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# Supplementary Materials for

## *Plasmodium falciparum* transmission stages accumulate in the human bone marrow

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**The PDF file includes:**

## 1. Supplementary material and methods

### *Plasmodium falciparum in vitro* culture

Parasites of the *P. falciparum* strains 3D7 and Pf2004 were used for this study. Parasites were maintained in fresh type O+ human erythrocytes (Research Blood Components, LCC) at 2% hematocrit in HEPES-buffered RPMI 1640 containing 10% human AB+ serum, 0.5 mL Gentamycin, 2.01 g sodium bicarbonate and 0.05 g Hypoxanthine at pH 6.74. Cultures were kept in a controlled environment at 37°C in a gassed chamber at 5% $CO_2$ and 1% $O_2$. To produce gametocytes, sorbitol synchronized ring-stage parasites from the 3D7 parasite line were seeded at 1-2% parasitemia and 6% hematocrit into 75 $cm^2$ vented flasks, and gametocytogenesis was triggered according to the Fivelman protocol (*24*). Briefly, asexual parasites were grown to a high parasitemia in the presence of partially spent ("conditioned") medium, and then sub-cultured at schizont stage into new dishes containing fresh media and RBCs.

### Purification of stage I gametocytes for TEM analysis

The protocol used to obtain >95% pure pigmented, round shaped stage I/early stage II gametocytes from a 3D7 parasite line producing fluorescent gametocytes is described in Silvestrini et al (*25*).

### Controls for immunohistochemistry

To determine antibody specificity for the parasite stages of interest, we generated formalin-fixed and paraffin-embedded mock tissue samples containing cultured RBCs infected with asexual and sexual parasites stages. Briefly, 100 µL of packed RBCs, derived either from asexual cultures or from individual time points of gametocyte development time courses, were mixed with thrombin

and fetal bovine plasma, wrapped in lens paper and fixed with formalin. Cell clots were then placed into a paraffin wax block with a composite of surgical tissue specimens obtained from the Brigham and Women's Hospital (Boston) for the 9 organs that are analyzed in the autopsy study, and sliced at 3 μm. Using these samples, parasite antibodies were titrated for parasite- and gametocyte-specificity and against host tissue background labeling (Supplementary figures S3 and S4).

**Parasite and host marker antibodies**

Working concentrations for each antibody were determined using the control samples as described above. The initial screen was performed using antibodies targeting pLDH, *Plasmodium* lactate dehydrogenase, which is constitutively expressed in parasites and previously identified as a useful marker of malaria parasites in autopsy tissue (*13*), and Pfs16, a gametocyte specific antigen expressed throughout gametocyte development (*14*). Here, we used a mouse monoclonal anti-pLDH antibody (kind gift from Dr. Michael Makler, FlowInc, Portland, OR) at 1:750 and a mouse polyclonal anti-Pfs16 antibody at 1:5000. The co-localization screen with CD31 was performed with these same parasite antibodies as well as a mouse anti-CD31 antibody (Clone JC70A, Dako) at 1:20. Due to the need to co-localize parasites within host cells labeled with CD163 and CD71 (both mouse antibodies), we optimized protocols for rabbit parasite antibodies to minimize cross-reactivity. We generated a polyclonal rabbit anti-Pfs16 antibody against the Pfs16-specific peptide DANDKAKKPAGKGSC (Genscript) for this purpose, and we used a rabbit antibody against BIP/Heat shock protein 70 (constitutively expressed in all blood stages, antibody provided by MR4; clone MRA19). The rabbit Pfs16 antibody was used at 1:4000 and the rabbit anti-BIP antibody was used at 1:300. Host proteins were labeled using

mouse anti-CD163 (Leica, clone 10D6) at 1:200 and mouse anti-CD71 (Invitrogen, clone H68.4) at 1:1000.

**Immunohistochemistry assays**

Formalin-fixed paraffin-embedded tissues and control blocks were cut into 3 micron sections and mounted on slides. Sections were dried overnight at 37°C and then processed through deparaffinization in xylene, and subsequently hydrated through a series of graded alcohols, finishing in water. Antigen retrieval was performed by incubating slides at approximately 95°C in a steamer for 20 minutes. One of two antigen retrieval solutions was used for this process (as determined based on antibody optimization conditions): 1 mM EDTA + 0.05% Tween at pH 8 or Retrieve-All-2 antigen retrieval buffer pH 10 (Covance Research Product, Dedham, MA). EDTA solution was used for the initial pLDH and Pfs16 screen, as well as for the double-labeling of pLDH and Pfs16 with platelet endothelial cell adhesion molecule (CD31). For the double-labeling of parasite antibodies with CD163 and CD71/Transferrin Receptor, Retrieve-All-2 buffer was used. Following antigen retrieval, slides were blocked using a universal blocking buffer (Thermo Scientific) for 20 minutes, followed by 10 minutes each of avidin and biotin blocking buffers (Invitrogen) to block endogenous biotin and avidin, respectively. Antibody incubation was then performed for either 2 hours (CD31) or 1 hour (all other antibodies), with each antibody diluted in blocking buffer. Wash steps, in between each step thereafter were performed with tris-buffered saline (TBS) + 5% Tween for 3 x 5 min.

For the initial screen, a secondary biotin-XX conjugate of the $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) antibody was used (Invitrogen), followed by streptavidin conjugated to alkaline phosphatase, AP (Thermo Scientific). For the development of signal, Fast Red

TR/Naphthol AS-MX substrate reagent (Sigma Aldrich) was applied. Slides were subsequently rinsed in water and counterstained in Mayer's hematoxylin, and mounted in aqueous mounting medium. Slides were blinded to patient ID and independently counted by two microscopists, counting parasites in 100 consecutive high power fields, starting in the upper left corner of each section. The average of the microscopists' counts were used for the analysis.

