Perturbation of *Plasmodium vivax* hypnozoite formation, growth and reactivation *in vivo* in a human-liver chimeric mouse

Erika L. Flannery

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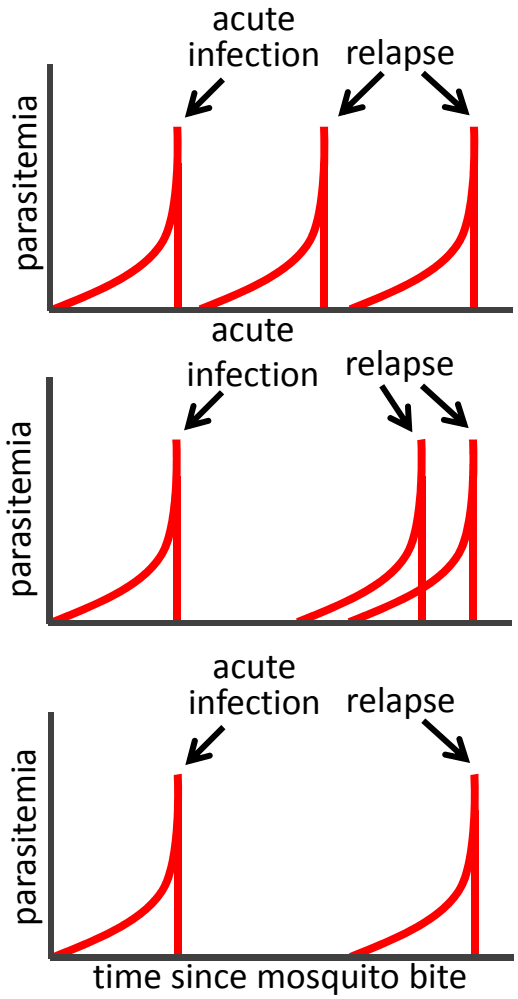
JITMM: Bangkok, Thailand

GOAL: predict radical cure activity using FRG huHep model

P. vivax radical cure _in vivo_ model development

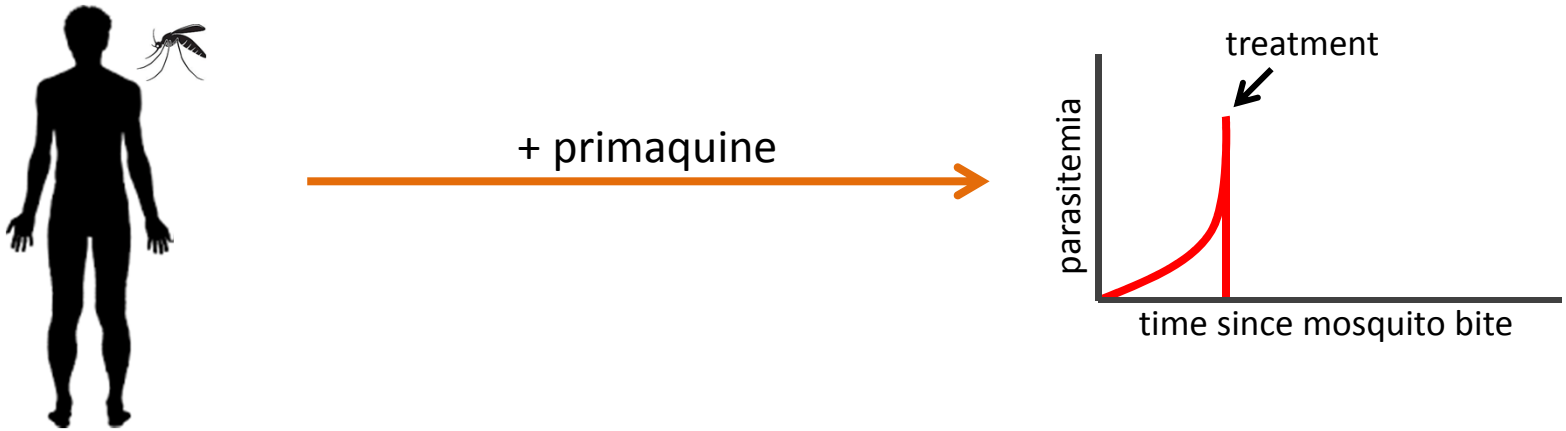
- Infection with *P. vivax* can result in a secondary, blood-stage infection (relapse)
  - Hypnozoites emerge from the liver and infect RBCs
- Relapse rates vary with geographic region
- Latent hypnozoite infection is a significant barrier to global malaria eradication efforts



GOAL: predict radical cure activity using FRG huHep model

P. vivax radical cure _in vivo_ model development

- _P. vivax_ infections are treated upon clinical detection (blood-stage)
- First-line treatment, in most countries
  - 3d of chloroquine (CQ) + 14d of primaquine (PQ) is used
- This prevents subsequent relapse (in most cases)
- Primaquine is the only anti-relapse drug and it has many liabilities



A liver-chimeric mouse to study human malaria infection

FRG huHep model

FRG huHep mouse

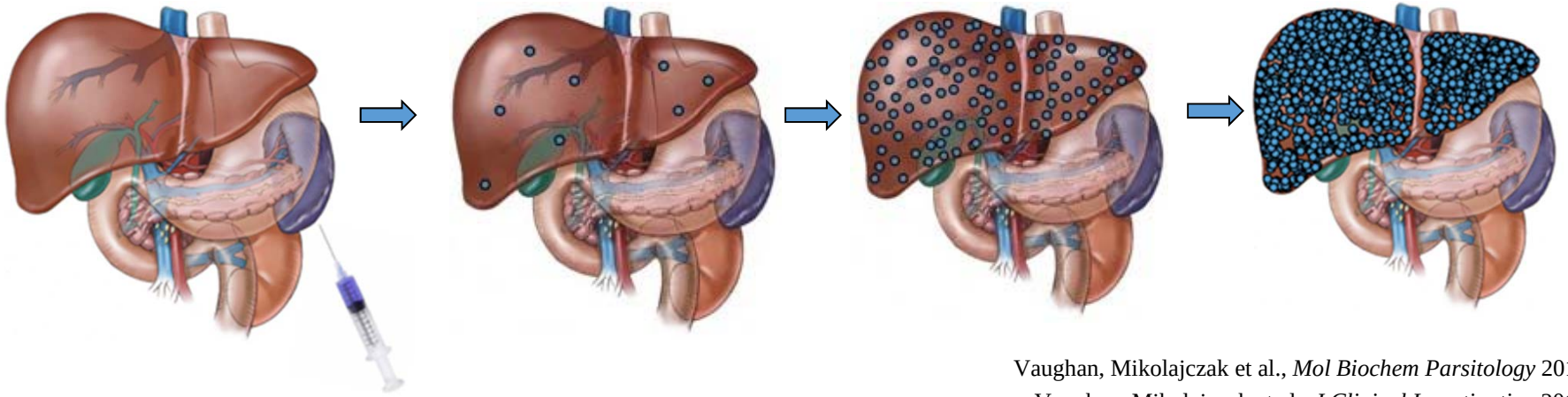
- **F**AH (fumaryl acetoacetate hydrolase) knockout: mouse hepatocyte death which can be controlled with drug
- **R**ag2 (recombination activating gene 2) knockout: T cell and B cell deficiency
- Il2r**g** (interleukin 2 subunit γ-chain) knockout: NK and NK(T) cell deficiency

