nature portfolio

Peer Review File

The artemisinin-induced dormant stages of Plasmodium falciparum exhibit hallmarks of cellular quiescence/ senescence and drug resilience.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The work submitted by Tripathi and colleagues focuses on the investigations of pyknotic forms of the Plasmodium parasite that arise in response to the stress conditions (eg. drug treatment). The authors present the protocol for the generation of these stages in laboratory culture and investigate their transcriptome as well as their morphology and drug responses. The presented data is consistent with previous reports of a "dormancy" state that parasites can enter into to avoid harmful conditions, only to resume the replication after a certain time leading to a potential relapse.

The topic of the investigation is of high interest to the parasitology community, the general methodology employed by the authors is sound and the presented experiments are well designed and controlled. In general, the manuscript is clearly written and definitely of publication quality. The discussion summarising the current knowledge about the connection between dormancy and ART resistance, in particular, is well-researched and presents the authors' data in a broader context without overinterpreting it.

There are however few issues that I would like to see addressed:

Major comments:

1. The two major stages present in the experiments (rings and pyknotic) are not distinguishable by flow cytometry and look similar on the Giemsa smears. Additionally, the intermediate parasite stages can be potentially present in some samples. The quantification of the stage composition through the dormancy induction and recovery relies therefore solely on a subjective assessment of a microscopist. If authors want to make statements like "proportion of the pyknotic parasites rose from 95.8 % to 98.9 %", a more objective measure of the stage composition should be used. A possible solution would be to report the parasite sizes or percentage of RBC taken. At a minimum, the assessments of two independent microscopists, performed without the knowledge of the samples' identity, should be used.

2. It is not clear based on the authors' description what percentage of their original culture undergoes the transformation into dormant forms. It can be presumably calculated based on the initial parasitaemia and final numbers of pyknotic cells generated. That information may be of crucial significance, particularly for the interpretation of the drug assay data (how many dormant cells per well are being subjected to the drug?)

3. The RNA-seq comparison to the existing Plasmodium datasets could be improved – for now this is the weakest part of the manuscript.
- The methods are described very briefly and do not mention which of the many available datasets was the data compared to - one can infer based on the spacing of time points that it is the microarray data available through PlasmodDB but the key publications should be cited.
- Importantly authors compare the data generated using two very different protocols (possibly different methodologies) and very different cell numbers. Just the fact that nearly all transcripts are present in the reference dataset and only ~50% in the generated dataset can result in a strong batch effect.
- the Pearson correlation coefficient assumes a linear correlation between data (often not true in heavily amplified libraries) and is not appropriate for heavily transformed datasets.
- The key comparison of interest – are the transcriptomes of the pyknotic form highly distinctive from ring stages from the same experiments – is not included in either the plots in Fig1C nor 3C. And Figure S4 is very hard to interpret but seems to show lack of very distinctive transcriptome shift.

Therefore to make the claim of "previously undefined developmental stage" credible, I would expect authors to either generate samples from different life stages using the same protocol or use more

sophisticated methods of transcriptome investigation eg. dimensionality reduction/principal component analysis using data from ~200 genes expressed in all samples etc.

4. It would be good to see examples of the fold changes in expression between the dormant stages and rings for the most informative genes. Ideally, the whole table of differential expression analysis between rings and pyknotics could be attached as supplementary material to allow the exploration of the dataset.
That would provide evidence of how strong the effect of quiescence is and maybe supply some markers that would differentiate between the rings and pyknotics and validate the authors' data and provide avenues for further investigation

5. The data presented by the authors suggests the existence of two different phenomena: 1) initial transformation into pyknotic stages, characterised by abnormal mitochondrial function, but still normal transcriptome taking place within 24h and 2) gradual gene expression shift – potentially in the response to the above. It is not clear how the two are connected. It would be interesting to hear how authors explain this phenomenon. Would the observed transcriptome change be a secondary reaction and the initial transformation happens purely at the protein level?

6. Following on the above – all drug assays were performed on day 5 parasites in which both morphology and transcriptome were modified. Would the treatment 24h after the induction of dormancy have the same effect? This is of key public health interest, as in standard combination therapy the two drugs are typically administered together. While the repeat of all of the assays on transcriptionally normal but already pyknotic parasites would be probably too laborious the validation of at least one of them would be useful. Otherwise, this limitation should be acknowledged in the discussion.

Minor comments:

1. What are the "small rings" mentioned in figure 1c but not appearing anywhere in the methods? Intermediate stages between "normal" rings and pyknotic forms? How are they defined and quantified?
2. It is unclear why samples were diluted with RBC for imaging in fig 1e? Was it to make better thin smears? It made it impossible to assess the purity of the prep (ie. were there any uninfected cells in the mix?) which may have been useful.
3. The materials and methods section is not clear and quite vague in places. It would be difficult to reproduce the experiments based on the current information provided. In particular, the data analysis part and clustering section could use more details – perhaps even the inclusion of the scripts used for data processing.
4. The TEM results seem a bit subjective. It would be useful to incorporate some form of objective measure into these. I.e. What do the authors mean when they say "well-defined," (Line 322)? At what point is a cuplike-shaped ring considered a round-shaped form? When is a mitochondrion considered enlarged? (Lines 329 – 353). Could measures be added to these in a way similar to figure S10?
5. On each correlation plot the custom colour scale is different (with arbitrary start and end) making the comparison of these plots difficult. In Fig S4 the colours through the scale are so similar that it is hardly readable.
6. Extra table with sequence quality metrics of the samples (to see how different it is between the data points) would be helpful.
7. On line 104 "day 5 mature dormant persisters (d5MDP)" is redefined on line 238 "the five (5)days Mature Dormant Parasite stage (5dMDP"). A mix of d5MDP and 5dMDP has been used throughout the paper. Minor detail, but it could be consistent.
8. Same for hours, hrs, hr or h; minutes, mins or min.in text and figures
9. Figure 1e: the dark blue and black arrows for rings and pyknotic stages are quite close. Perhaps different colour be chosen for one of them to make it easier to distinguish ?
10 Figure S1c: has no scale bars

11. Figure 5 and Figure S8: increase the size of the scale bars so they are readable.
12. Throughout the manuscript the dormant forms are discussed as associated with ART resistance – it may be worth mentioning that they appear also after treatment with other drugs or even in response to environmental factors and are likely to present a general stress response rather than specific recent adaptation (in fact the authors observe it with chloroquine as well)

Reviewer #2:
Remarks to the Author:

The MS entitled "The artemisinin-induced dormant stages of Plasmodium falciparum exhibit hallmarks of cellular senescence and drug resilience" by Tripathi and co-authors provides us with more insights into cellular pathways involved in maintaining dormant state (growth arrest) in Plasmodium falciparum parasites and provides valuable and comprehensive transcriptome data. The MS is well written and referenced.
At present as we face the wide spread of resistance to artemisinin-based combinations therapies (ACTs) in South East Asia, this topic is very important as dormancy underlies parasite recrudescence following treatment with ACTs. Deeper understanding of the pathways parasites use during the dormancy may assist in adjusting treatment regimens and selecting more suitable partners for ACTs.
The data presented in the MS is of sound quality and the methodology is well described.
The major novelty and contribution of this MS is providing high quality dormant parasite's transcriptome data and detailed analysis of the pathways that are active during the dormancy stages.
However, I have the following comments and questions.
1) Line 26 "Here, we developed a robust laboratory model for induction of dormant P. falciparum parasites…" and describe the laboratory model in lines 115-124
It is unclear what "marked modifications" in the experimental design authors are referring to. In fact, the culture protocol that Tripathi and co-authors used in this MS appears to be identical to that originally developed by Drs Kyle and Cheng's groups and originally described in Teuscher et al., 2010: "Artemisinin induced dormancy in Plasmodium falciparum: Duration, recovery rates and implications in treatment failure" as well as Tuescher, 2012, Peatey, 2016, Connelly 2021 and others. Could authors clarify what modifications to Tuescher's protocol were made.
2) Consistent with identical experimental design, Tripathi and co-authors describe observations (e.g, morphological changes in parasites, no change in parasitemia until parasites resume growth etc) identical to those previously described by Tuescher, 2010 and others from the same groups. However, there are several important differences in the interpretations and conclusions (discussed below) reached by the authors of the MS from seemingly same observations, which in my view are not sufficiently explained and discussed in the MS. Below are specific points
a). In the absence of the robust morphological markers, the exact appearance of dormant rings remains to be conclusively demonstrated. The findings by Tuescher, 2010 are consistent with the notion that the majority of parasites (up to 98%) is eventually killed following DHA exposure which would correspond to the majority of the pyknotic-looking parasites, whereas a small fraction is not killed and become dormant. As the time progresses this distribution may change as some dormant parasites may die and some (estimated 50%) resume growth.
In Lines 125-138 Tripathi and co-authors imply that "dormant" parasites have pyknotic appearance, which they describe as "81.8 % of the parasites (average of four biological replicates, Fig 1C) exhibited a pyknotic morphology, that is characterized by densely stained, small, round-shaped cell-like structures " and go on to say that this is analogous to those described in publications by Tuescher et al., 2010 and Tucker et al, 2012 :"The morphological transformation from ring-shape to pyknotic cells after exposure to artemisinin drugs is analogous to previous studies which have reported formation of rounded morphology with blue-stained cytoplasm and red stained chromatin in Giemsa smears of dormant parasites19,20. Importantly, the proportion of the pyknotic parasites rose from 95.8 % to 98.9 % (in four biological replicates) of the total parasitemia between Day 2 and Day 5; essentially dominating the entire parasite population within the culture (Figure 1C)."

In fact, in Tuescher's and Tucker's publications dormant parasites are distinguished (by shape and staining) and represent a small fraction (see below) of this overwhelming majority of "pyknotic" parasites observed after exposure to DHA. Over the next several days after drug exposure they progressively shrink to almost invisible dots and eventually disappear from the slides by Day 5-7. The parasites termed "dormant" in Tuescher, 2010 initially also appear condensed although stained differently to pyknotic forms. As early as on Day 3 some of them start enlarging and resume the live ring appearance. If authors are not making a distinction between dormant and pyknotic form they should clarify this and other points of differences with the previously published observations and results.

b). Tripathi and co-authors observed similar percentages of pyknotic parasites and assumed they are all dormant, which is not consistent with observations by Tuescher et al., where the fraction estimated as 0.044% to 1.313% of the initial ring populations. Tuescher and coauthors estimated that this number represents 50% of all initially observed dormant forms (not of all treated ring population), which would estimate the initial percentage of dormant rings at maximum of 2.626% of all treated rings. It follows, that the rest of 97.374% were killed by DHA (as they never recovered).

c). When describing recovery from dormancy in lines 140 -142 Authors state:

"This indicates re-emergence of the asexual growth that resumed fully by Day 10, with 60.2 % (average of four biological replicates) of the parasites in one of the asexual IDC forms (Figure 1C).

And in lines 369-371: "Subsequently, growth recovery occurred consistently at day 8-10 by which more than two thirds of the parasite population re-entered the IDC."

This statement may be interpreted as that 60.2% of the original population (from pyknotic parasites) resumed their growth is somewhat misleading and difficult to reconcile with findings by Tuescher and others, who observed only 0.044% to 1.313% of the initial ring populations parasites recovered under the same experimental conditions (for similar to 3D7 sensitive strains). It appears that the authors simply measured parasitemia in trohozoite/schizont gate by FACS on Days 8-10 but these parasites would represent a parasite population resulting from 1 to 4 cycles (3D7 parasites have shorter than 48 hours cycle in vitro) of asexual replication of parasites recovered from dormancy between Day 3 and Day 10. It also incorrect to say that recovery occurs on Day 8-10, when in fact growing parasites can be seen as soon as 50-60 h after treatment. These statements need to be corrected.

To estimate the recovery rates Tuescher and co-authors removed emerging live parasites using magnetic column every day for 20 days (Tuescher et al., 2010). This small fraction of the initial number (0.044% to 1.313%) of rings able to recover support supports the estimated fraction of 2.6% of "dormant" parasites estimated on Giemsa slides (Tuescher et al. 2010)

3) Unfortunately, the flow cytometry sorting used in the current study did not differentiate the dead, dormant and live rings. It is unfortunate that the authors did not isolate parasites based on MitoTracker Deep Red and Sybr Green staining as described in Connelly, 2021 publication, which provide a clear separation of dormant parasites from dead parasites.

The gate used to source the parasites for RNA-seq would therefore contain predominantly dead parasites, dormant ring parasites, but also some live ring stage parasites (not killed by DHA or already recovered from dormant). The recovery of some dormant parasites can start as early as Day 3 after the last Mag column (Tuescher, 2010). Presumably, contamination with live parasites (rings) on Days 1-3 and perhaps also on Days 4 and 5 is very negligible but it would certainly increase during the course of experiment (after the last MAC separation on Day 3) and could have affected RNA-seq results. This needs to be discussed, specifically in a context of defining different stages of dormant parasites. Also it would be beneficial for the readers not well familiar with RNA-seq methodology if authors could clarify if their methodology of sequencing of a "few" parasites is similar to single cell RNA-seq.

4). The definition of the dormant ring is very important for the selection of the parasites selected for electron microscopy. As authors selected "a pyknotic parasite" from a total population of all DHA-treated rings for electron microscopy and presented the data for live ring on Day 0, and treated rings on Day 1 as well as on Day 5, where the parasite appear to be shrinking and "loosing" organelles. It is not convincing if this parasite is dormant or simply dead. There are no electron micrographs showing the recovering "dormant" parasites after Day 5, which could have demonstrated that it was not a dead parasite presented and that the "dormancy" was temporary.

5). The drug susceptibility data presented are of interest and important. As for all drugs a 24- hour exposure time was selected for all compounds, I would suggest to discuss the findings in a context of drugs' stage specificity, mode of action, pathways and drugs' half-life.
6). The statement in the lines 427-428
"In vitro P. falciparum parasite line resistant to artelinic acid exhibit higher sensitivity to dormancy but also higher recovery rate" is incorrect. The parasites in this W2AL80 line exhibited a decreased propensity to fall dormant as they were able to better tolerate the drug exposure and continued to grow despite the drug treatment.
7). Lastly, based on the results described in the MS I do not think that authors can state, and especially in the title of the MS, that dormancy corresponds or "bearing hallmarks" of senescence in higher eukaryotes. The most common definition of senescence implies an irreversible stop in cellular division, originally described for fibroblasts by Hayflick in 1961 and attributed to multicellular higher eukaryotes, where different tissues and cells have to cooperate and communicate in order to prevent indefinite replication leading to cancerogenesis. In my view, the more appropriate analogies for the DHA-induced parasite growth arrest are "quiescence" or "cell cycle arrest", which typically implies a temporary, reversible arrest of growth in response to the stress factors, such as ROS or DNA-damage. With no clear homology/orthology relationship between Plasmodium cell cycle machinery involved in dormancy and that of higher eukaryotes, responsible for senescence, linking dormancy to senescence would be highly speculative and not based on the transcriptome findings described in MS.

