhibited *P. falciparum* growth *in vitro*,[4–6] with greater than 95% parasite death after 48 h.[6] Exposure to 41 °C significantly decreased parasite survival after only 2 h,[7] with parasite death noticed after as little as 30 min exposure.[8] The late developmental stages of trophozoites and schizonts were more susceptible to hyperthermal damage, whereas the ring stages were more resistant,[4–8] with exposures to 40 °C causing synchronization towards the ring stage.[4,8] Exposure to 41 °C has been shown to also inhibit the growth of these early stages.[7,8] Although febrile episodes are generally considered to benefit the host by suppressing parasite growth and assisting in clearance,[5] parasites may also derive benefit from increased temperatures. *In vitro* studies have shown that *P. falciparum* growth rates and development were accelerated when parasites were exposed to 40 °C for 2 h, incubated at 37 °C for 10 h and again exposed to 40 °C for 12–24 h.[9] The parasite life cycle was also accelerated after exposure to less severe temperatures of 38.5–39 °C.[10] Incubation at 40 °C for 1–2 h enhanced the cytoadherence of mature *P. falciparum*-infected erythrocytes (pRBC) to CD36 and ICAM-1 receptors and also caused adherence of ring-stage parasites, which do not normally bind to these receptors.[11] Febrile temperatures have also been shown to reduce the deformability of pRBC, which may aid in sequestration,[12] as well inducing phosphatidylserine (PS) externalisation in pRBC.[10]

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Keywords: *Plasmodium falciparum*; programmed cell death; febrile temperature; heat stress
Abbreviations: pRBC, *Plasmodium falciparum*-infected red blood cell; PS, phosphatidylserine; $\Delta\Psi$m, mitochondrial transmembrane potential; PCD, programmed cell death; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; HE, hydroethidine; ROS, reactive oxygen species; Atg, autophagy-related; TO, thiazole orange; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MCM, malaria culture medium; PBS, phosphate-buffered saline; PI, propidium iodide
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Heat shock triggers activation of the intrinsic apoptosis pathway in metazoans, resulting in permeabilisation of the mitochondrial outer membrane with concurrent depolarisation of the mitochondrial transmembrane potential ($\Delta\Psi$m) and the initiation of an enzyme cascade that ends in DNA fragmentation.[13] A growing body of evidence suggests that *P. falciparum* may undergo programmed cell death (PCD), although some conflicting results have suggested a range of cell death phenotypes, including apoptosis, autophagy-like cell death, necrosis or simply undetermined. Phenotypes may also not be exclusive and overlap may occur.[14] According to the best of our knowledge, data regarding the possible induction of PCD by heat stress are limited to two studies, which have offered conflicting conclusions, with an apoptosis-like form of PCD suggested on the one hand[7] and the other suggesting that cell death more closely resembled secondary necrosis, although some form of PCD was not ruled out.[6]

We present a comprehensive *in vitro* study that utilised a variety of biochemical and morphological markers of cell death, as well as heat stress of different duration and intensity, to provide extensive characterisation of the response of *P. falciparum* to conditions similar to febrile episodes experienced during malaria. *P. falciparum* exhibited markers of PCD, including DNA fragmentation, mitochondrial dysregulation, PS externalisation and cytoplasmic vacuolisation. However, early and late intra-erythrocytic stages differed in their response to heat stress and exhibited different phenotypes, which may represent different facets of a single PCD mechanism unique to *P. falciparum*. Febrile temperatures may induce self-limitation of parasite populations through PCD *in vivo* to the benefit of both the host and parasite. Elucidation of a PCD mechanism distinct from metazoans in *P. falciparum* may yield novel drug targets to be exploited in manipulating parasite fate.

Results

Heat stress inhibits *P. falciparum* growth and development. Parasitised cultures were exposed to heat stress to mimic either the extended fever periods experienced during prolonged malaria (40 °C for 6 or 24 h, Figure 1) or the occasional high peaks of fever paroxysms (41 °C for 2 h, Figure 2), and the response of the parasites was characterised with biochemical markers of PCD (Table 1). We observed time-dependant inhibition of growth and development by exposure to 40 °C, with late-stage parasites more severely affected. After 6 h exposure, an apparent delay was noted in the development of ring-stage parasites to late stages between 24 and 48 h (Figure 1). Ring-stage parasites became more vulnerable to heat stress at 41 °C and were more affected than previously thought: ~75% of early-stage parasites exposed to 41 °C for 2 h (Figure 2) failed to develop, compared with a 25% reduction in parasite survival previously shown under the same conditions.[7] The effect of heat stress on late-stage parasites also became more pronounced at 41 °C, with very few parasites observed 24 h after exposure, in comparison with control parasites maintained at 37 °C, which replicated to form new rings (Figure 2).

Heat stress induces markers of PCD in *P. falciparum*. *P. falciparum* exposed to heat stress exhibited a number of markers of PCD, including DNA fragmentation, mitochondrial dysregulation, PS externalisation and cytoplasmic vacuolisation, which varied with the developmental stage and in some instances with the temperature and duration of heat stress (see Table 1 for summary).

DNA fragmentation, quantified by the flow cytometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay, was observed in mixed-stage cultures exposed to 40 °C for 24 h (Figures 3aii and bii) but not for 6 h (Figures 3ai and bi). Therefore, 24 h was chosen for further experiments involving exposure to 40 °C. DNA fragmentation was also observed in synchronised ring-stage parasites exposed to 41 °C for 2 h (Figures 3aiii and biii). Surprisingly, late-stage parasites exposed to the same stress exhibited insignificant DNA fragmentation (Figures 3aiv and biv) at 24 h, despite a significant decrease in parasitaemia (Figure 2). Therefore, exposure to 41 °C may have caused the formation of nonviable merozoites that failed to invade new cells, whereas surviving parasites likely sustained little or no damage.

Mitochondrial depolarisation, generally considered to be characteristic of apoptosis,[15–20] was observed immediately following heat stress in early-stage parasites exposed to 40 °C for 24 h (Figures 4ai and bi), although it was not noted the following day (Figures 4aii and bii). Early-stage parasites exposed to 41 °C for 2 h exhibited slight mitochondrial depolarisation immediately after heat stress (Figures 4aiii and biii) that was significant the next day (Figures 4aiv and biv). Many of the ring-stage parasites exposed to 40 °C developed further (Figure 1), whereas ~75% of ring-stage parasites exposed to 41 °C did not (Figure 2aiii). Surprisingly, late-stage parasites exposed to 40 °C for 24 h exhibited mitochondrial hyperpolarisation (Figures 5ai and bi), that was less prominent the following day (Figures 5aii and bii). No change was observed in the $\Delta\Psi$m of late-stage parasites exposed to 41 °C (Figures 5aiii-iv and biii-iv). In accordance with previous studies,[10] increased PS externalisation was observed in late-stage pRBC exposed to 41 °C a day after heat stress was withdrawn (Figures 6avi and bvi). No increase was noted in synchronised ring-stage pRBC immediately after exposure to 41 °C for 2 h (Figures 6aiii and biii), with an apparent decrease noted at 48 h (Figures 6aiv and biv); however, when comparing the heat-stressed culture at both time points (iii and iv), no change was apparent. PS externalisation was also noted in mixed cultures immediately after 24 h exposure to 40 °C (Figures 6ai and bi).

Most late-stage parasites exposed to 41 °C for 2 h were observed outside erythrocytes on Giemsa-stained thin smears at 24 h, whereas many intracellular parasites exhibited jagged borders and prominent vacuoles within the cytoplasm (Figure 7). Real-time microscopy showed that many of the remaining intracellular parasites, despite not replicating to new ring stages, presented with continued rapid movement (Supplementary Videos) similar to that observed in 37 °C controls and considered characteristic of healthy parasites.[21] A clear recovery, evidenced by increased parasitaemia (Supplementary Figure 1), was noted 5 days after heat stress. Ring-stage parasites exposed to the same

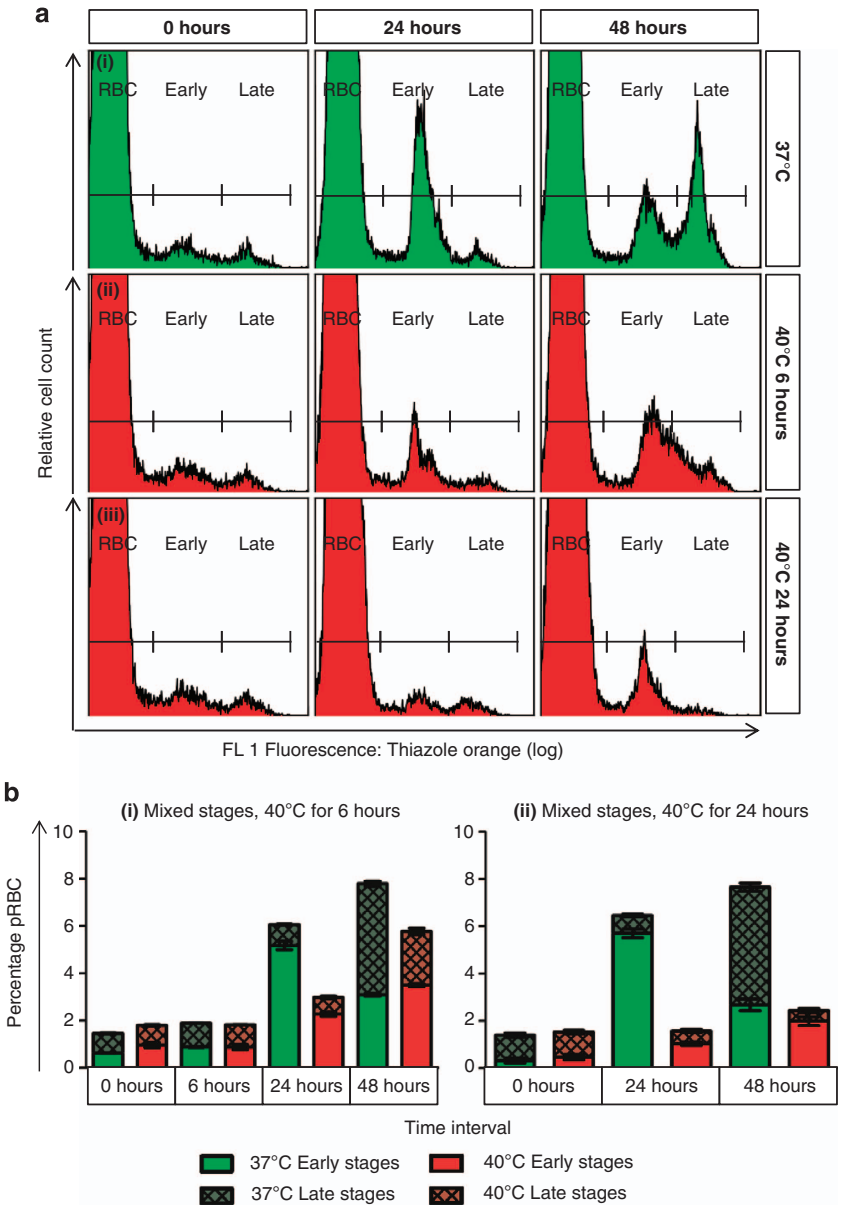

Figure 1 Exposure to 40 °C inhibited growth and development in mixed-stage *P. falciparum* in an exposure-dependant manner. Compared with control parasites kept at 37 °C (**ai**), cultures exposed to 40 °C for 6 or 24 h (**aii** and **iii**, respectively) showed an exposure-dependant inhibition in the formation of new ring-stage parasites (Early) from late-stage parasites (Late) at 24 h, with a correspondingly smaller late-stage population at 48 h (**b**). Late-stage parasites were more affected by heat stress. However, a delay in the development of ring-stage parasites to late stages was apparent between 24 and 48 h after 6 h exposure, which resulted in a broad, indiscrete peak at 48 h (**aii**), indicating a population of intermediate development. Furthermore, after 24 h exposure, few early-stage parasites at 24 h appeared to progress to late stages at 48 h (**aiii** and **bii**). Raw flow cytometry histograms (**a**) and stacked bar graphs (**b**) are shown. Stacked bar graphs show changes in parasitaemia (total bar height) and relative composition of the parasite population with respect to early- and late-stage parasites. For (**bi**) $n = 2$, for (**bii**) $n = 6$ at 24 h and $n = 4$ at 48 h. Data points represent arithmetic mean ± S.E.M. Heat stress was applied at 0 h

stress conditions also showed abnormal morphology on Giemsa-stained thin smears (Figure 7), although morphologically normal parasites were also observed.

Discussion

In vitro P. falciparum exhibited various biochemical markers of PCD, including DNA fragmentation, mitochondrial dysregulation and PS externalisation, as well as some abnormal morphological features including cytoplasmic vacuolisation, in response to heat stress. Different stages of the parasite life cycle exhibited a different combination of PCD markers, which also varied depending on the temperature and the duration of the heat stress.

Heat stress in *P. falciparum*: a single degree makes a difference. Several previous studies have shown that febrile temperatures inhibit *P. falciparum* growth *in vitro*[4,5,8,22] and

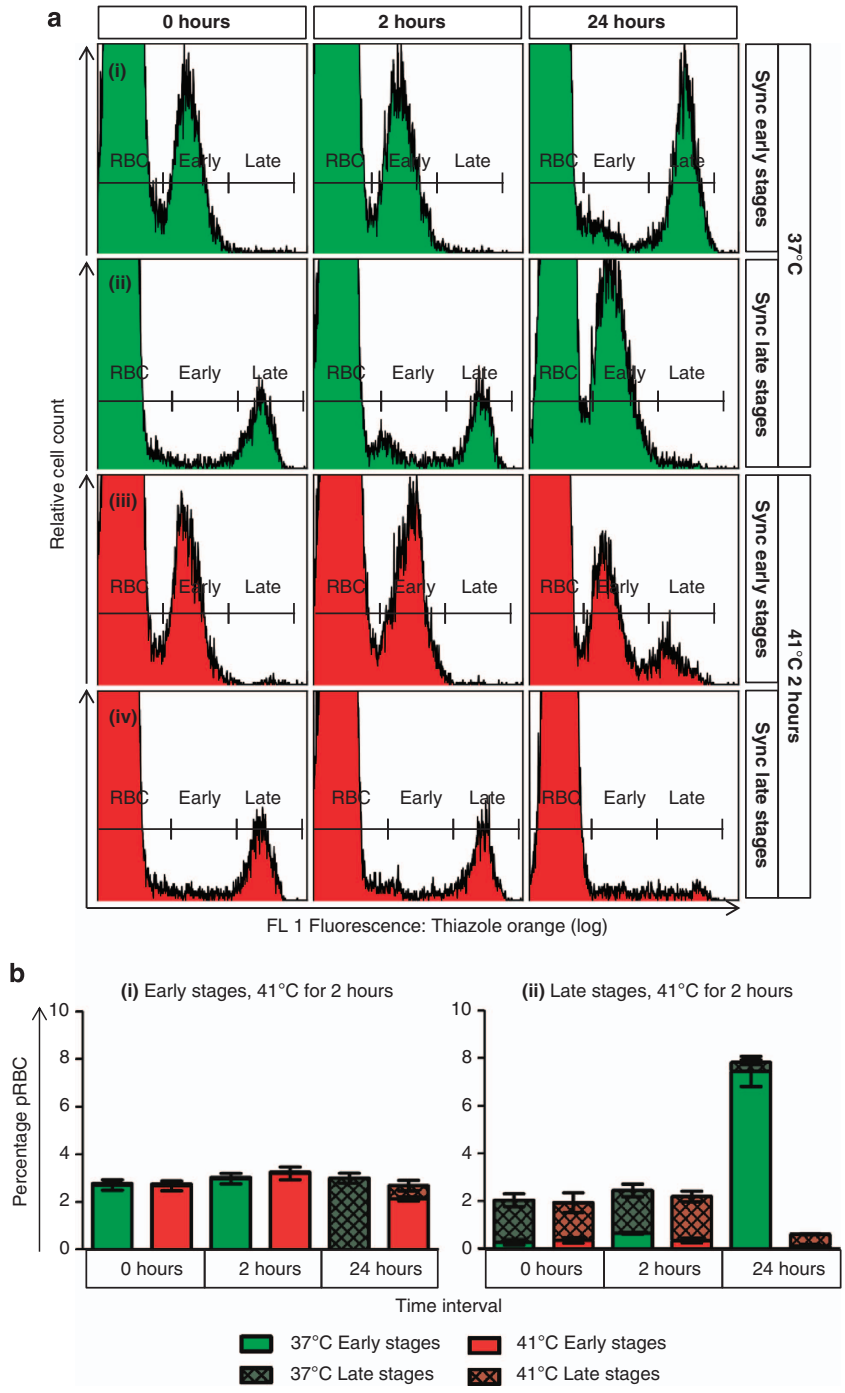

Figure 2 Exposure to 41 °C for 2 h inhibited replication and development of both early- and late-stage synchronised *P. falciparum*. Representative flow cytometry histograms (**a**) showed the progression of early (**i**) and late (**ii**) stage parasites kept at 37 °C, in comparison with early (**iii**) and late (**iv**) stage parasites exposed to 41 °C for 2 h. Time intervals are indicated on top, whereas synchronisation and treatment conditions are indicated on the right. (**b**) Stacked bar graphs show changes in parasitaemia (total bar height) and relative composition of the parasite population with respect to early- and late-stage parasites. A small portion of ring stage (Early) parasites exposed to 41 °C developed to late stages (Late), while most remained as ring-stage parasites. Late stages exposed to 41 °C failed to produce viable merozoites to infect erythrocytes and virtually no parasites were detected at 24 h, compared with 37 °C control parasites, which showed a large new ring population. Data points represent arithmetic mean ± S.E.M. ($n = 4$ for **bi** and $n = 5$ for **bii**). Heat stress was applied at 0 h

have noted that the effect varies with temperature.[5,8] However, only two studies have investigated the appearance of PCD markers in response to heat stress and those studies utilised different strains, cell death markers and heat stress conditions, and have offered conflicting conclusions.[6,7] We utilised heat stress conditions similar to both previous studies and scored a variety of biochemical and morphological markers to characterise the cell death phenotype in

Table 1 Summary of biochemical markers of cell death observed in *P. falciparum* after exposure to increased temperatures

Parasite life stage	Early stages at 0 h		Late stages at 0 h	
Exposure conditions	40 °C for 24 h	41 °C for 2 h		
Time of measurements	Immediately after heat stress	22 h after heat stress	Immediately after heat stress	22 h after heat stress
Growth and development	Slight growth inhibition. Progression to late stages was similar to 37 °C control parasites (Figure 1)	Inhibition of growth. A small number developed to late stages (Figure 2)	Severe growth inhibition with very few ring parasites observed (Figure 1)	>95% growth inhibition, with no new ring-stage parasites formed (Figure 2)
DNA fragmentation	*	Yes (Figure 3aiii)	*	Slight (Figure 3aiv)
Mitochondrial polarisation	Depolarised (Figure 4bi)	Depolarised (Figure 4bii)	Hyperpolarised (Figure 5bi)	No change (Figure 5bii)
PS externalisation	*	No (Figures 6c and d)	*	Yes (Figure 6f)

Parasite life stages, incubation conditions and relevant figures for each result are indicated. Asterisks (*) indicate no data, as effects were observed in mixed-stage cultures

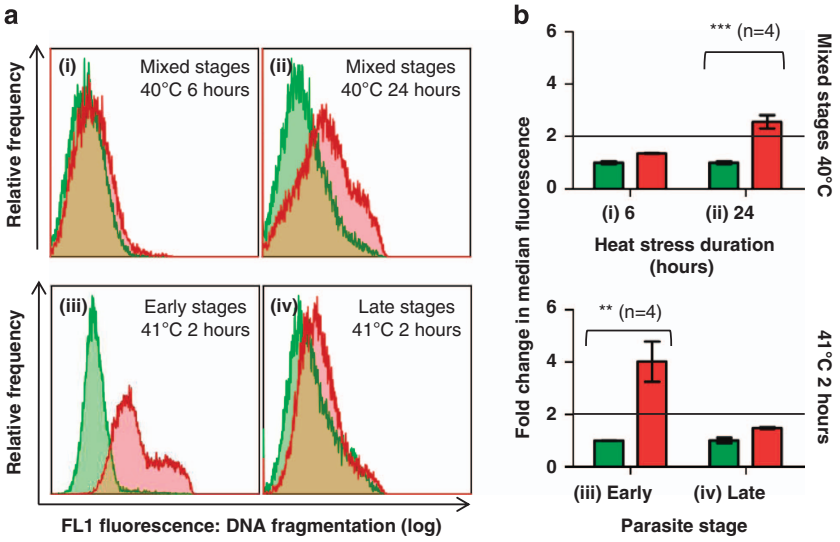

Figure 3 Heat stress induced DNA fragmentation in *P. falciparum*, as measured by the TUNEL assay. Control parasites, incubated at 37 °C, are indicated in green, whereas heat-stressed parasites are indicated in red. All samples were collected at 24 h. TUNEL results indicated significant DNA fragmentation in mixed-stage parasites exposed to 40 °C for 24 h and synchronised ring-stage parasites exposed to 41 °C for 2 h, with only slight fragmentation in synchronised late-stage parasites exposed to the same stress, compared with control parasites at identical time intervals. (**a**) Overlaid fluorescence histograms of the TUNEL assay indicate the FL1 (dUTP-FITC) fluorescence of isolated parasites from individual heat-stressed (red) or control (green) data sets. Fragmentation resulted in a higher fluorescence, indicated by a shift towards the right. Heat stress conditions are indicated in the top right corner of each overlay. Figure numbers (**i–iv**) match those of the bar graphs. (**b**) Bar graphs summarise TUNEL results for mixed-stage parasites exposed to 40 °C for 6 (**i**) and 24 (**ii**) hours, as well as synchronised early (**iii**) and late (**iv**) stage parasites exposed to 41 °C for 2 h. Changes in fragmentation are indicated as the mean change in median fluorescence for all samples. Differences of twofold or greater (indicated by a horizontal line on bar graphs) between heat-stressed and corresponding 37 °C control parasites are considered indicative of fragmentation. Data points indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.01 (**) or <0.001 (***)

intra-erythrocytic *P. falciparum* after heat stress. An apoptosis-like phenotype was previously suggested based on gene-expression studies in asynchronous parasites and DNA fragmentation detected in schizont-stage parasites by the *in situ* TUNEL assay, after exposure to 41 °C for 2 h.[7] After the same exposure, our data showed no significant DNA fragmentation in late-stage parasites; however, we did observe DNA fragmentation in ring-stage parasites, which Oakley *et al.*[7] did not consider. We also utilised flow cytometry to quantify the TUNEL assay, rather than fluorescence microscopy.[7] After exposure to 40 °C for various durations up to 48 h, Porter *et al.*[6] reported a lack of DNA fragmentation or caspase activation, mitochondrial depolarisation in late-stage parasites as well as food vacuole swelling and lysis. Although heat-induced PCD was not ruled out, it was suggested that the parasite cell death response more closely resembled secondary necrosis.[6] In contrast, we observed significant DNA fragmentation in mixed-stage cultures after exposure to 40 °C for 24 h, quantified by the TUNEL assay, which is more sensitive than the agarose gels utilised previously.[6] Furthermore, we surprisingly found mitochondrial hyperpolarisation in late-stage parasites, whereas only early-stage parasites showed depolarisation. It is worth noting that, although we utilised similar mitochondrial staining methods, we discriminated between early- and late-stage pRBC based on hydroethidine (HE) uptake, whereas Porter *et al.*[6] used synchronised cultures. Our data showed that differences in heat stress conditions may at