cell engraftment: 0.5-1 million hepatocytes

4 weeks: 1-5 million

8 weeks: 5-10 million

12+ weeks: 50-150 million

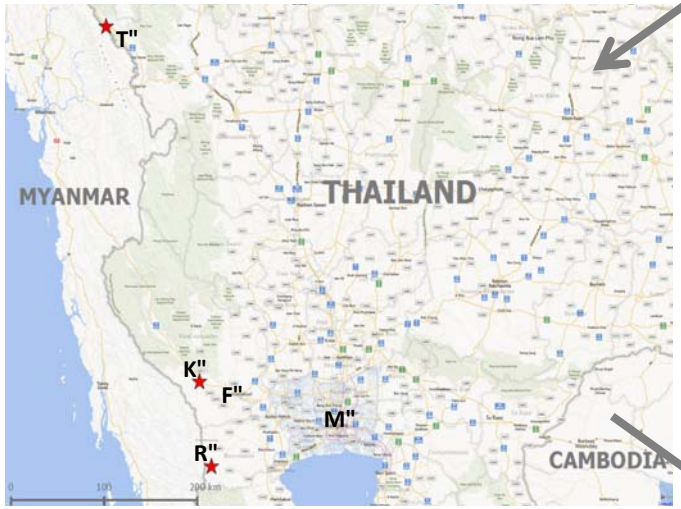


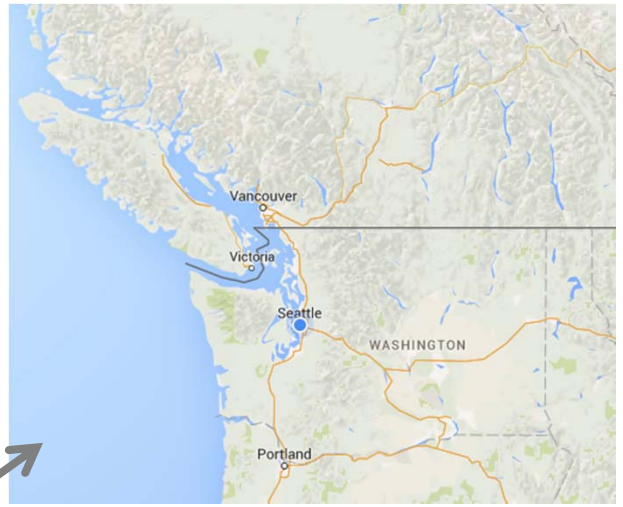
Vaughan, Mikolajczak et al., *Mol Biochem Parsitology* 2012

Vaughan, Mikolajczak et al., *J Clinical Investigation* 2012

Mikolajczak, Vaughan et al., *Cell Host Microbe* 2015

Workflow between CIDR and MVRU





Liver samples
(formalin fixed or trizol)

Contributors

Center for Infectious Disease Research

Sebastian Mikolajczak
Stefan Kappe
Eve Cheunchob
Carola Schaefer
Ashley Vaughan
Lander Foquet
Matthew Fishbaugher
Mary Jane Navarro
William Betz

Mahidol Vivax Research Unit

Jetsumon Sattabongkot
Niwat Kangwanrangsan
Narathatai Yimamnuavchok
Wanlapa Roobsoong
Rapatbhorn Patrapuvich

University of Washington

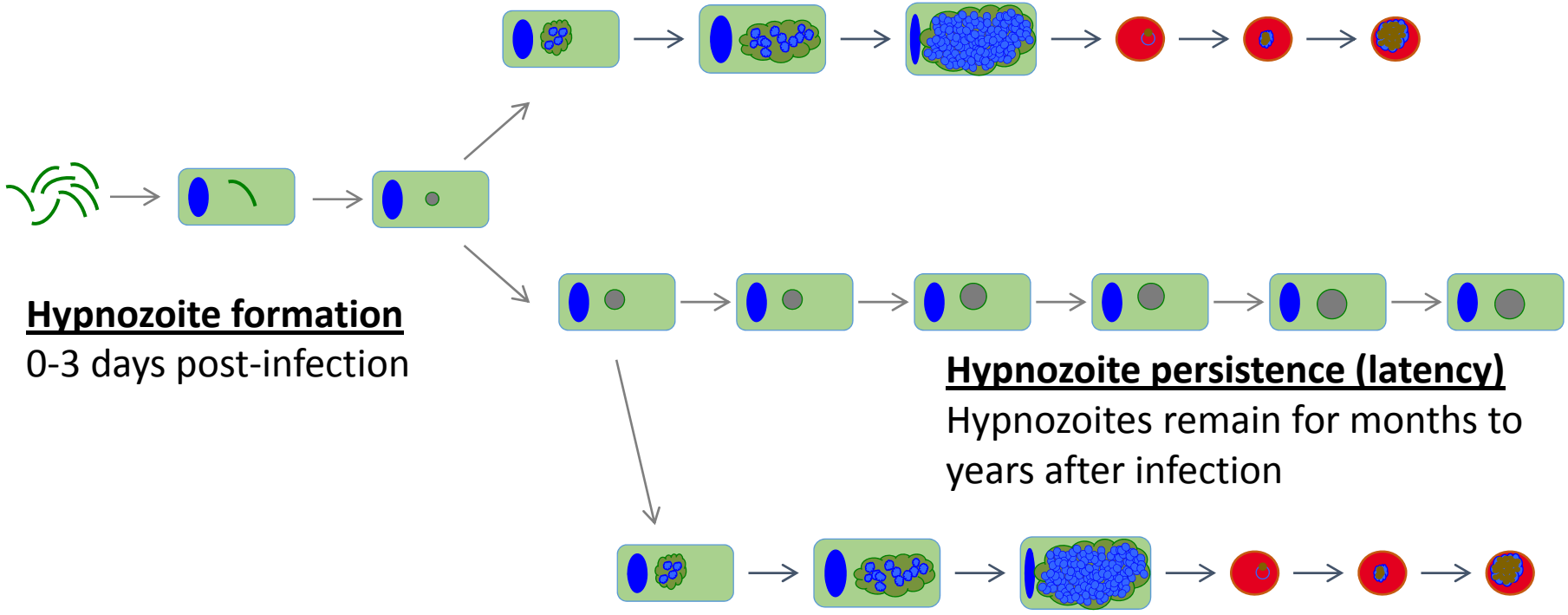
Sean Murphy
Zachary Billman

Liver stage efficacy testing against *P. vivax*

FRG huHep model

Primary liver stage infection
Schizonts mature approx. 7 days post-infection

Blood stage infection
> 7 days post-infection



Hypnozoite formation
0-3 days post-infection

Hypnozoite persistence (latency)
Hypnozoites remain for months to years after infection

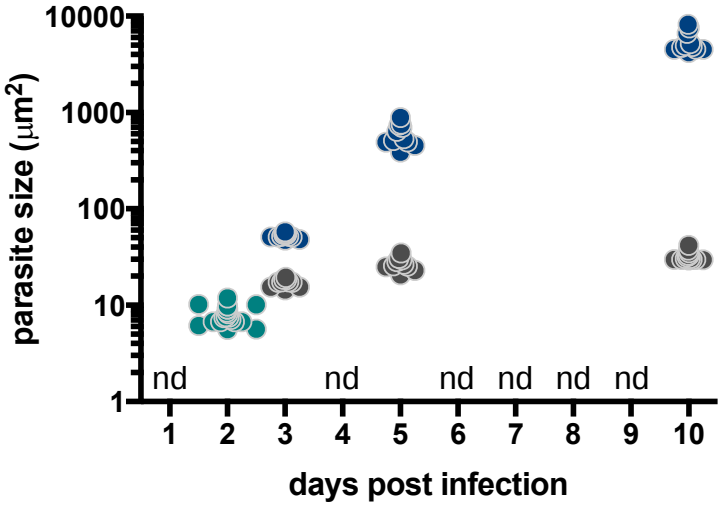
Hypnozoite activation (relapse)
Replication and blood-stage infection