Reviewer #3:
None

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The work submitted by Tripathi and colleagues focuses on the investigations of pyknotic forms of the Plasmodium parasite that arise in response to the stress conditions (eg. drug treatment). The authors present the protocol for the generation of these stages in laboratory culture and investigate their transcriptome as well as their morphology and drug responses. The presented data is consistent with previous reports of a "dormancy" state that parasites can enter into to avoid harmful conditions, only to resume the replication after a certain time leading to a potential relapse.

The topic of the investigation is of high interest to the parasitology community, the general methodology employed by the authors is sound and the presented experiments are well designed and controlled. In general, the manuscript is clearly written and definitely of publication quality. The discussion summarising the current knowledge about the connection between dormancy and ART resistance, in particular, is well-researched and presents the authors' data in a broader context without overinterpreting it.

We thank the reviewer for the positive comments and also for recognizing the impact of this study. Indeed, blood stage dormancy of malaria parasites has been debated in the field but yet limited data were generated so far. It is nice to see that this reviewer (and also reviewer2) acknowledging that our manuscript bring a fundamental shift forward in this topic.

Below we addressed all reviewers comments:

Major comments:

1. The two major stages present in the experiments (rings and pyknotic) are not distinguishable by flow cytometry and look similar on the Giemsa smears. Additionally, the intermediate parasite stages can be potentially present in some samples. The quantification of the stage composition through the dormancy induction and recovery relies therefore solely on a subjective assessment of a microscopist. If authors want to make statements like "proportion of the pyknotic parasites rose from 95.8 % to 98.9 %", a more objective measure of the stage composition should be used. A possible solution would be to report the parasite sizes or percentage of RBC taken. At a minimum, the assessments of two independent microscopists, performed without the knowledge of the samples' identity, should be used.

We agree with the reviewer that classifying of the pycnotic forms and distinguishing these from the rings stage parasites depends on the subjective assessment of the microscopist. This has been, however, a common practice in numerous *Plasmodium* studies distinguishing ring stages, characterized by a dense round shape body (nucleus) surrounded by a halo-like shape (cytoplasm) in a "diamond ring"-like shape; from pycnotic bodies lacking the cytoplasm (described in the text, also referred to as "densely stained, small, round-shaped cell-like structures" by reviewer #2). As suggested by the reviewer we now provide additional counts/assessment made by one additional microscopist (blinded to the samples) that in principle support our original counts. Although, carrying out microscopy-based cellular measurements of the cell volumes are possible, however, we respectfully argue that such studies are beyond the scope of this study. Such measurements were never done before and thus would require complete new sets of evaluations and cross referencing that are currently not feasible.

In the new version of the manuscript, we also avoid making statements about incremental changes in (pycnotic) parasites counts (e.g. from 95.8 % to 98.9 %). In principle, the main

purpose of counting of the pycnotic form is to demonstrate the inductions effect by DHA, linking these to previous studies.

In the new version we present the independent microscopist's measurements in **Figure S1B** and refer to it in line 140:

*"The proportion of the pyknotic parasites remain high (approx. 95% or higher in all four biological replicas estimated by two independent microscopists) between Day 2 and Day 5; essentially dominating the entire parasite population within the culture (**Figure 1C and Figure S1B**)."*

2. It is not clear based on the authors' description what percentage of their original culture undergoes the transformation into dormant forms. It can be presumably calculated based on the initial parasitaemia and final numbers of pyknotic cells generated. That information may be of crucial significance, particularly for the interpretation of the drug assay data (how many dormant cells per well are being subjected to the drug?).

Here we showed that the initial overall parasitaemia (% of infected erythrocytes prior to the DHA treatment) did not drop significantly immediately after the DHA treatment and remained at similar levels for up to 5 days. This suggest that the vast majority (close to 100%) of the parasites converted to pyknosis. To be more exact we calculated a ring-to-pyknosis conversion rate based on number of pyknotic parasites observed in culture on Day1, Day2 and Day3 when compared to Day 0 (prior to treatment) (**Figure S2B**).To clarify this further we provide precise counts of the parasitaemia prior and after treatment in a supplementary figure (**Figure S2A**).

Here we wish to stress that this conversion rate in considerably higher than all previously reported cases where typically only ~2% or less of the parasite population is converted to viable pyknosis (see comments and responses for reviewer #2). Although it is currently unclear what exactly accounts for this increased conversion, the main reason is likely a combination the usage of the *P. falciparum* strain (3D7) and minute but crucial details in our culturing techniques and/or materials (e.g. Albumax supplement instead of human serum) some of which might be beyond the description in previous publications. Nevertheless the high pyknosis conversion rate it the key feature of this study that allowed us to carry out all systems and cellular biology hat were not possible with previous experimental settings.

In the new version of the manuscript we stress this point in more detail; line 119.

*"Briefly, P. falciparum (3D7 strain) parasites, cultured in RPMI media supplemented with lipid-rich bovine serum albumin (Albumax) at 2% hematocrit were synchronized at the ring stage (6-12 hours post invasion, HPI) followed by exposure to 700 nM dihydroartemisinin (DHA) and subsequently maintained in the culture for 12 days (for further details see **Material and Methods**)."*

And discus this further in Discussion on line 401

*Here we modified the dormancy induction protocol using the 3D7 P. falciparum strain inducing dormancy at the mid-ring stages (6-12 HPI) and achieved considerably higher conversion rates that reached nearly ~100% (**Figure 1 & S2**). … Nevertheless, the dormant parasites induced in this study (d5MDPs) are most likely identical to those reported in the previous studies."*

3. The RNA-seq comparison to the existing *Plasmodium* datasets could be improved – for now this is the weakest part of the manuscript.

- The methods are described very briefly and do not mention which of the many available datasets was the data compared to - one can infer based on the spacing of time points that it is the microarray data available through PlasmodDB but the key publications should be cited.

The RNA seq data in this manuscript was compared to the high-resolution RNA seq data of the intraerythrocytic developmental cycle published in Kucharski et al, Malaria Journal, 2020, and the gametocyte data published in Zhu et al, Nature Communications, 2018, respectively for HPI correlations shown in Fig 2C and supplementary figure Supp Fig S4. The relevant articles are now cited. Details of SMARTseq2 reverse transcription, cDNA amplification and RNA sequencing are now further described in materials and methods **line 601-616** a Supplementary Protocol is provided.

- Importantly authors compare the data generated using two very different protocols (possibly different methodologies) and very different cell numbers. Just the fact that nearly all transcripts are present in the reference dataset and only ~50% in the generated dataset can result in a strong batch effect.

- the Pearson correlation coefficient assumes a linear correlation between data (often not true in heavily amplified libraries) and is not appropriate for heavily transformed datasets.

- The key comparison of interest – are the transcriptomes of the pyknotic form highly distinctive from ring stages from the same experiments – is not included in either the plots in Fig1C nor 3C. And Figure S4 is very hard to interpret but seems to show lack of very distinctive transcriptome shift.

Therefore to make the claim of "previously undefined developmental stage" credible, I would expect authors to either generate samples from different life stages using the same protocol or use more sophisticated methods of transcriptome investigation eg. dimensionality reduction/principal component analysis using data from ~200 genes expressed in all samples etc.

Indeed the transcriptomics analyses of the pyknotic cells in this study was carried out by "few cells RNA-Seq" approach in which transcriptome is analysed from exactly 500 cells sorted by FACS approach and amplified and sequenced using the SMARTseq2 method previously optimised for *Plasmodium* blood stages (Kucharski et al, 2020). The high-resolution intraerythrocytic developmental cycle (IDC) RNA seq data used for estimating HPI (in Fig 2C and Supp Fig S3) from Kucharski et al, 2020, was also generated using the SMART-seq2 method for bulk RNA. The correlation coefficients shown in Fig 2C and Supp Fig S3 were calculated from a pairwise comparison where only transcripts present in both datasets were included in the analysis. Furthermore, all correlation analyses were performed on log2FPKM values and NOT on the heavily transformed Fourier transformed datasets. Log transformation was performed for normal distribution of the dataset.

We agree that comparing our dataset to previously generated bulk-based transcriptomes could be confounded by a batch effect. Naturally, generating few cell transcriptomes for all *P. falciparum* life cycle would be ideal for better comparisons. This is unfortunately highly labour intensive and costly and as such unrealistic for the purpose of this study. Instead we take the

reviewers suggestion to use more sophisticated methods and all precautions to interpret our comparative analyses as suggested by the reviewer. We now present Principal Component Analysis in **Supp Fig S4A** to show three very distinct clusters formed by asexual forms, gametocytes and the dormancy development cycle (Day 1 to Day 12) indicating fundamental transcriptomic differences between the three developmental phases of the parasite.

Line 238

*"Principal component analysis further shows distinct clusters being formed by asexual, sexual and DHA-induced dormant parasite transcriptomes (**Figure S4A**) suggesting fundamental transcriptomic differences between these developmentally distinct stages."*

Moreover, to emphasize on the difference between rings and dormant stages, we would refer to the low transcriptome correlation (SPCC = 0.29 – 0.33 for three bioreps) between rings and dormant stages (d5MDP) (**line 219 to 222, Fig 2C and Fig S3**). To further explore the transcriptomic differences between rings and dormant forms, we have now provided a differential gene expression (DGE) analysis supported by z-scores and fold change values to show genes differentially expressed (DE) between rings and dormant parasites (line **278-288**, **Fig 3D, Supplementary Data**). Several of the key candidates from the mitochondrial pathway such as the putative cytochrome c oxidase subunit 2 (PF3D7_1430900), mitochondrial ribosomal protein S12 precursor (PF3D7_0412100) and ATP synthase F1, alpha subunit (PF3D7_0217100) were validated through qPCR to confirm several fold upregulation in d5MDP (Figure 3D).

4. It would be good to see examples of the fold changes in expression between the dormant stages and rings for the most informative genes. Ideally, the whole table of differential expression analysis between rings and pyknotics could be attached as supplementary material to allow the exploration of the dataset.

That would provide evidence of how strong the effect of quiescence is and maybe supply some markers that would differentiate between the rings and pyknotics and validate the authors' data and provide avenues for further investigation.

We welcome the reviewer's excellent suggestion and take advantage of this revision to select a narrow set of highly informative markers of pyknosis/dormancy through fold-change and z-score based analysis (Supplementary Data, line 278-288). Specifically, we generated quantitative RT-PCR results (Figure 3D) for a small subset of highly informative genes such as the putative cytochrome c oxidase subunit 2 (PF3D7_1430900), mitochondrial ribosomal protein S12 precursor (PF3D7_0412100) and ATP synthase F1, alpha subunit (PF3D7_0217100) and assess fold changes of their steady state mRNA levels in rings versus dormancy . Indeed this set of genes now provides a highly informative tool for further studies of the dormant parasite without the need of RNA-Seq.

We refer to this in text: line 290

"By visual inspection of the fold-change and statistical significance (z-score) of the transcriptional changes we identified several genes that could be used as transcriptomic markers differentiating between the rings and d5MDP. These include …could be used to

*detect the dormant parasites in various conditions using RNA based quantitative RT-PCR (**Figure 3D**)."*

5. The data presented by the authors suggests the existence of two different phenomena: 1) initial transformation into pyknotic stages, characterised by abnormal mitochondrial function, but still normal transcriptome taking place within 24h and 2) gradual gene expression shift – potentially in the response to the above. It is not clear how the two are connected. It would be interesting to hear how authors explain this phenomenon. Would the observed transcriptome change be a secondary reaction and the initial transformation happens purely at the protein level?

In principle, we agree with the reviewers assessment of the two phenomena including 1) induction of the pycnotic forms within 24 hours after the 6hr DHA treatment and subsequently 2) gradual gene expression shift over the 5-day maturation process. We also agree with the reviewer that the gradual transcriptional changes appear as a reaction to the initial induction of pyknosis (presumably driven by an abnormal mitochondrial function). However, we wish to argue that the transcriptional shifts are not just a "secondary reaction" but instead are highly relevant for a putative dormancy state; resembling senescence/quiescence. This is particularly demonstrated by downregulation of growth related functionalities and upregulation of mitochondria and DNA repair e.t.c.. This characterizes the presumed dormancy transcriptome that persist for at least 3 days (day 5-8). These are also parallel the morphological changes (shown by EM) where the parasites transition from a ring-like form on day 1 to highly irregular intracellular morphology on day 5.

Currently we do not know the exact mechanisms that underlines the gradual transition of the transcriptome into the dormancy state (over 5 days) and subsequently back to rings (over next 3-5 days). However, these transcriptional changes resemble other transcriptomics programs withing the *P. falciparum* life cycle such as the IDC and/or gametocyte development that are believed to be facilitated by a series of transcription factors such as the AP2 family. It is feasible to speculate that the dormancy related transcriptional shifts represent yet another hard-wired transcriptional transcriptome program within the *Plasmodium* life cycle driven by similar transcription factors; albeit in a different order.

We discuss this further in the text; Discussion section; line 473:

*"Taken together, the blood stage dormancy of P. falciparum appears combine molecular and cellular mechanism from both cellular quiescence and senescence in a unique set of processes leading to a robust but reversible growth arrest. This process seems to involve two seemingly independent but likely tightly interlinked steps: (i.) stress (DHA)-induced conversion from the ring stage to into pyknosis and (ii.) maturation process characterized by gradual **…** transcriptional regulators including the ApiAP2 family of transcription factors[46]. It will be also interesting to investigate whether the blood stage dormancy share common features with the preerythrocytic stages (hypnozoites) but also dormancy of other eukaryotic species including plants[57].*

6. Following on the above – all drug assays were performed on day 5 parasites in which both morphology and transcriptome were modified. Would the treatment 24h after the induction of

dormancy have the same effect? This is of key public health interest, as in standard combination therapy the two drugs are typically administered together. While the repeat of all of the assays on transcriptionally normal but already pyknotic parasites would be probably too laborious the validation of at least one of them would be useful. Otherwise, this limitation should be acknowledged in the discussion.

To some degree this was addressed in a previous studies by Tuscher, F. et al 2010 that demonstrated that treatments of dormant parasites 24 hours after induction by DHA or mefloquine lead to significant reductions of re-initiation rates and delays in growth recovery. Although, conducted in a fundamentally different experimental set up compared to ours, this suggests that the 24hr are substantially more susceptible to these and possibly other antimalarial drugs.

Nevertheless to fully address his here we conducted comparable drug sensitivity assay (12 days long) with pycnotic parasites treated 24 hours after induction for two drugs – lumefantrine and atovaquone (Figure S7B, line 340-342). Interestingly, we do not see parasite recovery for 10-100x IDC^{IC50} lumefantrine even when parasites were treated 24 hours after induction of dormancy with DHA, thus corroborating our previous findings for d5MDP (Figure 5) but also show that that pyknotic cells are somewhat more susceptible to lumefantrine after 24 hour compared to 5 days.