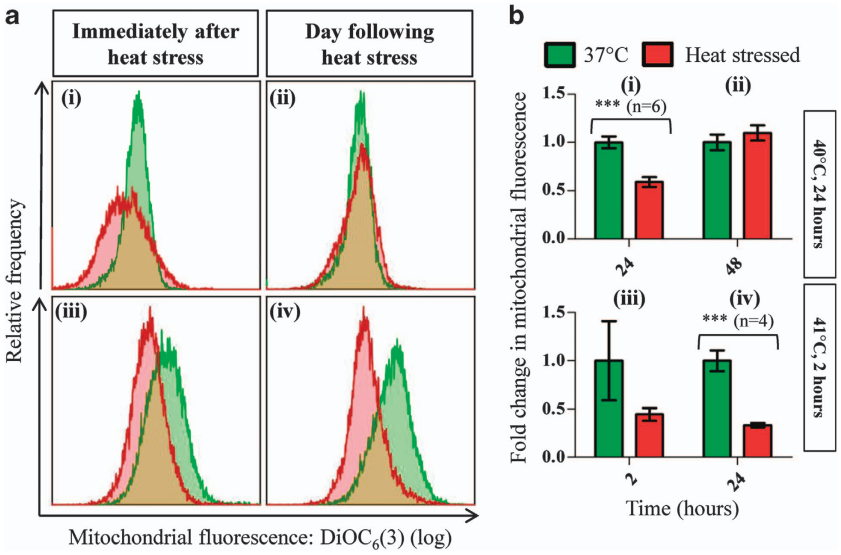

Figure 4 Heat stress induced mitochondrial depolarisation in early-stage parasites. Mitochondrial depolarisation was observed immediately following heat stress in early-stage parasites exposed to 40 °C for 24 h (**i**), although it was not noted the following day (**ii**). Early-stage parasites exposed to 41 °C for 2 h exhibited slight mitochondrial depolarisation immediately after heat stress (**iii**) that was significant the next day (**iv**). (**a**) Overlays of $DiOC_6(3)$ fluorescence histograms show representative merged data sets of at least two replicates for each time point and condition as an individual peak. A decrease in the median fluorescence of a population is indicated by a shift to the left (depolarisation). Fluorescence values were taken as the median fluorescence of individual populations. (**b**) Bar graphs indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.001 (***). Heat stress conditions are indicated on the right

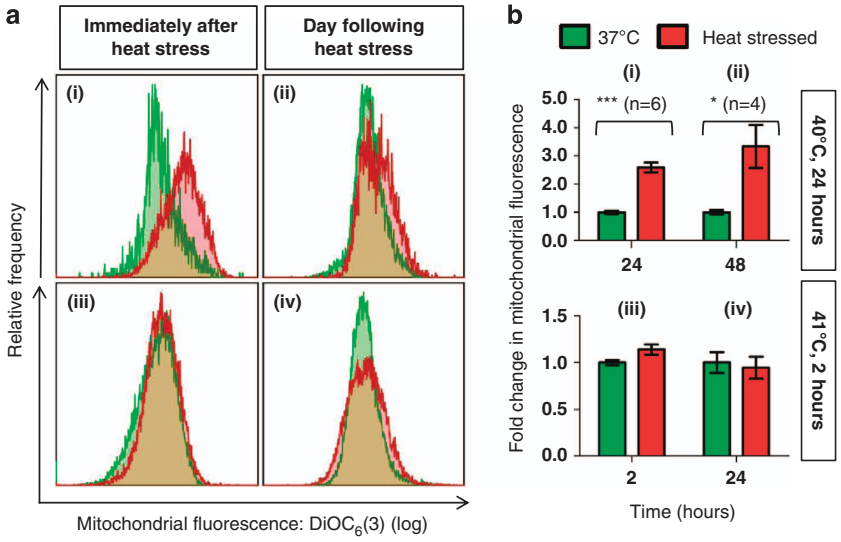

Figure 5 Heat stress-induced mitochondrial hyperpolarisation in late-stage parasites exposed to 40 °C for 24 h. Late-stage parasites exposed to 40 °C for 24 h showed mitochondrial hyperpolarisation after heat stress (**i**) and the following day (**ii**), whereas late-stage parasites exposed to 41 °C for 2 h showed practically no change in mitochondrial polarisation (**iii** and **iv**). (**a**) Overlays of $DiOC_6(3)$ fluorescence histograms show representative merged data sets of at least two replicates for each time point and condition as an individual peak. An increase in the median fluorescence of a population is indicated by a shift to the right (hyperpolarisation). Fluorescence values were taken as the median fluorescence of individual populations. (**b**) Bar graphs indicate the arithmetic mean of replicates ± S.E.M. Comparisons between control and heat-stressed parasites showed *P*-values that are significant at <0.05 (*) or <0.001 (***). Heat stress conditions are indicated on the right

least partly explain the conflicting conclusions of previous studies.[6,7] More significantly, however, our data suggested that the PCD phenotype depends on the intra-erythrocytic developmental stage. Early-stage parasites showed biochemical markers of PCD that were reminiscent of an apoptosis-like form of PCD. The phenotype observed in late-stage parasites, which exhibited a different combination of biochemical markers as well as cytoplasmic vacuolisation and continued survival after heat stress despite no replication, appeared similar to autophagy-like cell death.

Give and take: the potential mutual benefits of fever in malaria. Central to this study was the following question: who benefits most from fever during malarial illness – parasite or

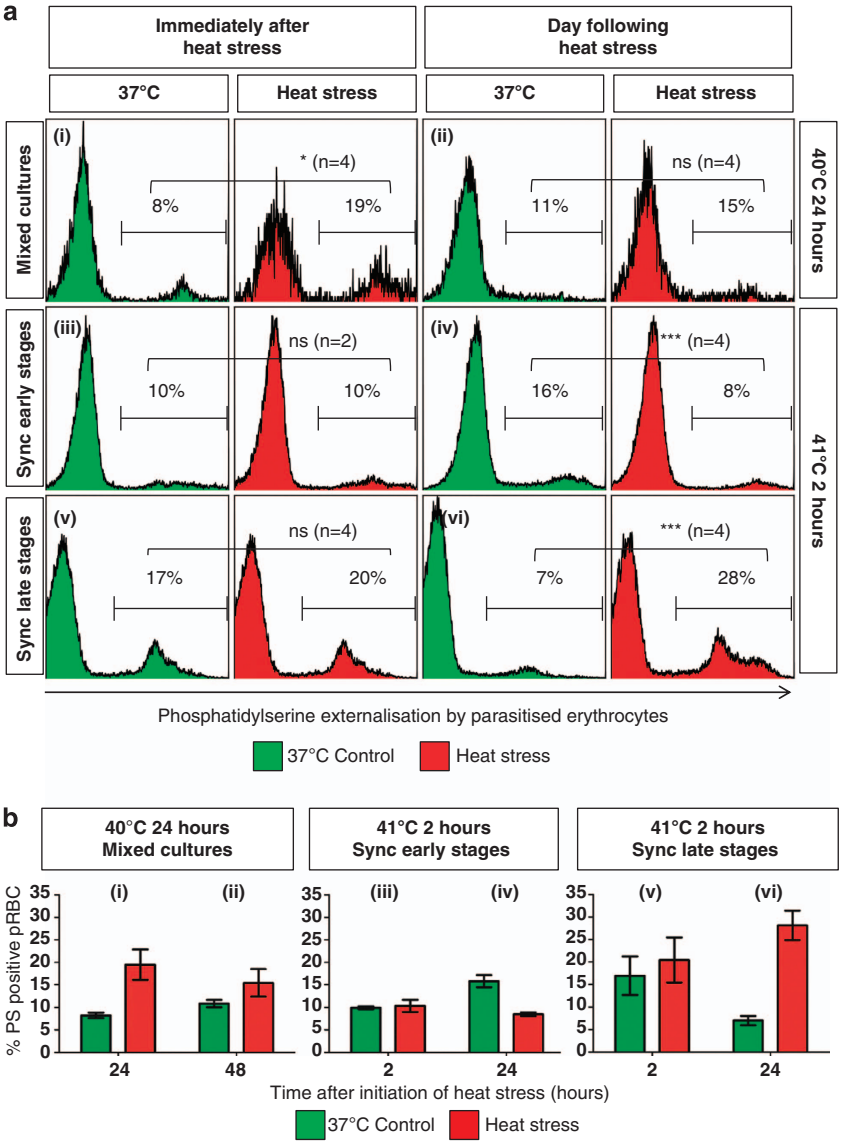

Figure 6 PS externalisation in pRBC exposed heat stress. (**a**) Fluorescence histograms and (**b**) bar graphs of pRBC showed an increase in PS externalisation by mixed-stage cultures immediately after exposure to 40 °C for 24 h (**i**) and was still apparent at 48 h (**ii**), although the latter difference was not statistically significant. No increase was noted in synchronised ring-stage pRBC immediately after exposure to 41 °C for 2 h (**iii**), with an apparent decrease noted at 48 h; however, it should be noted that the 37 °C control parasites used for comparison had developed to late stages, which typically have higher PS externalisation, even without stress. When comparing the heat-stressed culture at both time points (**iii** and **iv**), no change was apparent. Although PS externalisation was not seen in late-stage pRBC exposed to 41 °C for 2 h immediately after heat stress (**v**), an increase was observed a day later (**vi**). Representative histograms are merged data sets of at least two samples. Parasite stages are shown on the left as either mixed-stage cultures (Mixed) or synchronised (Sync) early- or late-stage cultures. Heat stress conditions are shown on the right. Gates on individual histograms indicate the percentage of infected erythrocytes that are PS positive. Comparisons between control and heat-stressed parasites have *P*-values that are significant at <0.05 (*), <0.001 (***) or not significant (ns)

host? On the one hand, fever may provide the host with a mechanism for parasite clearance.[5] Complete clearance would be most beneficial, but in this regard fever is relatively inefficient, as newly invaded parasites derived from the ruptured schizonts that caused the onset of fever are generally spared, even after high fever peaks.[23] It seems likely that parasites that survived exposure to 40 °C in this study were early-stage parasites. Our data showed that even after exposure to a high peak temperature of 41 °C, a significant number of ring-stage parasites and even some late-stage parasites survive, although a measurable increase in parasitaemia was only noted after several days of continuous culture. On the other hand, fever may provide a number of benefits to the parasite, including accelerated parasite maturation, as well as increased cytoadherence and reduced deformability of pRBC, thought to aid in the sequestration of pRBC to microvasculature.[9–12] With every asexual parasite capable of producing as many as 32 new merozoites at the end of a 48 h life cycle,[24] uncontrolled parasite replication would place the host at risk of death before the slow-forming gametocytes could be transmitted. A potential mutual benefit of exposure to increased

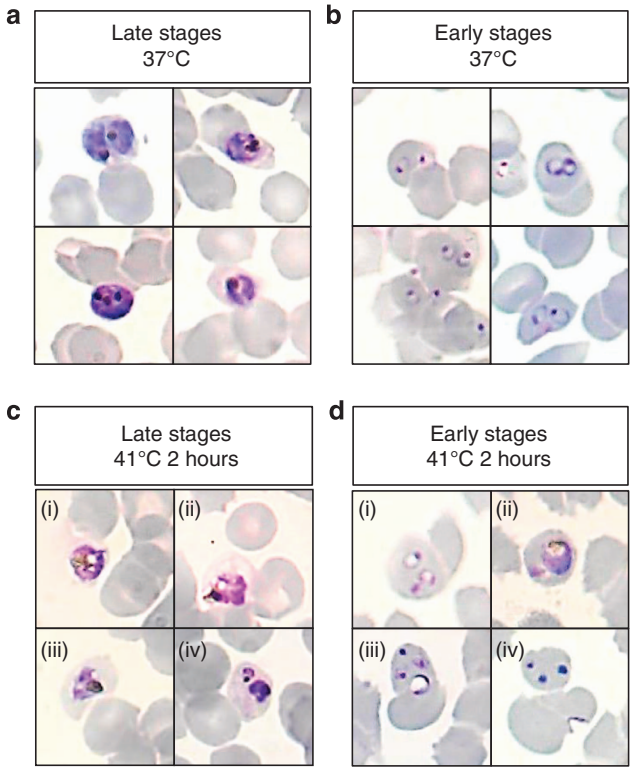

Figure 7 Heat stress induced abnormal morphology in *P. falciparum*. Control *P. falciparum* morphology showed trophozoites (**a**) that were large, full, well-rounded with clear and concentrated haemazoin and relatively even staining; and ring-stage parasites (**b**) that showed lightly-stained, delicate and rounded rings with single chromatin dots. In contrast, 24 h after exposure to 41 °C for 2 h, trophozoites (**c**) appeared unevenly stained with jagged borders and cytoplasmic vacuoles (**ci–iii**), or were condensed and very darkly stained (**civ**). Ring-stage parasites that were exposed to the same stress (**d**) showed variable morphology at 24 h. Some rings appeared slightly vacuolised (**di**) and remained as ring-stage parasites 24 h after exposure instead of progressing to trophozoites. Some morphologically normal trophozoites were also observed (**dii**). Many ring-stage parasites were abnormally large with a central vacuole (**diii**). Chromatin-like dots without any ring structure were also observed in many cells (**div**)

temperatures therefore lies in the induction of PCD: by providing the parasite with the means to self-limitation, the burden of infection on the host is reduced, although sufficient parasites are conserved for continued sublethal infection.[23,25] Our data suggest that intra-erythrocytic *P. falciparum* is likely to undergo PCD in response to heat stress.

PCD markers varied with life stages: different pieces, same puzzle? *P. falciparum* early and late developmental stages exhibited some intriguing differences in their response to the same stress conditions. Although ring-stage parasites exposed to 40 °C for 24 h exhibited the depolarisation that is expected to result from the permeabilisation of the outer mitochondrial membrane during apoptosis,[13] late-stage parasites exposed to the same stress surprisingly exhibited mitochondrial hyperpolarisation. Mitochondrial hyperpolarisation has been observed in protozoa other than *Plasmodium*. In heat-stressed *Leishmania*, hyperpolarisation formed an early part of an apoptosis-like cell death and was correlated with increased mitochondrial respiration, which drove superoxide radical production, in turn causing oxidative damage that mediated cell death.[26] However, during apoptosis-like cell death in *Trypansoma*, mitochondrial hyperpolarisation was associated with decreased mitochondrial respiration.[27] Both mitochondrial depolarisation and hyperpolarisation may be apoptosis responses in trypanosomatids.[28] In metazoans, a transient increase in the $\Delta\Psi m$ is thought to serve as a key checkpoint in determining cell fate,[27] and is correlated with increased production and accumulation of reactive oxygen species (ROS) by the mitochondria during apoptosis.[17,18] However, mitochondrial hyperpolarisation and increased ROS generation have also been implicated in the priming and occurrence of necrosis.[15,29,30] In *P. falciparum*, we observed continued mitochondrial hyperpolarisation of late-stage parasites after 24 h exposure to 40 °C and it is not entirely clear whether this marker is indicative of PCD or perhaps a necrosis-like form of cell death. Furthermore, the overall role of the mitochondrion in PCD in asexual *P. falciparum* may be questioned. Asexual stages of *P. falciparum* contain only a single, minimally active mitochondrion that functions primarily in *de novo* pyrimidine synthesis.[31]

We also observed differences between the PS externalisation of early- and late-stage pRBC exposed to 41 °C. In accordance with a previous study,[10] late-stage pRBC showed increased PS externalisation after exposure to heat stress. However, no change was observed in early-stage pRBC. During apoptosis, loss of plasma membrane asymmetry results in PS externalisation, which is often used as a marker of apoptosis and is thought to serve as a signal in mammalian cells for phagocytes to engulf and digest cellular remnants.[32] Would PS externalisation by pRBC be an advantage or disadvantage to the parasite residing within? Increased PS externalisation, observed both during parasite maturation[10,33,34] and after stress by febrile temperatures,[10] has been suggested to aid in the cytoadherence and sequestration of pRBC in microvasculature, thereby protecting parasites from splenic trapping and destruction.[34,35] In this study, PS externalisation was only observed in late-stage pRBC exposed to 41 °C a day after stress was withdrawn. PS externalisation also occurs during erythrocyte apoptosis,[36,37] and oxidative stress from *P. falciparum* infection has been shown to induce host cell apoptosis[38] and has been proposed to be the cause of PS externalisation in pRBC.[10] In this light, it is possible that increased PS externalisation by late-stage pRBC may simply be a by-product of oxidative stress from parasites.

Although biochemical markers of PCD are preferred over morphological criteria,[15] the assessment of autophagy is largely based on morphology.[39] Therefore, in light of the dissimilar responses of early- and late-stage parasites exposed to 41 °C, particularly the relative lack of biochemical markers of PCD exhibited by late-stage parasites despite a significant effect on parasite growth, morphological studies of both Giemsa-stained fixed slides and unstained, wet-mounted samples were also considered for parasites exposed to 41 °C.

Cytoplasmic vacuolisation was observed 1 day after heat stress for several subsequent days in late-stage parasites exposed to 41 °C for 2 h. Real-time microscopy showed that a

significant number of late-stage parasites exposed to 41 °C showed rapid movement within the food vacuole for several days after exposure to heat stress. Such movement is characteristic of healthy parasites[21] and has been shown to be decreased by the antimalarial drug artesunate, which also caused the appearance of 'pyknotic' parasites.[21,40] It has been suggested that loss of movement in the food vacuole is a very early indicator of an adverse parasite response to external stimuli that precedes other morphological changes.[40] Despite showing a significant number of parasites that were apparently still alive, parasites did not form new ring-stage parasites as would be expected of healthy parasites (Figure 2) and the appearance of new ring-stage parasites and increasing parasitaemia was delayed by several days (Supplementary Figure 1). The eventual recovery of parasitaemia and the formation of new ring-stage parasites indicate that at least a few synchronised late-stage parasites survived exposure to 41 °C. The appearance of cytoplasmic vacuolisation, along with the lack of growth exhibited by apparently living parasites, is suggestive of an autophagy-like form of PCD. Similar to Totino *et al.*,[41] who observed autophagy-like cell death in drug-treated *P. falciparum*, we also observed very little DNA fragmentation by the TUNEL assay, which was deemed not to be significant. 'Crisis form' morphology was also reported by Oakley *et al.*[7] under heat stress conditions similar to those employed by the present study.

Autophagy is a process of sequestration and subsequent degradation of cytoplasmic components that is regulated primarily by the sequential action of several autophagy-related (Atg) proteins. *Plasmodium* genomes encode a number of putative Atg orthologues,[42] although the most well-studied of these, the ubiquitin-like Atg8, has been shown to localise to the apicoplast, rather than the cytoplasm, in *P. falciparum*.[43] Therefore, it is unclear whether the *P. falciparum* proteome encompasses the tools required to undergo autophagy-like cell death, with very few observations of the phenomenon reported. However, it has been suggested that the sequestration of micronemes and rhoptries in double-membrane structures during the differentiation of sporozoites to trophozoites in hepatocytes may represent a type of functional autophagy in malaria parasites.[42] It is also not clear whether autophagy 'in dying cells is the cause of death or actually an attempt to prevent it',[39] particularly as autophagy often inhibits the induction of apoptosis or protects cells from exposure to apoptotic stimuli.[44] Our own observations reflect this conflicting nature: the slow decrease of living parasites observed by real-time microscopy suggests that parasites are undergoing cell death; however, the eventual recovery of parasitaemia suggests that at least some parasites survive this process. It has been suggested that apoptosis and autophagy-like cell death are forms of PCD that may share some common regulatory proteins and the two may be induced simultaneously.[44] We observed that the same stimulus that induced an apoptosis-like form of PCD in ring-stage parasites also induced autophagy-like cell death in late-stage parasites.

Apples and pears: the problem of identifying metazoan PCD phenotypes in *P. falciparum*. Despite mounting evidence suggesting that *P. falciparum* undergoes PCD in response to heat stress, the exact phenotype of cell death remains undetermined. Apoptosis and autophagy are recognised phenotypes of PCD, whereas necrosis is considered a non-PCD phenotype of cell death,[15] although some active form of necrosis might also be possible.[45] However, overlap between phenotypes may occur and many markers, including DNA fragmentation and mitochondrial depolarisation, may also occur during necrosis.[14] Our data provided evidence suggesting that the asexual blood stages of *P. falciparum* may be capable of undergoing both apoptosis-like and autophagy-like forms of PCD. However, *P. falciparum* may exhibit a unique PCD phenotype that could include such phenomena as both mitochondrial hyperpolarisation and depolarisation—observations that were attributed to apoptosis in other protozoans.[29] The origin of PCD may precede that of multicellularity[46] and thus with an organism as phylogenetically ancient and evolutionary distant from other taxa as *P. falciparum*,[47] attempting to impose metazoan PCD phenotypes may be misleading.

Conclusion

Intra-erythrocytic *P. falciparum* exhibited markers of an apoptosis-like PCD phenotype in ring-stage parasites and an autophagy-like cell death phenotype in late-stage parasites in response to heat stress. However, whether these are truly different phenotypes or simply different facets of a PCD phenotype unique to *P. falciparum* and distinct from metazoan apoptosis remains unclear. Elucidation of the underlying protein machinery responsible for the execution of PCD in *P. falciparum* will provide insight into the working of active cell death and the complex interactions between parasite and host.

Materials and Methods

Reagents. The APO-DIRECT TUNEL kit and FITC Annexin V Apoptosis Detection Kit II were obtained from Becton Dickinson (BD Pharmingen, San Diego, CA, USA). Thiazole orange (TO), HE, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumax II was obtained from Gibco (Gran Island, NY, USA).