Size and UIS4 staining differentiate hypnozoites

Hypnozoite formation

- Liver tissue sectioning and antibody staining in FRG huHep mice allows quantification of *P. vivax* infections

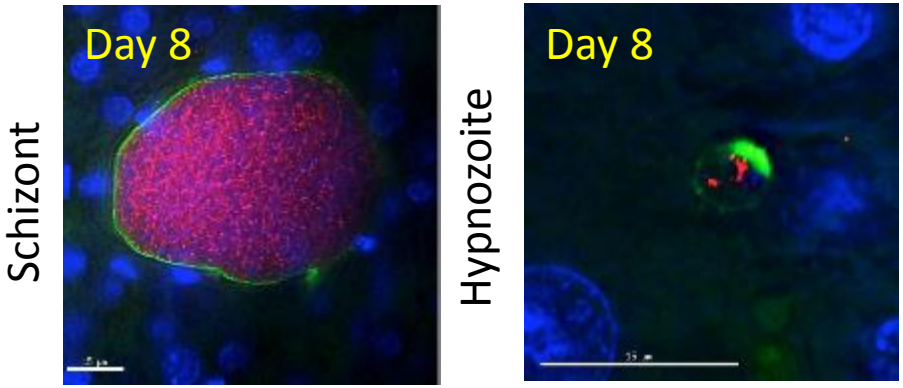
Parasite size



- trophozoite
- schizont
- hypnozoite

nd not determined

Morphological differentiation



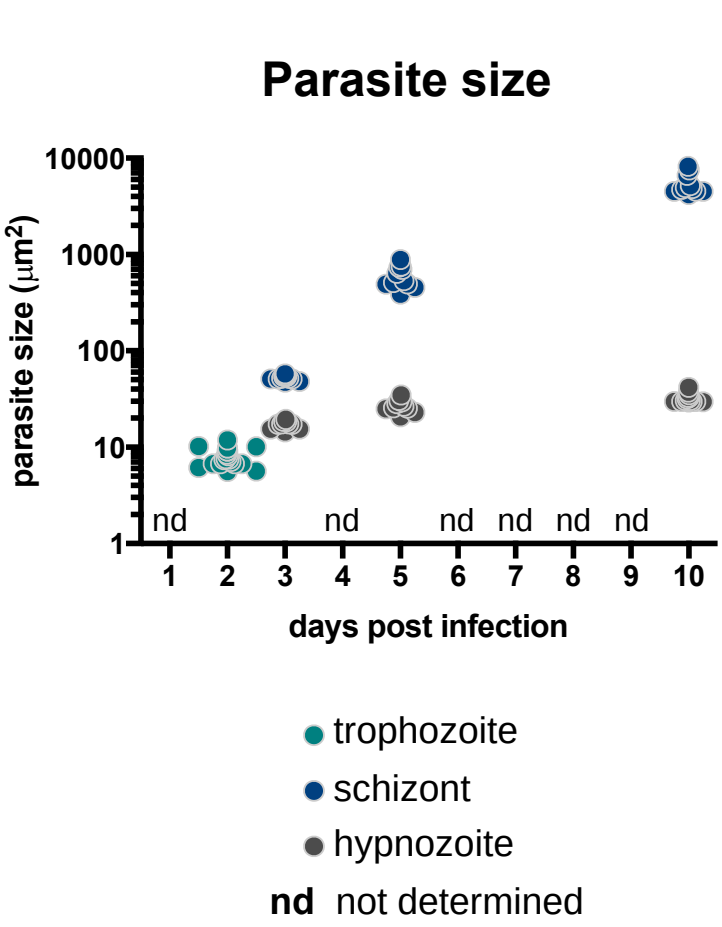
Scale bar: 15 µm

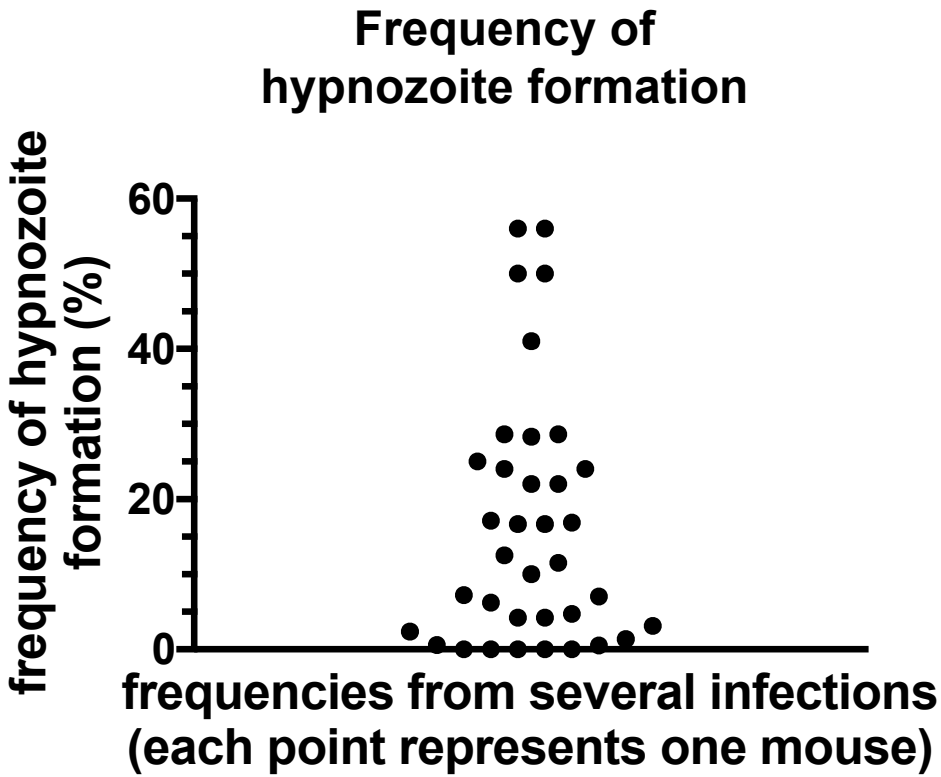
UIS4 **parasitophorous vacuole membrane marker**
ACP **apicoplast marker**
DAPI **DNA**

Size and UIS4 staining differentiate hypnozoites

Hypnozoite formation

- Liver tissue sectioning and antibody staining in FRG huHep mice allows quantification of *P. vivax* infections