We provide this information in Figure S7b and refer to it in text; line 347

*"The pattern of no parasite recovery was similar for 10-100x $^{IDC}IC_{50}$ LMF even when parasites were treated 24 hours after induction of dormancy with DHA, mimicking clinical treatment scenario (**Figure S7B**)."*

Minor comments:

1. What are the "small rings" mentioned in figure 1c but not appearing anywhere in the methods? Intermediate stages between "normal" rings and pyknotic forms? How are they defined and quantified?

"Small rings" are very early ring stages observed in culture. This is a minority in the culture as indicated by small proportion in Fig 1c. This is expected as the parasite cultures were synchronised with sorbitol to achieve 6-12 HPI rings. Any intermediate stages between rings and pyknotic parasites will be much higher in proportion given that >90% parasites turn pyknotic post DHA exposure.

2. It is unclear why samples were diluted with RBC for imaging in fig 1e? Was it to make better thin smears? It made it impossible to assess the purity of the prep (ie. were there any uninfected cells in the mix?) which may have been useful.

Yes, dilution of sorted infected RBCs was done for making smears. Regarding sorting, only Hoechst positive cells were sorted (Gates labelled R/P in Fig 1b), so uninfected RBCs (that are enucleated and have no DNA) will not be stained by Hoechst and will not be sorted. We have

uninfected, Hoechst-stained RBC control in the sorting experiments for the gating and avoid uninfected RBCs sorted in the sample.

3. The materials and methods section is not clear and quite vague in places. It would be difficult to reproduce the experiments based on the current information provided. In particular, the data analysis part and clustering section could use more details – perhaps even the inclusion of the scripts used for data processing.

Thanks for the suggestion. We have now added more details in the RNA sequencing sample preparation (line **590 to 600**). Method for Data Analysis is now clarified in line **668 to 688**. All the tools used in sequencing data processing are commonly used (HISAT2, Trim Galore etc.) and the scripts will be available upon request to the corresponding authors after publication. We have now included the script for Fourier Transformation analysis as Supplementary Data.

4. The TEM results seem a bit subjective. It would be useful to incorporate some form of objective measure into these. I.e. What do the authors mean when they say "well-defined," (Line 322)? At what point is a cuplike-shaped ring considered a round-shaped form? When is a mitochondrion considered enlarged? (Lines 329 – 353). Could measures be added to these in a way similar to figure S10?

We concur that the TEM results are highly subjective. This is due to the fact that no such ultrastructure was ever observed before. Indeed, it will require more work to define these new cellular structure that is beyond the scope of this study. Nevertheless we still believe that reporting these here to this extend does support the overall observation of the uniqueness of the dormancy parasite form. In the text we made an extra effort to avoid ambiguities.

5. On each correlation plot the custom colour scale is different (with arbitrary start and end) making the comparison of these plots difficult. In Fig S4 the colours through the scale are so similar that it is hardly readable.

The scales for each correlation plot are adjusted according to the maxima and minima for the particular dataset to get optimal dispersion of the colour scale visually. As such indeed, it is not practical to use the same colour scheme across different datasets as the dynamic range of correlation coefficients is different for different dataset. Instead, to make thigs clearer, we have now provided a 3 colour-scale for the correlation plot in Fig S4B for better visualization.

6. Extra table with sequence quality metrics of the samples (to see how different it is between the data points) would be helpful.

Please see Supp **Fig S11** for sequence quality metrics of the samples.

7. On line 104 "day 5 mature dormant persisters (d5MDP)" is redefined on line 238 "the five (5)days Mature Dormant Parasite stage (5dMDP"). A mix of d5MDP and 5dMDP has been used throughout the paper. Minor detail, but it could be consistent.

Thank you for highlighting this. We have now used d5MDP uniformly throughout the manuscript.

8. Same for hours, hrs, hr or h; minutes, mins or min.in text and figures

This is now made uniform throughout the manuscript.

9. Figure 1e: the dark blue and black arrows for rings and pyknotic stages are quite close. Perhaps different colour be chosen for one of them to make it easier to distinguish?

We chose blue colour arrow for rings and black (grey won't be visible) for pyknotic to keep colour scheme in Fig 1e corresponding to the **Fig 1b**, thus making it easier for the readers to visually compare and cross reference between the two figures.

10 Figure S1c: has no scale bars

Scale bars are now added to **Fig S1C.**

11. Figure 5 and Figure S8: increase the size of the scale bars so they are readable.

We have now added new legible scale bars to all EM images.

12. Throughout the manuscript the dormant forms are discussed as associated with ART resistance – it may be worth mentioning that they appear also after treatment with other drugs or even in response to environmental factors and are likely to present a general stress response rather than specific recent adaptation (in fact the authors observe it with chloroquine as well)

Indeed, there were several previous suggestions that other types of stresses such as other drugs and osmotic pressures (sorbitol) may induce similar dormancy like states. Although this is highly possible, with all due respect, in this manuscript we wish to refrain from such speculations as these could be misleading as no data currently exists about similarities and difference between the DHA and other stresses induced pyknotic parasites.

Reviewer #2 (Remarks to the Author):

The MS entitled "The artemisinin-induced dormant stages of Plasmodium falciparum exhibit hallmarks of cellular senescence and drug resilience" by Tripathi and co-authors provides us with more insights into cellular pathways involved in maintaining dormant state (growth arrest) in Plasmodium falciparum parasites and provides valuable and comprehensive transcriptome data. The MS is well written and referenced.

At present as we face the wide spread of resistance to artemisinin-based combinations therapies (ACTs) in South East Asia, this topic is very important as dormancy underlies parasite recrudescence following treatment with ACTs. Deeper understanding of the pathways parasites use during the dormancy may assist in adjusting treatment regimens and selecting more suitable partners for ACTs.

The data presented in the MS is of sound quality and the methodology is well described. The major novelty and contribution of this MS is providing high quality dormant parasite's

transcriptome data and detailed analysis of the pathways that are active during the dormancy stages.

We also thank this reviewer for recognizing the importance of our study in light of the current urgency of understanding malaria drug modes of action. We are humbly delighted by the reviewer's acknowledgment of the quality of our results, data and also their interpretations. The main concerns and resulting suggestions made by this reviewer aims at one key question; How do our results relate with previously published studies? In our new version we made an extra effort resolve this as we do believe that our results relate well to the previous discoveries.

Essentially all discrepancies pointed out by this reviewer relate to one single aspect of our results compared to the (all) previous work. This is the fundamentally higher conversion rate of *P. falciparum* to pyknosis that reached near 100%, which is in sharp contrast to the previous studies that saw conversion rates being ~2% or less. The high conversion rate is indeed the key featureof the entire study presented in this manuscript, enabling us to carry out all experimental assays that were not possible before. At the moment it is difficult for us to pinpoint exactly why we achieved such high conversion rate but assume that it is related to the parasite genetic background (here the 3d7 strain) as well as multiple aspects of techniques/materials for the *in vitro* culturing of *P. falciparum* utilized in this work. Given that these are typically not described in the previous studies to a great detail, at the moment it is hard for to pinpoint exactly the key aspect of the culturing techniques that mediate this high conversion rate. Nevertheless, below find our best effort to reconcile these differences to our best ability and knowledge.

However, I have the following comments and questions.

1) Line 26 "Here, we developed a robust laboratory model for induction of dormant P. falciparum parasites…" and describe the laboratory model in lines 115-124
It is unclear what "marked modifications" in the experimental design authors are referring to. In fact, the culture protocol that Tripathi and co-authors used in this MS appears to be identical to that originally developed by Drs Kyle and Cheng's groups and originally described in Teuscher et al., 2010: "Artemisinin induced dormancy in Plasmodium falciparum: Duration, recovery rates and implications in treatment failure" as well as Tuescher, 2012, Peatey, 2016, Connelly 2021 and others. Could authors clarify what modifications to Tuescher's protocol were made.

As mentioned in the text, our original experimental set up was based on the above-mentioned publications and in many aspects it is nearly identical. We acknowledge further in our manuscript. Nevertheless there are few small variations of the original setup that could account for the fundamentally higher conversion rate of *P. falciparum* to viable pyknotic forms and thus yields of the dormant cell fractions.
The main differences in our culture system compared to Teuscher et al, 2010, are mainly in the culturing conditions, stated as follows: I) We use Albumax which is a lipid-rich bovine serum albumin instead of human plasma (10%) for parasite culture. This may affect parasite growth rate (invasion, nuclear division) or recovery differently. II) We culture at 2% hematocrit versus 3% hematocrit used by Teuscher *et al*, this may lead to variations in nutrient diffusion to the infected RBCs and infection (merozoite) spread within the hematocrit. III) We use 3D7MR4 strain of *P. falciparum* for all our experiments whereas Teuscher *et al* used W2, HB3, S55, PH1 and D6 strains. Strain differences have previously been shown to affect the dormancy-recovery phenomenon. In fact, Teuscher *et al* report that recovery took 19 days for W2 and HB3 and 14 days for D6 (significantly longer than our observation for 3D7MR4). IV) Additionally, our

DHA treatment cultures were 6-12HPI rings with a parasitemia of ~2% to 8% (for four bioreplicates). Teuscher et al, do not specify what age rings were used for the recrudescence studies, and the authors conducted the recrudescence monitoring experiments at 2% parasitaemia. We have stated all details of our culturing system in the Methods Section line 556-574.
We also reiterate this in the result section; line 115:

"To gain further insights into the biological processes that drive induction and persistence of the dormant forms of P. falciparum parasites and the subsequent re-emergence asexual intraerythrocytic growth, we adopted the culturing protocol originally developed by Teuscher, F. et. al. 2010 for induction of P. falciparum dormancy[19] with few modifications. Briefly, P. falciparum (3D7 strain) parasites, cultured in RPMI media supplemented with lipid-rich bovine serum albumin (Albumax) at 2% hematocrit were synchronized at the ring stage (6-12 hours post invasion, HPI) followed by exposure to 700 nM dihydroartemisinin (DHA) and subsequently maintained in the culture for 12 days (for further details see ***Material and Methods****). "*

2) Consistent with identical experimental design, Tripathi and co-authors describe observations (e.g, morphological changes in parasites, no change in parasitemia until parasites resume growth etc) identical to those previously described by Tuescher, 2010 and others from the same groups. However, there are several important differences in the interpretations and conclusions (discussed below) reached by the authors of the MS from seemingly same observations, which in my view are not sufficiently explained and discussed in the MS. Below are specific points

In general we share the reviewer view that the dormant stage of *P. falciparum* characterized by this work is the same as that studied in the previous publications by Tuecher at al 2010 and others. In Tuescher et al , 2010, the authors observed "morphologically abnormal rings" following a single exposure to 200 ng/ml DHA. Unfortunately, Fig 2 in Tuescher et al 2010 (shown below for reference) did not show parasitemia from Day 0 onwards (even though the starting parasitemia is mentioned to be 2% in the Methods section). Later, on Day 7 (DHA, 3 columns data points) the parasitemia is very low (shown as zero on y-axis in Fig 2) followed by recovery and increase in parasitaemia in later days. At this point it is difficult to draw parallels to our data for Day 0 to Day 7 (for some strains recovery seen at Day4) and conclude that there was no drop in parasitemia in Tuescher et al, 2010, recovery experiments. Once again we strongly believe that the apparent higher viability of the pyknotic cells in this study stems from the substantially higher proportion of the total parasitaemia that is induced to dormancy after the DHA treatment compared to that published by Tuscher 2010 and others. This high conversion rate is likely responsible for most if not all differences between ours and the previous work due to the higher proportion of the induced viable pyknotic cells that remained in the *in vitro* culture. This is also causing the apparent higher rate of re-emergence; albeit not estimated in this study.

Similar to the reviewer 1 concern, we now discuss this in the text and supplementary **figure S2 a and b:** line 177

"This also contrasts previous studies that achieved only a small conversion rate (~2%) with most parasites being eliminated from the culture[18,19]. To investigate this further we estimated a ring-to-pyknosis conversion rate for additional three P. falciparum strains as well as the 3D7 strain induced by DHA at 0-3 HPI as done in the previous studies[18,19]. In our system, we achieve a ring to pyknotic conversion rate of close to 100% to up to 3 days post DHA treatment for all cases. The exception was the W2 strain, also mostly used in the previous studies, that

*even though fully converted to pyknosis, the overall parasitemia was declining rapidly over the next 3 days (**Figure S2A and S2B**). Taken together, the modifications of the culturing protocol combined with the choice of the 3D7 strain (and possibly other currently unknown factors) permitted an efficient transformations of ring stages to pyknosis that provides a suitable laboratory model for further characterizations of the putative blood stage dormancy parasites, in vitro."*

Specifically:

a). In the absence of the robust morphological markers, the exact appearance of dormant rings remains to be conclusively demonstrated. The findings by Tuescher, 2010 are consistent with the notion that the majority of parasites (up to 98%) is eventually killed following DHA exposure which would correspond to the majority of the pyknotic-looking parasites, whereas a small fraction is not killed and become dormant. As the time progresses this distribution may change as some dormant parasites may die and some (estimated 50%) resume growth. In Lines 125-138 Tripathi and co-authors imply that "dormant" parasites have pyknotic appearance, which they describe as "81.8 % of the parasites (average of four biological replicates, Fig 1C) exhibited a pyknotic morphology, that is characterized by densely stained, small, round-shaped cell-like structures " and go on to say that this is analogous to those described in publications by Tuescher et al., 2010 and Tucker et al, 2012 :"The morphological transformation from ring-shape to pyknotic cells after exposure to artemisinin drugs is analogous to previous studies which have reported formation of rounded morphology with blue-stained cytoplasm and red stained chromatin in Giemsa smears of dormant parasites19,20. Importantly, the proportion of the pyknotic parasites rose from 95.8 % to 98.9 % (in four biological replicates) of the total parasitemia between Day 2 and Day 5; essentially dominating the entire parasite population within the culture (Figure 1C)."

Indeed, we are aware that in the Tuescher *et al* study (as well as other previous studies), there was "a near complete disappearance of detectable parasitaemia" throughout first 5-10 days after induction (discussed above). As such, the reappearance of growth after 10 days is believed to originate from a small subfractions of presumed dormant/pyknotic parasites that remained viable while being nearly undetected. This was fundamentally different in our study where we could observe a high levels of pyknotic parasites (described by the reviewer: "densely stained, small, round-shaped cell-like structures") remaining in the culture through the entire course of the experiment. Moreover as we now demonstrate further essentially all pyknotic cells in our study remained viable exhibiting a strong mitochondrial membrane potential.

Once again altoough it is difficult to compare these two studies directly, we believe that this difference is most likely attributed to a near 100% conversion rate of the ring stage parasites to pyknosis that contrasts the Tuescher's (and other's) work in which only a small fraction of parasites converted to viable pyknosis (~2%) while most (~98%) were killed. Again this discrepancy might be due to subtle but important differences in the culturing setups that are difficult to trace.