For double labeling with CD31, a sequential double staining protocol was used, as previously described (*26*), as this is necessary when both antibodies have been raised in the same host. Here, following deparrafinization, rehydration, antigen retrieval and blocking steps, the primary host antibody (mouse anti-CD31) was incubated for 1 hour, followed by an additional blocking step in Peroxidase Suppressor (Thermo Scientific) to block endogenous peroxidase activity, secondary goat anti-mouse biotinylated antibody, streptavidin conjugated to horse radish peroxidase, HRP (Thermo Scientific) and development of signal with 3,3'-Diaminobenzidine (DAB) chromogen reagent (Thermo Scientific). After washing in TBS, biotin and avidin blocking steps are applied once again, followed by a 1 hour incubation with the parasite antibody (mouse anti-pLDH or mouse anti-Pfs16), followed by a secondary goat anti-mouse biotinylated antibody, streptavidin conjugated to alkaline phosphatase, and development of signal with Fast Red substrate. Slides were subsequently rinsed in water, counterstained in Mayer's hematoxylin, and mounted in aqueous mounting medium.

For the CD163 and CD71 double-labeling experiments, we used a double-staining protocol for mouse-rabbit combinations, as described in (*26*). Here, following deparrafinization, rehydration, antigen retrieval and blocking steps, the primary antibodies for both host protein (either mouse anti-CD163 or mouse anti-CD71) and parasite protein (rabbit anti-BIP or rabbit anti-Pfs16) were incubated simultaneously. This was followed by a peroxidase blocking step,

and the addition of both secondary antibodies: HRP conjugate of the $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) antibody and biotin-XX conjugate of the $F(ab')_2$ fragment of goat anti-rabbit IgG (H+L) antibody (both, Invitrogen). This was followed by incubation in streptavidin conjugated to AP. The HRP was developed by a brief incubation with 3,3',5,5'-Tetramethlybenzidine, TMB substrate (Vector Laboratories) and washed with TBS. The AP was subsequently developed by incubation with Fast Red. Slides were subsequently rinsed in water and mounted in aqueous mounting medium.

**Bone Marrow Histological Characterizations**

Blue hematoxylin and eosin stained sections of bone marrow were reviewed by an independent hematopathologist (blinded to IHC data and patient clinical information) and scored for myeloid to erythroid ratios, presence/absence of erythroid dysplasia, and presence/absence of marrow shift.

**Size Measurements**

Images of parasites in both control in vitro blocks and human tissue samples were taken using a digital microscope camera (Zeiss AxioCam IC). These images were processed using ImageJ software version 1.43 (NIH). In ImageJ, a threshold was applied to each image to create masks of each positively labeled cell in a binary black and white format. The Analyze Particles feature was subsequently applied to each mask, and measurements of area, diameter, perimeter and circularity in microns were recorded for each particle.

**Immunofluorescence assays**

For the evaluation of quantitative PCR markers and for localization of gametocytes in erythroid precursor cells, immunofluorescence assays were performed with cell monolayers on glass slides. For the purpose of evaluating PCR markers, slides were fixed in ice-cold methanol, blocked with 5% nonfat dry milk powder, incubated with polyclonal mouse antibody against Pfs16 (1:2500) (*14*), washed and incubated with a secondary antibody conjugated to Alexa 488. Parasite nuclei were labeled with DAPI and quantification was done on the proportion of FITC(+) parasites out of the total number of DAPI(+) parasites. For erythroid precursor cell quantification, slides were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde. Slides were subsequently washed in PBS and incubated for 10 min with wheat germ agglutinin (WGA) directly conjugated to Alexa 488 (8 µg/mL, Invitrogen). After washing, cells were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 min, blocked with 3% nonfat dry milk powder in PBS for 30 min, and incubated with rabbit anti-Pfs16 (1:40,000) for 1 hour. Slides were washed and primary antibodies detected by incubation for 1 hour with Alexa-594-conjugated anti-rabbit IgG (1:500, Invitrogen). All WGA and antibody dilutions were made in PBS supplemented with 3% bovine serum albumin. To avoid photobleaching, cells were mounted with Vectashield (Vector Laboratories) containing 1.5 mg/mL DAPI to stain for DNA. All incubations were performed at room temperature in a humid chamber. Microscopy imaging was performed using a 100x oil immersion objective in a Nikon E800 microscope and a Spot RT cooled CCD camera at the IDDRC Imaging Core, Children's Hospital Boston (NIH-P30-HD-18655). Images were captured with HCImage software (Hamamatsu) and processed with ImageJ version 1.46 (NIH).

**Transmission electron microscopy**

Transmission Electron Microscopy (EM) was performed on the bone marrow and brain from one patient for which well-preserved tissue was available for both organs, and for which both pLDH- and Pfs16-positive parasites were identified in the patient's brain and bone marrow during the IHC screen. Tissue taken at autopsy was fixed in 2% glutaraldehyde. Samples were then post-fixed in osmium tetroxide, dehydrated in ethanol, treated with prophylene oxide and embedded in epoxy resin. Thin sections were stained with 1% toluidine blue and examined under light microscopy to identify sections of the tissue that contained parasites. Ultra-thin sections were then made and stained with uranyl acetate and lead citrate just prior to imaging. Bone marrow sections were imaged using a Tecnai G2 Spirit BioTWIN conventional electron microscope. Brain sections were imaged using a JEOL 1200EX- 80kV conventional electron microscope. Protocols for the TEM analysis on the purified stage I gametocytes from in vitro culture are described in Tiburcio et al (*10*).

**RNA Extraction, DNAse Digest and Reverse Transcription.**

Tissue sections stored in RNAlater were processed through either grinding in liquid nitrogen using a mortar and pestle or homogenizing in TRI Reagent BD (Molecular Research Center). Peripheral blood samples and in vitro culture samples were added to TRI Reagent BD and vortexed. Following tissue homogenization and vortexing, all TRI Reagent BD samples were processed as per manufacturers instructions. Briefly, RNA was extracted by the phenol-chloroform method followed by DNAse digest (Ambion), and a second phenol-chloroform extraction for protein removal and sample concentration. First strand cDNA synthesis was performed using the SuperScript III First Strand Synthesis kit (Invitrogen).

**qRT-PCR assay**

To develop a qRT-PCR assay capable of differentiating between developmental gametocyte stages, we selected three established markers with peak transcription during early (*PF14_0748*), mid (*Pf4845*) and late (*Pfs25*) gametocyte development, respectively. We also included the constitutive marker ubiquitin conjugating enzyme (UCE, *PF08_0085*) (*27*). We tested the primer efficiency and sensitivity of each primer pair in dilution curves using pGEM-T Easy plasmids (Promega) containing the cloned target fragments as template. These experiments demonstrated that each pair was between 90-110% efficient and had a detection limit ranging between $10^1$ – $10^2$ copies for each primer pair. Finally, all primer pairs were tested for cross-reactivity with human cells using cDNA from human C32 cells and shown to be not cross-reactive.