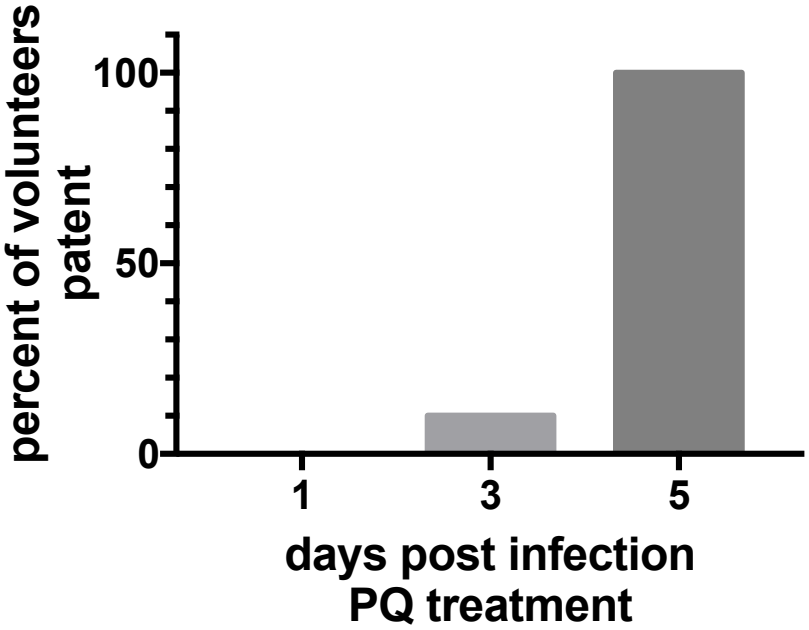




Timing of PQ treatment and efficacy in humans: a comparison

Hypnozoite functional experimentation

- Volunteers were bitten by 10 *P. falciparum* infected mosquitoes
- 30 mg single dose treatment with primaquine (PQ)

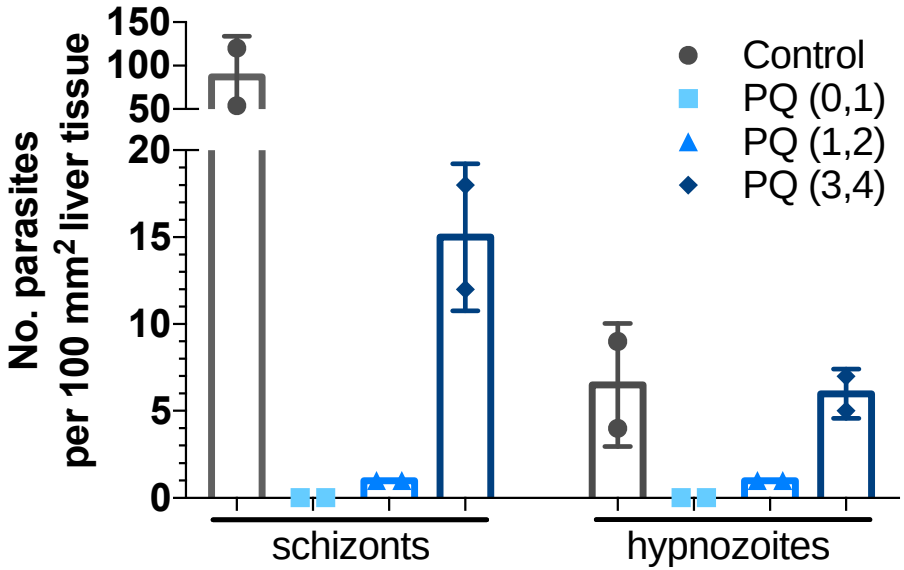


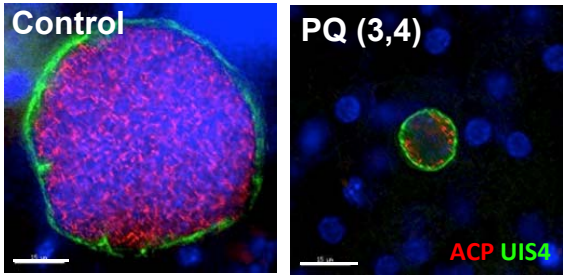
Adapted from: Arnold et al., 1955 *J Lab and Clin Med*

Mature hypnozoites respond differently to PQ

Hypnozoite functional experimentation

- **Primaquine prevents hypnozoite development**
- **Primaquine kills early stage hypnozoites and schizonts**
- **Primaquine is active in the FRG huHep mice**





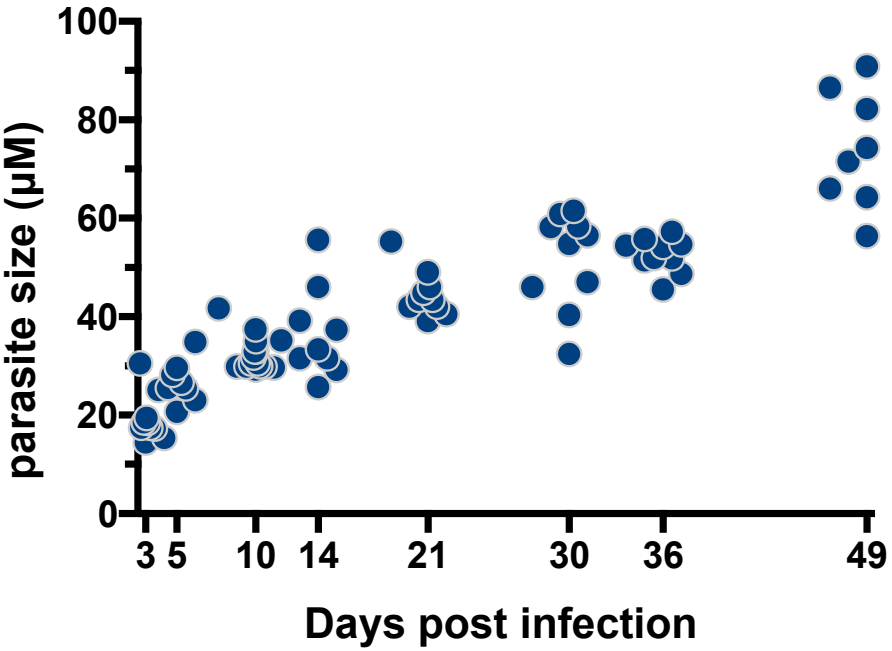
UIS4 parasitophorous vacuole membrane marker
ACP apicoplast marker
DAPI DNA

- Livers harvested 8 days after infection to allow surviving parasites to mature

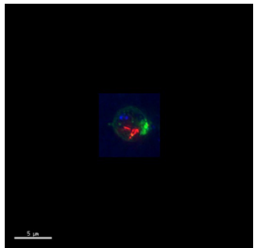
The complexity and size of hypnozoites change over time

Hypnozoite persistence

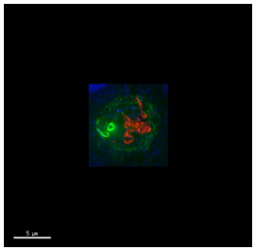
- Persistent hypnozoite infection present for at least 49 days



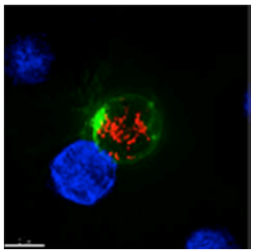
***Only labelled days were assayed**



Day 8



Day 24



Day 49

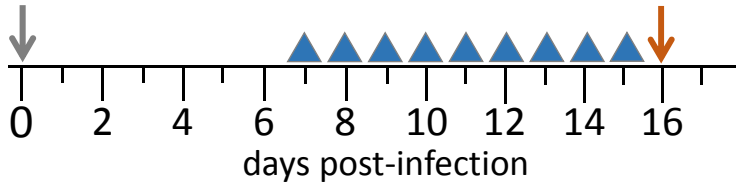
UIS4 parasitophorous vacuole membrane marker