)

In fact, in Tuescher's and Tucker's publications dormant parasites are distinguished (by shape and staining) and represent a small fraction (see below) of this overwhelming majority of "pyknotic" parasites observed after exposure to DHA. Over the next several days after drug exposure they progressively shrink to almost invisible dots and eventually disappear from the slides by Day 5-7. The parasites termed "dormant" in Tuescher, 2010 initially also appear condensed although stained differently to pyknotic forms. As early as on Day 3 some of them start enlarging and resume the live ring appearance.

We thank the reviewer for clarifying this in this review as these experimental aspects were not described in detail in the original publication. Once again, likely due to the initial high conversion rate to pyknosis (**Figure S2B**), this was not our experience with the DHA induction (see above). However, we did observe significant size reductions and death of pyknotic cells after induction/treatment with chloroquine (used as a negative control) as described in the Figure S2. This indicates that the induced pyknosis in our study is highly specific for DHA in the given experimental setting.

In the text we discuss this in somewhat in more detail; line 172

*"This contrasts the treatment of 6-12 HPI rings with chloroquine (CQ, 250nM (10xIC_{50}) for 24 hours) where a progressive reduction in parasitemia was observed from Day 0 (treatment) to Day 3 (**Figure S2A and S2C**). Mixed parasite morphology with both pyknotic forms and irregular/blebbed forms were observed as early as 24 hours after CQ treatment with progressive parasite elimination from the culture by Day 3 (**Figure S2D and S2E**)."*

If authors are not making a distinction between dormant and pyknotic form they should clarify this and other points of differences with the previously published observations and results.

Indeed, the initial FACS strategy did not differentiate between live and potentially dead pyknotic cells. In original version version, we carried out the fluorescence microscopy with MitoTracker staining showing that essentially all pyknotic cells in our cultures had a significant mitochondrial membrane potential and thus are alive (**Figure 4B**). In the new version we support this microscopy-based observation by flow cytometry using the MitoTracker staining; as suggested in the comment below. Indeed even by flow cytometry analysis, all DHA-induced pyknotic cells appear alive (**Figure 4A**). Similarly to above, this is likely due to the high conversion rate indicating high cell viability.

We note this in the text: line 308:

"Taken together, it is feasible to suggest that the d5MDPs represent the true dormant stages of P. falciparum that give rise to recrudescent malaria infection after treatment with artemisinins and possibly other conditions. As such we wished to investigate d5MDPs viability to assess their potential to give rise to re-new(ed) asexual growth. For that we investigated the intactness of the mitochondrial membrane potential (MMP) using the fluorescent indicator MitoTracker™. Using FACS we first observed that *essentially all cells sorted in the ring-pyknotic gate stained by Hoechst-Dihydroethidium (Hoechst-DHE) double-staining protocol at both day 1 and day 5 post DHA exposure were also MitoTracker™ positive (**Figure 4A**). "*

b). Tripathi and co-authors observed similar percentages of pyknotic parasites and assumed they are all dormant, which is not consistent with observations by Tuescher et al., where the fraction estimated as 0.044% to 1.313% of the initial ring populations. Tuescher and coauthors estimated that this number represents 50% of all initially observed dormant forms (not of all treated ring population), which would estimate the initial percentage of dormant rings at

maximum of 2.626% of all treated rings. It follows, that the rest of 97.374% were killed by DHA (as they never recovered).

c). When describing recovery from dormancy in lines 140 -142 Authors state: “This indicates re-emergence of the asexual growth that resumed fully by Day 10, with 60.2 % (average of four biological replicates) of the parasites in one of the asexual IDC forms (Figure 1C). And in lines 369-371: “Subsequently, growth recovery occurred consistently at day 8-10 by which more than two thirds of the parasite population re-entered the IDC.” This statement may be interpreted as that 60.2% of the original population (from pyknotic parasites) resumed their growth is somewhat misleading and difficult to reconcile with findings by Tuescher and others, who observed only 0.044% to 1.313% of the initial ring populations parasites recovered under the same experimental conditions (for similar to 3D7 sensitive strains). It appears that the authors simply measured parasitemia in trohozoite/schizont gate by FACS on Days 8-10 but these parasites would represent a parasite population resulting from 1 to 4 cycles (3D7 parasites have shorter than 48 hours cycle in vitro) of asexual replication of parasites recovered from dormancy between Day 3 and Day 10. It also incorrect to say that recovery occurs on Day 8-10, when in fact growing parasites can be seen as soon as 50-60 h after treatment. These statements need to be corrected. To estimate the recovery rates Tuescher and co-authors removed emerging live parasites using magnetic column every day for 20 days (Tuescher et al., 2010). This small fraction of the initial number (0.044% to 1.313%) of rings able to recover support supports the estimated fraction of 2.6% of “dormant” parasites estimated on Giemsa slides (Tuescher et al. 2010)

Addressing b) and c) together. the main question made by both points is: What percentage of pyknotic cells do eventually give rise to new asexual growth by re-emerging from dormancy? In the Teuscher’s study this was estimated beautifully by calculating a survivability and subsequent growth re-emergence rate that was no more than 2.626%. Indeed, as the reviewer suggests, currently we do not know what percentage of these putative dormant parasites do re-emerge back to the asexual growth. To estimate that we would need to carry out the suggested experiment in which the magnetic column sorting would be applied for 20 days. Currently we wish to opt out from such experiment as, we believe this to be beyond the scope of this study and moreover, strongly depends on growth conditions. Instead we correct our rough estimates of the growth emergence stating that by day 10, ~60% of the total parasitaemia accounts for asexual sages. We recognize that, the statement “two thirds of the parasite re-entered to asexual growth” is incorrect as we do not know the actual conversion rate.

Text Line 146

*“This indicates re-emergence of the asexual growth that resumed fully by Day 10, when 60.2 % (average of four biological replicates) of the parasitemia was represented by the asexual IDC forms (**Figure 1C**).”*

3) Unfortunately, the flow cytometry sorting used in the current study did not differentiate the dead, dormant and live rings. It is unfortunate that the authors did not isolate parasites based on MitoTracker Deep Red and Sybr Green staining as described in Connelly, 2021 publication, which provide a clear separation of dormant parasites from dead parasites. The gate used to source the parasites for RNA-seq would therefore contain predominantly dead parasites, dormant ring parasites, but also some live ring stage parasites (not killed by DHA or already recovered from dormant).

As mentioned above (coment 2a), we carried out the suggested additional experiments in which the pyknotic cells were assessed by flow cytometry using MitoTracker Deep Red and Hoechst staining (**Figure 4A**). The results are indeed compatible with our previous microscopy based observation in which most pyknotic cells are MitoTracker positive (**Figure 4B**). We hope that this results provide further confidence of our observations showing the viability of the pyknotic/dormant cells.

The recovery of some dormant parasites can start as early as Day 3 after the last Mag column (Tuescher, 2010). Presumably, contamination with live parasites (rings) on Days 1-3 and perhaps also on Days 4 and 5 is very negligible but it would certainly increase during the course of experiment (after the last MAC separation on Day 3) and could have affected RNA-seq results. This needs to be discussed, specifically in a context of defining different stages of dormant parasites.

Although this is technically possible, many aspects of our results suggest that this is not the case or at least it bears a minimal, likely negligible effect. First, we observed only a minute presence of asexual forms in the parasite counts between day 3 and day 7 of the time course. Second, the transcriptome of the d5MDPs is highly unique through day 5 to 8 with essentially no signs of asexual transcriptional pattern. Third, the MitoTracker staining remains positive with reduced intensity through these stages of dormancy (**Figure 4**B).

Also it would be beneficial for the readers not well familiar with RNA-seq methodology if authors could clarify if their methodology of sequencing of a “few” parasites is similar to single cell RNA-seq.

The “few cells” RNA seq methodology used in this study is similar to single cell RNA seq in that the cells are directly sorted into a lysis buffer and reverse transcription is performed on the lysate (no RNA isolation step). However, the reverse transcription, cDNA amplification and sequencing library preparation is based on the SMART-seq2 method originally published by Picelli *et al* in 2014 and optimised for *Plasmodium* blood stage samples by Kucharski et al, 2020. We optimised certain parameters of the original SMARTseq2 method to suit *Plasmodium* blood stage samples (Kucharski et al, 2020). We have now provided complete details of the steps in Methods section line **601 to 616**.

4). The definition of the dormant ring is very important for the selection of the parasites selected for electron microscopy. As authors selected “a pyknotic parasite“ from a total population of all DHA-treated rings for electron microscopy and presented the data for live ring on Day 0, and treated rings on Day 1 as well as on Day 5, where the parasite appear to be shrinking and “loosing” organelles. It is not convincing if this parasite is dormant or simply dead. There are no electron micrographs showing the recovering “dormant” parasites after Day 5, which could have demonstrated that it was not a dead parasite presented and that the “ dormancy” was temporary.

This is a very valid point questing the fact whether the irregular cellular infrastructure of the dormant parasites represents an intermediate step towards growth re-emergence and at what point it corresponds to parasite death. Here we wish to argue that capturing EM images of parasites past the recovery stage is unlikely going to resolve this problem. EM can capture only a subfraction of parasite cells that might not be necessarily representative to the whole populations. For this issue to be resolved we would consider a high resolution fluorescent microscopy of life cell imaging in which an individual cell can be followed though time. Although in the future we intend to conduct such studies, these are not feasible for this

population as it will require extensive optimizations. At the we agree with the reviewer that whether the parasites with irregular ultrastructure could revive to asexual growth remains an open question. Nevertheless, we believe that the EM studies presented are still of great interest as these are highly representative of the transition from rings to day 1 and subsequently day 5 pyknosis. We believe that our current wording clearly states this limitation but if reviewer finds it insufficient we are more than willing to modify our statements.

5). The drug susceptibility data presented are of interest and important. As for all drugs a 24-hour exposure time was selected for all compounds, I would suggest to discuss the findings in a context of drugs' stage specificity, mode of action, pathways and drugs' half-life.

We are thankful to the reviewer for inviting us to further discussion.

We add a paragraph in the discussion to address this comment: line 521 to the end.

"*The resilience of the d5MDPs to a broad spectrum of antimalaria drugs might indeed be a key factor of this phenomenon, allowing residual fractions of dormant parasites surviving an ACT course. The d5MDP appear to be fully resistant all or most tested antimalaria drugs when applied at ^{IDC}IC50 and 10x^{IDC}IC50 for the asexual growth, respectively. Only 100x^{IDC}IC50 of AMQ, LMF, MF and DOX ….."*

6). The statement in the lines 427-428"In vitro P. falciparum parasite line resistant to artelinic acid exhibit higher sensitivity to dormancy but also higher recovery rate" is incorrect. The parasites in this W2AL80 line exhibited a decreased propensity to fall dormant as they were able to better tolerate the drug exposure and continued to grow despite the drug treatment.

We thank the reviewer for this comment and we have now corrected this statement to "faster recovery rate"
This is now corrected in text; line 492
"Currently, there are two opposing studies associating (or not) dormancy in artemisinin resistance directly. In vitro, a P. falciparum parasite line resistant to artelinic acid (W2AL80) exhibited a decreased propensity to fall dormant as they were able to better tolerate the drug exposure and continued to grow despite the drug treatment[37]."

7). Lastly, based on the results described in the MS I do not think that authors can state, and especially in the title of the MS, that dormancy corresponds or "bearing hallmarks" of senescence in higher eukaryotes. The most common definition of senescence implies an irreversible stop in cellular division, originally described for fibroblasts by Hayflick in 1961 and attributed to multicellular higher eukaryotes, where different tissues and cells have to cooperate and communicate in order to prevent indefinite replication leading to cancerogenesis. In my view, the more appropriate analogies for the DHA-induced parasite growth arrest are "quiescence" or "cell cycle arrest", which typically implies a temporary, reversible arrest of growth in response to the stress factors, such as ROS or DNA-damage. With no clear homology/orthology relationship between Plasmodium cell cycle machinery involved in dormancy and that of higher eukaryotes, responsible for senescence, linking dormancy to senescence would be highly speculative and not based on the transcriptome findings described in MS.

We agree with the reviewers that the fact that the *P. falciparum* dormant parasite have the ability to re-enter regular growth is more compatible with the model of quiescence. At the same time however, our study suggest that some properties and features (e.g. transcriptomic

e.t.c.) of the d5MDP do not exhibit standard quiescence features but could resemble some form of a senesces; particularly that of therapy indices (TIS) that can also occasionally re-emerge to growth. In the new version we discuss this further arguing that the *P. falciparum* dormancy might be represented by a unique set of molecular and cellular process related to both quiescence and senescence.

Please see discussion part; line 423 – 454

"*The blood stage dormancy represents a physiological state of the P. falciparum parasite cells that are temporarily arrested in their development but can ultimately recover to full growth. This is analogous to quiescence that was reported for most (if not all) eukaryotic systems (reviewed in[39,40]). Cellular quiescence ranges*

…

. The ability of the P. falciparum to enter cellular quiescence can be also questioned by the fact that the canonical cell cycle regulatory network based on cyclins, cyclin-dependent kinases (CDK) and cyclin inhibitors and typically facilities quiescence[39] are not well conserved in the Plasmodium genomes[45]. Moreover, the asexual IDC is not a standard cell cycle but rather a developmental program[43] driven by series of transcription factors such as ApiAP2 gene family[46]. "

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
All my concerns have been addressed in a satisfactory way and I have no further comments regarding the manuscript.

I commend the authors on their interesting work and I look forward to seeing it in print.