To establish stage-specificity, a series of in vitro gametocyte time courses was performed using a gametocyte induction protocol, as previously described in Fivelman *et al* (*24*), and samples were collected daily during asexual growth and subsequent gametocyte development. Each time point sample was collected in TRI Reagent BD and processed by qRT-PCR for the expression of the three gametocyte genes and one constitutive marker. These results were compared with fluorescence microscopy images to confirm that expression of markers peak at different points along gametocyte development.

**qRT-PCR conditions**

Quantitative PCR was performed on cDNA in 20 μL reaction volumes in either an ABI 7300 or Viaa7 machine (both, Applied Biosystems). Amplification was performed using SYBR Green (BioRad), and primers for each of the genes of interest at 250 nM concentration. Reaction conditions were as follows: 1 cycle x 10 min at 95°C and 40 cycles x 30 sec at 95°C and 1 min at

58°C. Each cDNA sample was run in triplicate. As a positive control to ensure parasite RNA was present in our samples, we used primer pairs for *P. falciparum* ubiquitin conjugating enzyme transcript as a constitutively expressed parasite marker as previously described (*27*).

**Erythroid precursor cell culture**

Bone marrow-derived CD34+ hematopoietic stem cells (Lonza) were differentiated ex vivo as previously described (*21*), but in addition, RBC progenitors were co-cultured on murine MS-5 stromal cells from days 15-19 of ex vivo culture. On day 19, cultured RBCs (cRBCs) were collected for parasite growth assays. Of the harvested cRBCs, 41% were nucleated and 59% enucleated, and of the latter were 95% reticulocytes and 5% normocytes as determined by New methylene blue staining. Parasites of the strain Pf2004 were sorbitol-synchronized at ring stage and induced to form gametocytes as previously described (*24*). Late trophozoite/schizont stages were enriched by magnetic cell sorting (MACS), which yielded a parasitemia of 92%. Parasites were plated with cRBCs in triplicate at a final parasitemia of 1% and 0.7% hematocrit in 100 µL RPMI supplemented with 10% human AB+ serum. Following reinvasion, cells were collected by cytospin and stained with Giemsa to monitor parasite growth. Media was changed daily until day 5. On day 2 and 5, cells were collected by cytospin onto poly-L-lysine coated slides for immunofluorescence assays, and slides were air-dried and stored desiccated at -20°C.

## 2. Supplementary Figures S1-S8

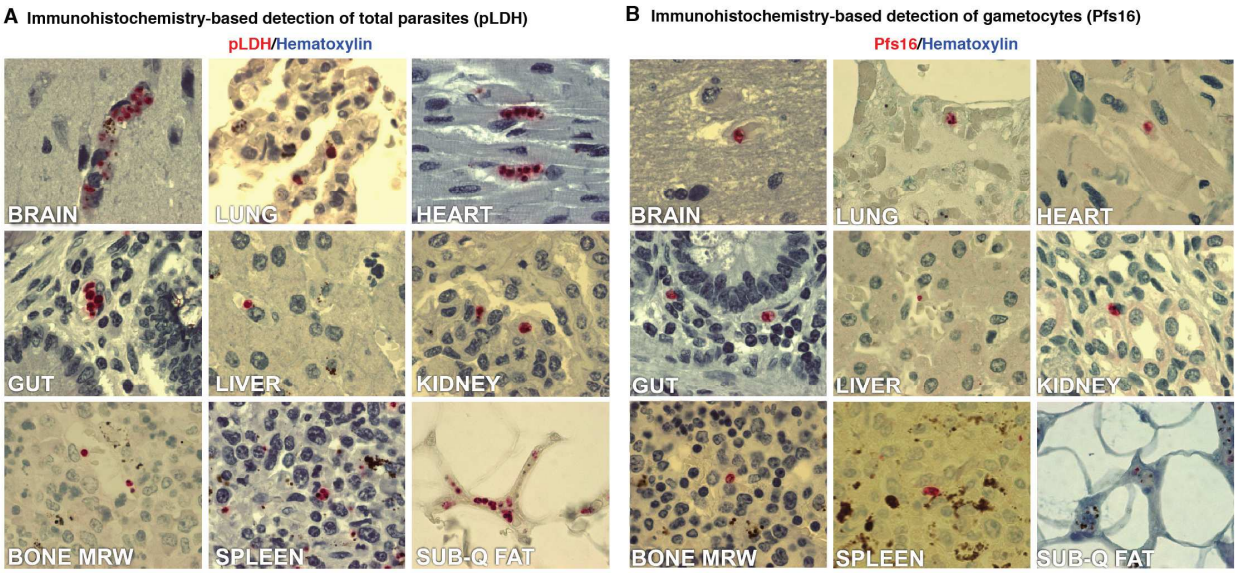


**Fig. S1. Representative pLDH and Pfs16 labeling across tissues. A. pLDH.** pLDH-labeled parasites were identified in all the 9 tissue types analyzed. In a subset of those tissues (i.e., brain, gut and heart) the classically described pathology of packed vessels could be observed. **B. Pfs16.** Pfs16-labeled parasites were observed in multiple tissues including those previously reported for gametocytes (i.e., bone marrow and spleen). All images were photographed at original 1000x (oil immersion) magnification.