***P. falciparum* culture.** The 3D7 strain of *P. falciparum* was maintained according to established methods,[48] with some modifications.[49] Briefly, parasites were maintained in malaria culture medium (MCM: RPMI 1640, 0.5% Albumax II and 0.21% sodium bicarbonate, supplemented with 50 mg/l gentamycin and 50 mg/l hypoxanthine) at 5% haematocrit in donor erythrocytes. MCM was changed daily. Optimal culture pH was maintained by daily gassing with a gas mixture of 2% O_2, 5% CO_2 and 93% N_2. Giemsa-stained smears were made daily to monitor parasite morphology. For studies involving synchronised cultures, parasite cultures were pelleted by centrifugation for 5 min at 1000 × *g* and 25 °C and the cell pellet incubated in 10 vol of 5% D-sorbitol for 5–10 min at 37 °C. Centrifugation was repeated and the cell pellet was resuspended to 5% haematocrit with MCM and returned to culture. This method resulted in synchronisation of parasites at the ring stage, similar to previously described.[50]

Heat treatment. Parasitised erythrocytes were seeded as 5 ml cultures at 1–5% parasitaemia in 25 cm^3 flasks. Control parasite cultures were maintained at 37 °C. Mixed-stage cultures were exposed to 40 °C for either 6 or 24 h and then maintained at 37 °C. Synchronised cultures were exposed to 41 °C for 2 h, and then maintained at 37 °C. Unless stated otherwise, samples for assays were collected immediately after heat stress as well as the following day.

Flow cytometry. Flow cytometric analyses were performed on a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Excitation for all assays was by 488 nm blue laser. Emission was detected with the use of 545/40BP (525 ± 20 nm, FL1) and, where indicated, 575/30BP (575 ± 15 nm, FL2) filters. Optical alignment was monitored daily with Beckman Coulter Flow Check Pro fluorospheres (Beckman Coulter Inc., Brea, CA, USA). Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

TO flow cytometry for parasitaemia. Parasitaemia was measured daily by flow cytometry with the DNA-binding dye TO, similar to a previous method.[51] Whole-culture samples (10 μl) were diluted 100-fold to 1 ml in Sorenson's phosphate buffer (47 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.2) with 1 μM TO final concentration (diluted from a 10 mM stock in methanol) and incubated at room temperature in the dark for 20 min. Stained cells were analysed by flow cytometry within 1 h. Erythrocytes were gated on a forward- *versus* side-scatter dot plot and analysed on a FL1 integral (log) histogram, with regions for uninfected, ring-infected and trophozoite- or schizont-infected erythrocytes delineated. Approximately 50 000 events in the erythrocyte gate were counted. Regions had previously been confirmed by microscopy of Giemsa-stained smears of synchronised cultures.

TUNEL assay for DNA fragmentation. The TUNEL assay was performed according to manufacturer's recommendations, with modifications similar to a previous study.[7] Briefly, *P. falciparum* cultures were centrifuged and the cell pellets fixed on ice for 60–90 min in 4% formaldehyde and phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), followed by permeabilisation with 0.1% tri-sodium citrate (w/v) and 0.1% Triton X-100 (v/v) in PBS for 3 min on ice. Labelling with DNA-staining solution (including TdT enzyme and FITC-dUTP) was performed according to the manufacturer's recommendations for 60–90 min at 37 °C, followed by staining with propidium iodide (PI) for 30 min at room temperature. Labelled cells were analysed by flow cytometry within 3 h. PI-positive parasites were acquired on a FL2 time-of-flight (lin) *versus* FL2 integral (lin) dot plot, with gated parasites analysed on a FL1 integral (log) histogram for DNA fragmentation, measured as FITC-dUTP fluorescence. At least 10 000 PI-positive events were counted. DNase-treated, non-treated and unlabelled parasites were used as positive, negative and staining controls.

DiOC6(3) flow cytometry for $\Delta\Psi$m. *P. falciparum* cultures were diluted 20-fold in PBS, stained with 10 nM $DiOC_6(3)$ (diluted from a 100 mM stock in DMSO) and 50 μM HE (diluted from a 10 mM stock in DMSO) and incubated at 37 °C for 45 min in the dark. Following incubation, cells were washed and suspended in 1 ml PBS and analysed by flow cytometry immediately. Erythrocytes were gated on a forward- *versus* side-scatter dot blot. HE-positive pRBC counted on a FL2 integral (log) histogram were analysed for $DiOC_6(3)$ fluorescence on a FL1 integral (log) histogram. At least 50 000 events in the erythrocyte gate were counted. Positive controls were treated with 200 nM CCCP for 1 h before staining. Unstained cells, cells stained with only HE or $DiOC_6(3)$ and non-parasitised erythrocytes were used as staining controls. Mitochondrial depolarisation by the uncoupling agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, data not shown) confirmed the integrity of the assay.

Annexin V-FITC for PS externalisation. *P. falciparum* cultures were diluted 50-fold in PBS and stained sequentially with a final concentration of 50 μM HE (diluted from a 10 mM stock in DMSO) in PBS for 15 min at 37 °C in the dark and annexin V-FITC in 1X annexin-binding buffer (provided with the kit), according to the manufacturer's recommendations with modifications similar to a previous study,[10] for 15 min at room temperature in the dark. Stained cells were diluted to 1 ml in 1X annexin-binding buffer and analysed by flow cytometry within 1 h. Erythrocytes were gated on a forward- *versus* side-scatter dot blot and pRBC were discriminated on a FL 2 integral (log) histogram for HE fluorescence. Gated pRBC were analysed for annexin V-FITC fluorescence on a FL1 integral (log) histogram. At least 50 000 events in the erythrocyte gate were counted. Parasitised cultures treated with recombinant annexin V before staining were used as a negative control and unstained parasite cultures and parasite cultures stained with only HE or annexin V-FITC were used as staining controls.

Real-time microscopy. Samples of 41 °C heat-stressed and 37 °C control parasitised cultures were wet-mounted by placing 5 μl sample on a glass slide and covering the sample with a coverslip. Samples were observed with an Olympus BX41 microscope under bright field and × 1000 magnification with an immersion oil type objective lens, at room temperature, with a halogen light source. Real-time microscopy was performed immediately after heat stress and for 5 subsequent days. Live images were captured with an Olympus XC50 digital camera. Parasites observed with rapid movement within the food vacuole were considered to be alive.[21]

Statistical analysis. Bar graphs were compiled using GraphPad Prism 5, with raw data values exported from analyses by Beckman Coulter Kaluza (v.1.1) software. Student's unpaired *t*-tests were performed with Microsoft Office Excel 2010 to test for significance between treated and control groups.

Conflict of Interest

The authors declare no conflict of interest.

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6 INTRODUCTION TO MANUSCRIPT 1

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Mob mentality: programmed cell death in *Plasmodium falciparum* at high population density

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Summary

During the asexual intra-erythrocytic cycle of *P. falciparum*, parasitaemia increases rapidly due to every mature dividing parasite giving rise to around 20 new parasites with every 48 hour cycle. This exponential growth may threaten the host survival long before slow-maturing sexual parasites could be transmitted. Therefore, this presents the ideal setting for the parasite to exercise self-limitation by inducing PCD in a portion of the parasite population. This study shows that *in vitro P. falciparum* undergoes an apoptosis-like form of PCD when left to replicate freely to a natural peak parasitaemia.

Mob mentality: programmed cell death in *Plasmodium falciparum* at high population density

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Running title: Peak parasitaemia triggers apoptosis in *P. falciparum*

Keywords: programmed cell death; apoptosis; *Plasmodium falciparum*; peak parasitaemia; DNA fragmentation; mitochondrial depolarisation; phosphatidylserine externalisation

Abstract

The asexual erythrocytic cycle of the protozoan parasite *Plasmodium falciparum* is responsible for the pathogenesis of malaria and causes the overwhelming majority of malaria deaths. Rapidly increasing parasitaemia during this 48 hour cycle threatens the survival of the human host and the parasite prior to transmission of the slow-maturing sexual stages to the mosquito host. The parasite may utilise programmed cell death (PCD) to achieve self-limitation in response to growing parasitaemia to aid its own survival and transmission. The occurrence of PCD in *P. falciparum* remains a controversial topic. We provide strong evidence for the occurrence of an apoptosis-like phenotype of PCD in *P. falciparum* under conditions of high parasite density. *P. falciparum* was maintained *in vitro* and stressed by allowing growth to an unrestricted peak parasitaemia. At peak parasitaemia, mitochondrial depolarisation was observed, together with phosphatidylserine externalisation in both parasitised- and neighbouring non-infected erythrocytes. DNA fragmentation coincided with a decline in parasitaemia. Growth recovery to near-peak parasitaemia was noted within 2 intraerythrocytic cycles. The combination and chronological order of the biochemical markers of cell death suggest the occurrence of apoptosis. The identification of a PCD pathway in *P. falciparum* may provide novel drug targets, particularly if the pathway differs from the host machinery.

Introduction

The asexual erythrocytic cycle of the protozoan parasite *Plasmodium falciparum* is responsible for the pathogenesis of malaria. With every mature schizont-infected erythrocyte capable of releasing approximately 20 new merozoites every 48 hours [1], a rapid increase in parasitaemia threatens to overwhelm the host prior to transmitting the slow-maturing gametocytes to the female *Anopheles* mosquito vector [1]. By controlling its own population density, the parasite may be capable of reducing the burden of infection on the host, thereby benefitting its own survival and transmission [2–4]. A number of mechanisms of self-limitation have been proposed, including: (i) increasing the rate of conversion to gametocytes, thereby relocating resources from reproduction to transmission; (ii) limiting the number of merozoites released per mature schizont, (iii) altering the invasion capabilities of merozoites; (iv) regulating population synchronicity and development time; or (v) altering the rate of cell death [2,4]. Programmed cell death (PCD), may offer the most effective mechanism of self-limitation by controlling parasite cell death independent of the host immune system [2].

Although an ever-proliferating body of evidence suggests that PCD does occur in *P. falciparum*, the phenotype remains controversial. Several studies have reported the occurrence of an apoptotic or apoptosis-like phenotype [2,5–11], an autophagy-like phenotype [12], necrosis [13], or simply a non-descript phenotype [8,9,12,14]. Some overlap between phenotypes has also been noted. Differences may be partly explained by the diverse combinations of strains, stimuli and biochemical markers used to characterise cell death [15]. Biochemical markers of

metazoan PCD have been extrapolated to protozoa, however, PCD may involve features of multiple cell death pathways [16], or may manifest as a unique pathway in protozoans, which may offer novel drug targets.

Although studies have generally focused on drug pressure, several natural stimuli encountered by *P. falciparum* during malaria illness have been proposed to induce PCD in the parasite, including heat stress similar to febrile episodes [5,13,17] and starvation [2]. However, only one other study has specifically addressed the occurrence of PCD under self-limiting population densities [11]. The present study confirms and expands those findings and shows the appearance of biochemical markers that support the occurrence of an apoptosis-like phenotype of PCD under the stress of high parasitaemia. Results indicated a permanent collapse of the mitochondrial transmembrane potential ($\Delta\Psi_m$) at peak parasitaemia, followed by extensive DNA fragmentation and a decline in parasitaemia. At peak parasitaemia, infected erythrocytes exhibited a large increase in phosphatidylserine (PS) externalisation, which dissipated somewhat with declining parasitaemia. Interestingly, neighbouring non-infected red blood cells (nRBC) consistently showed an increase in PS externalisation compared to parasitised cultures maintained at sub-peak parasitaemia. The specific combination of biochemical markers of cell death, as well as their order of appearance, provides strong evidence for an apoptosis-like phenotype of PCD.

Results

Self-limitation of P. falciparum at high population density

The growth and development of parasites were monitored daily by thiazole orange (TO) flow cytometry, with a difference in DNA content allowing discrimination between ring- and trophozoite- or schizont-infected erythrocytes [18].

Growth and development of asynchronous cultures between 0 and 24 hours showed a population of rings and trophozoites/schizonts that progressed to trophozoites/schizonts and newly invaded rings, respectively, the latter with multiplication in parasite numbers (**Fig 1 Ai and ii**). The growth rate of parasites maintained at low parasitaemia was ~4.6-fold over a single 48 hour cycle (data not shown). Following peak parasitaemia of ~9.5% at 24 hours (**Fig 1Aii and B**), parasitaemia in experimental cultures declined over the next 1.5 cycles to ~7.5% at 96 hours (**Fig 1 B**). Between 72 and 96 hours, a decrease in the percentage ring stage parasitised red blood cells (pRBC) along with an increase in trophozoite/schizont-stage pRBC could be seen, indicating growth recovery. Between 96 and 120 hours, a decrease in trophozoite/schizont pRBC was associated with a marked increase in new ring stage pRBC and total parasitaemia (**Fig. 1 B**).

High population density causes DNA fragmentation in P. falciparum

DNA fragmentation is a hallmark of cell death, both with PCD and non-PCD forms of cell death [16]. The flow cytometric TUNEL assay was used to quantify DNA

fragmentation. At peak parasitaemia, noted at 24 hours, no DNA fragmentation was detected in experimental cultures when compared to control cultures (**Fig 2 A & B, 24 hours**). The day after peak parasitaemia, substantial DNA fragmentation was observed (**Fig 2 A & B, 48 hours**) that coincided with a decline in total parasitaemia (**Fig 1 B**). The appearance of two discreet, albeit overlapping, peaks on flow cytometry histograms (**Fig 2 A, 48 hours**) indicates that only a portion of the population exhibited DNA fragmentation.

High population density causes early and permanent mitochondrial depolarisation in P. falciparum

Changes in the $\Delta\Psi_m$ were assayed as an indicator of PCD by measuring the accumulation of the cell-permeable cationic fluorescent dye 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], detected by flow cytometry. Changes in $\Psi\Delta_m$ are often considered unique to apoptosis, but may also occur during early necrosis [16,19,20]. Significant mitochondrial depolarisation occurred at peak parasitaemia (**Fig 3 B, 24 hours**), as indicated by a decreased median $DiOC_6(3)$ fluorescence (**Fig 3 A, 24 hours**). Mitochondrial depolarisation remained apparent at 48 hours (**Fig 3 A & B, 48 hours**), after parasitaemia had declined. Crucially, the manifestation of permanent mitochondrial depolarisation chronologically preceded DNA fragmentation and the decline in parasitaemia.

High population density causes PS externalisation in both pRBC and nRBC

Loss of membrane asymmetry leading to the externalisation of PS on the outer membrane leaflet is a common marker of apoptosis in mammalian cells, although it has also been utilised in unicellular lineages [21]. PS externalisation was monitored on the outer leaflets of pRBC as well as neighbouring nRBC. A significant increase in PS externalisation occurred at peak parasitaemia in both pRBC (**Fig 4 Aii & Bii**) and neighbouring nRBC (**Fig 4 Avi & Bvi**), compared to control cultures (**Fig 4 Ai & Bi, Av & Bv, respectively**). At 48 hours, after peak parasitaemia and with the first measurements of population decline, no significant difference in PS externalisation of pRBC was observed (**Fig 4 Aiv & Biv**) compared to control cultures (**Fig 4 Aiii & Biii**). Although data suggested PS externalisation in nRBC was higher at 48 hours (**Fig 4 Aviii & Bviii**) when compared to control cultures (**Fig 4 Avii & Bvii**), the t-test p-value of 0.057 was not statistically significant.

Discussion

Parasite culture “crash” due to excessively high parasitaemia is a well-known phenomenon amongst those who have cultured *P. falciparum in vitro*. The resultant decline in parasitaemia and altered growth may represent a self-preservation mechanism of the parasite, rather than simple random demise due to limited resources. By limiting and sustaining its population growth at a level that is sub-lethal to its host, the parasite may prolong its own transmission time. Altering the rate of cell death may provide the most efficient method of self-limitation [2]. It

is generally postulated that PCD in *P. falciparum* developed by group-level selection, with closely-related kin benefiting from the controlled demise of some members of the same population [2].

Growth characteristics at self-limiting population density

Using asynchronous cultures, experimental cultures showed ring stage parasites that progressed to late stages until peak parasitaemia was achieved. Following a decline in parasitaemia the day after peak parasitaemia, the majority of the remaining parasites were detected as ring-stage parasites by TO flow cytometry. It has been previously demonstrated that the detection of apparent ring stage parasites with a nucleic acid-binding dye following a parasitaemia "crash" may be misleading, as dead parasites that remain within erythrocytes and contain nucleic acid matter may overestimate parasite numbers [22]. However, as significant growth recovery was noted within the relatively short time of 1.5 asexual cycles, a considerable number of parasites must have survived and continued to develop.

Growth recovery was observed as a simultaneous decrease in ring stage parasites and increase in trophozoite/schizont stage parasites between 72 and 96 hours, followed by increased new ring stage parasites and total parasitaemia at 120 hours (**Fig. 1 B**). Late stage parasites (trophozoites and schizonts) at 96 hours, that divided to form new rings at 120 hours and were thus responsible for the increase in parasitaemia, showed a very low replication rate, with only ~1.5-fold multiplication, compared to ~4.6-fold observed in control cultures (data not shown). Thus, it seems that despite growth recovery, self-limitation remains in effect while

the population density is near peak parasitaemia. However, it is unclear whether the low multiplication resulted from fewer merozoites or whether the invasion efficiency of merozoites was somehow restricted, for instance through the formation of non-viable merozoites. Both explanations could account for self-limitation [2,4]. It is also uncertain whether surviving parasites may have experienced some form of growth stagnation in the time between peak parasitaemia and growth recovery, thus explaining the slow decline in parasitaemia before resurgence was noted.

Late manifestation of DNA fragmentation suggests an active cell death process

DNA fragmentation is considered a characteristic feature of PCD and non-PCD cell death phenotypes [16] and TUNEL-positive results could be indicative of apoptosis, autophagy or necrosis [23–26]. Crucially however, DNA fragmentation was not manifested at peak parasitaemia, but only observed 24 hours later. The late occurrence of DNA fragmentation, especially when considered holistically with other markers such as mitochondrial depolarisation (discussed below), suggests that it was indeed the end product of an active death process.

Crucially, a significant portion of the parasite population in experimental cultures had no DNA fragmentation, indicated by a discreet peak with low TUNEL fluorescence, supporting the occurrence of PCD, which would require some members of the population to survive and benefit from the process.

Chronology matters: mitochondrial depolarisation precedes DNA fragmentation in the cell death process

Mitochondria play an essential role in the occurrence of PCD. During apoptosis, loss of $\Delta\Psi_m$ precedes complete permeabilisation of the mitochondrial membrane, in turn causing the release of pro-apoptotic proteins such as cytochrome c from the mitochondria [16,19,27,28], that ends in the activation of caspases and DNA degradation [29–32]. Our data showed mitochondrial depolarisation starting at peak parasitaemia, indicating that this was an early step in the cell death process. Crucially mitochondrial depolarisation preceded DNA fragmentation in the execution of parasite PCD. However, it is unclear whether the loss of $\Delta\Psi_m$ causes downstream DNA fragmentation as would be the case in apoptosis. Such a model of apoptosis has been proposed in *P. falciparum* [9], although others have suggested that early and permanent loss of $\Delta\Psi_m$ may fit better with secondary necrosis [13]. It is worth noting that *P. falciparum* has no known caspases [2]. Plant-like metacaspases have been identified in *P. falciparum* and suggested to be involved in parasite PCD [7,33,34], however the function of metacaspases in cell death remains a matter of debate [35,36].

PS externalisation coinciding with peak parasitaemia indicates the onset of stress

PS is normally maintained nearly exclusively within the inner leaflet of viable erythrocytes [37]. Loss of membrane asymmetry leading to PS externalisation occurs during various red blood cell membrane disorders and senescence [37]. In mammalian cells, PS externalisation is often used as a characteristic marker of

apoptosis and is thought to serve as an “eat-me” signal to phagocytic cells to engulf cellular remains [38,39]. In *P. falciparum*, PS externalisation has been observed both during parasite maturation [40–42] and after exposure to heat stress [17,40]. Erythrocyte PS externalisation during malaria has been attributed to the activation of a non-selective Ca^{2+}-permeable cation channel that is essential for intracellular growth of the pathogen, through oxidative stress generated by the parasite [43,44]. PS externalisation on the surface of pRBC may contribute to the cytoadherance of infected cells [45,46] , although it has also been postulated to serve as a host defense mechanism to signal the destruction of infected cells by phagocytosis prior to the release of merozoites [43,44,47].

Interestingly, PS externalisation was only noted at peak parasitaemia and in both pRBC and neighbouring nRBC. In fact, PS externalisation appeared high in nRBC even after a decline in parasitaemia was evident, but this was not statistically significant. Apoptosis in nRBC, with concurrent PS externalisation has been shown to be a causative agent of anaemia during malaria [48]. Simultaneous PS externalisation in both pRBC and neighbouring nRBC was likely caused by a massive release of toxic haemozoin or other reactive oxygen species (ROS) from degraded parasites inflicting oxidative damage, known to cause apoptosis-like death in erythrocytes [49]. In this case, the daily replacement of medium would remove ROS from dead or dying parasites, thus explaining the observed normalisation of PS externalisation after peak parasitaemia.

PS externalisation during stress induced by high parasitaemia may benefit *P. falciparum* either by promoting clearance of parasites undergoing PCD, or by

aiding cytoadherance of infected cells. It may also represent a host defense mechanism, or simply a by-product of oxidative stress. Of course, these causes are not mutually-exclusive. It is interesting to note that the majority of falciparum malaria patients are positive for anti-phospholipid antibodies, which was posited to represent a response against PS-expressing cells [50], suggesting that the host immune system has at least some involvement with cells externalising PS. It seems likely that oxidative stress is the initial trigger, and that a host immune response follows.

Multiple possible cell death pathways?

We have demonstrated *P. falciparum* self-limitation manifesting as parasite cell death characterised by mitochondrial depolarisation coinciding with PS externalisation, but preceding DNA fragmentation. Our data support the findings of another study, which used synchronous Dd2 parasites and reported DNA fragmentation measured by the TUNEL assay, loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the expression of a metacaspase gene demonstrated by RT-qPCR [11]. We observed higher levels of DNA fragmentation in both stressed and control parasites, as well as a higher peak parasitaemia. Differences could be attributed to different strains used or subtle differences in methodology.

We have elsewhere demonstrated parasite cell death in heat-stressed *P. falciparum* that was characterised by mitochondrial depolarisation and DNA fragmenation in ring stage parasites, but hyperpolarisation and cytoplasmic

vacuolisation in trophozoite and schizont stage parasites [17]. The manifestation of cell death markers that differs with the type of stress stimulus, despite being monitored with the same methods and using the same parasite strain, suggests that *P. falciparum* may exhibit either multiple active cell death mechanisms, or different facets of a unique cell death phenotype under various conditions.

The conundrum of self-limitation: communication between unicells

In multicellular organisms, the adaptive advantages of PCD are easily understood, where the sacrifice of some cells contributes to the development and maintenance of a higher-level organism (reviewed in [51]). However, the idea of a suicidal mechanism is difficult to reconcile with a unicellular organism such as *P. falciparum* (reviewed in [51]). It is generally argued that PCD in *P. falciparum* may have developed by group-level selection [2]. This raises the question of how individual parasites are triggered to undergo PCD [15], and poses the intriguing possibility of a communication mechanism between parasites in a clonal population, similar to quorum sensing in bacteria [52]. Quorum sensing results in the regulation of gene expression in response to increasing population density in bacteria, allowing a population of unicellular organisms to function similarly to a multicellular organism (reviewed in [53]). Signalling molecules involved may be small organic molecules, such as homoserine lactones, or small oligopeptides (reviewed in [53]). The elucidation of similar molecules responsible for within-population communication in *P. falciparum* would greatly advance our understanding of PCD in unicellular parasites.