Reviewer #2:
Remarks to the Author:
I would like to thank the¬ authors for providing the answers and comments. Although the MS has been significantly improved, the major problem I still have with the MS is, in my view, incorrect interpretation of what is "dormant" and what is "pyknotic" (dead or dying) ring stage parasite and the lack of distinction between "pyknotic" and "dormant" forms in the MS. The major assumption in the MS that all pyknotic parasites (close to 100% of all DHA- treated rings) are in fact dormant parasites with 60.2% of them were able to recover.
The definition of "pyknosis" used for higher eukaryotes is a process of chromatin and nucleus shrinkage, which may occur both in apoptosis and necrosis. Typically, pyknosis is irreversible and associated with cell death, whereas the term "dormant" implies a cell cycle (temporary) arrest. These definitions are consistent with the terminology used in the previously published literature referenced well in the MS.
In previously published work cited by the authors (Teuscher, 2010, Peatey, 2021 and others) indeed the majority of DHA-treated rings (>90%) also became "pyknotic" with dark red stained nuclei and NO blue cytoplasm, identical to those observed by the authors also observe in their experiments: pyknotic forms shown in Fig 1 of the MS. However, these pyknotic forms were presumed dead or dying parasites in the previously published studies (Tuescher, 2010, Tucker, 2012, Peatey, 2015, Connelly, 2021, Peatey, 2021, etc.). These are dead or dying rings and therefore, they are not "analogous" as claimed in line 138 to dormant rings in the previously published work.
The definition of a dormant ring is well described in Tucker 2012, with Fig 2 showing microphotographs of the dormant vs dead pyknotic paraites "Comparison of dormant and dead parasites after a typical exposure to artemisinin drugs. (A) After ring-stage parasites were treated with artemisinin drugs, they became dormant, developed a rounded morphology, and retained blue cytoplasm and red chromatin on a Giemsa smear. (B) Dead parasites lacked distinct chromatin and cytoplasm, appearing globular, and they appeared red/purple on a Giemsa smear. Also in Peatey , 2021 "The dormant ring morphology shows condensed blue cytoplasm and condensed red chromatin as opposed to a normal ring with red chromatin dot and open cytoplasm. Dead parasites have a pyknotic morphology with no cytoplasm evident". This description and depiction of dead pyknotic parasites appears to match those in Fig 1C of the MS.
The authors claim that they have developed "a robust in vitro (laboratory) model, where they achieved "a near 100% conversion to pyknotic presumed dormant rings", which they explain by specific culture conditions and using 3D7 strain as opposed to W2 strain, which is, in my view, improbable. In addition to Tuescher et al., 2010, used D6 parasites, which are of similar background to 3D7 parasites. In fact, 3D7 strain was used in infection studies and observation of dormant parasites in ex vivo cultures in Peatey, 2021 with similar to the previous studies' outcomes. Another point difference is much higher initial parasitemia 4-8% in combination with lower hematocrit (2%) and Albumax, which may have additionally add to the stress on parasites caused by DHA. The graphs in Fig S1A show very similar to previous studies recovery, with duration somewhat proportional to the initial parasitemia in 4 experiments.
The authors present 2 lines of evidence of all pyknotic rings being dormant: 1) the microcopy images shown in Fig 1C and 2) staining with Mitotracker Red to demonstrate that parasites are live. Unfortunately, it is not possible to clearly see if there are any dormant rings (as described in Teuscher,

2010, Tucker, 2012, Peatey, 2021 and others) with differential staining of the blue cytoplasm and the red nucleus could be seen in those images of "pyknotic" parasites in Fig 1c of the MS.
Also it would help if the FACS images in Fig 4A included double stained with Hoescht and Mitotracker parasites and uninfected RBC rather than Mito vs FSC plots. Authors also state that "Third, the MitoTracker staining remains positive with reduced intensity through these stages of dormancy" in reference to Fig 4A. It is not clear from the images.
In contrast to the majority of observed pyknotic (dead) forms, dormant rings were only a small fraction ~2% of all DHA-treated rings, with only about half of that recovered, which was actually quantified by Tuescher, 2010 using limited dilution cloning and removing emerging live parasites by using magnetic columns. This is much more accurate quantification that the formula used by the authors to arrive at 60.2% of nearly 100% presumed dormant parasites. Authors acknowledge, that they observe live growing trophozoites as early as after day 3 in minute quantities. With exponential nature of parasite amplification (4-8-fold every 48 h or even faster in cultures), without removing live parasites by magnetic columns it is impossible to differentiate parasites, which have just recovered from the progenies of the recovered earlier parasites.
I believe, it would be very important for the MS to be convinced of what was the composition of parasite populations taken for RNA-seq. If the majority of these cells are "pyknotic" (dying) cells, it is plausible that the transcripts (at least in part, for days 1-5) may correspond to the transcriptome associated with pyknosis caused by DHA rather than dormancy.

Reviewer #3:
Remarks to the Author:
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

All my concerns have been addressed in a satisfactory way and I have no further comments regarding the manuscript.

We are delighted that this reviewer found the new version of the manuscript addressing all his/her concerns and as such suitable for publication. We thank the reviewer for all the effort in evaluating the results but also point out the importance of the discoveries made.

I commend the authors on their interesting work and I look forward to seeing it in print.

Reviewer #2 (Remarks to the Author):
I would like to thank the authors for providing the answers and comments. Although the MS has been significantly improved, the major problem I still have with the MS is, in my view, incorrect interpretation of what is "dormant" and what is "pyknotic" (dead or dying) ring stage parasite and the lack of distinction between "pyknotic" and "dormant" forms in the MS. The major assumption in the MS that all pyknotic parasites (close to 100% of all DHA- treated rings) are in fact dormant parasites with 60.2% of them were able to recover.

We thank the reviewer for acknowledging the progress we made from the previous version of the manuscript and are glad about the overall positive attitude toward this study. We acknowledge that our current version is still somewhat vague in the definitions of the cellular states. In the current version, we made the utmost effort to clarify it (please see below).

The main effort in the new version of the manuscript went to clarifying the persistent misunderstanding about the asexual recovery rate. We would like to reiterate that the 60.2% of asexual stages in the final cultures is not a direct representation of the recovery rate but instead an accumulation of asexual stage parasites in contrast to the fraction of the dormant parasites (39.8%). This ratio is a result of a cumulative rise of asexual stages by the recovery from dormancy and continual multiplication of the asexual stages.
In the new manuscript, we attempted to establish a closer estimate of a presumed intrinsic recovery rate by limited dilution analyses. Here we monitored recovery rate estimation in which 250,000 through 10,000 cells were plated on day 5, following the recovery to up to 31 days. Crucially we observed that the recovery rate was highly dependable on the initial cell density with the highest density (250,000 cells per well) showing the highest recovery rates. For this experiment, we estimated that on day 19 the asexual growth corresponds initial 4.16% +/- 3.86 initial parasitemia. These observations do not support the exact estimation of the intrinsic recovery rate given the lack of information about the multiplication rate of asexually multiplying parasites (especially in low cell densities). Nevertheless, the low recovery cell numbers observed in these dilution analyses do resemble the previous studies by Teucher, F. et al 2010 that estimated a range of 0.044% to 1.313%.

The limited dilution recovery experiment is described in the text. (line160-187):

"*As mentioned above, in these initial bulk culturing experiments we observed a considerable representation of asexual IDC stages on day 10 which corresponds to ~60% of the total parasitemia compared to ~40% that remains in the dormancy-like condensed cell morphology (**Figure 1A**). This morphological stage distribution is likely a result of multiple factors including accumulation of asexual stages from growth recovery particularly aided by erythrocyte supplementation on day 5 (**Figure S1a**). Hence, … However, the dynamic of the dilution*

recovery rate do resemble previously reported estimates between 0.044% to 1.313% established by Teucher, F. et al 2010[19]. Nonetheless, here we show that initial cell density can affect the apparent recovery rates in in vitro conditions indicating that this parameter also contributed to the high asexual parasitemia (~60.2% on day 10) in our initial bulk cultures."

The definition of "pyknosis" used for higher eukaryotes is a process of chromatin and nucleus shrinkage, which may occur both in apoptosis and necrosis. Typically, pyknosis is irreversible and associated with cell death, whereas the term "dormant" implies a cell cycle (temporary) arrest. These definitions are consistent with the terminology used in the previously published literature referenced well in the MS.

With apologies, this confusion that was caused by us referring to the induced cells as "pyknotic". Here we were referring to their morphological appearance rather than their cellular state. We did not mean to imply that the induced dormant parasites in this study are in the state of cellular pyknosis that indeed, reflects a process of chromatin and nucleus shrinkage, during apoptosis or necrosis. Instead, we showed that all dormant parasites in this study are NOT in the state of pyknosis as these carry cytoplasm with active mitochondria. Hence in the new version of the manuscript, we avoid the term Cellular Pyknosis and instead refer to the dormant parasite-induced state as "condensed cells".

In previously published work cited by the authors (Teuscher, 2010, Peatey, 2021 and others) indeed the majority of DHA-treated rings (>90%) also became "pyknotic" with dark red stained nuclei and NO blue cytoplasm, identical to those observed by the authors also observe in their experiments: pyknotic forms shown in Fig 1 of the MS. However, these pyknotic forms were presumed dead or dying parasites in the previously published studies (Tuescher, 2010, Tucker, 2012, Peatey, 2015, Connelly, 2021, Peatey, 2021, etc.). These are dead or dying rings and therefore, they are not "analogous" as claimed in line 138 to dormant rings in the previously published work.

Referring to our dormant parasites being "analogous" to previously reported results was referring purely about the living dormant parasites, not the presumably dying pyknotic cells. Indeed, in the previous reports, the authors observed the majority of the DHA-induced parasite in the state of pyknosis, hence a cell death-like state, with only a minority of the cells in a truly dormant viable parasite. We believe that the dormant parasites induced in this study are analogous to the latter: viable dormant parasites.

We correct this in the discussion (line 432-435)

"Nevertheless, the dormant parasites induced in this study (d5MDPs) are most likely identical to the subfraction of the dormant parasites that were shown to give rise to the re-emergence of asexual growth reported in previous studies."

The definition of a dormant ring is well described in Tucker 2012, with Fig 2 showing microphotographs of the dormant vs dead pyknotic paraites "Comparison of dormant and dead parasites after a typical exposure to artemisinin drugs. (A) After ring-stage parasites were treated with artemisinin drugs, they became dormant, developed a rounded morphology, and retained blue cytoplasm and red chromatin on a Giemsa smear. (B) Dead parasites lacked distinct chromatin and cytoplasm, appearing globular, and they appeared red/purple on a Giemsa smear. Also in Peatey , 2021 "The dormant ring morphology shows condensed blue cytoplasm and condensed red chromatin as opposed to a normal ring with red chromatin dot and open cytoplasm. Dead parasites have a pyknotic morphology with no cytoplasm evident".

This description and depiction of dead pyknotic parasites appears to match those in Fig 1C of the MS.

Taking this advice, in the new version of the manuscript, we investigated the presence of cytoplasm in the dormant parasites induced in this study (d5MDP). In **Figure S2a & b,** we show that essentially all condensed cells, representing the dormant parasite forms, d5MDP, contain a cytoplasm; demonstrated by the blue Giemsa stain as defined by Peatey , 2021. To support the Giemsa smear microscopy, however, we also now show that all 5dMDP carry functional mitochondria characterized by active membrane potential indicator MitoTracker imaged by high-resolution microscopy (**Figure 4**). This result supports the main point of this manuscript demonstrating that the generated transcriptional profile comes from viable cells representing the dormant developmental stage.

We clarify this in the text (lines 331 through 353)

*"Our initial Giemsa stain-based evaluations suggested that essentially all condensed forms of the parasites across the experimental time course (Day1-Day5) exhibit the presence of compressed chromatin (red/purple stain) along with condensed but visible cytoplasm (blue stain) (**Figure S2a** & **b**)…. compartments present in each d5MDP juxtaposed to the nucleus, similarly to the ring stage parasites (**Figure 4C**). This is consistent with previous suggestions that the P. falciparum dormant stages contain enlarged mitochondria located near the nucleus[25]."*

The authors claim that they have developed "a robust in vitro (laboratory) model, where they achieved "a near 100% conversion to pyknotic presumed dormant rings", which they explain by specific culture conditions and using 3D7 strain as opposed to W2 strain, which is, in my view, improbable. In addition to Tuescher et al., 2010, used D6 parasites, which are of similar background to 3D7 parasites. In fact, 3D7 strain was used in infection studies and observation of dormant parasites in ex vivo cultures in Peatey, 2021 with similar to the previous studies' outcomes.

First, the high level of dormancy conversion is indeed the main difference between ours and previously reported studies. At the moment it is unclear what was the main reason for this but, here we wanted to find all possible differences some of which could indeed explain this difference. We agree that the choice of the 3D7 might have not been the main factor, however, such a possibility cannot be completely ruled out either. The D6 strain is indeed related to 3D7 but not completely identical.

Another point difference is much higher initial parasitemia 4-8% in combination with lower hematocrit (2%) and Albumax, which may have additionally add to the stress on parasites caused by DHA. The graphs in Fig S1A show very similar to previous studies recovery, with duration somewhat proportional to the initial parasitemia in 4 experiments.

We thank the reviewer for this comment and an important suggestion. The high parasitemia could indeed contribute to the increased conversion rates that in turn result in the apparent higher recovery rates in the bulk cultures. This comment is also in line with our recovery experiment stressing the importance of initial cell density for the overall outcome of the experiment.

Although we did comment on the high parasitemia in our previous version, here we elaborate somewhat more in Discussion (line 447-451):

*"… and possibly other experimental parameters. One of such parameter could be the higher starting parasitemia (**Figure S1a**), compared to the previous report that can enhance the DHA-*

mediate strass due to high cell density. It will be interesting to explore various experimental parameters to further improve the P. falciparum dormancy model for future studies."

The authors present 2 lines of evidence of all pyknotic rings being dormant: 1) the microcopy images shown in Fig 1C and 2) staining with Mitotracker Red to demonstrate that parasites are live. Unfortunately, it is not possible to clearly see if there are any dormant rings (as described in Teuscher, 2010, Tucker, 2012, Peatey, 2021 and others) with differential staining of the blue cytoplasm and the red nucleus could be seen in those images of "pyknotic" parasites in Fig 1c of the MS.

Referring to the comment above, in the new version of the manuscript we provide extensive Giemsa smear staining images (**Figure S2a & b**). We hope that the presence of the blue-stained cytoplasm is more clear in these. Nevertheless, to support the evidence of persisting cytoplasm in the dormant parasite, we also conducted high-resolution microscopy showing the presence of active mitochondria in essentially all dormant parasite cells (**Figure 4**). This strongly suggests that the dormant parasite population generated in this study on day 5 (d5MDP) represents viable cells. As such, these results support the main discovery made in this manuscript; the transcriptome of the dormant parasites in a viable state.

Also it would help if the FACS images in Fig 4A included double stained with Hoescht and Mitotracker parasites and uninfected RBC rather than Mito vs FSC plots. Authors also state that "Third, the MitoTracker staining remains positive with reduced intensity through these stages of dormancy" in reference to Fig 4A. It is not clear from the images.

In the new version of the manuscript, we provide the double-stained FACS analysis showing that all dormancy-induced parasites are MitoTracker positive. This is supported by microscopy imaging (**Figure S9**) that was analyzed quantitatively (**Figure 4b**). To reiterate, the reduced MitoTRacker staining originates from these quantitative analyses. To support these analyses we carried out identical MitoTracker staining life cell imaging, this time visualized by high-resolution microscopy (**Figure 4c**). We trust that this provides sufficient evidence for the presence of active mitochondria in all dormant parasites induced in this study.

In contrast to the majority of observed pyknotic (dead) forms, dormant rings were only a small fraction ~2% of all DHA-treated rings, with only about half of that recovered, which was actually quantified by Tuescher, 2010 using limited dilution cloning and removing emerging live parasites by using magnetic columns. This is much more accurate quantification that the formula used by the authors to arrive at 60.2% of nearly 100% presumed dormant parasites. Authors acknowledge, that they observe live growing trophozoites as early as after day 3 in minute quantities. With exponential nature of parasite amplification (4-8-fold every 48 h or even faster in cultures), without removing live parasites by magnetic columns it is impossible to differentiate parasites, which have just recovered from the progenies of the recovered earlier parasites.