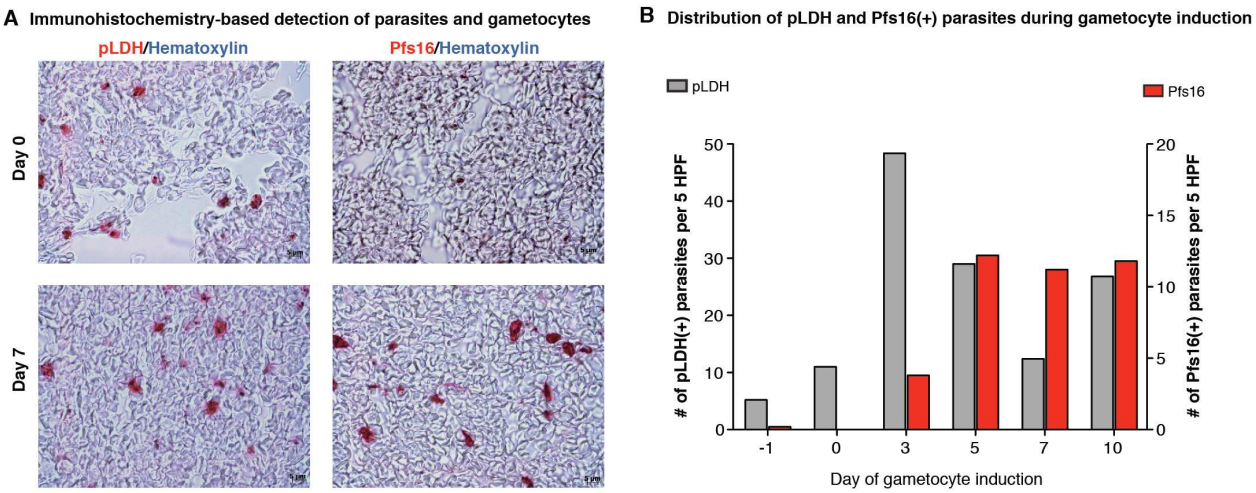


**Fig. S2. Stage specificity of antibodies by immunohistochemistry. A. Representative images of cells stained with either pLDH or Pfs16.** Gametocytes were induced according to Fivelman et al (*24*), with Day 0 being the first day of gametocyte development. Infected RBCs from the time course were fixed in formalin, embedded in paraffin (FFPE), labeled with either pLDH (all parasites) or Pfs16 (gametocytes) and detected using AP and Fast Red. Images are shown from Day 0 and Day 7. **B. Quantification of antibody labeling across gametocyte development.** Shown is the number of labeled parasites observed in 5 high power fields of the in vitro cultured cells. Pfs16 labeling is present at Day 3 of gametocyte development but not at Day 0 (where gametocytes are still at ring stage).

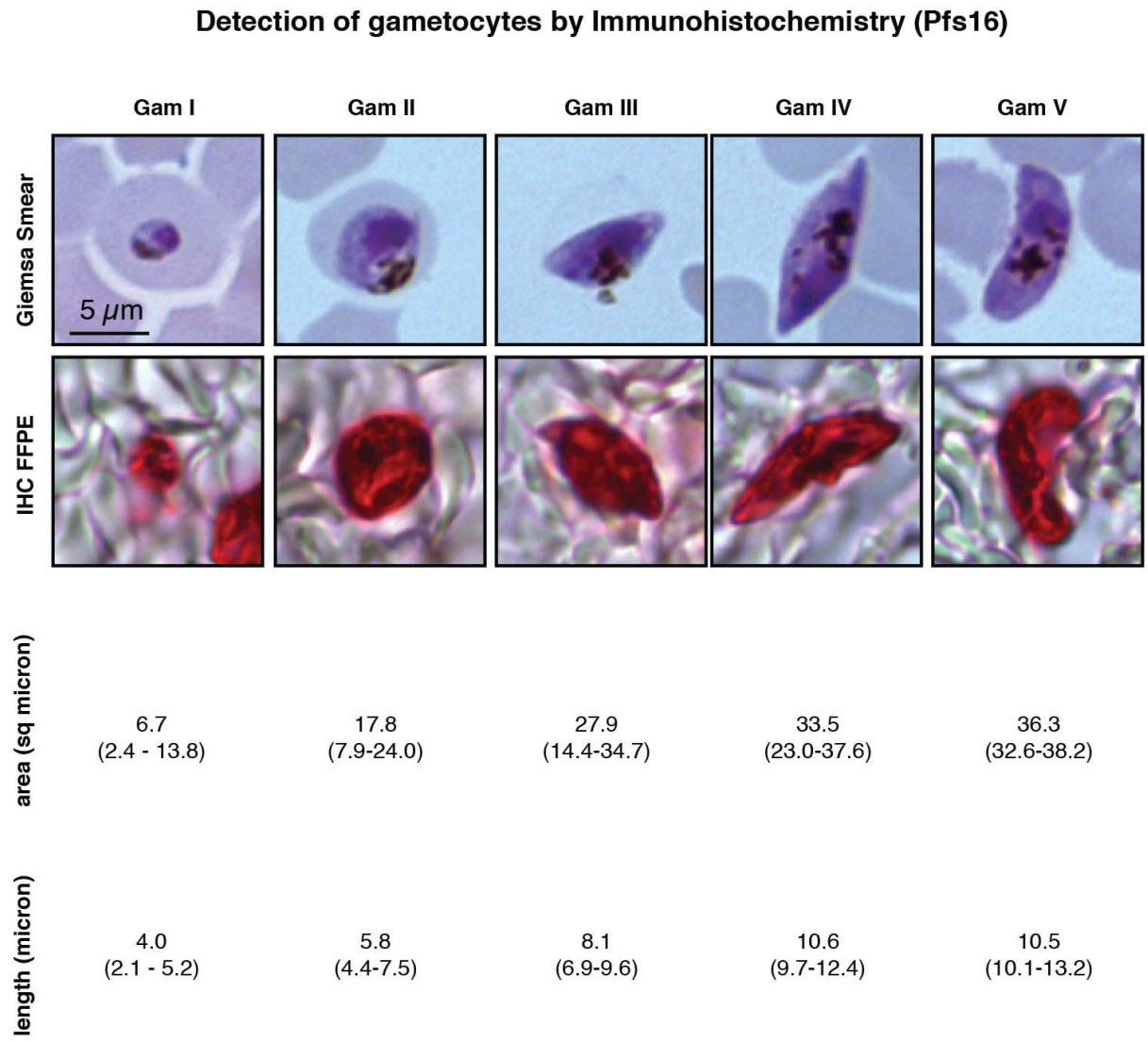


**Fig. S3. Morphology and dimensions of parasites by immunohistochemistry.** Shown are in vitro derived gametocytes prepared using blood smear, methanol fixation, and Giemsa staining (top panel) and FFPE, IHC-labeling with Pfs16 antibodies, and detection with AP and Fast Red (bottom panel). The mean (and range) of ImageJ measurements for cell area and length are shown for Pfs16-labeled gametocyte stages observed in IHC sections, classified as stage I, II, III, IV or V morphologically, as previously described. Stage-specific dimensions are similar to previous measurements using live cells (*11*).