Conclusion

In vitro P. falciparum exhibited self-limitation of parasite numbers at high parasitaemia that manifested with mitochondrial depolarisation followed by DNA fragmentation, as well as PS externalisation in both pRBC and nRBC. The combination and chronology of these markers suggest that *P. falciparum* undergoes an apoptosis-like form of PCD to control its own parasite density. These findings shed light on an important and poorly understood feature of *P. falciparum* biology and highlight unique aspects of parasite PCD.

Materials and Methods

Reagents

The APO-DIRECT TUNEL kit and FITC Annexin V Apoptosis Detection Kit II were obtained from Becton Dickinson (BD Pharmingen, San Diego, CA, USA). TO, hydroethidine (HE), $DiOC_6(3)$, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumax II was obtained from Gibco (Gran Island, NY, USA).

P. falciparum *culture*

The 3D7 strain of *P. falciparum* was maintained asynchronously according to established methods [54] with some modifications [55]. Briefly, parasites were maintained in complete culture medium (RPMI, 0.5% Albumax and 0.21 % sodium

bicarbonate, supplemented with 50mg/L gentamycin and 50mg/L hypoxanthine) at 5% haematocrit in washed donor erythrocytes. Cultures were seeded as 5 ml cultures in 25 cm^2 sealed culture flasks. Complete culture medium was changed daily and cultures were maintained continually at 37^oC in the dark. Optimal pH was maintained by daily gassing with a mixture of 2% O_2, 5% CO_2 and 93% N_2 for 1 min per flask, at 2.5 L/min flow rate. Parallel to parasitised cultures, non-infected erythrocytes taken from the same erythrocyte stock were maintained under identical conditions. Giemsa-stained smears were made daily to monitor parasite morphology.

Achieving in vitro self-limitation

Asynchronous cultures were seeded from the same stock at different starting parasitaemia levels, with experimental cultures designated to undergo high population stress seeded above 5%, while non-stressed control cultures were diluted with erythrocytes to a parasitaemia of ~1.5%. Samples of 1.5 ml were taken from each culture at 24 and 48 hours. Duplicate experimental and control cultures were maintained for the assessment of biochemical markers of cell death.

Flow Cytometry

Flow cytometric analyses were performed on a Beckman Coulter FC500 flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Excitation for all assays was by 488 nm argon laser. Emission was detected with the use of 545/40BP (525±20 nm, FL1) and, where indicated, 575/30BP (575±15 nm, FL2) filters. Optical

alignment was monitored daily with Beckman Coulter Flow Check Pro fluorospheres (Beckman Coulter Inc., Brea, CA, USA). Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

Thiazole orange flow cytometry for parasitaemia

Parasitaemia was determined daily with the use of the nucleic acid-binding dye TO, according to an established method [18]. Samples of 10 µl were collected from each culture and diluted to 1 ml in Sorenson's phosphate buffer (47 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.2) with a final TO concentration of 1 µM (diluted from a 10 mM stock in methanol). Samples were incubated at room temperature in the dark for 20 min and analysed within 1 hour. Erythrocytes were gated on a forward- versus side-scatter dot plot and analysed on a single-parameter FL1 integral (log) histogram, with regions for uninfected, ring-infected and trophozoite- or schizont-infected erythrocytes delineated. Approximately 50 000 events in the erythrocyte gate were counted. Regions had previously been confirmed by microscopy of Giemsa-stained smears of synchronised cultures.

TUNEL assay for DNA fragmentation

The terminal deoxynucleotidyltransferase-mediated nick end labelling (TUNEL) assay was performed according to manufacturer's recommendations, with modifications [5]. Briefly, *P. falciparum* cultures were fixed on ice for 60–90 min in 4% formaldehyde and phosphate-buffered saline (PBS: 10mM Na_2HPO_4, 1.5mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), followed by permeabilisation with

0.1% tri-sodium citrate (w/v) and 0.1% Triton X-100 (v/v) in PBS for 3 min on ice. Cells were labelled with TUNEL reagent (including TdT enzyme and FITC-dUTP) for 60-90 min on ice, according to manufacturer's recommendations. Lastly, propidium iodide (PI) staining was performed for 30min at room temperature. Labelled cells were analysed by flow cytometry within 3 hours. PI-positive parasites were acquired on a FL2 time-of-flight vs. FL2 integral (log) dot blot, with gated parasites analysed on a single-parameter FL1 integral histogram for DNA fragmentation. At least 10 000 PI positive events were counted. DNase-treated, non-treated and unlabelled parasites were used as positive, negative and staining controls. The FL1 arithmetic median fluorescence values of experimental and control parasites were compared, with all values normalised to control parasites and histograms overlaid for comparison.

$DiOC_6(3)$ flow cytometry for $\Delta\Psi_m$

Asynchronous *P. falciparum* culture samples (50 µl) were diluted to 1 ml PBS with 10 nM $DiOC_6(3)$ and 50 nM HE final concentrations and incubated at 37°C for 45 min in the dark. Following incubation, cells were pelleted, re-suspended in 1 ml PBS and analysed by flow cytometry immediately. Erythrocytes were gated on a forward- vs. side-scatter dot blot. HE-positive parasites acquired on a FL2 integral (log) histogram were analysed on a FL1 integral (log) histogram. Positive controls were treated with 200 nM CCCP for an hour before staining. Unstained cells, cells stained with only HE or $DiOC_6(3)$ and non-parasitised erythrocytes were used as staining controls. The FL1 arithmetic median fluorescence values of experimental

and control parasites were compared, with all values normalised to control parasites and histograms overlaid for comparison.

FITC Annexin V for PS externalisation

Asynchronous *P. falciparum* culture samples (20 µl) were diluted to 1 ml in PBS with a final concentration of 50 nM HE and incubated in the dark for 15 min at 37°C. Following staining, cells were pelleted and labelled with FITC Annexin V in 100 µl 1X annexin binding buffer for 15 min in the dark at room temperature. Stained cells were diluted to 1 ml in 1X annexin binding buffer and analysed by flow cytometry within an hour. Erythrocytes gated on a forward- vs. side-scatter dot blot were further analysed on a FL1 integral (log) vs. FL2 integral (log) dot blot with density colouring. Percentages of PS positive values from non-parasitised erythrocyte cultures were deducted as background. Parasitised cultures treated with recombinant Annexin V prior to staining were used as a negative control. Unstained parasite cultures and parasite cultures stained with only HE or FITC Annexin V were used as staining controls.

Statistical analysis

Bar graphs were compiled using GraphPad Prism 5, with raw data values exported from analyses by Beckman Coulter Kaluza (v.1.1) software. Student's unpaired t-tests were performed with Microsoft Office Excel 2010 to test for significance between experimental and control groups. Data distributions were not tested for normality.

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Figure legends

Figure 1: Growth and development of *in vitro P. falciparum* at self-limiting density. Raw TO flow cytometry data (**A**) indicate pre-determined regions for ring stage (**R**) and trophozoite or schizont stage (**T/S**) pRBC. Growth is indicated by a line graph (**B**). Peak parasitaemia of ~9.5% was achieved at 24 hours, whereafter it declined. Between 72 and 96 hours, a decline in the number of ring stage parasites and coinciding increase in trophozoite/schizont stage parasites indicated developmental progression. An increase in new rings and total parasitaemia between 96 and 120 hours indicated growth recovery. Corresponding regions from non-infected erythrocyte cultures maintained in parallel were subtracted as background.

Figure 2: Self-limitation at high population density causes DNA fragmentation in *P. falciparum*. Overlays of raw flow cytometry data were constructed from merged data sets for duplicate samples (**A**). Statistical analyses, indicated as bar graphs (**B**), showed no differences in DNA fragmentation at peak parasitaemia (**24 hours**) between control (**green**) and high parasitaemia (**red**), cultures. At **48 hours**, significant DNA fragmentation was observed in self-limiting parasitised cultures. The increase in DNA fragmentation was calculated as the fluorescence median of duplicate samples, relative to the corresponding control

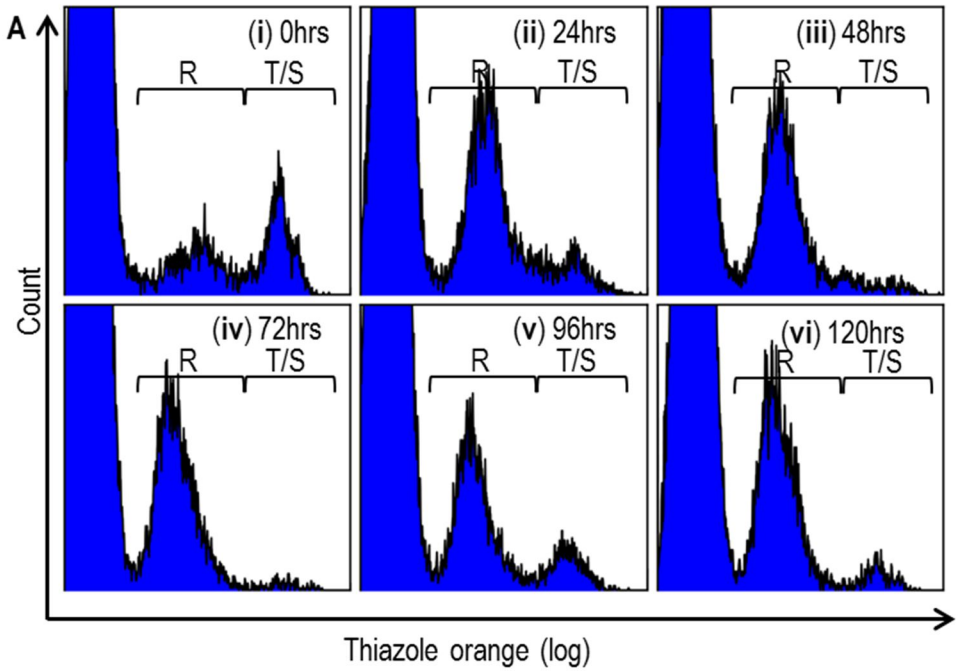
cultures. For each set of duplicates, the arithmetic mean +/- S.E.M. is indicated. Statistical comparisons were not significant (ns) or significant at $p < 0.01$ (**).

Figure 3: Self-limitation at high population density causes early and permanent mitochondrial depolarisation in *P. falciparum*. Overlays of raw flow cytometry data were constructed from merged data sets for duplicate samples (**A**) Statistical analyses (**B**), showed mitochondrial depolarisation, indicated by decreased $DiOC_6(3)$ fluorescence in self-limiting cultures (**red**) when compared to control cultures (**green**), at peak parasitaemia (**24 hours**) and the following day (**48 hours**). Differences in $DiOC_6(3)$ fluorescence were calculated as the fluorescence median of duplicate samples, relative to the corresponding control cultures. For each set of duplicates, the arithmetic mean +/- S.E.M. is indicated. Statistical comparisons were significant at $p < 0.05$ (*) or $p < 0.01$ (**).

Figure 4: Self-limitation at high population density causes phosphatidylserine externalisation in both pRBC and nRBC at peak parasitaemia. Raw flow cytometry histograms of representative samples (**A**) show the **PS+** gate used to quantify phosphatidylserine-externalising erythrocytes. Statistical analyses (**B**) indicated a significant increase in PS externalisation in self-limiting pRBC and nRBC at 24 hours (**ii and vi, respectively**) compared to control cultures at the same time intervals (**i and v, respectively**). At 48 hours, no difference in PS externalisation was noted for pRBC (**iii-iv**), and although nRBC from self-limiting cultures (**viii**) appeared to show more PS externalisation than the corresponding control cultures (**vii**), the difference was not statistically significant. PS externalisation values on bar graphs are indicated as the arithmetic mean of

duplicates +/- S.E.M. Statistical comparisons were significant not significant (ns), significant at $p < 0.01$ (**), or at $p < 0.001$ (***).

Figure 1:


A
(i) 0hrs
R
T/S
(ii) 24hrs
R
T/S
(iii) 48hrs
R
T/S
(iv) 72hrs
R
T/S
(v) 96hrs
R
T/S
(vi) 120hrs
R
T/S
Count
Thiazole orange (log)

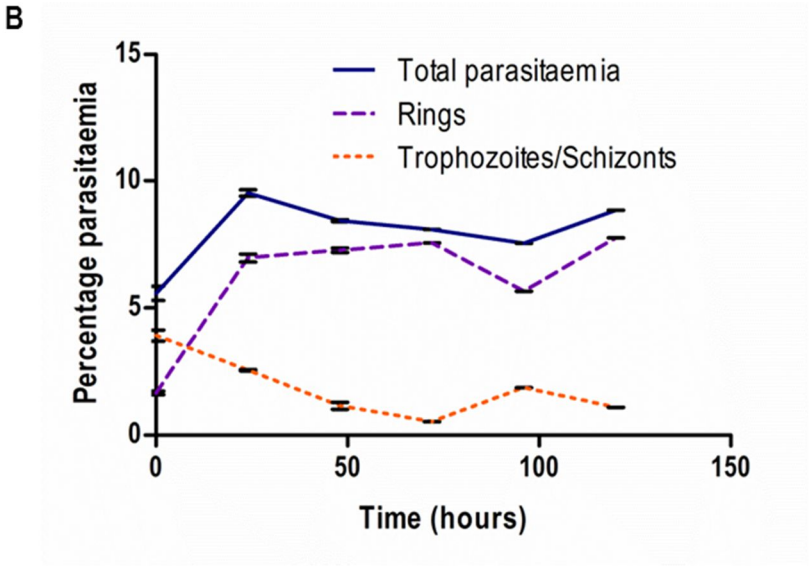

B
Total parasitaemia
Rings
Trophozoites/Schizonts
Percentage parasitaemia
15
10
5
0
0
50
100
150
Time (hours)

Figure 2:

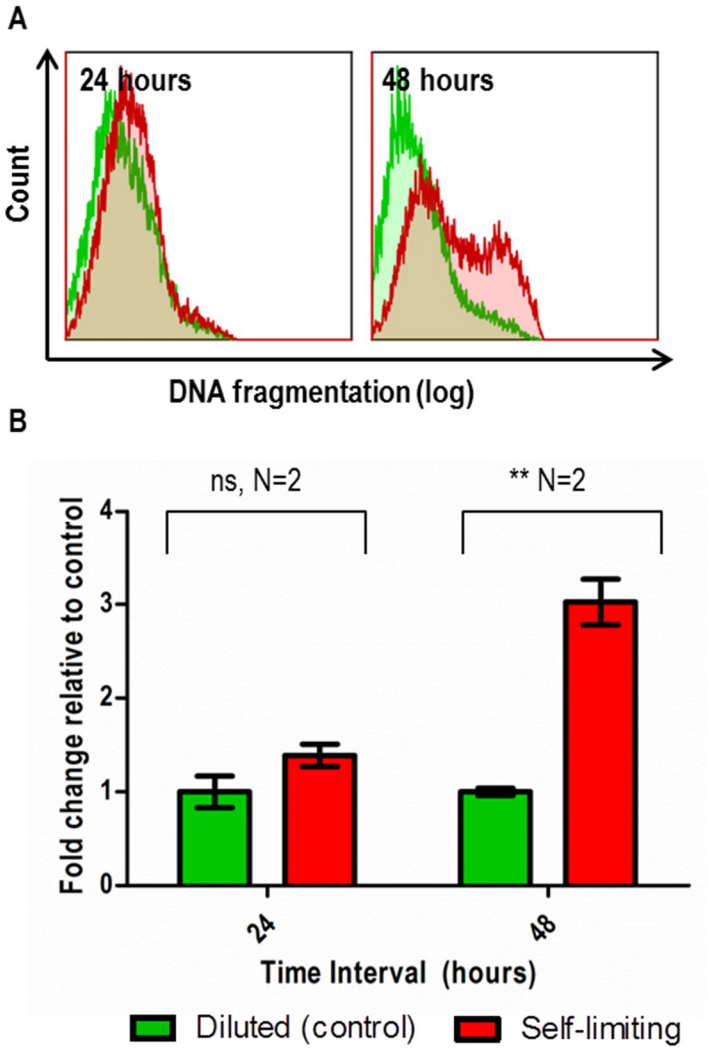

A
24 hours
48 hours
Count
DNA fragmentation (log)
B
ns, N=2
** N=2
Fold change relative to control
0
1
2
3
4
24
48
Time Interval (hours)
Diluted (control)
Self-limiting

Figure 3:

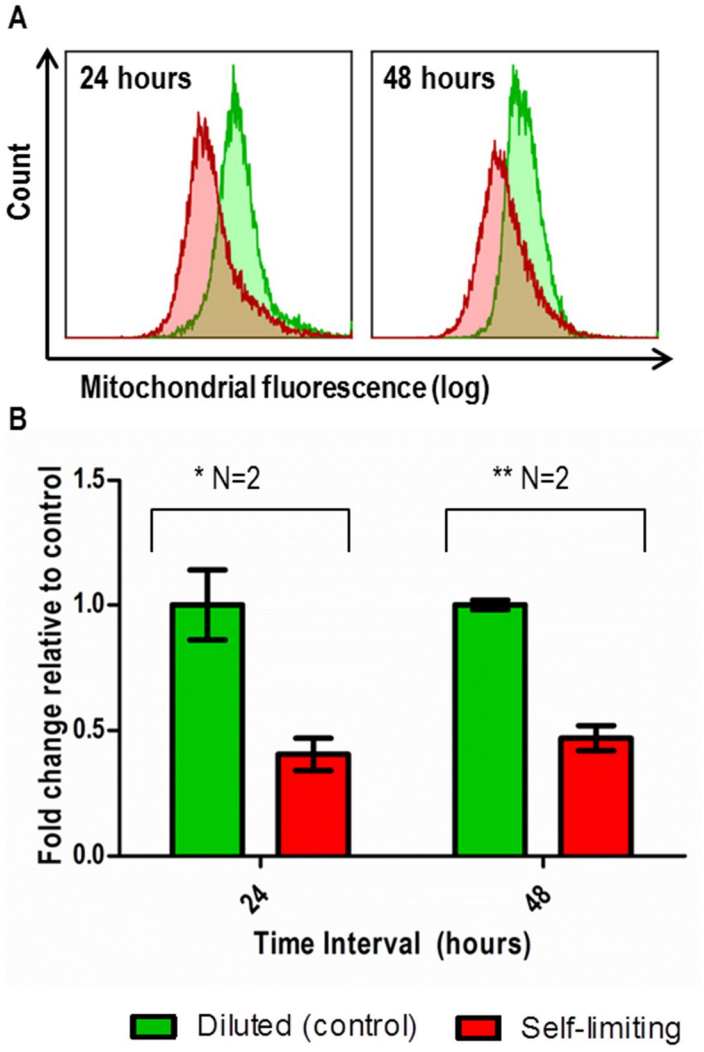
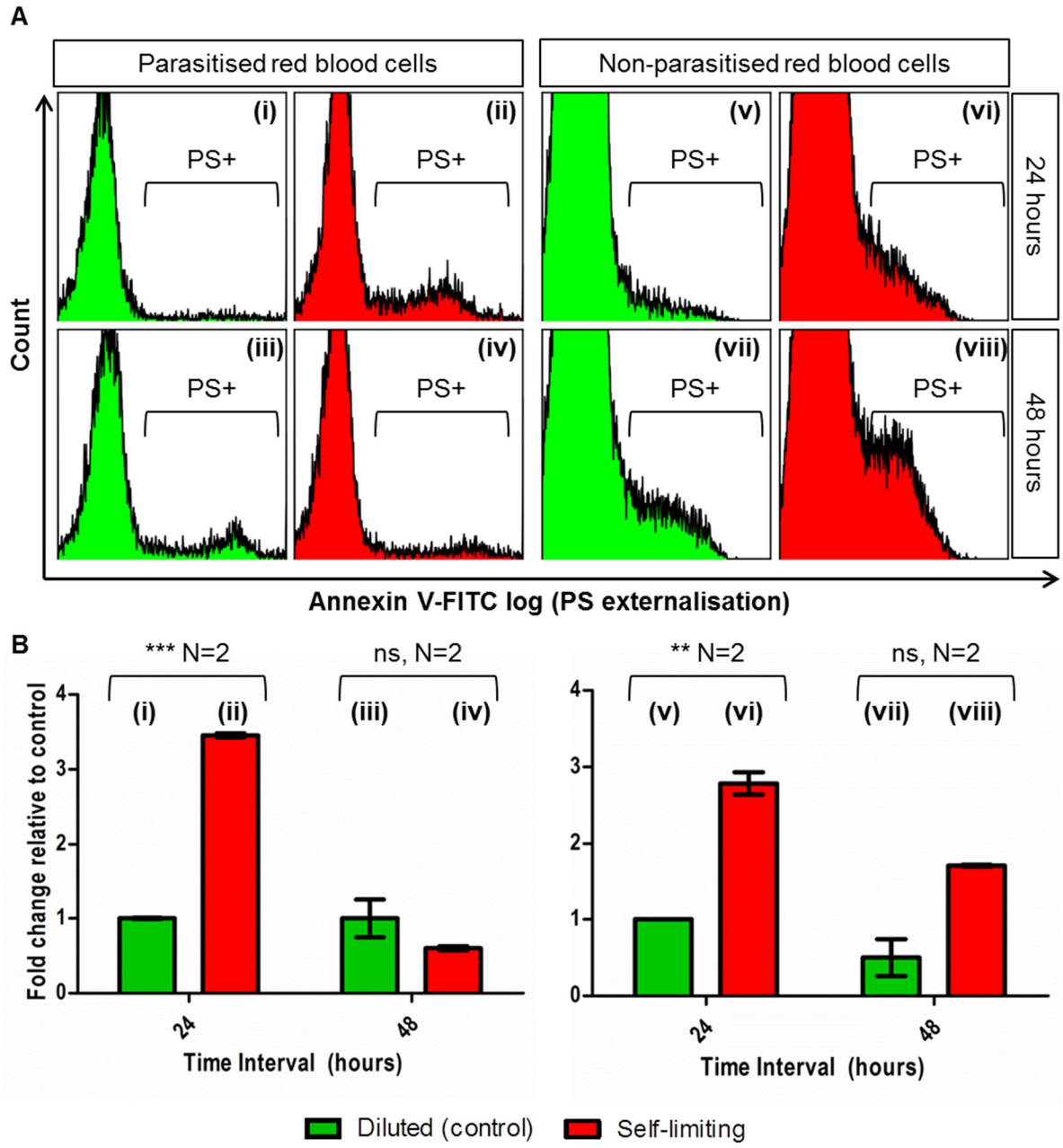

A
24 hours
48 hours
Count
Mitochondrial fluorescence (log)
B
* N=2
** N=2
Fold change relative to control
1.5
1.0
0.5
0.0
24
48
Time Interval (hours)
Diluted (control)
Self-limiting

Figure 4:


A
Parasitised red blood cells
Non-parasitised red blood cells
(i)
(ii)
(v)
(vi)
(iii)
(iv)
(vii)
(viii)
PS+
24 hours
48 hours
Count
Annexin V-FITC log (PS externalisation)
B
*** N=2
ns, N=2
** N=2
ns, N=2
Fold change relative to control
Time Interval (hours)
Diluted (control)
Self-limiting

7 INTRODUCTION TO MANUSCRIPT 2

Engelbrecht D and Coetzer TL

Sunlight inhibits growth and induces markers of programmed cell death in *Plasmodium falciparum in vitro*

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Summary

The effect of sunlight exposure on *P. falciparum* is practically entirely overlooked. Although previous data from our laboratory has shown that sunlight can inhibit *P. falciparum* growth, this study is the first to characterise parasite cell death in response to natural sunlight. Results showed that early stage parasites were unaffected by sunlight exposure. On the other hand, late stage parasites exhibited biochemical markers of PCD including DNA fragmentation that preceded mitochondrial hyperpolarisation. Data also suggested phosphatidylserine externalisation in mixed stage parasites, but results were not statistically significant. This publication agrees with other data in this thesis that show that *P. falciparum* undergoes PCD. However, unlike other data, the phenotype of cell death after sunlight exposure was not clear.