I believe, it would be very important for the MS to be convinced of what was the composition of parasite populations taken for RNA-seq. If the majority of these cells are "pyknotic" (dying) cells, it is plausible that the transcripts (at least in part, for days 1-5) may correspond to the transcriptome associated with pyknosis caused by DHA rather than dormancy.

In agreement with the reviewer, we also believe that it is important to demonstrate the nature of the parasite cell population that was taken for the RNA-Seq analysis. Based on all results collectively we can say that an overwhelming proportion (possibly all) of dormant cells

analyzed by RNA-Seq are NOT in the state of pyknosis but instead are viable. This is based on the presence of the blue-stained cytoplasm by Giemsa staining (**Figure S2a & b)** but also active mitochondria in essentially all induced dormant parasites shown by FACS analysis (**Figure 4a**), live cell imaging **(Figure 4b & Figure S9**) and finally high-resolution microscopy (**Figure 4 c)**. Although, in this manuscript, we did not establish the exact recovery rate of these parasites, we provide a close approximation by our limited dilution experiment that in their number resembles the previous studies. We however also demonstrate that the apparent recovery rate is strongly dependent on growth conditions, particularly on cell density.
With all due respect, we wish to argue that establishing such recovery rates in *in vitro* cultures can be highly precarious and as such do not necessarily reflect the overall viability of the dormant parasite population. Here we opted for a more direct approach, showing the presence of active mitochondria as the key measure of cell viability. Nevertheless, we agree that developing more assays for estimation of the potential of dormant parasite recovery will be important in the future. This will be particularly important for studies of the potential if recrudesce of malaria infection in vivo. We believe that our manuscript provides a good foundation for such work.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
I would like to thank the authors for all improvements they have made to the MS and for conducting extra experiments to provide an additional evidence to the percentage of viable parasites, able to recover that exist in the population of dormant parasites on Day 5 (d5MDP).
Authors used limiting dilution cloning technique, and plated d5MDP parasites (infected RBC only) at densities ranging from 10,000 to 250,000 parasites per well (Fig 1c). The objective of limiting dilution method is to use the dilution of the parasites (cells) that would produce ~65% of positive wells on the plate. This calculation is based on Poisson distribution statistical analysis (Coller and Coller, Methods of Enzymology, 1986). The dilution that produces ~65% positive wells contains 1 viable parasite per well. From Fig 1c of the MS it is clear that 100,000 parasite per well dilution results in ~60% of positive wells by day 31. As we get to less than 1 parasite per well dilutions, wells can only be either positive or negative and the number of positive wells (%) on the plate should not change over the time assuming that all parasites start growing at the same time. Clearly in this case we see several plateaus for each density graphs, presumably corresponding to more parasites recovering from dormancy after day 5, before plateauing by day 31. Based on the best case scenario, in this experiment there is only 1 viable parasite out of 100,000 iRBC per well. This means that the % of viable parasites would be ~ 0.001% of total iRBC in d5MDP. This estimate is even lower than that reported by Tuescher et al., 2010. The authors, however, calculated the rate differently, recalculating the original % of viable parasites based on parasites reaching an arbitrary 0.2% parasitemia over 7 cycles. Indeed, this calculation is based on a lot of assumptions (i.e. multiplication rate etc. duration of the cycle, etc). Nevertheless, these results also demonstrate, that consistent with all previous studies, there is only a small fraction of initial population that is able to recover and give rise to recrudescence.
How would authors account for microscopy results of the Fig 4b in the previous version of the MS (now removed) where all Day 5 parasites appear to be positive by Mitotracker Deep Red staining, which would imply that all cells in d5MDP were live, whereas only 4.16% or perhaps even less, 0.001% of them recover. The intensity of the staining is significantly lower for parasites on Day 1 compared to live rings (Fig 4b current version). Is it possible that there may be residual low intensity staining of dead cells by Mitotracker Deep Red?
In addition, the statement (lines 200-201) that in previous publications authors reported that parasitemia was declining following DHA treatment is incorrect. In Chen et al the Fig 1 demonstrates that parasitemia remains stable at the same level of 2% for at least 6 days following DHA exposure.
The major statement and the finding in this MS is a new robust model that allows to produce close to 100% dormant rings by using essentially the same experimental conditions with exception of different P. falciparum laboratory strain (3D7),. This statement contradicts results from all previous studies (well referenced in the MS). The subsequent transcriptome analysis and the electron microscopy images of "dormant" rings are all based on this assumption. If this is correct, this would, indeed, constitute a "paradigm shift" in as it is in stark contrast to the previous findings produced by different research groups. This whole model with all conclusions that follow is based only on results of one type of assay showing that ~100% of DHA-treated parasites are identified as "live" by positive Mitotracker DeepRed staining, which also contradicts results of similar experiments obtained by Connelly, et al., 2021. Another line of evidence, microscopy analysis of the Giemsa stained slides does not differentiate between live and dead and is subjective in interpretations of "dormant" or "dead" ring stage parasites.
Given a very high potential impact of this paper for the malaria field and future interpretations of the transcriptome analysis it would be critical to provide additional lines of evidence to substantiate Mitotracker DeepRed staining results. For an example, P. falciparum lactate dehydrogenase (LDH) enzyme activity assay (Sigma-Aldrich) and ATP measurement assay (CellTiter-Glo Luminescent Cell Viability Assay kit (Promega), as described in ref 22, would strengthen authors statements.
With all due respect, in my view, the additional evidence would substantially enhance the quality of this publication and validity of overall conclusions. Regretfully, I do not believe that the authors have sufficiently addressed my concerns, which I have raised in my previous reviews and I do not believe that the MS should be published in the current version.

Reviewer #4:
Remarks to the Author:
NCOMMS-23-06681B
The artemisinin-induced 1 dormant stages of Plasmodium falciparum exhibit hallmarks of cellular quiescence/senescence and drug resilience.
Corresponding author: Jaishree Tripathi & Zbynek Bozdech
The manuscript I was able to review, submitted by Jaishree Tripathi et al, reports some important findings on P. falciparum parasite recrudescence, which may be due to artemisinin-induced dormant parasites. This is an important issue and a poorly understood biological form of the parasite that may have huge implications for malaria control and elimination strategies.

As suggested by the editor, I have carefully read the manuscript, reviewer 2's comments and the authors' responses.
Here are my comments:

Reviewer #2 (Remarks to the Author):
I would like to thank the authors for providing the answers and comments. Although the MS has been significantly improved, the major problem I still have with the MS is, in my view, incorrect interpretation of what is "dormant" and what is "pyknotic" (dead or dying) ring stage parasite and the lack of distinction between "pyknotic" and "dormant" forms in the MS. The major assumption in the MS that all pyknotic parasites (close to 100% of all DHA- treated rings) are in fact dormant parasites with 60.2% of them were able to recover.

We thank the reviewer for acknowledging the progress we made from the previous version of the manuscript and are glad about the overall positive attitude toward this study. We acknowledge that our current version is still somewhat vague in the definitions of the cellular states. In the current version, we made the utmost effort to clarify it (please see below).

The main effort in the new version of the manuscript went to clarifying the persistent misunderstanding about the asexual recovery rate. We would like to reiterate that the 60.2% of asexual stages in the final cultures is not a direct representation of the recovery rate but instead an accumulation of asexual stage parasites in contrast to the fraction of the dormant parasites (39.8%). This ratio is a result of a cumulative rise of asexual stages by the recovery from dormancy and continual multiplication of the asexual stages.

In the new manuscript, we attempted to establish a closer estimate of a presumed intrinsic recovery rate by limited dilution analyses. Here we monitored recovery rate estimation in which 250,000 through 10,000 cells were plated on day 5, following the recovery to up to 31 days. Crucially we observed that the recovery rate was highly dependable on the initial cell density with the highest density (250,000 cells per well) showing the highest recovery rates. For this experiment, we estimated that on day 19 the asexual growth corresponds initial 4.16% +/- 3.86 initial parasitemia. These observations do not support the exact estimation of the intrinsic recovery rate given the lack of information about the multiplication rate of asexually multiplying parasites (especially in low cell densities). Nevertheless, the low recovery cell numbers observed in these dilution analyses do resemble the previous studies by Teucher, F. et al 2010 that estimated a range of 0.044% to 1.313%.

The limited dilution recovery experiment is described in the text. (line160-187):

"As mentioned above, in these initial bulk culturing experiments we observed a considerable representation of asexual IDC stages on day 10 which corresponds to ~60% of the total parasitemia

compared to ~40% that remains in the dormancy-like condensed cell morphology (Figure 1A). This morphological stage distribution is likely a result of multiple factors including accumulation of asexual stages from growth recovery particularly aided by erythrocyte supplementation on day 5 (Figure S1a). Hence, … However, the dynamic of the dilution recovery rate do resemble previously reported estimates between 0.044% to 1.313% established by Teucher, F. et al 201019. Nonetheless, here we show that initial cell density can affect the apparent recovery rates in in vitro conditions indicating that this parameter also contributed to the high asexual parasitemia (~60.2% on day 10) in our initial bulk cultures."

I am quite sceptical because the main problem here is that there is no evidence that the live parasites (R, T and S) observed at day 12 are from 'condensed forms' as claimed by the authors. As shown in Figure 1A, it seems possible that they are derived from parasites that were able to survive until the DHA pulse (~2% at day 5, ~8% at day 7, ~40% at day 9 and ~70% at day 11). This is consistent with a multiplication rate of x4-5.
In my opinion, the data presented do not provide evidence that the condensed cell parasites are dormant parasites. Dormant parasites could be typical ring stage forms.

The definition of "pyknosis" used for higher eukaryotes is a process of chromatin and nucleus shrinkage, which may occur both in apoptosis and necrosis. Typically, pyknosis is irreversible and associated with cell death, whereas the term "dormant" implies a cell cycle (temporary) arrest. These definitions are consistent with the terminology used in the previously published literature referenced well in the MS.

With apologies, this confusion that was caused by us referring to the induced cells as "pyknotic". Here we were referring to their morphological appearance rather than their cellular state. We did not mean to imply that the induced dormant parasites in this study are in the state of cellular pyknosis that indeed, reflects a process of chromatin and nucleus shrinkage, during apoptosis or necrosis. Instead, we showed that all dormant parasites in this study are NOT in the state of pyknosis as these carry cytoplasm with active mitochondria. Hence in the new version of the manuscript, we avoid the term Cellular Pyknosis and instead refer to the dormant parasite-induced state as "condensed cells".

I agree that the author's response clarifies the definition and the terms used in the MS.

In previously published work cited by the authors (Teuscher, 2010, Peatey, 2021 and others) indeed the majority of DHA-treated rings (>90%) also became "pyknotic" with dark red stained nuclei and NO blue cytoplasm, identical to those observed by the authors also observe in their experiments: pyknotic forms shown in Fig 1 of the MS. However, these pyknotic forms were presumed dead or dying parasites in the previously published studies (Tuescher, 2010, Tucker, 2012, Peatey, 2015, Connelly, 2021, Peatey, 2021, etc.). These are dead or dying rings and therefore, they are not "analogous" as claimed in line 138 to dormant rings in the previously published work.

Referring to our dormant parasites being "analogous" to previously reported results was referring purely about the living dormant parasites, not the presumably dying pyknotic cells. Indeed, in the previous reports, the authors observed the majority of the DHA-induced parasite in the state of pyknosis, hence a cell death-like state, with only a minority of the cells in a truly dormant viable parasite. We believe that the dormant parasites induced in this study are analogous to the latter: viable dormant parasites.

We correct this in the discussion (line 432-435)

"Nevertheless, the dormant parasites induced in this study (d5MDPs) are most likely identical to the subfraction of the dormant parasites that were shown to give rise to the re-emergence of asexual growth reported in previous studies."

This answer is quite speculative. There is no clear evidence in the MS to show that 'the dormant parasites induced in this study are most likely identical to the subset of dormant parasites that have been shown to cause the reappearance of asexual growth reported in previous studies'. I appreciate the use of 'most likely'.

The definition of a dormant ring is well described in Tucker 2012, with Fig 2 showing microphotographs of the dormant vs dead pyknotic paraites "Comparison of dormant and dead parasites after a typical exposure to artemisinin drugs. (A) After ring-stage parasites were treated with artemisinin drugs, they became dormant, developed a rounded morphology, and retained blue cytoplasm and red chromatin on a Giemsa smear. (B) Dead parasites lacked distinct chromatin and cytoplasm, appearing globular, and they appeared red/purple on a Giemsa smear. Also in Peatey, 2021 "The dormant ring morphology shows condensed blue cytoplasm and condensed red chromatin as opposed to a normal ring with red chromatin dot and open cytoplasm. Dead parasites have a pyknotic morphology with no cytoplasm evident". This description and depiction of dead pyknotic parasites appears to match those in Fig 1C of the MS.

Taking this advice, in the new version of the manuscript, we investigated the presence of cytoplasm in the dormant parasites induced in this study (d5MDP). In Figure S2a & b, we show that essentially all condensed cells, representing the dormant parasite forms, d5MDP, contain a cytoplasm; demonstrated by the blue Giemsa stain as defined by Peatey , 2021. To support the Giemsa smear microscopy, however, we also now show that all 5dMDP carry functional mitochondria characterized by active membrane potential indicator MitoTracker imaged by high-resolution microscopy (Figure 4). This result supports the main point of this manuscript demonstrating that the generated transcriptional profile comes from viable cells representing the dormant developmental stage.

We clarify this in the text (lines 331 through 353)

"Our initial Giemsa stain-based evaluations suggested that essentially all condensed forms of the parasites across the experimental time course (Day1-Day5) exhibit the presence of compressed chromatin (red/purple stain) along with condensed but visible cytoplasm (blue stain) (Figure S2a & b)…. compartments present in each d5MDP juxtaposed to the nucleus, similarly to the ring stage parasites (Figure 4C). This is consistent with previous suggestions that the P. falciparum dormant stages contain enlarged mitochondria located near the nucleus25."

I am not convinced by the microphotographs of dormant parasites, where the presence of cytoplasm in dormant parasites is not clearly visible. However, I am more convinced by the data showing that 5dMDP parasites carry functional mitochondria, characterised by the active membrane potential indicator, MitoTracker, imaged by high resolution microscopy.

The authors claim that they have developed "a robust in vitro (laboratory) model, where they achieved "a near 100% conversion to pyknotic presumed dormant rings", which they explain by specific culture conditions and using 3D7 strain as opposed to W2 strain, which is, in my view, improbable. In addition to Tuescher et al., 2010, used D6 parasites, which are of similar background to 3D7 parasites. In fact, 3D7 strain was used in infection studies and observation of dormant parasites in ex vivo cultures in Peatey, 2021 with similar to the previous studies' outcomes.