ACP apicoplast marker

DAPI DNA

Unexpected results in FRG huHep mice following PQ treatment

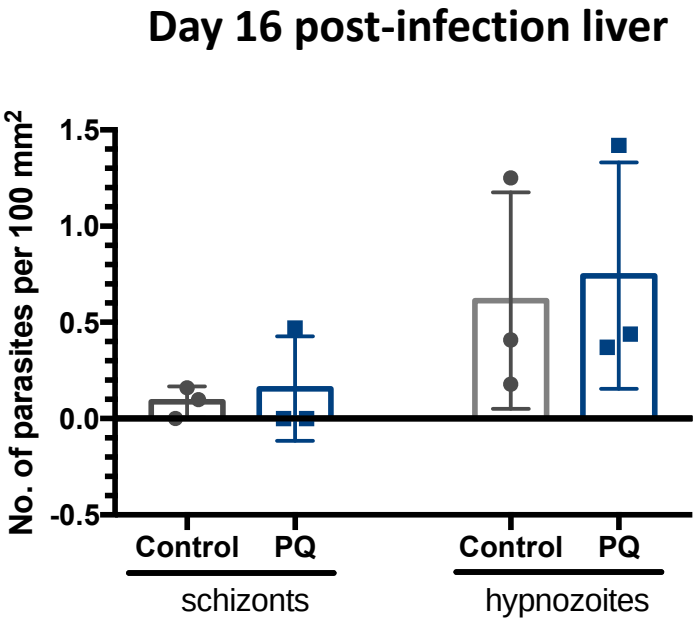
Hypnozoite persistence



↓ Infection by 1M spz i.v. (VUNL-23)

↓ liver harvest for IFA

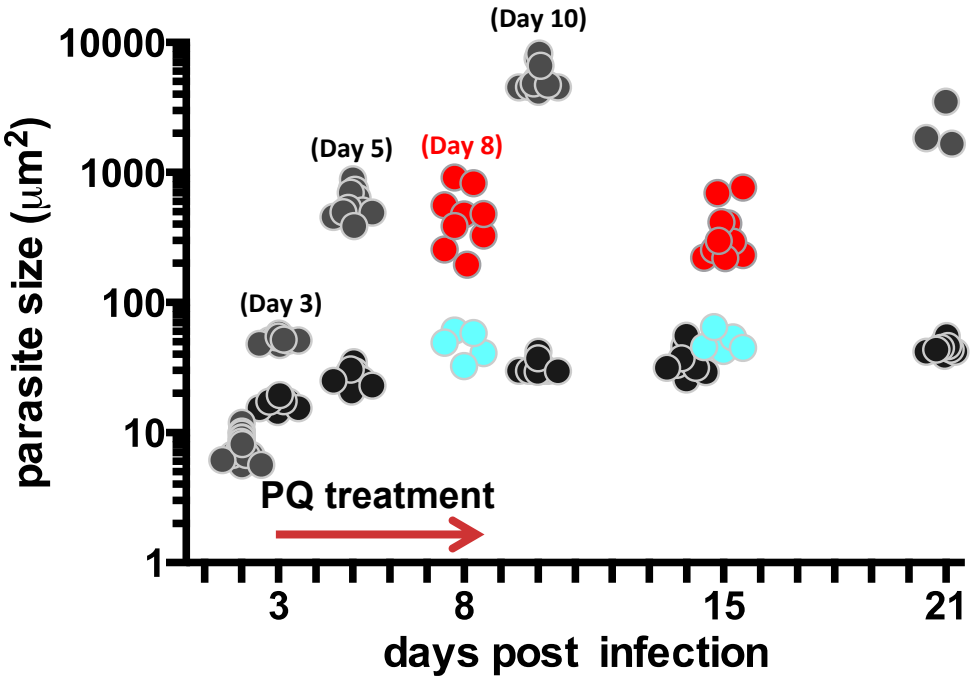
▲ 30 mg/kg primaquine (PQ) p.o.



Long-duration PQ treatment arrests schizont growth

Hypnozoite persistence

- **Primaquine arrests parasite growth, but parasites are not immediately cleared from the cell**



- schizont
- hypnzoite
- PQ treated schizont
- PQ treated hypnozoite

- 60 mg/kg PQ treatment days 3-8
- Liver harvest at day 8 or day 16

Single-dose PQ is effective, but does not clear gametocytes from the blood

Hypnozoite persistence

- Single dose treatment prevents transmission
- 24 hours post treatment gametocytes there is no reduction in the presence of gametocytes by blood smear
- At the same time, there is a large reduction in transmission

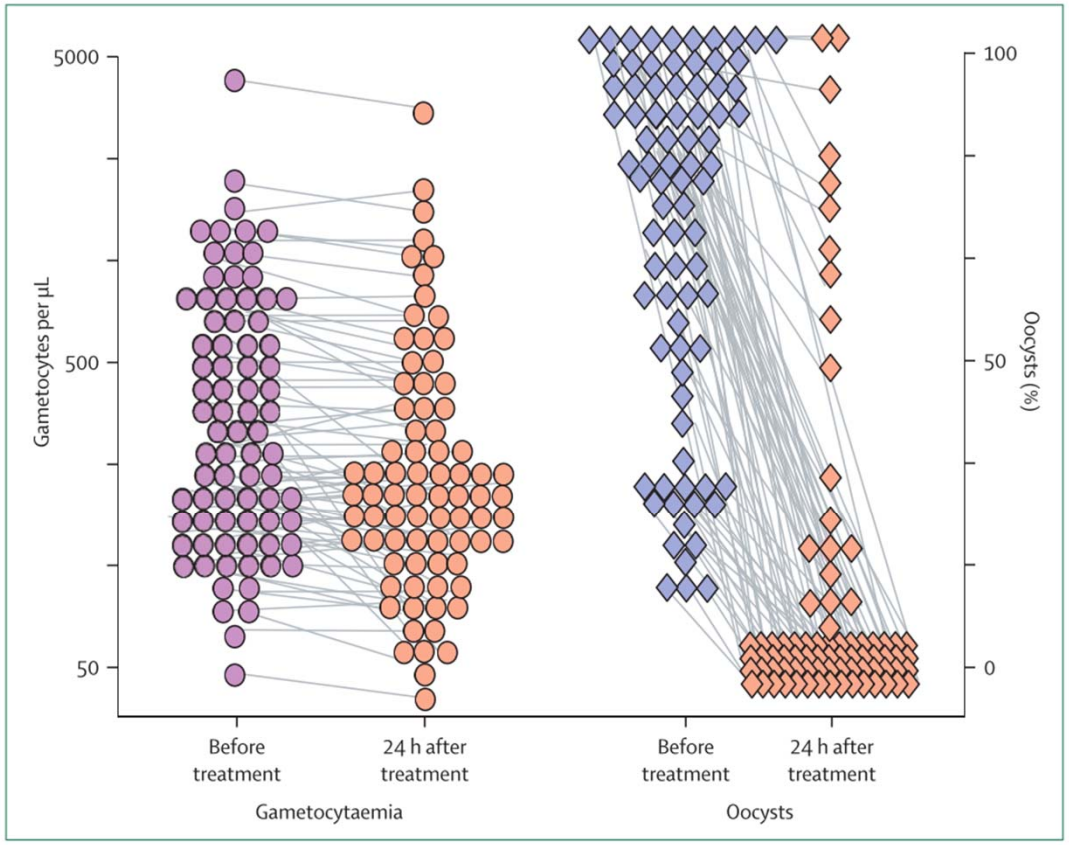


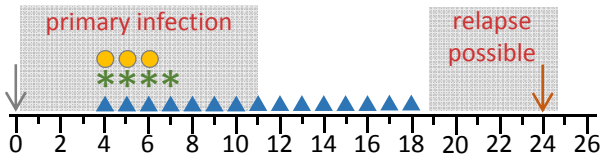
Figure 3: **Infectivity to mosquitoes that fed 24 h after patients with falciparum malaria and gametocytaemia were treated with plasmoquine or primaquine**

Oocysts were typically assessed in ten to 20 mosquitoes 6–7 days after they had fed. Each pair of circles or diamonds represents one patient.[21–38] Gametocytaemia changed little in 24 h, although it generally declined rapidly thereafter, but oocyst numbers fell rapidly to zero in most mosquito batches. When assessed later in parallel batches, sporozoites were correspondingly absent.