Sunlight inhibits growth and induces markers of programmed cell death in *Plasmodium falciparum in vitro*

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Abstract

Background: *Plasmodium falciparum* is responsible for the majority of global malaria deaths. During the pathogenic blood stages of infection, a rapid increase in parasitaemia threatens the survival of the host before transmission of slow-maturing sexual parasites to the mosquito vector to continue the life cycle. Programmed cell death (PCD) may provide the parasite with the means to control its burden on the host and thereby ensure its own survival. Various environmental stress factors encountered during malaria may induce PCD in *P. falciparum*. This study is the first to characterise parasite cell death in response to natural sunlight. **Methods:** The 3D7 strain of *P. falciparum* was cultured *in vitro* in donor erythrocytes. Synchronised and mixed stage parasitised cultures were exposed to sunlight for 1 hour and compared to cultures maintained in the dark, 24 hours later. Mixed stage parasites were also subjected to a second 1 hour exposure at 24 hours and assessed at 48 hours. Parasitaemia was measured daily by flow cytometry. Biochemical markers of cell death were assessed, including DNA fragmentation, mitochondrial membrane polarisation and phosphatidylserine externalisation. **Results:** Sunlight inhibited *P. falciparum* growth *in vitro*. Late stage parasites were more severely affected than early stages. However, some late stage parasites survived exposure to sunlight to form new rings 24 hours later, as would be expected during PCD whereby only a portion of the population dies. DNA fragmentation was observed at 24 and 48 hours and preceded mitochondrial hyperpolarisation in mixed stage parasites at 48 hours. Mitochondrial hyperpolarisation likely resulted from increased oxidative stress. Although data suggested increased PS externalisation in mixed stage parasites, results were not statistically significant. **Conclusion:** The combination of biochemical markers and the survival of some parasites, despite exposure to a lethal stimulus, support the occurrence of PCD in *P. falciparum*.

Keywords: *Plasmodium falciparum*; programmed cell death; sunlight

Background

During the recurring erythrocytic stages of *Plasmodium falciparum* malaria, each mature schizont stage parasite produces around 20 new infective merozoites [1], resulting in an exponential increase in parasite load every 48 hours. Severe parasitaemia threatens the survival of the human host before transmission of the slow-maturing gametocytes to the *Anopheles* mosquito, and thus careful regulation of parasite density may be advantageous to both the parasite and its host. Several mechanisms are postulated to allow the parasite to regulate its own parasitaemia, including altering the rate of division, the efficiency of invasion and the rate of cell death [2]. Programmed cell death (PCD) may provide the parasite with the most effective means to regulate parasitaemia [2]. In multicellular organisms PCD fulfils essential roles in development, immunity and the maintenance of homeostasis [3–5], but it has also been shown in unicellular protozoa [6, 7], including *P. falciparum* (reviewed in [8]). Although the exact phenotype remains debated, a growing body of evidence suggests that *P. falciparum* exhibits one or several PCD phenotypes.

PCD may be induced by a number of environmental stress factors encountered by the parasite during malaria illness, including high parasite population density [9] and febrile episodes [10–12]. However, the effect of sunlight is almost entirely overlooked. The considerable cardiac output delivered to cutaneous circulation [13] means that at any one time, a significant number of intra-erythrocytic parasites are located in the superficial blood vessels, and are thus exposed to penetrating solar radiation. This proportion may further increase as a result of vasodilation during fever paroxysms [14].

Solar radiation is a potent inducer of apoptosis in various eukaryotic cell types. Natural sunlight is composed of UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) radiation [15], although UV-C radiation is filtered out by the atmosphere [16, 17]. UV-B

radiation directly damages nuclear DNA by causing lesions such as cyclobutane pyrimidine dimers and pyrimidine 6-4 pyrimidone photoproducts [18–20]. However, UV-B may also indirectly induce apoptosis through the generation of reactive oxygen species (ROS) that in turn triggers mitochondrial cytochrome-c release [20–22]. UV-B radiation may also cause apoptosis via other cytoplasmic or membrane targets, such as direct activation of membrane-bound death receptors (reviewed in [22]). UV-A radiation causes oxidative stress that damages and permeabilises lipid membranes (reviewed in [23]). In murine lymphoma cells, UV-A radiation was shown to induce apoptosis in less than 4 hours, while the apoptotic effects of UV-B and UV-C wavelengths were delayed (reviewed in [23]).

The *in vitro* photosensitivity of *P. falciparum* has previously been noted in the authors' laboratory [24], but data on *Plasmodium* spp. are otherwise limited to the effect of UV radiation on the murine host [25]. In other protists, UV-B exposure did not affect the viability of *Leishmania major* parasites either *in vitro* or *in vivo* [26] and had no impact on parasite infectivity [26, 27].

The present study is the first to investigate biochemical markers of PCD in *P. falciparum* parasites in response to natural sunlight, a physiologically relevant stress factor. It should be noted that the present study focused on the effect of natural sunlight, rather than a specific wavelength spectrum. The use of culture flasks that were not UV transparent may have prevented light in the UV spectrum from reaching parasites, whereas physiological exposure would include UV light. However, even with the exclusion of the UV spectrum, the effects observed in this study derived from natural sunlight. Exposure to sunlight caused growth inhibition and induced PCD in a sub-population of parasites. Late stage parasites – trophozoites and schizonts – were far more affected than ring stage parasites. Sunlight caused DNA fragmentation that preceded mitochondrial hyperpolarisation, suggesting a unique form of PCD in *P. falciparum* that is not initiated by the

mitochondrion. These findings provide important new information on cell death mechanisms that are utilised by the parasite to limit its population and thereby prevent premature death of the human host and thus ensure its transmission to the mosquito vector.

Methods

Reagents. The APO-DIRECT TUNEL kit and FITC Annexin V Apoptosis Detection Kit II were obtained from Becton Dickinson (BD Pharmingen, San Diego, CA, USA). Thiazole orange (TO), hydroethidine (HE), 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumax II was obtained from Gibco (Gran Island, NY, USA).

***P. falciparum* culture**. The 3D7 strain of *P. falciparum* was maintained according to established methods [28] with some modifications [29]. Briefly, parasites were maintained at 37°C in malaria culture medium (RPMI 1640, 0.5% Albumax II and 0.21% sodium bicarbonate, supplemented with 50 mg/l gentamycin and 50 mg/l hypoxanthine) at 5% haematocrit in donor erythrocytes. Medium was changed daily. Optimal culture pH was maintained by gassing cultures daily for 60 seconds with a mixture of 2% O_2, 5% CO_2 and 93% N_2. Parasite morphology was monitored by Giemsa-stained smears and parasitaemia and staging were assessed by TO flow cytometry.

Synchronisation of parasites to ring stages. For studies involving synchronised parasites, synchronisation was performed similar to an established method [30]. Briefly, *P. falciparum*-infected red blood cells (pRBC) were centrifuged for 5 min at 1000 x *g* and 25°C. The resulting cell pellet was incubated in 10 volumes of 5% D-sorbitol for 5-10 min at 37°C. Centrifugation was repeated and the cell pellet was resuspended to 5% haematocrit with medium, returned to a clean 25 cm^2 culture flask and incubated at 37°C.

This method synchronised parasites to ring stages. For studies involving late stage parasites, pRBC cultures were allowed to mature for 20-24 hours.

Exposure to natural sunlight. pRBC were seeded as 5 ml cultures in 25 cm^2 optically-clear, sealed tissue culture flasks. Sunlight exposure was performed for 1 hour in the middle of the day (between 10h00 and 14h00) on clear, sunny days in Johannesburg, South Africa, with an elevation of 1753 m above sea level. Culture flasks were maintained at 37°C in a circulating waterbath throughout exposure. Control cultures were covered with thick cardboard to prevent exposure to sunlight, while experimental cultures were left uncovered in direct sunlight. Following exposure, all cultures were returned to the dark in an incubator at 37°C.

Flow cytometry. Flow cytometric analyses were performed on a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Excitation for all assays was by 488 nm blue laser. Emission was detected with the use of 545/40BP (525±20 nm, FL1) and, where indicated, 575/30BP (575±15 nm, FL2) filters. Optical alignment was monitored daily with Beckman Coulter Flow Check Pro fluorospheres (Beckman Coulter Inc., Brea, CA, USA). Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

TO flow cytometry for parasitaemia. Parasitaemia was measured daily by flow cytometry with the DNA-binding dye TO, similar to a previous method [31]. Whole-culture samples (10 µl) were diluted 100-fold to 1ml in Sorenson's phosphate buffer (47 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.2) with 1 µM TO final concentration (diluted from a 10 mM stock in methanol) and incubated at room temperature in the dark for 20 min. Stained cells were analysed by flow cytometry within 1 hour. Erythrocytes were gated on a forward- versus side-scatter dot plot and analysed on a FL1 integral (log) histogram, with regions for uninfected, ring-infected and trophozoite- or schizont-infected erythrocytes

delineated. Regions had previously been confirmed by microscopy of Giemsa-stained smears of synchronised cultures. Approximately 50 000 events in the erythrocyte gate were counted.

TUNEL assay for DNA fragmentation. The terminal deoxynucleotidyltransferase (TdT)-mediated nick end labelling (TUNEL) assay was performed according to manufacturer's recommendations, with modifications similar to a previous study [11]. Briefly, pRBC were pelleted and fixed on ice for 60–90 min in 4% formaldehyde and phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.5mM KH_2PO_4, 137 mMNaCl, 2.7 mMKCl, pH 7.4), followed by permeabilisation with 0.1% tri-sodium citrate (w/v) and 0.1% Triton X-100 (v/v) in PBS for 3 min on ice. Labelling with DNA-staining solution (including TdT enzyme and FITC-dUTP) was performed according to the manufacturer's recommendations for 60–90 min at 37°C, followed by staining with propidium iodide (PI) for 30min at room temperature. Labelled cells were analysed by flow cytometry within 3 hours. PI-positive parasites were acquired on a FL2 time-of-flight (lin) versus FL2 integral (lin) dot plot, with gated parasites analysed on a FL1 integral (log) histogram for DNA fragmentation, measured as FITC-dUTP fluorescence. At least 10 000 PI-positive events were counted. DNase-treated, non-treated and unlabelled parasites were used as positive, negative and staining controls, respectively.

$DiOC_6(3)$ flow cytometry for mitochondrial transmembrane potential. *P. falciparum* culture samples (50 µl) were diluted to 1 ml in PBS containing 10 nM $DiOC_6(3)$ (diluted from a 100 mM stock in DMSO) and 50 µM hydroethidine HE (diluted from a 10 mM stock in DMSO) and incubated at 37°C for 45 min in the dark. Following incubation, cells were washed and suspended in 1ml PBS and analysed by flow cytometry immediately. Erythrocytes were gated on a forward- versus side-scatter dot blot. HE-positive pRBC counted on a FL2 integral (log) histogram were analysed for $DiOC_6(3)$ fluorescence on a FL1 integral (log) histogram. At least 10 000 HE-positive events were counted. Positive

controls were treated with 200 nM CCCP for 1 hour before staining. Unstained cells, cells stained with only HE or $DiOC_6(3)$ and non-parasitised erythrocytes were used as staining controls.

Annexin V-FITC for PS externalisation. *P. falciparum* culture samples (20 µl) were diluted 50-fold to 1 ml in PBS and stained with a final concentration of 50 µM HE (diluted from a 10 mM stock in DMSO) in PBS for 15 min at 37°C in the dark. Cells were then pelleted, suspended to 100 µl 1X annexin-binding buffer (provided with the kit) and stained with annexin V-FITC for 15 min in the dark according the manufacturer's recommendations with modifications similar to a previous study [32]. Stained cells were diluted to 1ml in 1X annexin-binding buffer and analysed by flow cytometry within 1 hour. Erythrocytes were gated on a forward- versus side-scatter dot blot and pRBC were discriminated on a FL 2 integral (log) histogram for HE fluorescence. Gated pRBC were analysed for annexin V-FITC fluorescence on a FL1 integral (log) histogram. At least 50 000 events in the erythrocyte gate were counted. pRBC treated with recombinant annexin V before staining were used as a negative control and unstained parasite cultures and parasite cultures stained with only HE or annexin V-FITC were used as staining controls.

Statistical analysis. Bar graphs were compiled using GraphPad Prism 5, with raw data values exported from analyses by Beckman Coulter Kaluza (v.1.1) software. Student's unpaired *t*-tests were performed with Microsoft Office Excel 2010 to test for significance between treated and control groups. Data distributions were not tested for normality.

Results

Sunlight inhibited *P.falciparum* growth *in vitro*

Mixed and synchronised parasite cultures were exposed to 1 hour of sunlight and assessed for growth 24 hours later. Mixed stage cultures were exposed to another 1 hour

of sunlight at 24 hours and assessed again 24 hours later, at 48 hours. Sunlight decreased the *in vitro* growth of both mixed (**Figure 1**) and synchronised (**Figure 2**) parasite cultures.

Early stage parasites were unaffected by sunlight. Twenty-four hours after a single exposure, ring stage parasites in both mixed stage (**Figure 1Aii and Bii**) and synchronised (**Figure 2Aii and Bii**) parasites progressed to trophozoite stages (**Figure 1Aiv, Biv and Figure 2Aiv, Biv, respectively**) similar to control cultures maintained in the dark (**Figure 1Aiii, Biii and Figure 2Aiii, Biii, respectively**).

In contrast, sunlight decreased the growth of late stage parasites in both mixed (**Figure 1**) and synchronised (**Figure 2**) cultures. Fewer new rings were formed (**Figure 1Aiv, Biv and Figure 2 Aviii, Bviii**) from late stage parasites exposed to sunlight (**Figure 1Ai, Bi and Figure 2Avi, Bvi**) than from corresponding cultures maintained in the dark. However, growth inhibition was only partial, as evidenced by increased parasitaemia between 0 and 24 hours, albeit less than in control cultures.

In mixed stage cultures, a decrease in both early and late stage parasites was seen at 48 hours (**Figure 1Avi and Bvi vs. Av and Bv**), after a second dose of sunlight was delivered at 24 hours. A decrease in late stage parasites, compared to control cultures at 48 hours, is attributed to the initial exposure of trophozoites at 0 hours causing fewer rings at 24 hours and correspondingly fewer late stage parasites at 48 hours. As sunlight had no effect on the growth of early stage parasites, only mixed stage parasites and synchronised late stage parasites were used for cell death assays.

Exposure to sunlight caused DNA fragmentation in *P. falciparum*

DNA fragmentation was quantified from fixed and isolated parasites by the flow cytometric TUNEL assay, 24 hours after every sunlight exposure. DNA fragmentation was seen at 24

hours in synchronised late stage parasites (**Figure 3B**) and at 48 hours in mixed stage parasites (**Figure 3C**).

Mixed stage parasites showed suggestive but insignificant DNA fragmentation at 24 hours (**Figure 3A**), likely due to the negating effect of early stage parasites that were unaffected by sunlight that reduced the fragmentation of the population as a whole. Therefore, it seems likely that late stage parasites in mixed cultures exhibited DNA fragmentation in the same manner that synchronised late stage parasites did.

Exposure to sunlight caused mitochondrial hyperpolarisation in mixed stage parasites

Mixed stage parasites showed increased $DiOC_6(3)$ fluorescence, suggesting mitochondrial hyperpolarisation, the day following a second exposure to sunlight (**Figure 4C**). No mitochondrial dysregulation was observed in synchronised late stage parasites or in mixed stage parasites 24 hours after the first exposure to sunlight (**Figure 4A and B**). DNA fragmentation therefore preceded mitochondrial dysregulation, with DNA fragmentation observed in late stage parasites at 24 hours, and mitochondrial hyperpolarisation only observed at 48 hours in mixed stage parasites. Mitochondrial hyperpolarisation was likely due to increased reactive oxygen species (ROS) [10, 24], but it is not clear whether there is any causative relationship between DNA fragmentation and the appearance of mitochondrial hyperpolarisation.

Exposure to sunlight caused no change in phosphatidylserine externalisation

Flow cytometry datashowed increased PS externalisation in mixed stage pRBC on the day following both the first (**Figure 5A**) and second exposure (**Figure 5C**) to natural sunlight, but the difference was not statistically significant. No increase in PS externalisation was observed in synchronised late stage pRBC exposed to sunlight (**Figure 5B**).

Discussion

Shedding light on a killer: Sunlight inhibits *P. falciparum* growth

Since ancient times humans have linked sunlight and malaria and one of the myths was that staying out in the sun would cause malaria [33]. However, sun worship in early cultures may have in fact conferred a survival advantage to its practitioners, by reducing the burden of disease [Mendelow and Coetzer unpublished observations]. Our data showed that exposure to sunlight inhibited the *in vitro* growth of intra-erythrocytic *P. falciparum.* Early stage parasites were resistant to damage caused by sunlight and although late stage parasites were more severely affected, some late stage parasites survived sunlight exposure. These may have been early trophozoites at the time of exposure, rather than late trophozoites or schizonts. The conservation of some parasites despite exposure to a lethal stress stimulus supports the occurrence of PCD, which requires a portion of the population to survive. It is not clear whether decreased parasite growth was due to fewer schizonts being produced or if some of the newly released merozoites were non-viable and hence could not invade new erythrocytes.

To further investigate the decline in parasitaemia after exposure to sunlight, biochemical markers of cell death were evaluated. DNA fragmentation is one of the characteristics of PCD and was observed in late stage parasites, confirming that the reduced growth resulted from the death of some parasites, rather than the reduced reproduction of the population as a whole. DNA fragmentation has been widely reported in *P. falciparum* in response to antimalarial drugs [2, 34–37], heat stress [10, 11], high parasite density [9] bilirubin [38], etoposide [35] and staurosporine [36]. Some studies have attributed the occurrence of DNA fragmentation, without other biochemical markers of cell death, to apoptosis in *P. falciparum* [2, 11, 35], although it has also been attributed to an undefined pathway [39].

The effect of sunlight on another key element of PCD, the polarisation of the mitochondrial membrane, was also investigated. Mitochondrial hyperpolarisation was observed in mixed stage parasites the day following a second sunlight exposure. Although mitochondrial depolarisation, resulting from permeabilisation of the outer mitochondrial membrane, is expected during apoptosis [40–45], both mitochondrial depolarisation and hyperpolarisation may form part of an apoptosis response in other protozoa [46–48].The asexual stages of *P. falciparum* contain only a single mitochondrion that is chiefly involved in pyrimidine synthesis [49]; however, previous studies have shown the importance of mitochondria in parasite programmed cell death [50]. It should also be noted that transient hyperpolarisation in metazoans may occur as an early checkpoint in deciding cell fate [47]; however, hyperpolarisation in this case was not observed immediately after exposure to sunlight, but only a day after the second exposure and two days after the first. Mitochondrial hyperpolarisation was likely the result of increased accumulation of ROS, which has previously been suggested to be the cause of parasite death after sunlight exposure [24].

The externalisation of PS in pRBC was evaluated as a third marker of PCD. PS externalisation has previously been noted to result from parasite maturation or stress [32, 51]. Although results suggested increased PS externalisation in mixed stage cultures, the results were not statistically significant. The absence of increased PS externalisation, particularly in late stage pRBC, supports the notion of a unique PCD pathway in *P. falciparum*.

Our combined data therefore showed that sunlight exposure induced DNA fragmentation followed by mitochondrial hyperpolarisation in the absence of PS externalisation. This unusual manifestation of biochemical markers suggests a cell death phenotype that does not entirely fit with any metazoan PCD models and might be unique to *P. falciparum*.

Sunlight and heat stress: cooperative effect

P. falciparum utilises a unique method of erythrocyte modification to allow adhesion of late stage asexual and immature sexual parasites to endothelium, thereby sparing parasites from splenic clearance [1]. Heat stress, such as febrile episodes characteristic of malaria [1], increases cardiac output to cutaneous circulation to as much as 60% of total vascular conductance [13] and further increases the cytoadherence of mature *P. falciparum*-infected RBC [52]. Thereforea large number of late stage parasites would be present in the microvasculature near the skin and thus be exposed to sunlight. Heat stress reduces *P. falciparum* numbers *in vitro* [10–12, 53–55] and has been suggested to cause PCD in the parasite [10, 11].Sunlight and heat stress may therefore have a cooperative effect in parasite clearance.

Sunlight and heat stress: different stress, different phenotype

In contrast to the effect of sunlight on late stage pRBC, PCD induced by heat stress was characterised by mitochondrial hyperpolarisation and PS externalisation without any DNA fragmentation [10]. Interestingly, heat stress also induced PCD in some early stage parasites that exhibited DNA fragmentation and mitochondrial depolarisation [10], whereas sunlight did not affect ring stages. Therefore, *P. falciparum* shows unique combinations of cell death markers that differ depending on the type of stress.

Early stage parasites appear to be well-adapted to surviving lethal stress stimuli. *In vivo*, the rupture of pRBC and the egress of merozoites are responsible for the onset of fever, but the merozoites would have reinvaded and be present as ring stage parasites by the time heat stress occurs and will thus not be affected [10, 56]. In the case of sunlight, early stage parasites are exposed as a result of continuous circulation, but since they do not sequester as late stage parasites do, they presumably receive less sunlight than late stage parasites.