First, the high level of dormancy conversion is indeed the main difference between ours and previously reported studies. At the moment it is unclear what was the main reason for this but, here we wanted to find all possible differences some of which could indeed explain this difference. We agree that the choice of the 3D7 might have not been the main factor, however, such a possibility cannot be completely ruled out either. The D6 strain is indeed related to 3D7 but not completely identical.

The authors' response is somewhat evasive and does not explain the data presented and the claimed high level of dormancy conversion in MS.

Another point difference is much higher initial parasitemia 4-8% in combination with lower hematocrit (2%) and Albumax, which may have additionally add to the stress on parasites caused by DHA. The graphs in Fig S1A show very similar to previous studies recovery, with duration somewhat proportional to the initial parasitemia in 4 experiments.

We thank the reviewer for this comment and an important suggestion. The high parasitemia could indeed contribute to the increased conversion rates that in turn result in the apparent higher recovery rates in the bulk cultures. This comment is also in line with our recovery experiment stressing the importance of initial cell density for the overall outcome of the experiment.

Although we did comment on the high parasitemia in our previous version, here we elaborate somewhat more in Discussion (line 447-451):

"… and possibly other experimental parameters. One of such parameter could be the higher starting parasitemia (Figure S1a), compared to the previous report that can enhance the DHA-mediate stress due to high cell density. It will be interesting to explore various experimental parameters to further improve the P. falciparum dormancy model for future studies."

This is an excellent point raised by the reviewer and could explain the high dormancy conversion rate. I have to say that the authors 'jump' on this point to provide a possible explanation for their data without convincing evidence.

The authors present 2 lines of evidence of all pyknotic rings being dormant: 1) the microcopy images shown in Fig 1C and 2) staining with Mitotracker Red to demonstrate that parasites are live. Unfortunately, it is not possible to clearly see if there are any dormant rings (as described in Teuscher, 2010, Tucker, 2012, Peatey, 2021 and others) with differential staining of the blue cytoplasm and the red nucleus could be seen in those images of "pyknotic" parasites in Fig 1c of the MS.

Referring to the comment above, in the new version of the manuscript we provide extensive Giemsa smear staining images (Figure S2a & b). We hope that the presence of the blue-stained cytoplasm is more clear in these. Nevertheless, to support the evidence of persisting cytoplasm in the dormant parasite, we also conducted high-resolution microscopy showing the presence of active mitochondria in essentially all dormant parasite cells (Figure 4). This strongly suggests that the dormant parasite population generated in this study on day 5 (d5MDP) represents viable cells. As such, these results support the main discovery made in this manuscript; the transcriptome of the dormant parasites in a viable state.

Same comment than above.

Also it would help if the FACS images in Fig 4A included double stained with Hoescht and Mitotracker parasites and uninfected RBC rather than Mito vs FSC plots. Authors also state that "Third, the MitoTracker staining remains positive with reduced intensity through these stages of dormancy" in reference to Fig 4A. It is not clear from the images.

In the new version of the manuscript, we provide the double-stained FACS analysis showing that all dormancy-induced parasites are MitoTracker positive. This is supported by microscopy imaging (Figure S9) that was analyzed quantitatively (Figure 4b). To reiterate, the reduced MitoTRacker staining originates from these quantitative analyses. To support these analyses we carried out identical MitoTracker staining life cell imaging, this time visualized by high-resolution microscopy (Figure 4c). We trust that this provides sufficient evidence for the presence of active mitochondria in all dormant parasites induced in this study.

I agree that these data provide sufficient evidence for the presence of active mitochondria in artemisinin-induced dormant parasites, but I am sceptical that all parasites exposed to artemisinin are MitoTracker positive and therefore dormant parasites.

In contrast to the majority of observed pyknotic (dead) forms, dormant rings were only a small fraction ~2% of all DHA-treated rings, with only about half of that recovered, which was actually quantified by Tuescher, 2010 using limited dilution cloning and removing emerging live parasites by using magnetic columns. This is much more accurate quantification that the formula used by the authors to arrive at 60.2% of nearly 100% presumed dormant parasites. Authors acknowledge, that they observe live growing trophozoites as early as after day 3 in minute quantities. With exponential nature of parasite amplification (4-8-fold every 48 h or even faster in cultures), without removing live parasites by magnetic columns it is impossible to differentiate parasites, which have just recovered from the progenies of the recovered earlier parasites.

I believe, it would be very important for the MS to be convinced of what was the composition of parasite populations taken for RNA-seq. If the majority of these cells are “pyknotic” (dying) cells, it is plausible that the transcripts (at least in part, for days 1-5) may correspond to the transcriptome associated with pyknosis caused by DHA rather than dormancy.

In agreement with the reviewer, we also believe that it is important to demonstrate the nature of the parasite cell population that was taken for the RNA-Seq analysis. Based on all results collectively we can say that an overwhelming proportion (possibly all) of dormant cells analyzed by RNA-Seq are NOT in the state of pyknosis but instead are viable. This is based on the presence of the blue-stained cytoplasm by Giemsa staining (Figure S2a & b) but also active mitochondria in essentially all induced dormant parasites shown by FACS analysis (Figure 4a), live cell imaging (Figure 4b & Figure S9) and finally high-resolution microscopy (Figure 4 c). Although, in this manuscript, we did not establish the exact recovery rate of these parasites, we provide a close approximation by our limited dilution experiment that in their number resembles the previous studies. We however also demonstrate that the apparent recovery rate is strongly dependent on growth conditions, particularly on cell density.

With all due respect, we wish to argue that establishing such recovery rates in in vitro cultures can be highly precarious and as such do not necessarily reflect the overall viability of the dormant parasite population. Here we opted for a more direct approach, showing the presence of active mitochondria as the key measure of cell viability. Nevertheless, we agree that developing more assays for estimation of the potential of dormant parasite recovery will be important in the future. This will be particularly important for studies of the potential if recrudesce of malaria infection in vivo. We believe that our manuscript provides a good foundation for such work.

I agree with the authors' response. Even if their data are not completely convincing, the manuscript presents some very interesting data that may open new perspectives on a new mechanism for survival (and not resistance) of Plasmodium falciparum to antimalarial drugs.
I think the authors should insist on the limitations of their work in the discussion to put into perspective the work they have done, but also the potential technical biases raised by reviewer 2.

I have several comments, which I list below:

Abstract.

Introduction.
Lines 47-49. ACT should be replaced by artemisinin. I suggest rewording the sentence as follows:
"Unfortunately, the reduced susceptibility of P. falciparum to artemisinins, which emerged in the

eastern part of the Greater Mekong Subregion as early as 20092,3 and is now emerging in other parts of the world, including sub-Saharan Africa, is a major concern for the future.

Line 51. ACTs should be replaced by artemisinins.

Line 76. I suggest adding 'ring stages' to the sentence, which could read: 'Indeed, exposure of P. falciparum ring stages to artemisinins in vitro induces dormancy-like forms that may represent this residual parasite (sub)population.

Results

Fig1. Please reconcile figure legends and fig1 (i.e. B -> C and C-> B, there is no panel E in fig1).

Lines 366_367. This is very surprising that d5MDPs appear resistant to high IC50 concentrations of atovaquone and gibberellic acid (GA) did not affect the recrudescence and delay of d5MDPs.

Discussion
Line 449. Stress not strass

Additional question. Why did the authors use the smart-seq2 protocol and perform bulk transcriptome analysis rather than single cell transcriptome analysis? This would have allowed to deconvolute the dormant and ring stage populations.

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for all improvements they have made to the MS and for conducting extra experiments to provide an additional evidence to the percentage of viable parasites, able to recover that exist in the population of dormant parasites on Day 5 (d5MDP).

Authors used limiting dilution cloning technique, and plated d5MDP parasites (infected RBC only) at densities ranging from 10,000 to 250,000 parasites per well (Fig 1c). The objective of limiting dilution method is to use the dilution of the parasites (cells) that would produce ~65% of positive wells on the plate. This calculation is based on Poisson distribution statistical analysis (Coller and Coller, Methods of Enzymology, 1986). The dilution that produces ~65% positive wells contains 1 viable parasite per well. From Fig 1c of the MS it is clear that 100,000 parasite per well dilution results in ~60% of positive wells by day 31. As we get to less than 1 parasite per well dilutions, wells can only be either positive or negative and the number of positive wells (%) on the plate should not change over the time assuming that all parasites start growing at the same time. Clearly in this case we see several plateaus for each density graphs, presumably corresponding to more parasites recovering from dormancy after day 5, before plateauing by day 31. Based on the best case scenario, in this experiment there is only 1 viable parasite out of 100,000 iRBC per well. This means that the % of viable parasites would be ~ 0.001% of total iRBC in d5MDP. This estimate is even lower than that reported by Tuescher et al., 2010. The authors, however, calculated the rate differently, recalculating the original % of viable parasites based on parasites reaching an arbitrary 0.2% parasitemia over 7 cycles. Indeed, this calculation is based on a lot of assumptions (i.e. multiplication rate etc. duration of the cycle, etc). Nevertheless, these results also demonstrate, that consistent with all previous studies, there is only a small fraction of initial population that is able to recover and give rise to recrudescence.

Indeed, here we reiterate that in terms of recovery rate, in agreement with reviewer 2, the generated dormant parasites in our study are very similar, if not identical, to those generated in previous studies. There is indeed only a small proportion of the condensed parasites that give rise to new growth recovery, which we estimate at a maximum of ~4%. One might argue about the correct way of calculating the rate of recovery. Indeed, our calculation is based on several assumptions reflecting the average parameters of basic parasite cultures. Respectfully, we can also argue that this is the case for all other previous studies. Using the Coller and Coller method, which is based on the multiplication of mammalian cells, could also give a skewed view of the dormancy recovery rate. *Plasmodium* parasites multiply in a fundamentally different way compared to mammalian cells.

Nevertheless, here we argue that the rate of recovery is NOT necessarily the direct reflection of the dormant parasite viability. From our study and previous work, it appears that what portion of the condensed parasite gives rise to asexual growth largely depends on the parasite's genetic background and growth/induction conditions. Nevertheless, here we showed that the condensed parasites at day 5 post-induction (hence d5MDP) are at some form of a viable state. This is demonstrated by the intact mitochondria and several other viability indicators (more provided in this new version). Last but not the least, the fact that the d5MDPs carry a highly specific transcriptomic profile that is soundly reproducible, reflecting a well-defined set of biological processes up- or down-regulated, should be considered as an additional viability feature.

How would authors account for microscopy results of the Fig 4b in the previous version of the MS (now removed) where all Day 5 parasites appear to be positive by Mitotracker Deep Red staining, which would imply that all cells in d5MDP were live, whereas only 4.16% or perhaps even less, 0.001% of them recover. The intensity of the staining is significantly lower for parasites on Day 1 compared to live rings (Fig 4b current version). Is it possible that there may be residual low intensity staining of dead cells by Mitotracker Deep Red?

To address this, in the new version of the manuscript, we first provide a more detailed description of the distribution of the MitoTracker signal in both live cell imaging experiments and FACS. For the live cell imaging, we included >100 cells for each timepoint (i.e., rings (day 0), day 1, and day 5) that carried positive DAPI (nuclear) signal indiscriminately. For these,

we carried out automated MitoTracker quantifications using ImageJ showing that 100% of DHA treated cells (i.e. Day 1 and Day 5) contained well-detectable MitoTracker signal albeit at a lower intensity range than rings (Day 0). This is now depicted as violin plots in Fig 4b, showing the distribution of cells at each intensity. To further confirm the MitoTracker signal was coming from a specific cellular compartment, we peformed 3D volume rendering by Imaris software of detectable MitoTracker signals and show that this came from a mitochondrial-like compartment (Fig 4c) as shown previously in Connelly et al, 2021. To further address the lower Mitotracker staining observed in Day 1 and Day 5 parasites, it is possible that dormant/growth arrested parasites have a lower mitochondrial membrane potential than actively replicating parasites. In fact, previous research in stem cells has shown that quiescent hematopoietic stem cells (HSC) have a lower mitochondrial membrane potential compared to primed (activated) HSCs (Umemoto et al 2010; Ghaffari,2024, etc.). Since, MitoTracker cannot label mitochondria that have lost their membrane potential due to damage or dysfunction, it is highly unlikely we see any residual or non-specific staining. Also, we do not see any staining in RBC cytosol (which can be taken as a negative control in this case), thus excluding any non-specific signal. Moreover, in the FACS analysis, we observed one contiguous population with the MitoTracker signal indicating that all cells produced at least some mitochondrial potential, similar to what we observed through live cell confocal microscopy. Analogously to Connelly et al (2021), we applied a gate threshold at the edge of the uninfected erythrocyte, which was suggested to provide the most conservative threshold between the dormant and dead parasites. In our study, at least 74.7% of condensed parasites on Day 5 that passed this threshold. We would like to highlight, that even for untreated healthy ring stage parasites, we observed 70.6% parasites that passed the gating threshold suggesting a lower range of MitoTracker signal in rings. Lower Mitotracker signal in rings (compared to trophozoites and schizonts) is possibly a reflection of single, smaller mitochondrion found in ring stage parasites (Evers et al, 2021, Nat. Comm.) whereas the wide distribution of Mitotracker signal could reflect natural variability in mitochondrial membrane potential as observed previously in other systems (Pires das Neves et al, 2010, Plos Biology). Overall, our results suggests that with these minimum criteria, at least three quarters of the condensed cell population carry an active mitochondrial potential. This is higher but fundamentally not very different from the Connelly study where ~30% (0.7% parasitemia) for the 803 strain was gated in this subpopulation.

Finally, in addition to the MitoTracker staining we now carried out Propidium Iodide(PI) staining as an additional viability assay. As shown in Fig 5, on Day 5, 98.7% of parasites are PI negative thus confirming their viability as compared to 99.9% PI positive parasites in the death control (paraformaldehyde and Glutaraldehyde treated). This confirms that on day 5 post DHA treatment, parasites are still viable as corroborated by multiple lines of evidence.

In addition, the statement (lines 200-201) that in previous publications authors reported that parasitemia was declining following DHA treatment is incorrect. In Chen et al the Fig 1 demonstrates that parasitemia remains stable at the same level of 2% for at least 6 days following DHA exposure.

We agree that the statement about previous studies is not exactly correct. This statement should be: "…*more than half of previous publications*…". To our knowledge, there are 8 previous publications in which total parasitemia was plotted across the time 10-25-day courses after the DHA exposures (all cited in this manuscript). In 5 of these, the overall parasitemia was shown to drop within the first day to zero or near zero and recovery occurred past 7-10 days. (Nakazawa, Kanbara et al. 1995, Teuscher, Gatton et al. 2010, Teuscher, Chen et al. 2012, Breglio, Rahman et al. 2018, Connelly, Manzella-Lapeira et al. 2021) In our statement, we referred to these, assuming this is the general common view. Indeed, in three publications, the total parasitemia appeared to persist at the original level until the growth recovery that occurred anywhere between 7 to 10 days (Tucker, Mutka et al. 2012, Chen, LaCrue et al.