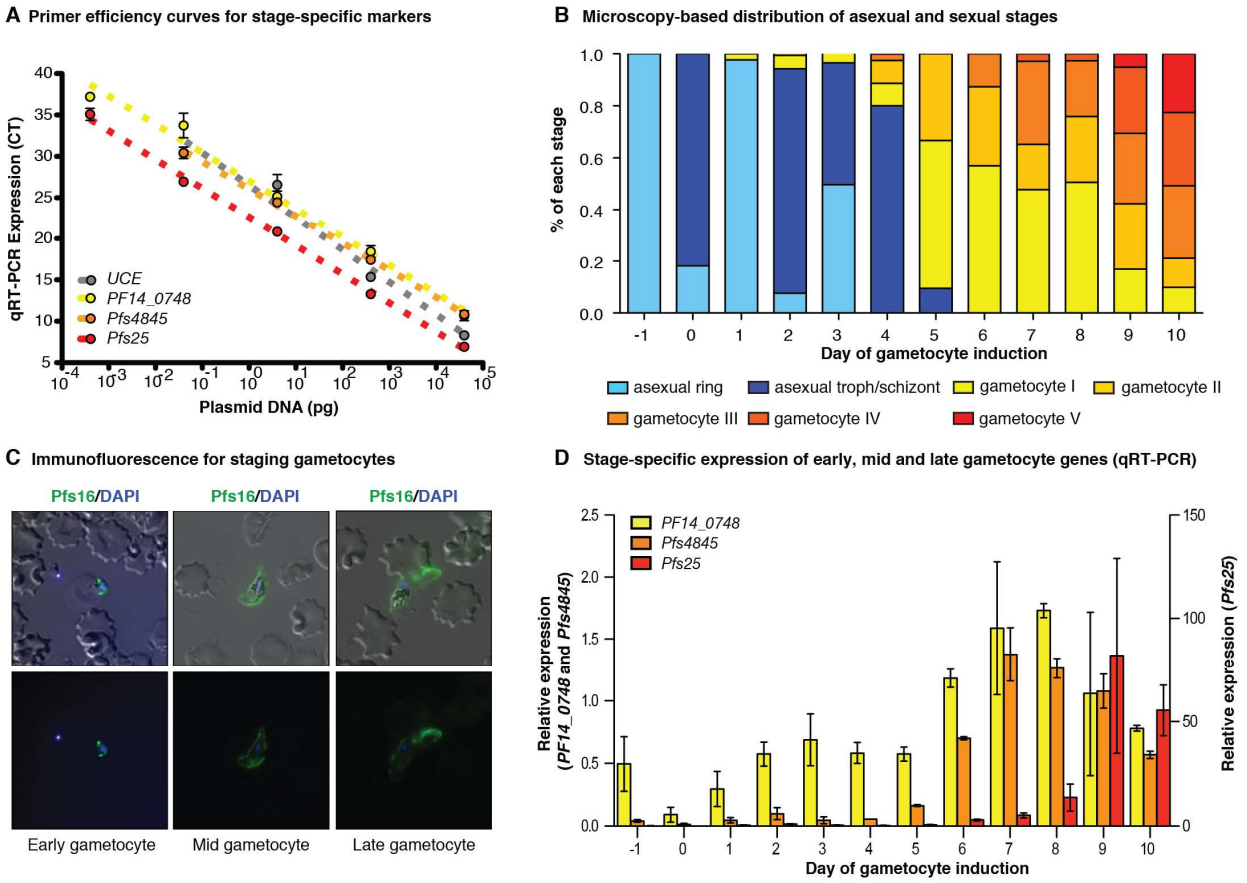


**Fig. S4. Development of qRT-PCR assay. A. Primer efficiency.** Each PCR amplicon was inserted into a pGEM-T Easy plasmid and primer efficiency curves were generated using serial dilutions of plasmid DNA (see Table S2 for primer sequences). **B. Gametocyte time course and staging.** Gametocytes were induced according to Fivelman *et al* (*24*) and samples collected every day for 12 days. Parasite nuclei were labeled with DAPI and gametocytes were stained with antibodies against Pfs16. Gametocytes were identified based on Pfs16-labeling and categorized into the five known stages I-V, according to Sinden (*28*). **C. Representative images from the time course experiments.** Shown are early, mid stage and late gametocytes, labeled with Pfs16 antibody and DAPI. **D. Stage-specific expression of markers.** In contrast to the constitutive marker *UCE*, the gametocyte markers increase upon induction according to their

previously described expression peaks: *PF14_0748* peaks at days 3 and 4, *Pfs48/45* peaks at days 7-9 and *Pfs25* peaks at days 9 and 10.