White, 2012 *Lancet Infectious Diseases*

An *in vivo* relapse model of infection

Hypnozoite activation



infection with 1.0M spz i.v. (Vxxx-xxx:VK2xx)

liver harvest for IFA and rtPCR

▲ 30 mg/kg primaquine p.o.

* 30 mg/kg MMV048 p.o.

● 10 mg/kg chloroquine p.o.

Same treatment as in the clinic

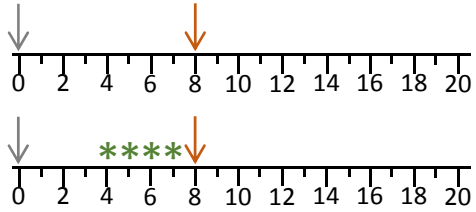
- 14d PQ treatment + 3d CQ
- We start at day 4 because we know these are true hypnozoites.
- Follow with blood sampling for weeks

Treatment with PQ alone at day 4 would arrest schizonts confounding day 24 readouts

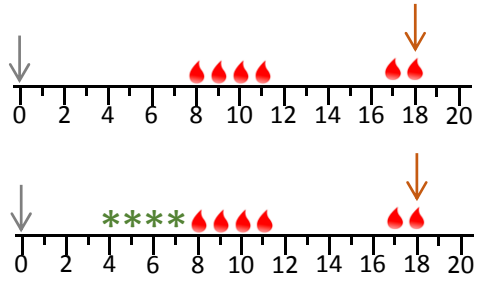
Demonstration that late timepoint schizonts are relapses

Hypnozoite activation

Primary infection



Latent/relapse infection



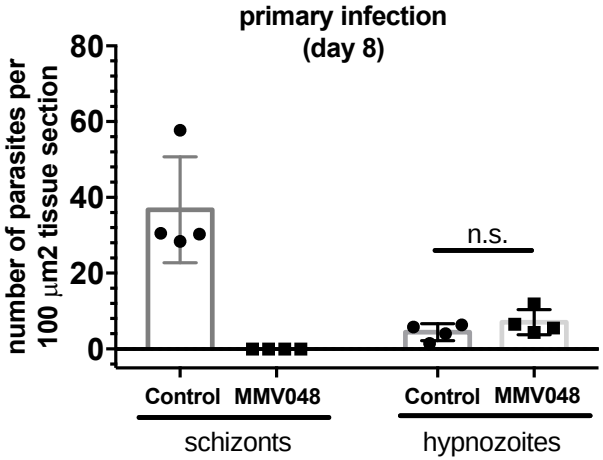
↓ infection with 0.6M spz i.v. (VTTY-111)

↓ liver harvest for IFA and rtPCR

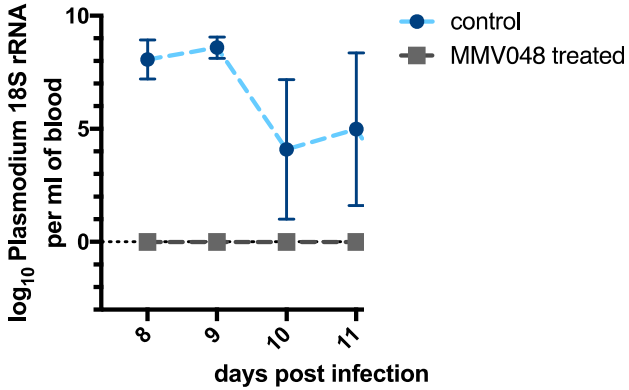
* 30 mg/kg MMV048 p.o.

bleed to detect merosome release

Absence of schizonts in the liver (primary infection)



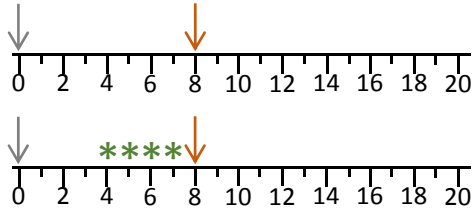
Absence of merozoites in the blood (primary infection)



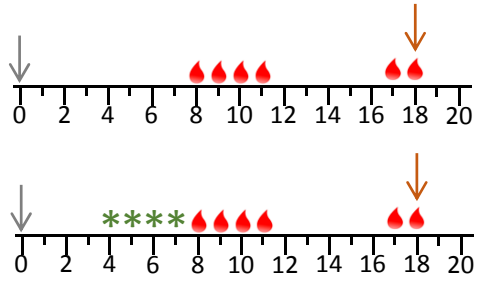
Demonstration that late timepoint schizonts are relapses

Hypnozoite activation

Primary infection



Latent/relapse infection



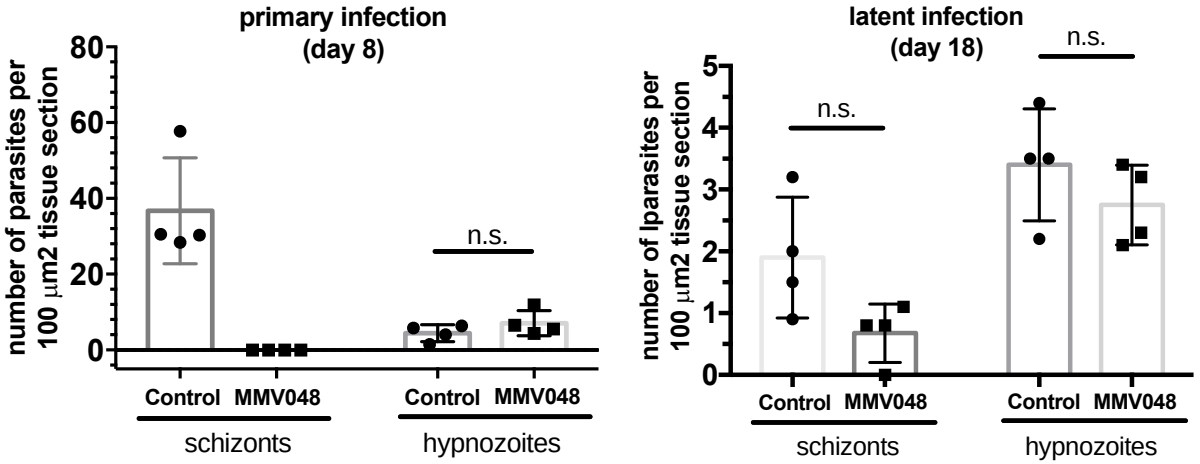
↓ infection with 0.6M spz i.v. (VTTY-111)