Conclusion

In vitro exposure to natural sunlight reduced the growth of *P. falciparum* due to the death of a portion of the parasite population. The occurrence of DNA fragmentation prior to mitochondrial depolarisation suggests a cell death mechanism that is not initiated by the mitochondrion. This combination of biochemical markers may offer clues to a unique phenotype of PCD in *P. falciparum*. Although conflicting data concerning the phenotype of PCD in *P. falciparum* might be attributed to the use of various strains and cell death markers, it is also apparent that *P. falciparum* may vary its response to different potentially fatal stimuli. However, whether this should be attributed to diverse underlying mechanisms or simply different facets of the same pathway remains unclear. Furthermore, while this study focused on sunlight as a whole future studies may narrow the wavelength spectrum responsible for the induction of cell death in *P. falciparum*.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; HE, hydroethidine; PBS, phosphate-buffered saline; PCD, programmed cell death; PI, propidium iodide; pRBC, *Plasmodium falciparum*-infected red blood cells; PS, phosphatidylserine; ROS, reactive oxygen species; TdT, terminal deoxynucleotidyltransferase; TO, thiazole orange; TUNEL, TdT-mediated nick end labelling.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DE participated in the study design and conception as well as data interpretation, and was responsible for all data collection, statistical analyses and drafted the manuscript. TLC participated in the design, conception and coordination of the study, as well as data interpretation and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1: Mixed stage *P. falciparum* growth decreased after exposure to sunlight *in vitro*. Flow cytometry histograms (**A**) and statistical analyses (**B**) showed that late stage parasites (**Late**) were more affected by exposure to solar radiation than early stage parasites (**Early**). After equivalent cultures were seeded (**i** and **ii**), experimental cultures (**Sunlight**) were exposed to sunlight for 1 hour, while control cultures (**Dark**) were maintained in the dark. After exposure, experimental cultures were maintained in the dark until measurements were taken at 24 hours. Control cultures showed a large population of new early stage parasites (**iii**), whereas the formation of early stage parasites was significantly reduced in experimental cultures (**iv**), indicating that late stage parasites exposed to sunlight produced fewer new rings than those maintained in the dark. Equivalent late stage populations at 24 hours between experimental and control cultures (**iii** and **iv**, respectively), indicated that early stage parasites were not significantly affected by sunlight exposure. At 24 hours, experimental cultures were subjected to another 1 hour sunlight exposure and assessed again at 48 hours, when both early and late stage parasites were reduced in experimental cultures (**vi**), compared to control cultures at the same time (**v**). Statistical comparisons were made between total parasitaemia values and were not significant (ns) or significant at $P < 0.01$ (**). For all comparisons, $n = 2$.

Figure 2: Sunlight decreased the growth of late stage *P. falciparum*, but early stages were unaffected. Flow cytometry histograms (**A**) and statistical analyses (**B**) showed that early stage parasites (**i-iv**) were unaffected by exposure to solar radiation,

whereas late stage parasites (**v-viii**) were significantly affected. Synchronised early stage parasites maintained in the dark (**i**) or exposed to sunlight for 1 hour and maintained in the dark for the remainder of 24 hours (**ii**) progressed to late stages at 24 hours with no difference between control (**Dark**) and experimental (**Sunlight**) cultures (**iii** and **iv**, respectively). On the other hand, synchronised late stage parasites showed significantly reduced growth at 24 hours (**viii**), compared to control cultures maintained in the dark (**vii**). Statistical comparisons were made between total parasitaemia values and were not significant (ns) or significant at $P < 0.001$ (***). For all comparisons, n = 4.

Figure 3: Exposure to sunlight caused DNA fragmentation in *P. falciparum in vitro*. Although DNA fragmentation was not observed in mixed stage parasites after a single exposure to sunlight (**A**), synchronised late stage parasite did show DNA fragmentation at 24 hours (**B**). DNA fragmentation was also seen in mixed stage parasites at 48 hours, after two exposures to sunlight (**C**), measured the day after the second exposure. Histograms of the flow cytometric TUNEL assay (upper panels) show overlaid results from control (green) and experimental parasites (red), with fragmentation analysed as the change in median fluorescence of the experimental parasite population, relative to control parasites at the same time (bottom panels). Statistical comparisons were not significant (ns),or significant at $P < 0.05$ (*) or at $P < 0.01$ (**).n = 2 for (**A**) and (**B**); n = 4 for (**C**).

Figure 4: Sunlight caused changes in mitochondrial polarisation in *P. falciparum* only after 48 hours. No change was seen was seen in the mitochondrial polarisation of mixed stage parasites (**A**) or synchronised late stage parasites (**B**) exposed to sunlight for 1 hour and maintained in the dark for the remainder of 24 hours, compared to control parasites kept in the dark. After a second exposure to solar radiation, mixed stage parasites showed an apparent increase in mitochondrial polarisation at 48 hours, compared to parasites maintained in the dark (**C**). Flow cytometry histograms (upper panels) show overlaid results from control (green) and experimental parasites (red), with

changes in mitochondrial polarisation analysed as the change in median fluorescence of the experimental parasite population, relative to control parasites at the same time (bottom panels). Statistical comparisons were not significant (ns) or significant at $P < 0.01$ (**).n = 2 for (**A**) and (**B**); n = 4 for (**C**).

Figure 5: No significant change was seen in the phosphatidylserine externalisation of parasitised erythrocytes after exposure sunlight. Flow cytometry histograms (upper panels) showed an apparent increase in PS externalisation in mixed stage pRBC at both 24 (**A**) and 48 (**C**) hours after one and two exposures to sunlight, respectively. However, statistical analyses of bar graphs (bottom panels) showed no significant differences (ns). Synchronised late stage pRBC exposed to solar radiation for 1 hour and maintained in the dark for the remainder of 24 hours also showed no change in PS externalisation, compared to control cultures maintained in the dark (**B**). n = 2 for (**A**) and (**B**); n = 4 for (**C**).

Figure 1

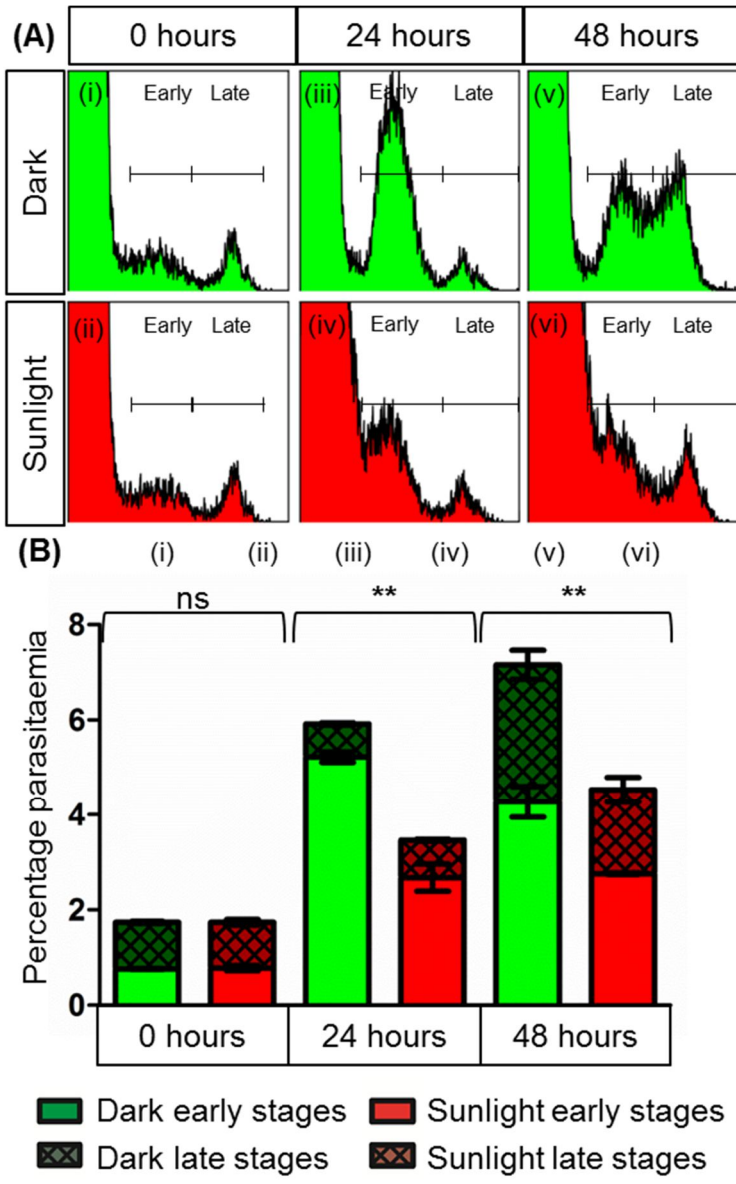

(A)
0 hours
24 hours
48 hours
Dark
Sunlight
(i)
(ii)
(iii)
(iv)
(v)
(vi)
Early
Late
(B)
ns
**
**
Percentage parasitaemia
8
6
4
2
0
0 hours
24 hours
48 hours
Dark early stages
Dark late stages
Sunlight early stages
Sunlight late stages

Figure 2

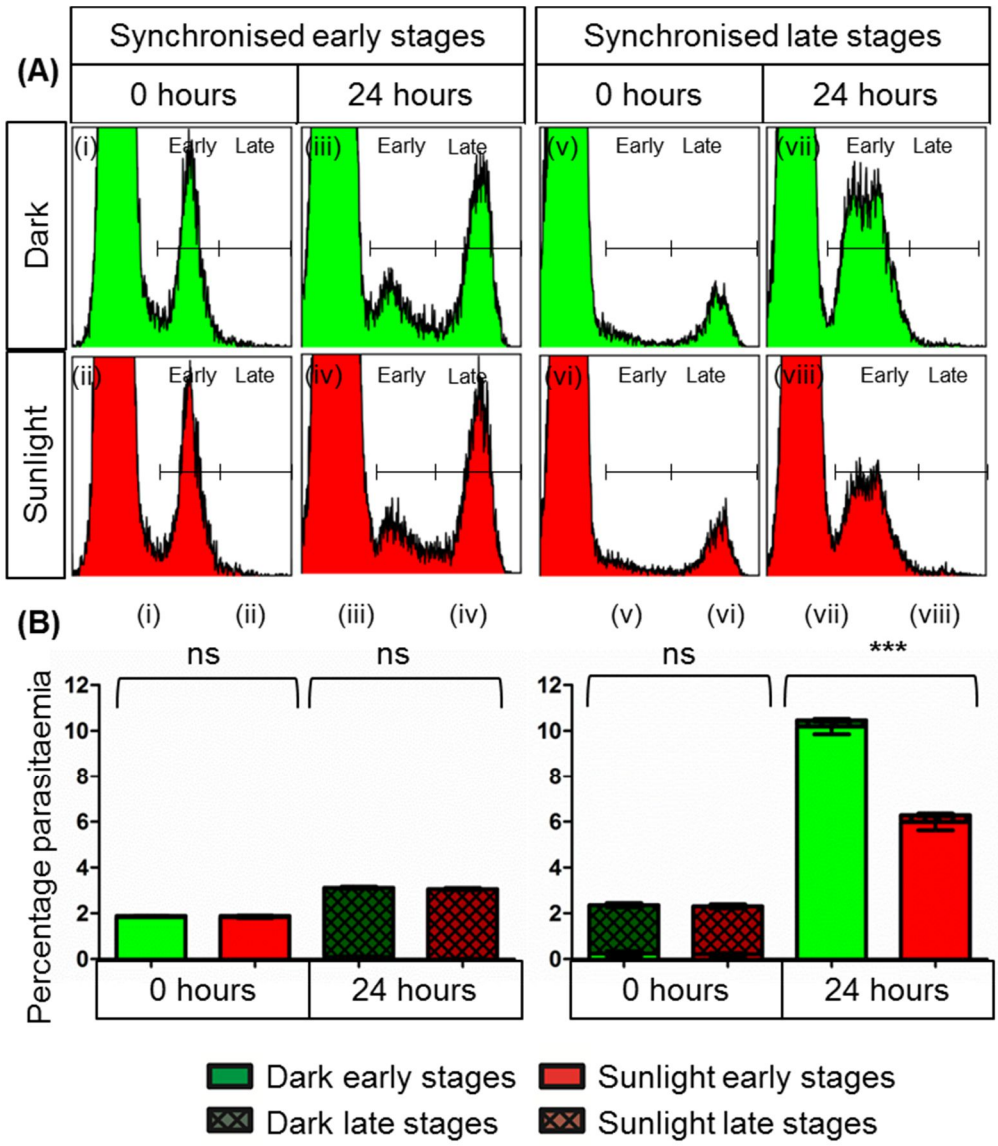

(A)
Synchronised early stages
Synchronised late stages
0 hours
24 hours
0 hours
24 hours
Dark
Sunlight
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
Early
Late
(B)
ns
ns
ns
***
Percentage parasitaemia
0
2
4
6
8
10
12
0 hours
24 hours
0 hours
24 hours
Dark early stages
Dark late stages
Sunlight early stages
Sunlight late stages

Figure 3

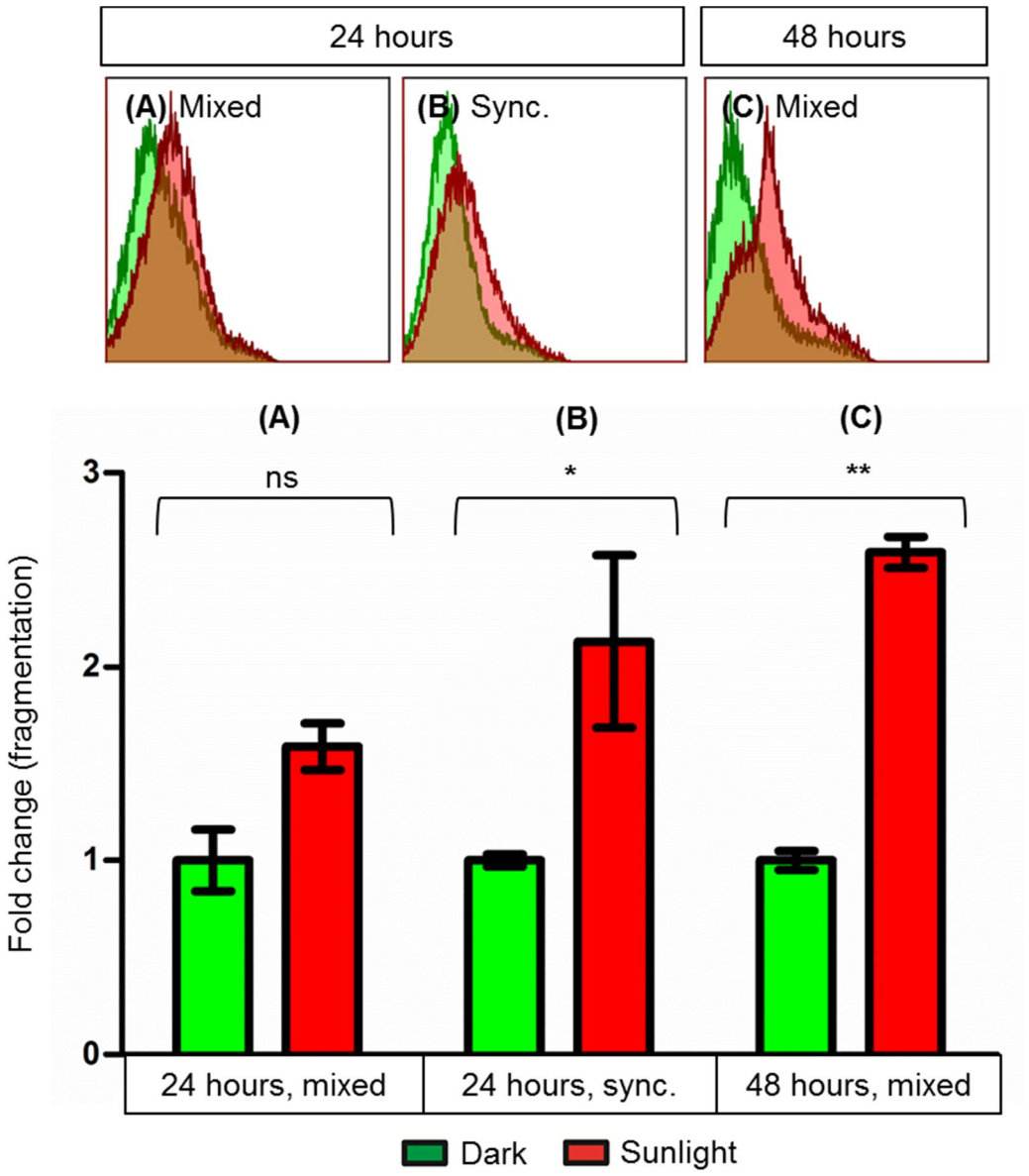

24 hours
48 hours
(A) Mixed
(B) Sync.
(C) Mixed
(A)
(B)
(C)
ns
*
**
Fold change (fragmentation)
3
2
1
0
24 hours, mixed
24 hours, sync.
48 hours, mixed
Dark
Sunlight

Figure 4

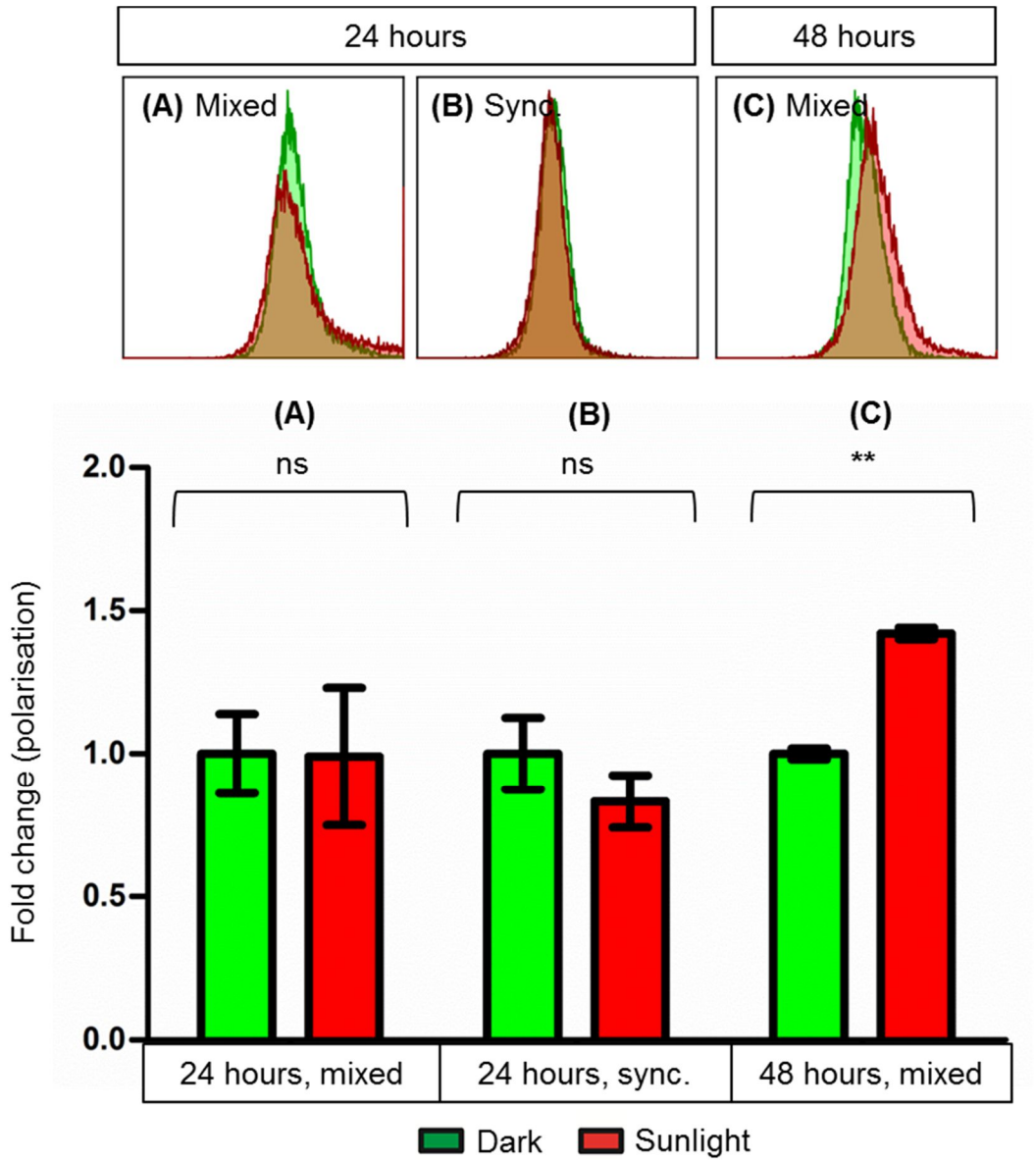
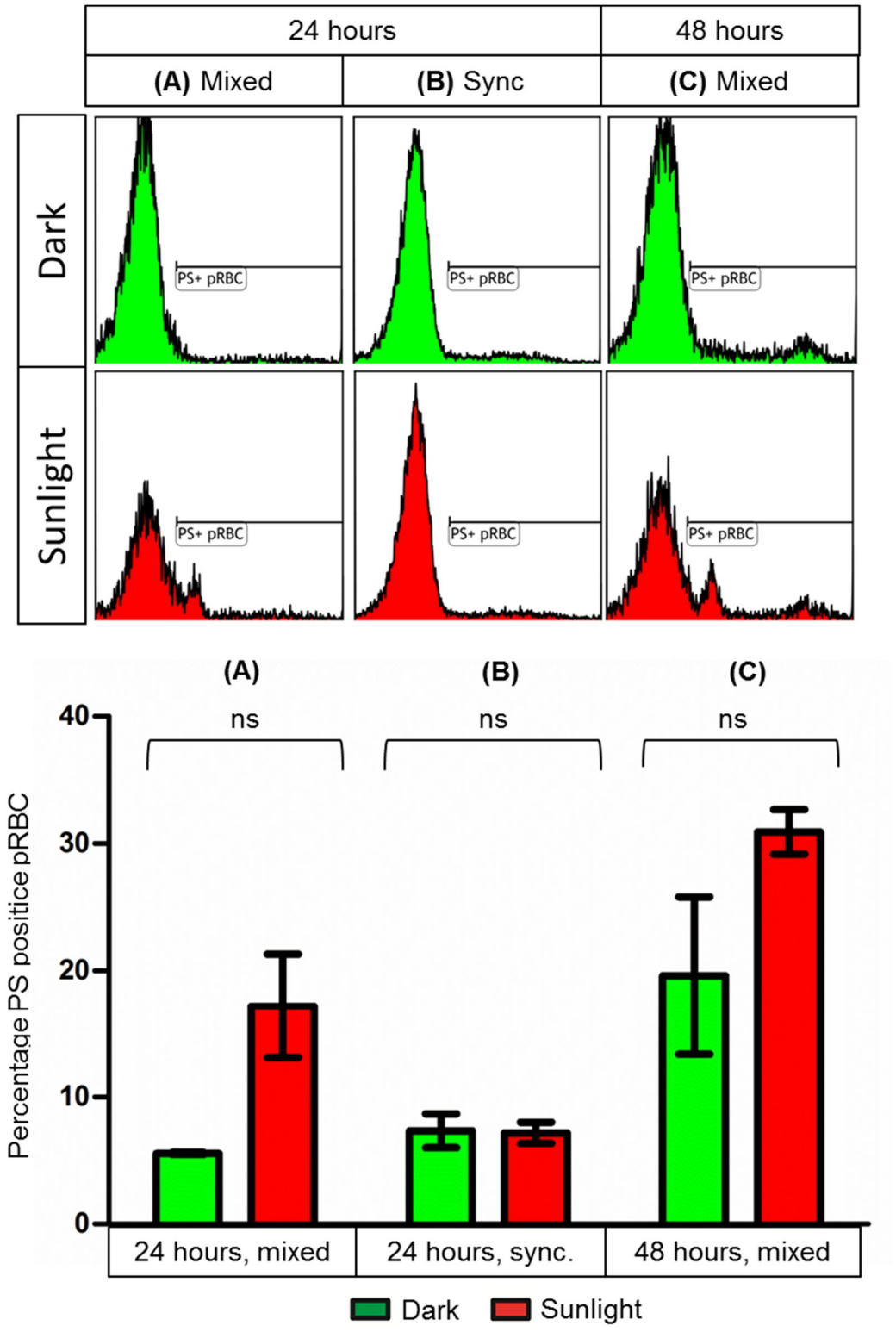

24 hours
48 hours
(A) Mixed
(B) Sync.
(C) Mixed
(A)
(B)
(C)
ns
ns
**
2.0
1.5
1.0
0.5
0.0
Fold change (polarisation)
24 hours, mixed
24 hours, sync.
48 hours, mixed
Dark
Sunlight

Figure 5


24 hours
48 hours
(A) Mixed
(B) Sync
(C) Mixed
Dark
Sunlight
PS+ pRBC
(A)
(B)
(C)
ns
Percentage PS positice pRBC
40
30
20
10
0
24 hours, mixed
24 hours, sync.
48 hours, mixed
Dark
Sunlight

8 CONCLUSION

At the outset, this study had what appeared to be a fairly simple goal: to prove that intra-erythrocytic *P. falciparum* undergoes PCD by showing a number of biochemical markers of one or another phenotype of PCD, such as apoptosis. Various phenotypes of PCD are well-characterised from mammalian studies, and the line between PCD and non-PCD cell death mechanisms, such as necrosis, was clearly drawn. However, phenotype definitions quickly proved to be anything but clear, with most biochemical markers of cell death not being exclusive to any one phenotype and overlap existing not only between different PCD phenotypes, but also between PCD and cell death mechanisms previously considered as non-PCD. In *P. falciparum*, the matter is even more confounding and no clear consensus could be reached from previous studies of PCD in *P. falciparum.*

In light of a gathering body of conflicting evidence, this study identified a number of possible areas that could account for the often stark differences in the results and conclusions of various previous studies. Amongst these, the use of different strains, stress stimuli and markers of cell death were suggested to be potential obstacles to cohesive cell death characterisation. In this regard, this study represents a fundamental step forward in understanding the inner workings of cell death in *P. falciparum*. This study utilised a range of biochemical markers of cell death, rather than depending on a single "hallmark" feature on its own. Furthermore, the same set of markers was assayed for a variety of vastly different inducers of cell death. Importantly, physiologically relevant stress stimuli that the parasite would normally encounter during malaria illness were chosen above drug pressures often applied to PCD studies in *P. falciparum*. All these factors together ensured a comprehensive and holistic characterisation of cell death phenotypes.