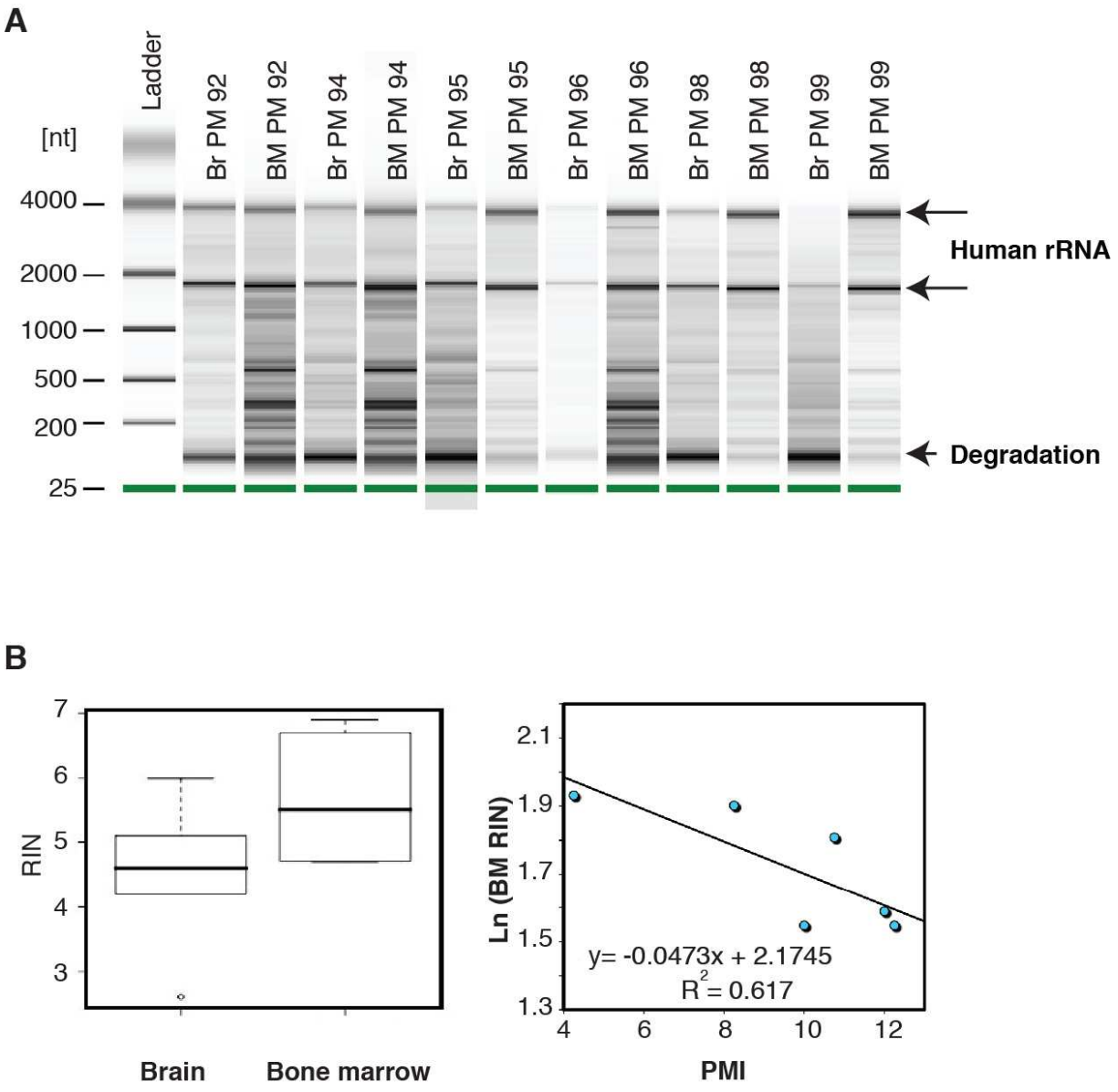


**Fig. S5. RNA quality from brain and bone marrow tissue samples. A. RNA size distribution per sample.** Purified and DNAse digested RNA from 6 brain (BR) and 6 bone marrow samples (BM) were run on a Bioanalyzer (Agilent) to determine the length distribution of RNA fragments. The most prominent bands are human 28s and 18s ribosomal RNA (rRNA) bands (at app 4000 and 2000 nt, respectively, see arrows). **B. RIN and PMI.** Based on the average RNA integrity number (RIN, a measure of RNA integrity based on Bioanalyzer results), the RNA in these tissues is considered partially degraded but sufficient for amplifying the small fragments (100 -

200 nt) of the qRT-PCR assay. A positive correlation is observed between the RIN and post mortem intervals (PMI, the time between death and autopsy), suggesting that RNA degrades more with increased PMI. In addition RNA from brain samples appears generally more degraded than from bone marrow samples**.**