↓ liver harvest for IFA and rtPCR

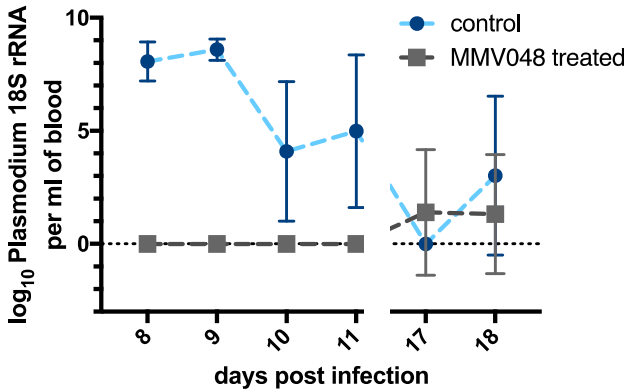
* 30 mg/kg MMV048 p.o.

bleed to detect merosome release

Presence of schizonts in the liver (latent infection)



Presence of merozoites in the blood (latent infection)



Additional tools to advance *P. vivax* studies

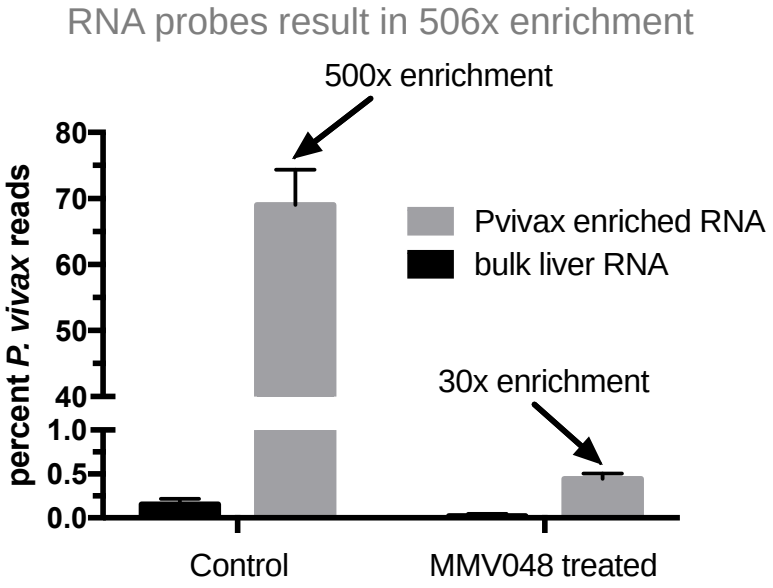
***P. vivax* radical cure *in vivo* model development**

Completion of the full *P. vivax* transmission cycle in FRG huHep mice

- Inject human reticulocytes
- Egress of liver merosomes into the blood stream
- Feed mosquitoes directly on mice
- This would allow repeated experiments with the same *P. vivax* isolate

Molecular methods

- Contamination: interspecies and intraspecies (hypnozoites v. schizonts)
- Tools for enrichment: *P. vivax* specific RNA probes
- Exosome enriched serum (in collaboration with Hernando del Portillo, ISB global)

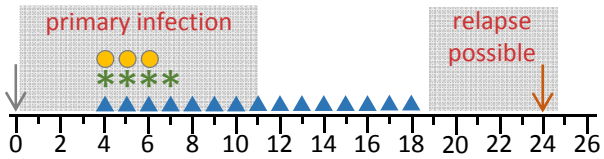


Summary

- Complete *P. vivax* liver stage development and blood stage transition can be recapitulated in the FRG huHep mouse
- *P. vivax* hypnozoites appear metabolically active because they grow and mature over time
- Relapse propensity of hypnozoites can be assessed using the FRG huHep model
- Genomics tools can inform drug discovery
  - Assay design
  - Biomarkers of infection
- Potential to complete the transmission cycle in FRG huHep mice
  - Source of *P. vivax* sporozoites

In vivo P. vivax radical cure model – modeling relapse

Pv radical cure model



infection with 1.0M spz i.v. (Vxxx-xxx:VK2xx)

liver harvest for IFA and rtPCR

▲ 30 mg/kg primaquine p.o.

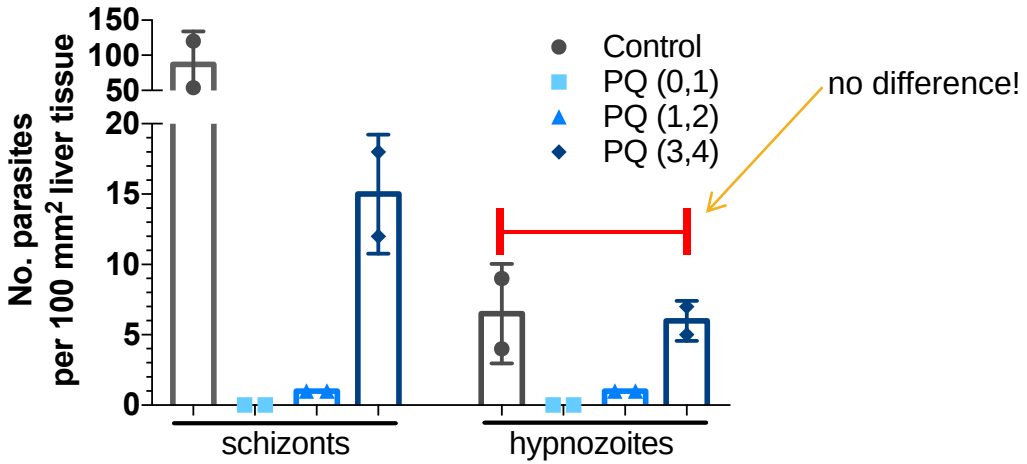
* 30 mg/kg MMV048 p.o.

● 10 mg/kg chloroquine p.o.

Same treatment as in the clinic

- 14d PQ treatment + 3d CQ
- We start at day 4 because we know these are true hypnozoites.
  - Begin at day first symptomatic? (10)
  - Earlier shortens the overall study period

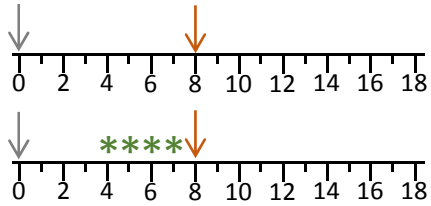
Treatment with PQ alone at day 4 would arrest schizonts confounding day 24 readouts



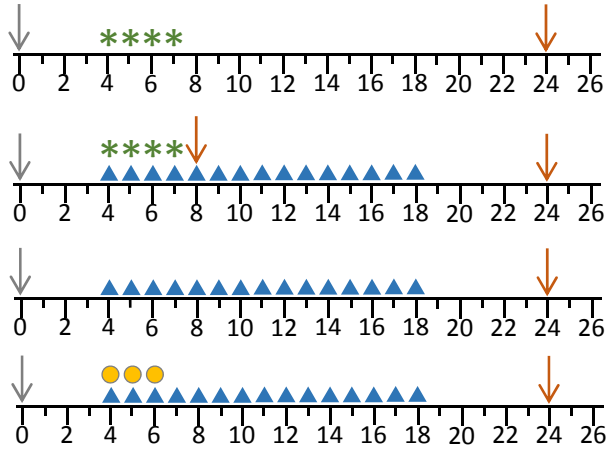
May 2017 experiment used to inform November experiment