However, instead of providing a clear-cut conclusion of the cell death mechanism, the data implied a smorgasbord of cell death phenotypes. Under stress from high parasitaemia, results clearly indicated apoptosis, with mitochondrial depolarisation and PS externalisation followed by DNA fragmentation. This combination of PCD markers undeniably draws parallels to the intrinsic pathway of mammalian apoptosis, although proteins associated with this pathway, such as the Bcl-2 family or Smac, have not been identified in *P. falciparum*. After exposure to sunlight, mitochondrial hyperpolarisation occurred after DNA fragmentation and no significant PS externalisation was observed. The greatest surprise though, came from heat-stressed *P. falciparum* – here the cell death mechanism differed for different intra-erythrocytic stages of the parasite. Early stage parasites were more severely affected than previous studies suggested and showed clear markers of apoptosis, including mitochondrial depolarisation and DNA fragmentation, albeit without PS externalisation. In contrast, late stage parasites showed only slight DNA fragmentation and mitochondrial hyperpolarisation as well as cytoplasmic vacuolisation, suggestive of an autophagy-like response. The latter was the only instance in which morphological criteria were combined with biochemical markers, as the morphology was unique and essential to clarify the phenotype after heat stress. Late stage pRBC at the time of heat stress also showed PS externalisation the following day, when parasites were expected to reinvade to form new rings. Furthermore, some late stage parasites exposed to heat stress were shown to be alive by real-time microscopy, despite no increase in parasitaemia for 2.5 life cycles, with growth recovery only observed after 5 days.

It should be noted that PS externalisation was measured on the outer membrane of pRBC and nRBC in the same culture, rather than on the plasma membrane of parasites enclosed

in erythrocytes. While this does not serve the same purpose as PS externalisation in apoptotic mammalian cells, it is used as a surrogate marker to understand the state of stress of the underlying parasite and how it might modify the host cell membrane to its own advantage. However, PS externalisation may also be induced by the host as a defence mechanism. Nonetheless, its correlation with other PCD markers makes it a valuable tool in studying parasite self-regulation of cell death.

It is therefore clear that *P. falciparum* displays markers of PCD, the combination and timing of which differs with the stress stimulus and also with the intra-erythrocytic developmental stage of the parasites. DNA fragmentation was the only marker that appeared after all stimuli. Arguably, the most important consistent feature was survival of some parasites despite potentially lethal stimuli and the death of significant portions of the parasite population. This observation is crucial to our understanding of PCD: in order for it to serve the purpose of self-limitation, a portion of the parasite population must survive to continue and transmit the infection.

Apoptosis has been documented in *P. falciparum* ookinetes in the mosquito midgut after cell death presented with loss of the $\Delta\psi_m$, nuclear chromatin condensation, DNA fragmentation and PS externalisation (Arambage *et al*., 2009). It has been suggested that PCD in ookinetes may protect the mosquito from being overwhelmed by ingested parasites and limit the competition for resources amongst developing ookinetes (Hurd and Carter, 2004). However, extracellular mosquito stages have drastically different biology than the intracellular erythrocytic stages.

Data from this study suggest that blood stage *P. falciparum* exhibited various phenotypes of PCD, including apoptosis and autophagic-like cell death. In isolation, some markers might also be attributed to necrosis-like cell death, although this seems less likely when all the data are combined into a holistic picture of death. It is worth noting that an apoptosis-like cell death phenotype was concluded twice, based on DNA fragmentation and mitochondrial dysregulation, but that PS externalisation only occurred in one instance, under conditions of high parasitaemia. This may indicate that PS externalisation during high parasite density was caused by a factor other than parasite death. More likely however, it simply points again to the diverse strategies that *P. falciparum* may utilise to live and die. One must then conclude that the well-classified mammalian cell death phenotypes might not apply to an organism as evolutionary distant and distinct from other taxa (Durand, 2010) as *P. falciparum*. It then follows that by trying to force the dying parasite into one phenotype or another, critical and fascinating aspects of how *P. falciparum* regulates the number of asexual intra-erythrocytic parasites might be overlooked. Certainly, *P. falciparum* undergoes a regulated cell death mechanism. Throughout the thesis this has been referred to as PCD, although it might equally be called active cell death or regulated cell death. However, the phenotype is most likely unique to the parasite and will not align entirely with classical definitions.

Set against a backdrop of ever-evolving nomenclature and definitions of cell death, it is clear that the paradigm of PCD in *P. falciparum* is a dynamic one that will challenge our thinking and understanding of how the world's deadliest parasitic killer can also induce its own death to limit its damage on the host. In response to the NCCD's recommendations that morphological assessments of cell death be replaced by biochemical criteria, the author would like to offer the following amendment: in instances such as that of *P.*

falciparum, which exhibits a unique phenotype of cell death, the elucidation of genomic elements responsible for the execution of cell death may be as important as the comprehensive phenotypical characterisation of that cell death.

8.1 Future Directions

With this in mind, the next step doubtlessly entails the in-depth investigation into those genomic elements of PCD. To accomplish this, one might select either of two approaches. One might select a number of mammalian proteins involved in PCD, such as ataxia telangiectasia mutated, p53 DNA-binding domain, inhibitor of apoptosis or peptidase C14 and identify homologues in *P. falciparum* by bioinformatic manipulations (Coetzer *et al*., 2010). The expression of these genes may then be monitored after stimuli eliciting PCD. However, with the difficulty presented by *P. falciparum*'s unusually A/T-rich genome and large number of hypothetical proteins (Gardner *et al*., 2002), a reverse genetics approach might be considered. The advent of high-throughput next-generation sequencing (NGS) technologies, along with the availability of both the *P. falciparum* genome (Gardner *et al*., 2002) and transcriptome (Bozdech *et al*., 2003), has opened up new possibilities in gene expression studies. Thus, in the case of *P. falciparum*, the expression of all genes after appropriate stimuli can be examined and the resulting expression data can be used to identify the players involved in the execution of cell death. As part of transitioning this study to a post-doctoral fellowship, mRNA was extracted from heat-stressed and control parasites and sequenced by the Agricultural Research Council (ARC), Onderstepoort Veterinary Institute, Pretoria, South Africa using an Illumina HiSeq platform. Primary analysis and differential gene expression has already been performed by the Centre for Proteomic and Genomic Research (CPGR), Cape Town, South Africa. Preliminary data

indicated 38% and 39% of all genes were differentially expressed by heat-stressed parasites 2 and 4 hours after heat stress, respectively, compared to parasites maintained at 37°C. Secondary analyses and pathway construction is planned. As part of these analyses, careful attention will be paid not only to identifying proteins involved directly in *P. falciparum* PCD, but also to selected non-PCD pathways, such as the heat shock pathway. The levels of change in heat shock proteins (Hsp) will be correlated with the degree of heat stress. This may shed light on the role Hsp in PCD after heat stress.

Selected genes expected to be involved in *P. falciparum* PCD, including previously-identified putative metacaspase (PfMCA1), ataxia telangiectasia mutated (ATM) and peptidase C14, as well as some identified during transcriptome sequencing, will be utilised in double-homologous crossover gene knock-out studies to determine whether such proteins are essential for parasite survival.

Inhibition of proteins associated with mammalian PCD may also be used in combination with transcriptome sequencing and/or phenotypical characterisation to further confirm the described phenotype and identify PCD homologues in *P. falciparum*. For instance, although *P. falciparum* lacks true caspases, the application of pan-caspase or cysteine protease inhibitors to stressed parasites may identify proteases in *P. falciparum* with similar function when compared to stressed parasites lacking the inhibitors. Similarly, chemical inhibition of autophagy may further confirm the occurrence of this phenomenon in *P. falciparum*. Data obtained will be used to advance our understanding of life and death decisions in *P. falciparum*.

APPENDIX A: METHOD VALIDATIONS

A1 The Crime Scene: Methods for Investigating Cell Death in *Plasmodium falciparum*

This section details the rationale, protocols and controls to validate the assay methods used to monitor the biochemical markers of cell death, namely DNA fragmentation, mitochondrial dysregulation and PS externalisation as well as commenting on the reproducibility of experimental data from these assays. Descriptions of parasite culture methods, performed according to established methods (Trager and Jensen, 1976) with some modifications (Mphande *et al*., 2008), are described elsewhere (Publication 2-5).

A2 DNA Fragmentation: the Autopsy of Apoptosis

The assessment of DNA fragmentation is essential for comprehensive cell death characterisation, as both the amount and type of fragmentation can provide useful data. In *P. falciparum*, two methods are used to assess DNA fragmentation: (1) agarose gel electrophoresis involving the electrophoretic resolution and visualisation of isolated DNA by ethidium bromide or SYBR green staining, or more commonly (2) the TUNEL (Terminal deoxynucleotidyltransferase-mediated dUTP Nick End Labelling) assay.

Apoptotic cells produce a characteristic ladder pattern of multiples of ~200 bp on agarose gels (Duke *et al*., 1983; Elmore, 2007; Kroemer *et al*., 2009), coinciding with the nucleosome size (Arends *et al*., 1990). In contrast, non-apoptotic forms of cell death, such as necrosis, produce a smear on agarose gels. At first glance, it would appear that agarose gel electrophoresis is the ideal method to analyse apoptosis, as the appearance of an

oligonucleosomal laddering pattern would be conclusive evidence of PCD. However, the method presents a number of major disadvantages. Critically, agarose gels are not quantitative (Jonker *et al*., 1993). Furthermore, due to low sensitivity of ethidium bromide-stained gels, detection of apoptosis requires a high number of apoptotic cells, in excess of 10^6 in the case of mammalian cells (Jonker *et al*., 1993). Genomic DNA extractions required for separation of DNA by agarose gels have no fixation step. This may cause loss of low molecular weight DNA fragments that cannot be easily sedimented by centrifugation and could account for as much as 30% of cellular DNA in apoptotic cells (Arends *et al*., 1990; Darzynkiewicz *et al*., 1992). The challenge of low sensitivity has been addressed in the past by expensive and labour-intensive modifications, such as radiolabelling followed by Southern blotting (Picot *et al*., 1997). Arguably the greatest drawback to the use of agarose gels to detect PCD in *P. falciparum* derives from white blood cell contamination in the culture. The problem was demonstrated by Porter and co-workers who showed oligonucleosomal laddering in chloroquine-treated whole blood (Porter *et al*., 2008). However, after extensive leukocyte depletion by affinity chromatography, oligonucleosomal laddering was absent in *P. falciparum* under chloroquine drug pressure (Porter *et al*., 2008). In *P. falciparum* cultures, contamination by a small number of leukocytes could easily produce misleading results.

The single-cell specificity of the TUNEL assay (Jonker *et al*., 1993) eliminates the dilemma presented by leukocyte contamination, since the number of contaminating cells is insignificant when analysed by the TUNEL assay, as illustrated by the assessment of the assay's performance and protocol setup with purified erythrocyte cultures. The TUNEL assay is also the most commonly used method to quantify DNA fragmentation in *P. falciparum* (Ch'ng *et al*., 2010; Deponte and Becker, 2004; López *et al*., 2010; Meslin *et*

al., 2007; Mutai and Waitumbi, 2010; Oakley *et al*., 2007; Totino *et al*., 2008). The assay utilises *in situ* nick translation (Kerem *et al*., 1983) to label and detect DNA fragmentation in apoptotic cells. The terminal deoxynucleotidyltransferase (TdT) enzyme (Gorczyca *et al*., 1993) adds fluorochrome-conjugated deoxynucleotides to free 3'OH-ends of fragmented DNA in a single-step reaction (Gold *et al*., 1993; Li *et al*., 1995), in combination with propidium iodide (PI) staining of cellular DNA (Jonker *et al*., 1993). In applying this assay to *P. falciparum,* PI is not used to distinguish dead cells based on membrane permeability, but to detect parasites, as all cells are permeabilised. Prior to labelling, cells are fixed with a cross-linking agent to retain small DNA fragments and permeabilised to allow access to cellular DNA (Wijsman *et al*., 1993). DNA fragmentation is detected as a fluorescent signal by flow cytometry, fluorescence microscopy or light microscopy with the appropriate filters (Elmore, 2007; Vermes *et al*., 2000). The TUNEL assay offers a number of advantages, including the ability to detect early DNA breaks during apoptosis (Darzynkiewicz *et al*., 1992) and single-cell specificity (Jonker *et al*., 1993). For the above reasons, the TUNEL assay was selected as the method of choice for assessing DNA fragmentation in PCD experiments.

A2.1 TUNEL assay method

The TUNEL assay was performed with the APO-Direct kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions, with modifications similar to a previous study (Oakley *et al*., 2007). The method is described in full below as performed for control and treated experimental samples. For each experiment, control samples equivalent to experimental samples, except lacking exposure to the chosen stress stimulus,

were collected from parallel unstressed control cultures and assayed in the same manner as treated experimental samples.

One to 1.5 ml whole *P. falciparum* culture was centrifuged for 5 min at 300 xg and 20°C. The supernatant was aspirated and the cell pellet resuspended in 4% paraformaldehyde in phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and briefly vortexed. The solution was incubated on ice for 60-90 min to allow fixation, after which the sample was again centrifuged for 5 min at 300 xg and 20°C. The supernatant was aspirated and the cell pellet was washed once with 1 ml PBS and the centrifugation repeated. The supernatant was aspirated and the cell pellet was resuspended in permeabilisation solution of 0.1% tri-sodium citrate (w/v) and 0.1% TritonX-100 (v/v) in PBS. The solution was incubated on ice for 3 min and centrifuged for 5 min at 1000 xg and 4°C. The supernatant was removed and the cell pellet resuspended in 1 ml PBS. **Overnight step**: at this point, samples were refrigerated at 4°C overnight. **Wash step:** Samples were centrifuged for 5 min at 1000 xg and 4°C. The cell pellet was resuspended in 1 ml Wash Buffer (supplied with the kit) and the solution centrifuged for 5 min at 1000 xg and 4°C. **Labelling step:** The supernatant was discarded and the pellet resuspended in 50 µl freshly made DNA labelling solution (10 µl Reaction buffer, 0.75 µl TdT enzyme solution, 8 µl FITC dUTP, all supplied with the kit, and 32.25 µl nuclease-free water per reaction) and incubated for 60 min at 37°C in the dark in a waterbath. Following staining, 1 ml Rinse Buffer (supplied with the kit) was added and the sample was centrifuged for 5 min at 1000 xg and 4°C. **Staining step:** The resulting cell pellet was resuspended in 500 µl PI/RNase solution (supplied with the kit) and incubated in the dark at room temperature for 30 min. Samples were analysed on a FC500 or Gallios flow cytometer (Beckman Coulter Inc., Miami, FL, USA) within 3 hours after staining. PI-positive parasites were acquired on

a FL2 time-of-flight (lin) versus FL2 integral (lin) dot plot, with gated parasites analysed on a FL1 integral (log) histogram for DNA fragmentation, measured as FITC-dUTP fluorescence.

A2.2 Controls used for validation

A2.2.1 Positive control: DNase-treated, TUNEL-positive for fragmentation

Positive control samples were prepared as above until completion of the **overnight step**. Following overnight refrigeration, the samples were centrifuged for 5 min at 1000 xg and 4°C. The supernatant was discarded and the cell pellet resuspended in 50µl DNase 1 digestion solution (30 U DNase 1, 10 mM Tris-HCl at pH 7.25, 2.5 mM $MgCl_2$ and 100 nM $CaCl_2$ in nuclease-free water). The solution was incubated at 37°C for 1 hour and centrifuged for 5 min at 1500 xg and 4°C. The protocol was continued from the **wash step** onwards as indicated above. DNase-treated parasites were acquired as PI-positive events (**Fig. A1A DNase**), the same as control samples (**Fig. A1A Control**), but showed an increased TUNEL stain (FL1) fluorescence (**Fig. A1B DNase**), producing a population with a shift towards the right when compared to control samples (**Fig. A1Cii**).

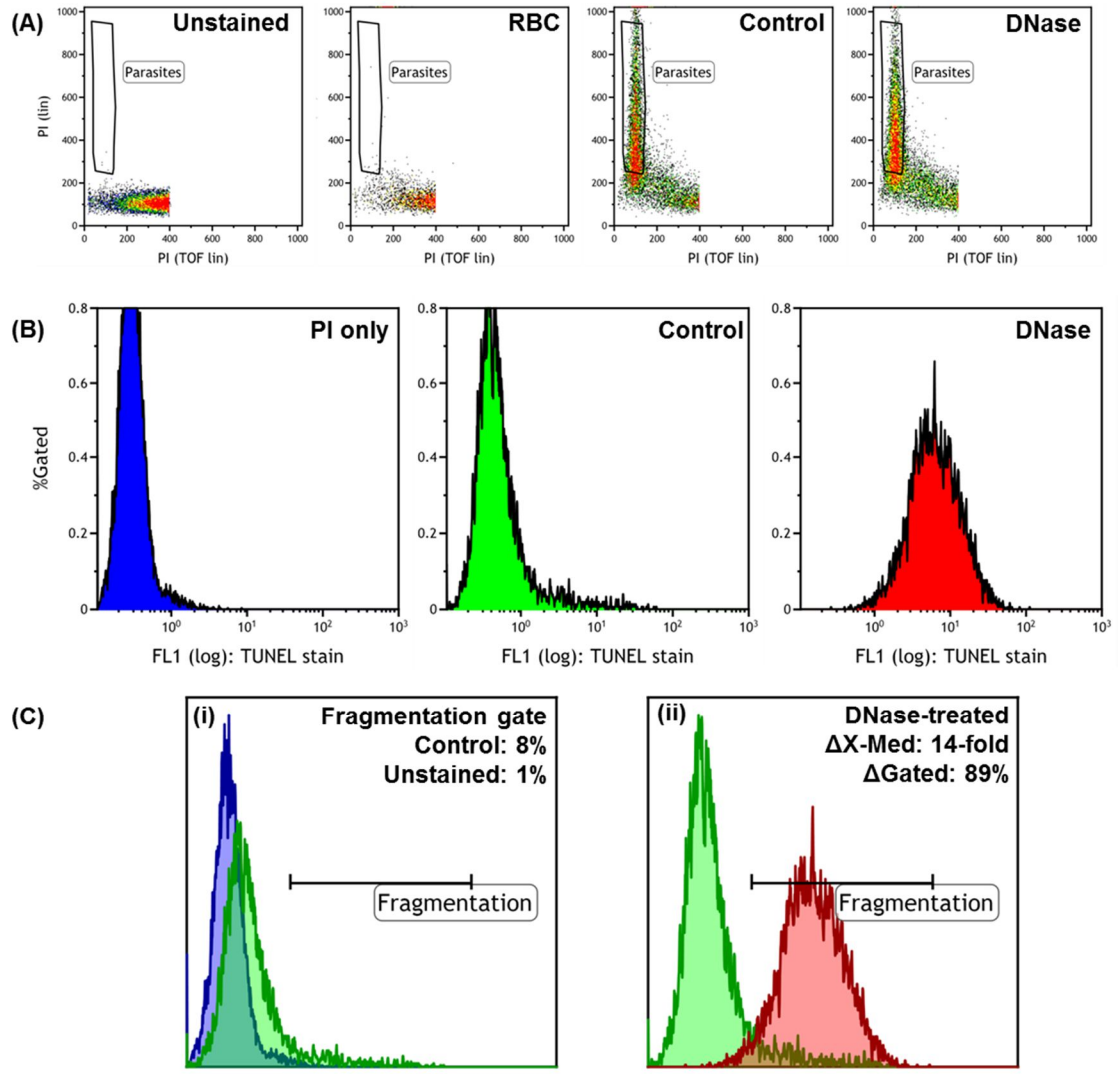


Figure A1: Gating strategy and staining controls for the TUNEL assay.
(A) Fixed, stained and isolated parasites are initially acquired as PI-positive events on a PI integral linear (FL2 INT lin) vs. PI time-of-flight (FL2 TOF lin) density plot. **Unstained** and non-parasitised erythrocyte (**RBC**) controls show negligibly few events in the PI-positive [Parasites] gate. **DNase**-treated and untreated **Control** parasites show similar acquisition in the same gate.
(B) PI-positive events are interrogated on a single parameter histogram for TUNEL (FITC-dUTP) fluorescence (FL1 INT log). Control parasites without TUNEL staining, but with PI (**PI only**) show a population with FITC fluorescence below 10^0. **Control** parasites show the expected TUNEL fluorescence of an overwhelmingly TUNEL-negative (but stained) parasite population. **DNase**-treated positive control parasites show a large increase in FITC fluorescence, as indicated by a population shift towards the right.
(C) Overlays of histograms indicate low levels of background positivity for FITC-unstained and stained control parasites **(i)**; and the differences in the median FITC fluorescence (**ΔX-Med**) and gated population percentages (**ΔGated**) between control and DNase-treated parasites **(ii)**. For Control, DNase-treated and PI-only staining control, at least 5 000 parasites were acquired. No TUNEL histograms **(B)** are shown for **Unstained** (no PI, no FITC) and **RBC** controls, as interrogation of events on FITC histograms are dependent on prior acquisition of PI-positive events, which those controls lack.