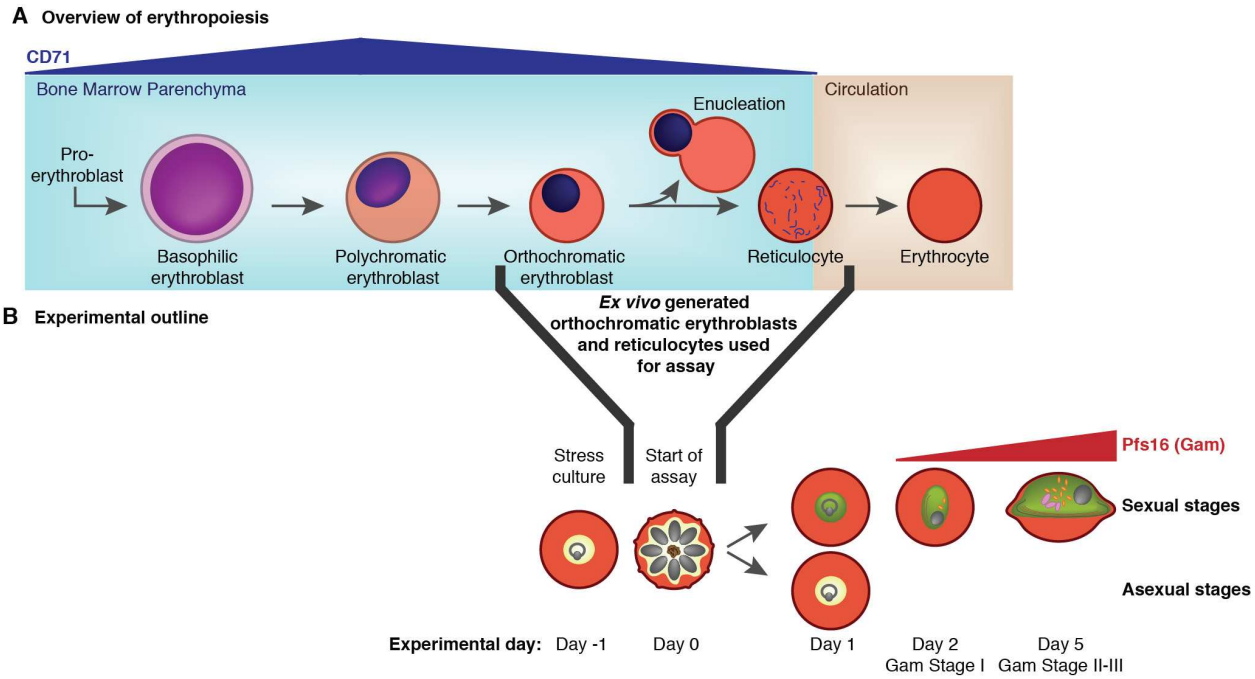


**Fig. S6. Invasion and development of gametocytes in erythroid precursor cells.**

**A. Schematic overview of erythropoiesis starting from pro-erythroblasts.** CD71 (shown in dark blue triangle) is expressed in erythroid cells in the bone marrow, but is lost in reticulocytes as they are released from the bone marrow during maturation. **B. Experimental outline.** Starting on day -1 of the in vitro experiment, RBCs infected with ring stage parasites were induced to form gametocytes. When reaching schizont stage (day 0), iRBCs were enriched and incubated with a mixture of ex vivo generated orthochromatic erythroblasts and reticulocytes. In order to visualize gametocyte development, antibodies towards Pfs16 were used. Pfs16 expression (shown in red triangle) can be detected by antibodies in gametocytes starting at approximately 30 hours post invasion, i.e., at stage I.

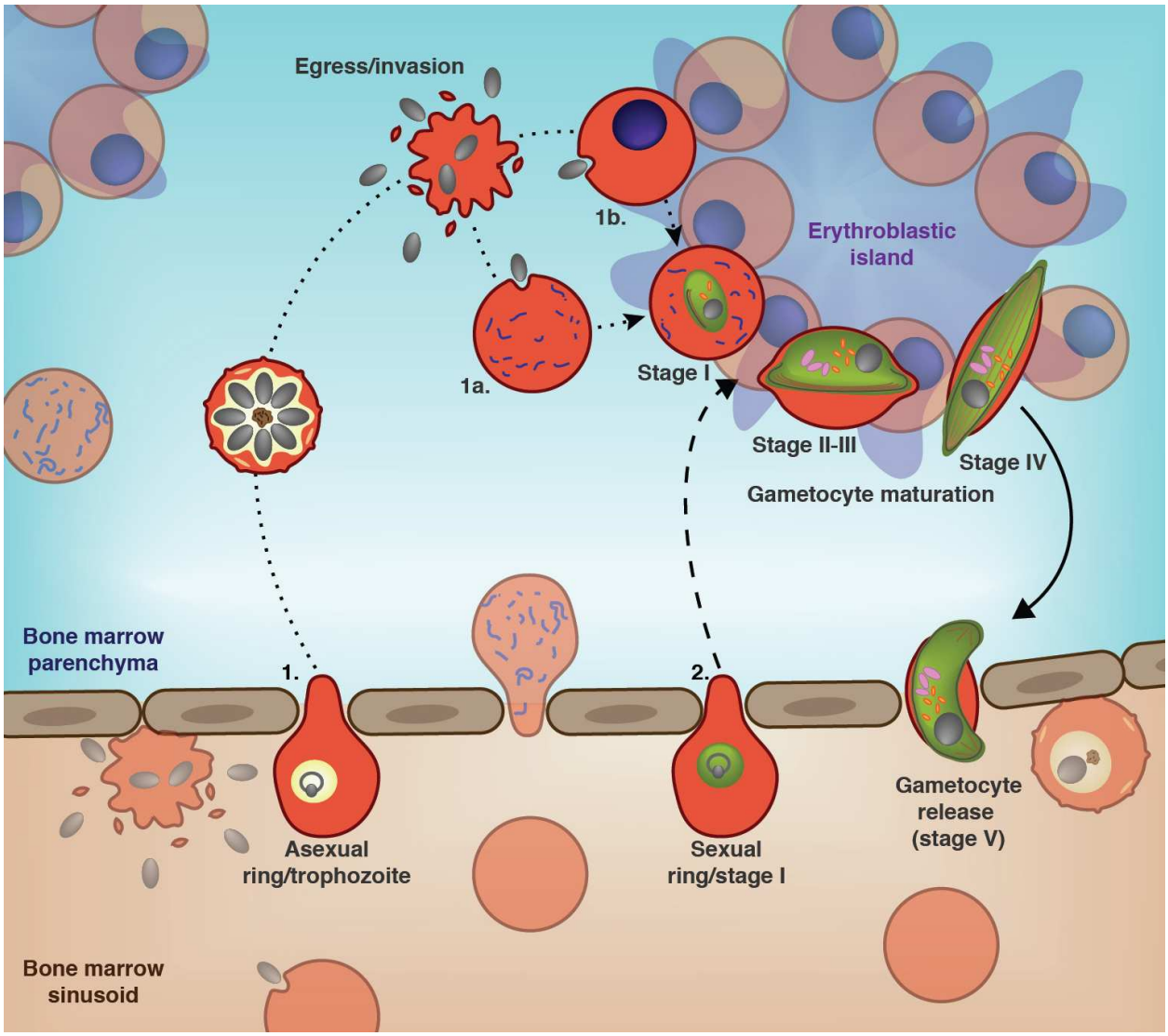


**Fig. S7. Proposed models for *P. falciparum* gametocyte development.** Identification of developing gametocytes in the external space of the bone marrow implies that some stage(s) of the parasite are able to cross the endothelial barrier. Young asexual parasites may cross through the endothelial barrier **(1)** due to their high deformability compared to more mature parasites (*11*, *29*), and their progeny may invade either reticulocytes (1a) or nucleated erythroid precursors (1b) within this space. Alternatively, young circulating gametocytes (stage I or earlier) may enter the hematopoietic system and directly develop in this environment **(2)**. The observed specific enrichment of developing gametocytes in the extravascular environment of the bone marrow

suggests that i) these stages are retained in this location and ii) mature gametocytes are able to cross the endothelial barrier to reenter the circulation. Binding interactions of immature gametocytes with cells of the hematopoietic system (e.g. erythroblastic islands) could provide one possible explanation, as the gametocyte would simply have to hijack a system already in place to retain erythroid precursor cells as they mature. Alternatively, or as a redundant mechanism, the increased rigidity of immature gametocyte-infected RBCs (*10, 11*) could lead to the retention of gametocytes within the bone marrow during development. Finally, restoration of deformability in mature gametocytes may contribute to their exit from the bone marrow and reentry into the peripheral circulation.