Pv radical cure model

Primary infection



Latent/relapse infection



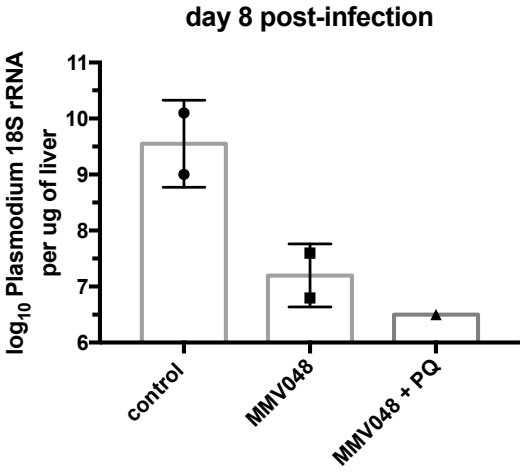
infection with 0.85M spz i.v. (VYBN-14:VK210)

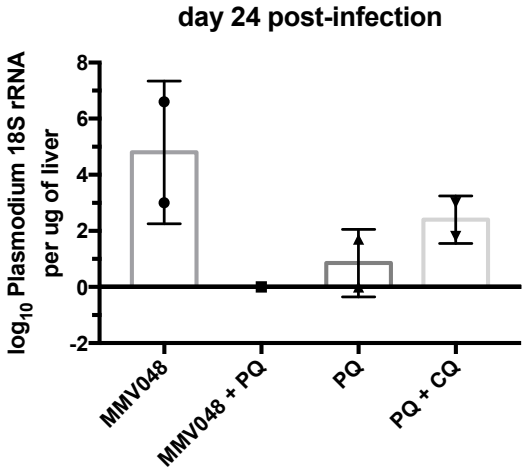
liver harvest for IFA and rtPCR

▲ 30 mg/kg primaquine p.o.

* 30 mg/kg MMV048 p.o.

● 10 mg/kg chloroquine p.o.





November – full relapse model experiment

Pv radical cure model

Objective: reproduce relapse model using same dosing regimen as used in the clinic

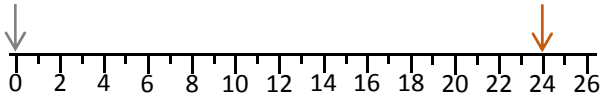
- start primaquine treatment on day 4 because we know these are true hypnozoites
- 5 mice per group
- Treatment with primaquine alone would arrest schizonts confounding results
  - MMV048 is used to clear out schizonts

↓ infection with 1.0M spz i.v. (Vxxx-xxx:VK2xx)

↓ liver harvest for IFA and rtPCR

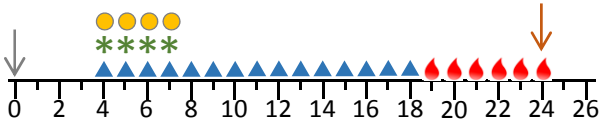
🩸 bleed to detect merosome release, i.e. relapse

1.



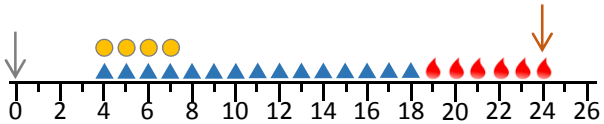
Control no drug treatment

2.



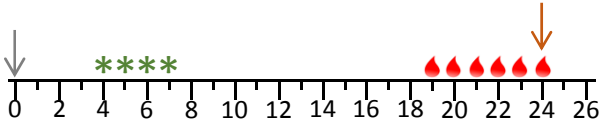
* 30 mg/kg MMV048 p.o.
▲ 30 mg/kg primaquine p.o.
● 10 mg/kg chloroquine p.o.

3.



▲ 30 mg/kg primaquine p.o.
● 10 mg/kg chloroquine p.o.

4.



* 30 mg/kg MMV048 p.o.

November – full relapse model experiment

Pv radical cure model

Results:

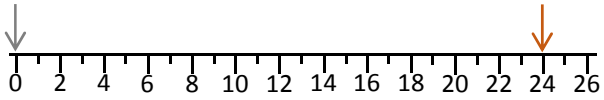
- Mouse death 4/22
- All treatments and bleeding performed as scheduled
- Will ship to CIDR next week

infection with 1.0M spz i.v. (Vxxx-xxx:VK2xx)

liver harvest for IFA and rtPCR

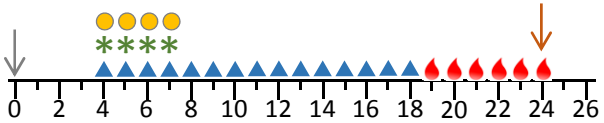
bleed to detect merosome release, i.e. relapse

1.



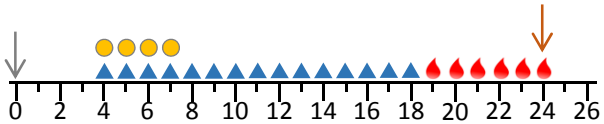
Control no drug treatment

2.



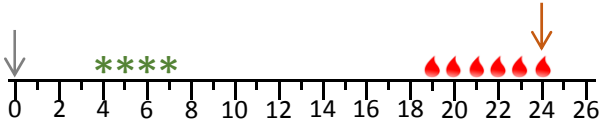
* 30 mg/kg MMV048 p.o.
▲ 30 mg/kg primaquine p.o.
● 10 mg/kg chloroquine p.o.

3.



▲ 30 mg/kg primaquine p.o.
● 10 mg/kg chloroquine p.o.

4.



* 30 mg/kg MMV048 p.o.

Future experiments

Pv radical cure model

- Repeat relapse model – add PK study

- Novel drug testing
  - Monensin
    - Formulation can be a problem (MMV assessment)
    - Days of dosing

- Timing of relapse
- Repopulation with reticulocytes to model viability
- Activation of relapse
  - HDAC inhibitors
  - Pf co-infection
  - RBC lysis

Thank-you

CIDR

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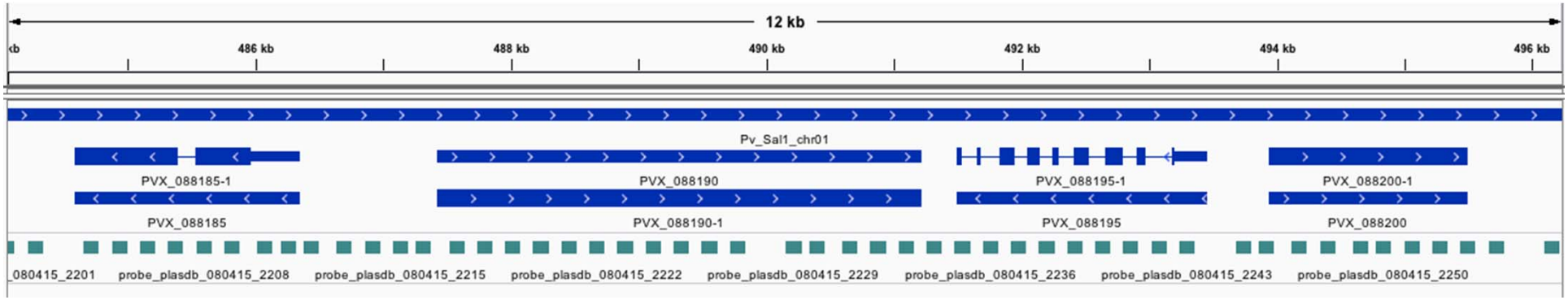
Towards a molecular marker of hypnozoites

***P. vivax* radical cure *in vivo* model development**



- *in silico* probe design
  - tiled across full genome
- 85,000 120 bp probes
  - tiled every 100bp
- Shear size of 350-400bp
- BLAT'd against human genome
- 30% mismatch is allowed