A2.2.2 Staining control: unstained sample

This control established that none of the reaction components other than the labelling (FITC-dUTP) and staining (PI) fluorochromes used (whether cells, cell components or reagents) would exhibit auto-fluorescence in the channels used (FL1 and FL2). The samples were prepared as indicated above, except that the FITC-dUTP during the **labelling step** was replaced with nuclease-free water and the PI/RNase solution during the **staining step** was replaced with PBS. The sample showed negligibly few events in the PI-positive acquisition gate (**Fig. A1A Unstained**).

A2.2.3 Staining control: PI only sample

This control established that the acquisition of DNA-containing, isolated and fixed parasites in the FL2 channel was due to PI uptake and that PI did not interfere with the detection of FITC in the FL1 channel. The sample was prepared as indicated above, except that the FITC-dUTP during the **labelling step** was replaced with nuclease-free water. As expected, this sample showed very low FL1 fluorescence (**Fig. A1B PI only**).

A2.2.4 Sample acquisition control: Non-parasitised erythrocyte sample

This control was included to show that acquisition of parasites as PI-positive events on the FL2 channel was capable of excluding all other cell components (uninfected erythrocytes) except parasites. The sample was prepared as indicated above, except that 1 to 1.5 ml non-parasitised erythrocytes suspended in culture medium at 5% haematocrit (RBC culture) was initially collected instead of whole *P. falciparum* culture. Stained RBC showed negligibly few events in the PI-positive gate (**Fig. A1A RBC**), confirming that non-infected erythrocytes could be excluded by PI staining.

A3 Detecting Changes in Mitochondrial Polarisation in *P. falciparum*:

The mitochondrial membrane potential is normally evaluated with lipophilic cationic probes (Kroemer *et al*., 1998), such as $DiOC_6(3)$, that bind mitochondria with intact $\Delta\psi m$ with high affinity, resulting in a strong fluorescent signal (López *et al*., 2010; Nyakeriga *et al*., 2006; Porter *et al*., 2008). Due to high background $DiOC_6(3)$ staining from dye accumulation in non-parasitised RBC (nRBC), $DiOC_6(3)$ staining was combined with hydroethidine (HE) to distinguish DNA-containing pRBC.

A3.1 Mitochondrial transmembrane potential ($\Delta\psi_m$) assay method

The $\Delta\psi_m$ assay was performed similar to previous descriptions (Srivastava and Vaidya, 1999), with some modifications. *P. falciparum* whole culture samples (50 µl) were diluted to 1 ml in PBS. **Staining step:** Samples were stained with a final concentration of 10 nM $DiOC_6(3)$ and 50 µM HE in 5% (v/v) DMSO for 45 min at 37°C in the dark. Following incubation, the solution was centrifuged for 5 min at 300 xg and 20°C. Supernatant was discarded and the cell pellet resuspended in 1 ml PBS. Samples were analysed immediately by flow cytometry with a Gallios (Beckman Coulter Inc., Miami, FL, USA). Erythrocytes were gated on a forward- versus side-scatter dot blot. HE-positive pRBC counted on a FL2 integral (log) histogram were analysed for DiOC6(3) fluorescence on a FL1 integral (log) histogram.

A3.2 Controls used for validation

A3.2.1 Positive control: CCCP-treated

The positive control was used to establish that mitochondrial depolarisation could be observed as decreased FL1 fluorescence, measuring $DiOC_6(3)$ staining. Prior to the **staining step**, the positive control was treated with the mitochondrial uncoupling agent carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP: 200 nM in 0.2% [v/v] DMSO) in PBS and incubated for 60 min at 37°C. Thereafter, the sample was centrifuged for 5 min at 1000 xg and 20 °C, the cell pellet resuspended in 1ml PBS and the staining was completed as described above. As expected, the positive control showed decreased $DiOC_6(3)$ (FL1) staining, indicated as a population shift towards the left, compared to control samples (**Fig. A2Dii**).

A3.2.2 Negative control: untreated

The negative control sample was untreated and stained with both $DiOC_6(3)$ and HE, with the aim of establishing the staining of pRBC with intact $\Delta\psi_m$ (**Fig. A2A & B Control**). Negative control samples were collected for each experiment from unstressed cultures maintained in parallel to stressed cultures, to establish a baseline staining for each study. For the purpose of protocol setup, prior to the **staining step**, the negative control was treated with 0.2% (v/v) DMSO and incubated for 60 min at 37°C to provide equal treatment to the positive control, except for the addition of CCCP. Following incubation, the sample was centrifuged for 5 min at 1000 xg and 20 °C, the cell pellet resuspended in 1ml PBS and the staining was completed as described above.

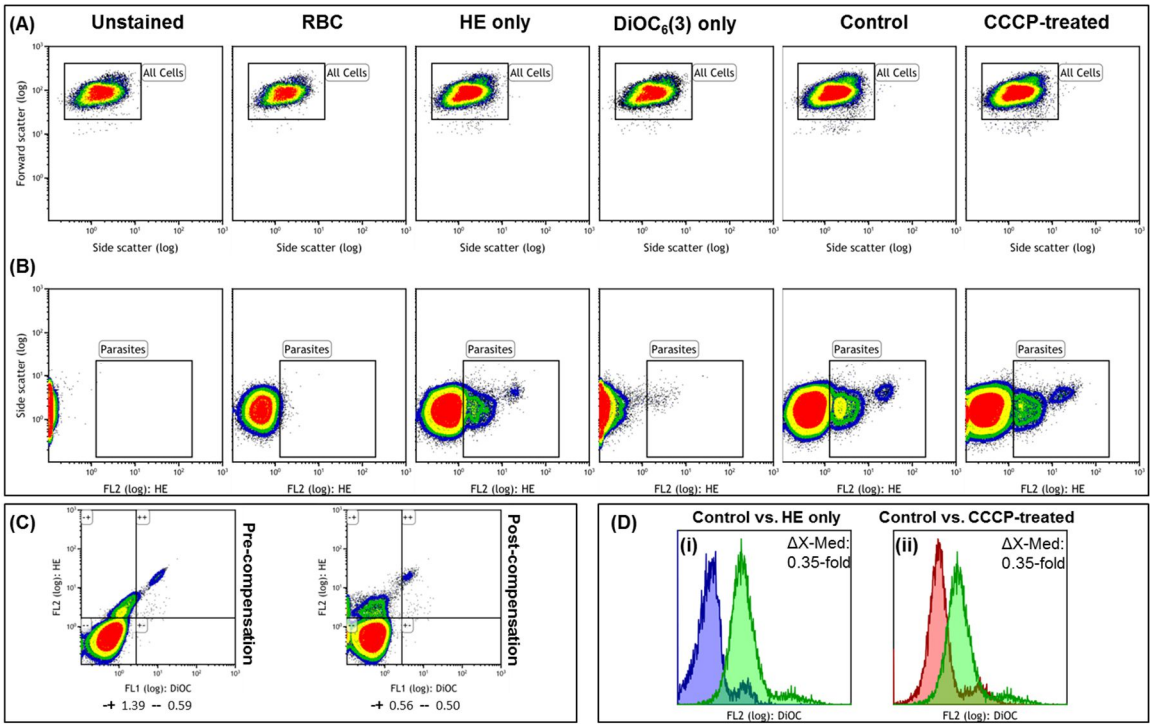


Figure A2: Gating strategy and staining controls for DiOC$_6$(3) assay for mitochondrial transmembrane potential.

(A) Forward- vs. side-scatter density plots were used to acquire erythrocyte populations in the [All Cells] gate, with no differences between the acquisition of various staining or treated controls.

(B) Gated cells were interrogated on a side scatter (SS log) vs. DiOC$_6$(3) fluorescence (FL2 INT log) density plot to discriminate HE-positive pRBC in the [Parasites] gate. **Unstained** controls, without HE or DiOC$_6$(3) stains showed minimal fluorescence. Non-parasitised erythrocytes (**RBC**) stained with HE and DiOC$_6$(3) showed the expected HE-negative population. **HE only** staining control (pRBC, no DiOC$_6$(3) stain) was used to gate HE-positive pRBC in the [Parasites] gate. **DiOC$_6$(3)** only staining control (pRBC, no HE) showed low background fluorescence in the [Parasites] gate, caused by slight spill-over of DiOC$_6$(3) (FL1) into the HE (FL2) channel. No differences were observed between the acquisition of **Control** and **CCCP-treated** pRBC, stained with both HE and DiOC$_6$(3).

(C) Significant spill-over of HE fluorescence (FL2) into the DiOC$_6$(3) channel (FL1) necessitated compensation. The **HE only** staining control is shown on a two parameter (HE: FL2 INT log vs. DiOC$_6$(3): FL1 INT log) density plot before (**Pre-compensation**) and after (**Post-compensation**) compensation. The x-axis median fluorescence for the left upper (-+) and left lower (--) quadrants is indicated below the plots. Both quadrants should have equal x-axis fluorescence as all events are DiOC$_6$(3) negative (x-axis). A large fluorescence difference between the two quadrants before compensation (1.39 vs. 0.59) was corrected after compensation (0.56 vs. 0.50). **(D)** Overlays of DiOC$_6$(3) (FL1 Int log) histograms from individual samples, acquired as HE-positive events in the [Parasites] gate show the change in DiOC$_6$(3) fluorescence (**ΔX-Med**) for HE-only and CCCP-treated controls relative to the stained, untreated control (**green**). HE-positive, DiOC$_6$(3)-negative events (**blue**) showed expected low fluorescence (**i**), while the CCCP-treated control (**red**), showed a decrease in DiOC$_6$(3) fluorescence, indicated by a population shift to the left (**ii**), that is expected from mitochondrial transmembrane depolarisation. Approximately 100 000 events total events were acquired for each sample.

A3.2.3 Staining controls: unstained; HE only; and $DiOC_6(3)$ only

Staining controls were utilised to set up correct detector voltages on flow cytometry channels and to perform compensation to eliminate spill-over effects of fluorochromes. The unstained control was used to show that no reaction components, such as parasites or erythrocytes, exhibited auto-fluorescence that would interfere with fluorochrome detection. The unstained control was prepared as described above, except for the addition of HE or $DiOC_6(3)$. For the HE only control, $DiOC_6(3)$ was substituted with PBS, while for the $DiOC_6(3)$ only control, the addition of HE was omitted and replaced with PBS. Otherwise, staining was performed as described. The unstained control (**Fig. A2B Unstained**) showed negligible fluorescence by HE staining (FL 2), confirming the absence of autofluorescence in the FL2 channel. The HE only control (**Fig. A2A & B HE only**) showed lower fluorescence than the control pRBC population (**Fig. A2Di**) in the $DiOC_6(3)$ channel (FL2) after compensation had been performed (**Fig. A2C**), confirming that spill-over could be eliminated.

A3.2.4 Sample acquisition control: Non-parasitised erythrocyte sample

Non-parasitised erythrocyte culture at 5% haematocrit was used to establish that non-parasitised cells could be excluded by detection and gating of HE-stained pRBC. The sample (**Fig. A2 RBC**) was stained as described, except that the starting material was collected from an uninfected erythrocyte culture, instead of a parasitised culture.

A4 Detecting PS Externalisation in *P. falciparum*

PS externalisation in apoptotic cells is commonly detected by a flow cytometric assay that utilises FITC-conjugated annexin V to bind translocated PS, in conjunction with a membrane-impermeable vital dye such as PI, to exclude demised cells that have become permeable (Koopman *et al*., 1994; van Engeland *et al*., 1996; Vermes *et al*., 1995). Annexin V is a 319 amino acid, ~36 kDa protein with a high proportion of charged amino acids that functions physiologically as a vascular anticoagulant that inhibits thromboplastin-initiated blood plasma coagulation (Reutelingsperger *et al*., 1988) and regulates inflammation during apoptosis (Reutelingsperger and Van Heerde, 1997). Annexin V binds PS with a very high affinity in a calcium-dependant reaction, although it may also bind to other anionic phospholipids (Andree *et al*., 1990; Casciola-rosen *et al*., 1996; Reutelingsperger *et al*., 1988; Van Zandbergen *et al*., 2006). In *P. falciparum* studies, the membrane-impermeable vital dye is substituted with a membrane-permeable nucleic-acid binding dye such as HE, to allow the discrimination of DNA-containing pRBC (Pattanapanyasat *et al*., 2010).

A4.1 PS externalisation assay method

The PS externalisation assay was performed with the FITC Apoptosis Detection Kit II (BD Pharmingen, San Diego, CA, USA), with modifications similar to a previous study (Pattanapanyasat *et al*., 2010). *P. falciparum* whole culture (10 µl) was diluted to 1 ml in PBS containing 50 µM HE and 0.5% DMSO (v/v). The sample was placed in an incubator at 37°C in the dark for 20 min. Following incubation, the sample was centrifuged for 3 min at 800 xg and 20°C. **Labelling step:** The supernatant was discarded, the cell pellet

resuspended in 100 µl freshly-made 1X Annexin binding buffer containing 5 µl FITC Annexin V (both supplied with the kit) and the sample incubated in the dark at room temperature for 15 min. Following incubation, 500-1000 µl 1X Annexin binding buffer was added to dilute cells to an appropriate concentration for flow cytometry analysis. Samples were analysed within 1 hour of staining on a Gallios or FC500 flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Erythrocytes were gated on a forward- versus side-scatter dot blot and pRBC were discriminated on a FL 2 integral (log) histogram for HE fluorescence. Gated pRBC were analysed for annexin V-FITC fluorescence on a FL1 integral (log) histogram.

A4.2 Controls used for validation

A4.2.1 Binding control: recombinant Annexin V

In order to exclude the possibility of non-specific binding of Annexin V-FITC, a binding control (**Fig. A3A & B rAnnexin**) was used that was treated with recombinant Annexin V (without conjugated FITC) prior to staining with Annexin V-FITC (with conjugated fluorochrome). The control sample was treated the same as other samples, except that immediately prior to the **labelling step**, cells were incubated with 15 µg recombinant Annexin V (supplied with the kit) and incubated in the dark at room temperature for 20 min. The sample was then centrifuged for 3 min at 800 xg and 20°C. The preparation was then continued normally from the **labelling step** onwards. For the purposes of protocol setup, all other control samples, including the positive control (**Fig. A3 Control**) of pRBC stained with both Annexin V-FITC and HE, were also incubated for 20 min at room temperature in the dark prior to the labelling step.

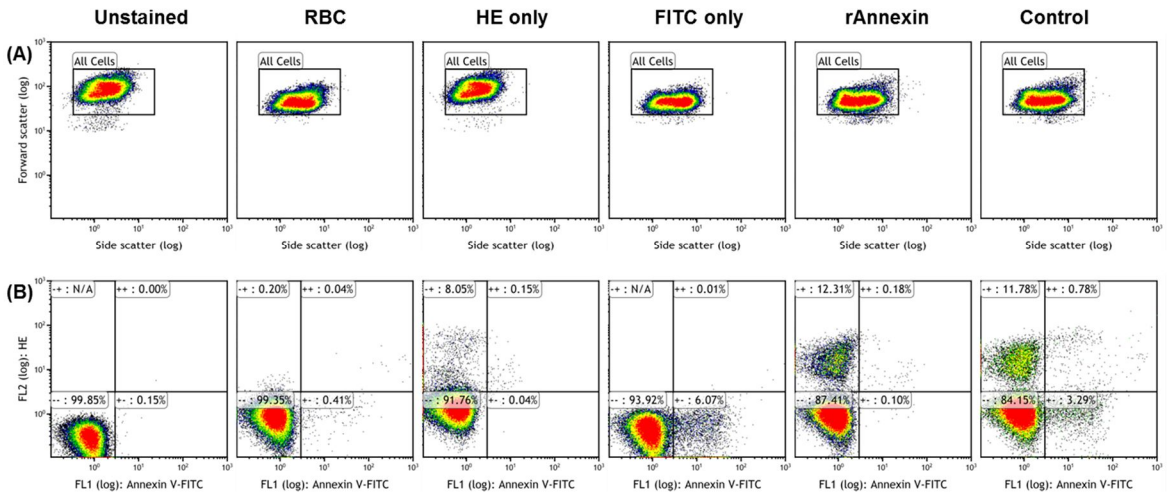


Figure A3: Gating strategy and staining controls for Annexin V-FITC method for PS externalisation.
(**A**) Forward- vs. side-scatter density plots were used to acquire erythrocyte populations in the [All Cells] gate. Placement of [All Cells] gate allows the exclusion of debris occasionally noted below the gate.
(**B**) Acquired cells were then interrogated on a two-parameter HE fluorescence (FL2 INT log) vs. Annexin V-FITC fluorescence (FL1 INT log) density plot. The **Unstained** sample, without HE or Annexin V-FITC stains showed very low fluorescence for both parameters. The stained, non-parasitised erythrocyte (**RBC**) sample allowed the gating of the double negative (HE- FITC-) population. Slight, but negligible background positivity was noted in other quadrants. The **HE only** sample (without Annexin V-FITC) allowed compensation of HE (FL2) spill-over into the FITC (FL1) channel. HE-positive pRBC can be seen with increased FL2 fluorescence, indicated by an upward population shift. Cells with increased PS externalisation could be seen to shift towards the right in the **Annexin V-FITC only** sample (without HE). A binding control treated with recombinant Annexin V (**rAnnexin**) before staining with HE and Annexin V-FITC showed negligible fluorescence in the FITC channel (FL1). Double-stained pRBC positive **Control** (both HE and Annexin V-FITC) showed populations in all four quadrants, albeit with only a small double-positive (upper right quadrant) population. Some experimental samples showed larger populations in this quadrant as PS externalisation by pRBC increased. For each sample, approximately 50 000 events were acquired in the [All Cells].

A4.2.2 Staining controls: HE only, FITC only

Staining controls were utilised to set up correct detector voltages on flow cytometry channels and to perform compensation to eliminate spill-over effects of fluorochromes. The unstained control (**Fig. A3A & B Unstained**) was used to show that no reaction components, such as parasites or erythrocytes, exhibited auto-fluorescence that would interfere with fluorochrome detection. The unstained control was prepared as described above, except for the addition of HE or Annexin V-FITC. For the HE only control

(**Fig. A3A & B HE only**), Annexin V-FITC was substituted with PBS, while for the FITC only control (**Fig. A3A & B FITC only**), HE was omitted and replaced with PBS. Otherwise, staining was performed as described.

A4.2.3 Sample acquisition control: Non-parasitised erythrocyte sample

Non-parasitised erythrocyte culture at 5% haematocrit was used to establish that non-parasitised cells could be excluded by detection and gating of HE-stained pRBC (**Fig. A3A & B RBC**). The sample was stained as described, except that the starting material was collected from an uninfected erythrocyte culture, instead of a parasitised culture.

A5: Replicates and Reproducibility: the Sanity of the Methods

"Insanity: doing the same thing over and over again and expecting different results."

-Albert Einstein

To paraphrase Einstein, the assay methods described above are only useful if one can do the same thing over and over again and expect the same results. To this extent, replicates of all data presented are indicated in figure legends. These replicates (n) always refer to independent experiments representing biological replicates, and not technical replicates, unless otherwise stated. As data presented are used to make qualitative, rather than quantitative statements, a comparatively small number of biological replicates were required to determine reproducibility. The generally small standard error of the mean (S.E.M.) of replicated data points further illustrate the reproducible nature of these experiments and assays. Thus, in this case sanity is indeed repeating the same experiment and obtaining the same result.

APPENDIX B: REAL-TIME MICROSCOPY

The following appendix contains a description of real-time microscopy data discussed in the publication "**Turning up the heat: heat stress induces markers of programmed cell death in *Plasmodium falciparum in vitro***" in *Cell Death and Disease* (2013) 4, e971, referred to on pg. 49 of this thesis.

The data can also be found at the following URL:

http://www.nature.com/cddis/journal/v4/n12/suppinfo/cddis2013505s1.html?url=/cddis/journal/v4/n12/full/cddis2013505a.html

B1 Movies Contained in the Digital Supplementary Folder Appendix B

Heat-stressed late stage parasites remain active despite growth inhibition. Real time microscopy of late stage *P. falciparum* exposed to 41°C for two hours showed numerous parasites (Movies 1-4) that exhibited movement of the hemozoin, indicating that parasites were still alive. Many trophozoites were observed that did not show such movement, suggesting that those parasites were dead (Movie 5). Many extracellular trophozoites were also observed (not shown).

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

29 May 2013

Professor Theresa Coetzer
Head: Red Cell Membrane Unit
Head: Plasmodium Molecular Research Unit
National Health Laboratory Service
University

Sent by email to Theresa.coetzer@nhls.ac.za

Dear Professor Coetzer

RE: **Protocol M031106: 'An Investigation of the Biology of the Malaria Parasite'**
Protocol amendment-Request for Renewal

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved the following amendments on the abovementioned protocol as detailed in your letter dated 20 June 2011:

- Renewal of ethics clearance M031106 now M130569 (a copy of the certificate is attached)
- Blood sampling (6-12 ml) from staff and students in the Unit

Thank you for keeping us informed and updated

Yours sincerely,

Anisa Keshav
Administrator
Human Research Ethics Committee (Medical)

R14/49 Professor Theresa Coetzer

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130569

NAME: Professor Theresa Coetzer
(Principal Investigator)

DEPARTMENT: Molecular Medicine & Haematology/RCMU
National Laboratory Services

PROJECT TITLE: An Investigation of the Malaria Parasite
(previously M031106)

DATE CONSIDERED: Ad hoc

DECISION: Renewal approved

CONDITIONS:

SUPERVISOR:

APPROVED BY: _____
Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 29/05/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

_____ _____
Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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