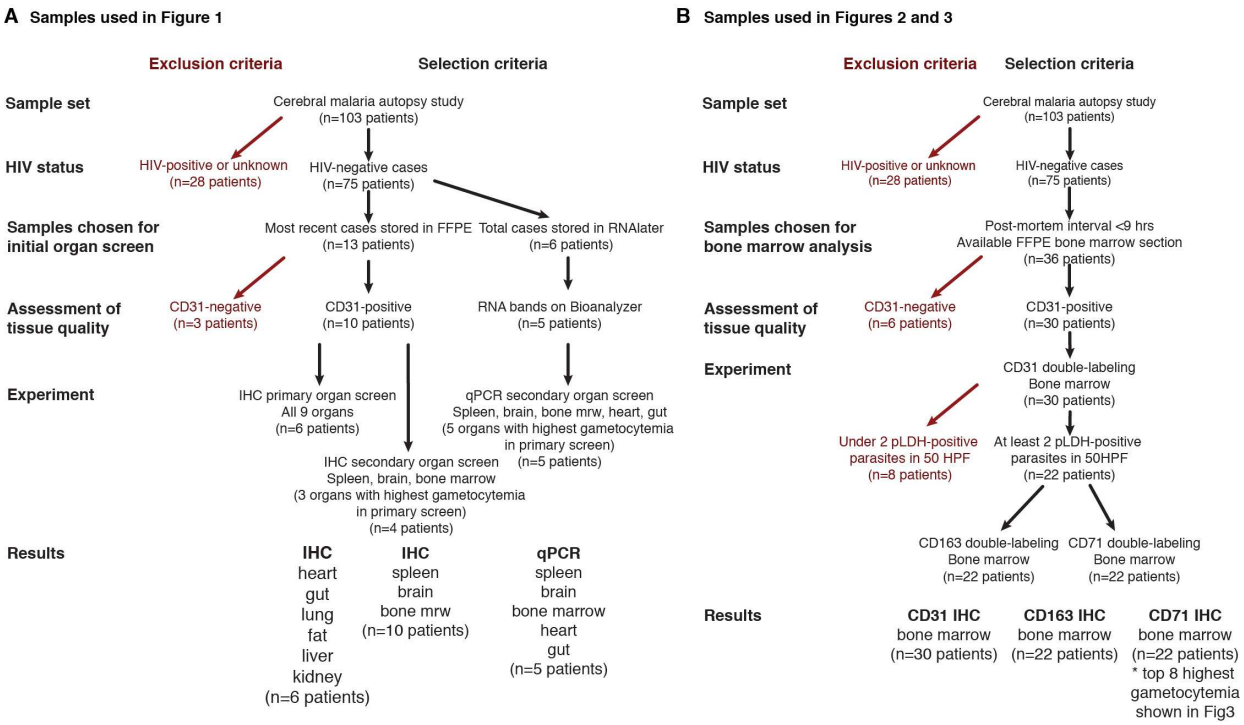


**Fig. S8. Flow chart of samples used in Figs. 1 to 3. A. Selection criteria for organ screen (Figure 1).** For the initial organ screen, we assessed the 13 most recent HIV(-) cases in which all nine tissues were available in FFPE blocks, and the 6 HIV(-) cases in which all tissues were available in RNAlater. Out of those, a subset of 10 cases was determined to have tissue quality sufficient for IHC and 5 for qRT-PCR. **B. Selection criteria for bone marrow analysis (Figures 2/3).** For co-localization of host cells and parasites/gametocytes in the bone marrow, we started with the entire archived collection of autopsy cases and filtered according to the following criteria: HIV-negative status, PMI under 9 hours and available FFPE bone marrow section. We then focused our bone marrow analysis on a final set of 30 patients for which CD31 labeling was successful, indicating sufficient tissue quality.

## 3. Supplementary Tables

**Table S1. Gametocyte fraction in bone marrow versus clinical parameters**. Clinical data for the 22 patients in the study for whom a gametocyte fraction (ratio of Pfs16/pLDH) was determined in the bone marrow; grouped by above and below 33%. Means, standard deviation and p-values for t-tests comparing the means of each group are shown. Parasitemia was log transformed prior to analysis. All cases are from Blantyre, Malawi and surrounding areas, and autopsies were performed between 1996 and 2011.

| | < 33% | > 33% | p-value |
|---|---|---|---|
| Age (months) | 27.8 ± 18.2 | 25 ± 11.0 | 0.3502 |
| Duration of Fever (hours) | 62 ± 37 | 48 ± 38 | 0.2318 |
| **Time to Death (hours)** | **7.8 ± 7.1** | **33.9 ± 26.6** | **0.0058** |
| Hematocrit | 20 ± 6.1 | 24.4 ± 9.4 | 0.1259 |
| Parasitemia (log p/uL) | 3.8 ± 1.9 | 2.8 ± 2.4 | 0.1815 |
| Erythroid Marrow (%) | 44 % ± 27% | 43 % ± 22% | 0.4825 |

**Table S2. Primer pairs used in qRT-PCR assay.** The same primer pairs were used for subcloning fragments into pGEM-T Easy plasmids and for the qRT-PCR assay.

| Name | | Stage-Specificity | Primer Efficiency | Primer sequences (listed 5' to 3') |
|---|---|---|---|---|
| *Plasmodium exported protein (PHISTa)* | *PF14_0748* | early - mid gametocyte | 97.6% | ATTCAAGGGTAGTTCCTAGAGCAGTGTGG<br>AGCACTCGTAATTCTAACACTGGG |
| *6-cysteine protein (Pfs48/45)* | *PF13_0247* | mid - late gametocyte | 101.4% | GTAAGCCTAGCTCTTTGA ATAGTGA<br>GACCTACGTTCACGCATATCTGGCT |
| *25 kDa ookinete surface antigen precursor* | *PF10_0303* | late gametocyte | 93.1% | GGA AATCCCGTTTCATACGCTTGT<br>TCTTGTACATTGGGAACTTTGCCT |
| *Ubiquitin-conjugating enzyme* | *PF08_0085* | all stages | 100.5% | GGTGTTAGTGGCTCACCAATAGGA<br>GTACCACCTTCCCATGGAGTATCA |