2014, Gray, Gresty et al. 2016). Moreover, there is one additional publication (Hott, Casandra et al. 2015) that reports a persistence of condensed-form parasites for up to 72 hrs past the DHA treatment, albeit in a subsequent figure, the total parasitemia was depicted at zero (not clear if condensed forms were counted). Our experiment resembles the three latter publications in which a substantial number of parasites remained in the culture for at least 7 days until the stage of growth recovery. Our experiment resembles most that of (Tucker, Mutka et al. 2012) where initial parasitemia (~2%) remained constant for up to 140 hrs (~6 day) after which the asexual growth resumed.

Overall, this indicates a certain degree of inconsistency in the studies of malaria dormancy reported up until today. This is to be expected, given how little we still know about it. It is feasible that these inconsistencies are due to multiple factors, which could include the parasite's genetic background and other various culture parameters, mainly at the induction stage but also possibly during the prolonged culturing. In the future, it will be interesting to carry out a comparative analysis to pinpoint the key parameters that influence the viability of parasites after DHA treatment. Unfortunately, this is beyond the scope of our study. Our main focus was to characterize the transcriptional profile of the dormancy induction, showing the 5-day maturation process after which the dormant parasites exhibit a specific transcriptional profile carrying a combination of quiescence and senescence.

The major statement and the finding in this MS is a new robust model that allows to produce close to 100% dormant rings by using essentially the same experimental conditions with exception of different P. falciparum laboratory strain (3D7),. This statement contradicts results from all previous studies (well referenced in the MS). The subsequent transcriptome analysis and the electron microscopy images of "dormant" rings are all based on this assumption. If this is correct, this would, indeed, constitute a "paradigm shift" in as it is in stark contrast to the previous findings produced by different research groups. This whole model with all conclusions that follow is based only on results of one type of assay showing that ~100% of DHA-treated parasites are identified as "live" by positive Mitotracker DeepRed staining, which also contradicts results of similar experiments obtained by Connelly, et al., 2021.

In our view, this manuscript does not directly contradict these previous publications but instead provides the next step in the studies of the dormant stage of *Plasmodium* parasites. As described in the point above, there are some discrepancies between all 8 previous publications in terms of the dynamics of the time course dormancy induction, including the persistent parasitemia, time of reemergence, e.t.c. Here, we explored these previous works and managed to improve the system, allowing us to analyze more biological aspects of the dormancy stages, such as transcriptome. In that aspect our manuscript is indeed, potentially paradigm-shifting. This is done in many ways, thanks to the previous studies that provided a lot of foundation knowledge. In lines 115-116, we now mention that we adopted the culturing protocol originally developed by Teuscher, F. et. al. 2010 with few modifications.

Regarding specifically the study of Connelly et al., our manuscript does not contradict this work but, on the contrary, shares many components. In particular, similar to Connelly et al, we observed a substantial fraction of the condensed forms carrying mito tracker staining in the condensed forms that correspond to the mitochondria membrane potential. In our study, that proportion was ~75% compared to ~30% that passed the gate threshold of FACS staining and subsequent confocal mitochondria imaging. In the new version of the manuscript, we acknowledge the percentage of viable cells in the d5MDPs that reflect the fluorescence-based measurements being ~75% or greater.

Another line of evidence, microscopy analysis of the Giemsa stained slides does not differentiate between live and dead and is subjective in interpretations of "dormant" or "dead" ring stage parasites. Given a very high potential impact of this paper for the malaria field and future interpretations of the transcriptome analysis it would be critical to provide additional lines of evidence to substantiate Mitotracker DeepRed staining results. For an example, P. falciparum lactate dehydrogenase (LDH) enzyme activity assay (Sigma-Aldrich) and ATP

measurement assay (CellTiter-Glo Luminescent Cell Viability Assay kit (Promega), as described in ref 22, would strengthen authors statements.

We included the Giemsa-stained condensed parasite forms as previous reviewers suggested that dead and dormant parasites have been shown to look different when stained with Giemsa (Kyle group). However, we agree this is a rather subjective method and hence we adopted modern fluorescent staining techniques (Mitotracker, PI) to distinguish the viable from the non-viable. To provide more evidence of viability, we carried out FACS analysis with PI staining as shown in Fig 5 depicting 98.7 % of d5MDP are PI negative (hence viable) and show a distinct staining as compared to the dead control (PFA and GA treated parasites).

We are also willing to conduct the LDH and ATP assays if the editor agrees with yet more additions to this manuscript. However, here, we wish to argue that interpreting the measurements of bulk LHD and bulk ATP levels in the dormant parasite will unlikely shed more light on the percentage of viable cells. From that perspective, these assays will be redundant to the MitoTracker studies.

With all due respect, in my view, the additional evidence would substantially enhance the quality of this publication and validity of overall conclusions. Regretfully, I do not believe that the authors have sufficiently addressed my concerns, which I have raised in my previous reviews and I do not believe that the MS should be published in the current version.

We are sad to hear that despite all our efforts, our revisions and additional evidence failed to convince this reviewer about the validity and importance of these results. We are somewhat perplexed, given that we always addressed all the reviewers' comments in the three rounds of the previous reviews with a positive result. Perhaps this view can be changed with the new version and additional evidence.

Reviewer #4 (Remarks to the Author):

I am quite sceptical because the main problem here is that there is no evidence that the live parasites (R, T and S) observed at day 12 are from 'condensed forms' as claimed by the authors. As shown in Figure 1A, it seems possible that they are derived from parasites that were able to survive until the DHA pulse (~2% at day 5, ~8% at day 7, ~40% at day 9 and ~70% at day 11). This is consistent with a multiplication rate of x4-5.

Indeed, it is correct to assume that not all growth recovery in the initial bulk culture experiment comes from the re-emergence of the dormant parasites, and at least a proportion of these may originate from the residual surviving asexual stage parasites. The residual asexual stages in these cultures represent the limit of the Magnetic Assisted Cell Sorting (MACS), which cannot remove all mature forms of the parasites reported in the previous studies. Like in essentially all studies of *Plasmodium* dormancy, the growth recovery is likely a combination of both the dormancy re-emergence and continual residual asexual multiplication that is ongoing, albeit at very low levels. In particular, Connelly et al 2021, reported that asexual parasitemia persisted throughout the time course, reaching up to 8.04% in the 803 strain. Similarly, we carried out a limited dilution assay (outgrowth assay, Connely 2021), showing that only a small fraction of the dormant parasites (4.16% ± 3.86%) has the propensity to recover to asexual growth, which is comparable to the previous work. This suggests that in the given *in vitro* growth conditions, only a small fraction of the condensed parasites re-emerged to asexual growth within the time frame of the experiment while most remained dormant. These dormant parasites may persist for prolonged periods of time and eventually die along with the host erythrocyte, whose life span is also limited.

Nevertheless, here we show that at Day 5, most, if not all, condensed form parasites (d5MDPs) are in a viable state (i.e. Mitotracker positive, Propidium Iodide negative) exhibiting unique biological functions. First, we showed that a minimum of ~75% of the d5MDP exhibits the mitochondria membrane potential based on the FACS analysis with MitoTracker staining. This is supported by the high-resolution confocal microscopy 3D volume rendering showing that all MitoTracker signal comes from a distinct organelle adjacent to the nucleus, hence mitochondrion. In the new version of the manuscript, we carried out an additional viability assay using propidium iodine (PI) that was previously shown to penetrate dead cells with compromised membrane integrity. In the new version of the manuscript, we showed that dead cells killed by paraformaldehyde-glutaraldehyde (PFA-GA) treatment exhibit strong PI staining while 98.69% of d5MDP remains PI negative. Crucially, the PFA-GA treatment can kill the d5MDP, making them PI-positive, further suggesting their viability prior to the treatment (**Figure 5A and Supp Figure 10**).

Last but not least, the d5MDPs exhibit a highly specific transcription profile characterized by a unique pattern of biological functions upregulated but downregulated. This profile can be readily reproduced in multiple biological replica experiments. Here, we wish to reiterate that the transcriptomic results presented here do represent a bulk experiment in which all cells in the culture were analyzed regardless of their status. Instead, here we carried out a “few-cell” RNA-Seq in which exactly 500 condensed cells were sorted by FACS as the input. In this type of experiment, the specificity and reproducibility of the generated transcription would not be achieved if most of the cells were dead. We hope this is now clear in the text of our new manuscript version.

With apologies, this confusion that was caused by us referring to the induced cells as “pyknotic”. Here we were referring to their morphological appearance rather than their cellular state. We did not mean to imply that the induced dormant parasites in this study are in the state of cellular pyknosis that indeed, reflects a process of chromatin and nucleus shrinkage, during apoptosis or necrosis. Instead, we showed that all dormant parasites in

this study are NOT in the state of pyknosis as these carry cytoplasm with active mitochondria. Hence in the new version of the manuscript, we avoid the term Cellular Pyknosis and instead refer to the dormant parasite-induced state as “condensed cells”.

I agree that the author's response clarifies the definition and the terms used in the MS.

We are delighted that reviewer 4 agrees with our definitions.

“Nevertheless, the dormant parasites induced in this study (d5MDPs) are most likely identical to the subfraction of the dormant parasites that were shown to give rise to the re-emergence of asexual growth reported in previous studies.”

This answer is quite speculative. There is no clear evidence in the MS to show that 'the dormant parasites induced in this study are most likely identical to the subset of dormant parasites that have been shown to cause the reappearance of asexual growth reported in previous studies'. I appreciate the use of 'most likely'.

Indeed, we used “most likely” as there is no definite way to provide such evidence. Unfortunately, we cannot make direct comparisons between our experiment and the cells generated by the previous studies. As discussed in the responses to reviewer 2, and in the discussion section, there are discrepancies even between these (8) previous studies in terms of the conversion rate to condensed forms, time to recovery e.t.c.. We believe that it is feasible to speculate about the analogy between our studies and the previous works since we used a similar induction protocol (700nM DHA for 6hr) and observed similar parasite morphology and stage transition dynamics.

We discuss this further in the first paragraphs of Discussion.

“As shown in the earlier studies, in vitro exposures of ring-stage parasites to clinically relevant concentrations of DHA (~700nM) can transform varying proportions of the parasite population into dormant stages[17,19,20,25,38]. The parasites remain dormant with little or no apparent signs of multiplication for at least 8 days, after which a growth recovery was reported as early as Day 10 but sometimes as late as Day 25[21,25,27,31,39]. These time differences were generally attributed to the parasite’s genetic background (including drug resistance) and growth conditions at the induction time. The growth initiation was suggested to originate largely from the “re-awakening” of a small portion of the dormant parasites to asexual growth, which in one study was estimated to range between 0.044% and 1.313%[19]. Nevertheless, neither of the studies could exclude a contribution of the residual asexual stage parasites that remained in the bulk cultures at small levels such as 0.9% and 8.04% for GB4 and 803 strains, respectively, in the study of Connelly S. V. et al (2021)[25]. Finally, in approximately half of the previous reports, the total parasitemia (including the condensed forms) remained constant through the DHA-induced dormancy time course[20,22,23,31,39] . In other studies, however, the total parasitemia was reported to be near zero before the asexual growth re-emergence[19-21,25,38]. Taken together, these results indicate overall variability of the artemisinin induction of the dormant parasites form as well their persistence and growth reemergence.”

The definition of a dormant ring is well described in Tucker 2012, with Fig 2 showing microphotographs of the dormant vs dead pyknotic paraites “Comparison of dormant and dead parasites after a typical exposure to artemisinin drugs. (A) After ring-stage parasites were treated with artemisinin drugs, they became dormant, developed a rounded morphology, and retained blue cytoplasm and red chromatin on a Giemsa smear. (B) Dead parasites lacked distinct chromatin and cytoplasm, appearing globular, and they appeared red/purple on a Giemsa smear. Also in Peatey, 2021 “The dormant ring morphology shows condensed blue cytoplasm and condensed red chromatin as opposed to a normal ring with red chromatin dot and open cytoplasm. Dead parasites have a pyknotic morphology with no cytoplasm evident”. This description and depiction of dead pyknotic parasites appears to match those in Fig 1C of the MS.

Taking this advice, in the manuscript's new version, we investigated cytoplasm's presence in the dormant parasites induced in this study (d5MDP). In Figure S2a & b, we show that essentially all condensed cells, representing the dormant parasite forms, d5MDP, contain a cytoplasm; demonstrated by the blue Giemsa stain as defined by Peatey , 2021. To support the Giemsa smear microscopy, however, we also now show that all 5dMDP carry functional mitochondria characterized by active membrane potential indicator MitoTracker imaged by high-resolution microscopy (Figure 4). This result supports the main point of this manuscript demonstrating that the generated transcriptional profile comes from viable cells representing the dormant developmental stage. We clarify this in the text (lines 331 through 353)

"Our initial Giemsa stain-based evaluations suggested that essentially all condensed forms of the parasites across the experimental time course (Day1-Day5) exhibit the presence of compressed chromatin (red/purple stain) along with condensed but visible cytoplasm (blue stain) (Figure S2a & b)…. compartments present in each d5MDP juxtaposed to the nucleus, similarly to the ring stage parasites (Figure 4C). This is consistent with previous suggestions that the P. falciparum dormant stages contain enlarged mitochondria located near the nucleus25."

I am not convinced by the microphotographs of dormant parasites, where the presence of cytoplasm in dormant parasites is not clearly visible. However, I am more convinced by the data showing that 5dMDP parasites carry functional mitochondria, characterised by the active membrane potential indicator, MitoTracker, imaged by high resolution microscopy.

We thank the reviewer for acknowledging the significance of the MitoTracker result.

The authors claim that they have developed "a robust in vitro (laboratory) model, where they achieved "a near 100% conversion to pyknotic presumed dormant rings", which they explain by specific culture conditions and using 3D7 strain as opposed to W2 strain, which is, in my view, improbable. In addition to Tuescher et al., 2010, used D6 parasites, which are of similar background to 3D7 parasites. In fact, 3D7 strain was used in infection studies and observation of dormant parasites in ex vivo cultures in Peatey, 2021 with similar to the previous studies' outcomes.

First, the high level of dormancy conversion is indeed the main difference between ours and previously reported studies. At the moment it is unclear what was the main reason for this but, here we wanted to find all possible differences some of which could indeed explain this difference. We agree that the choice of the 3D7 might have not been the main factor, however, such a possibility cannot be completely ruled out either. The D6 strain is indeed related to 3D7 but not completely identical.

The authors' response is somewhat evasive and does not explain the data presented and the claimed high level of dormancy conversion in MS.

We are very confident about the high conversion rate in our study and describe these with full assertion. We might sound evasive in comparing our conversion rate to the previous studies as (again) we do not have insight into such parameters in these previous studies. It is important to note that this study is the first in which a conversion rate was actually estimated specifically. Nonetheless, we speculate that the conversion rate was likely similar to ours in the four previous studies where the overall parasitemia remained high.

We discuss this in the overall context of the variability of dormancy induction that currently exist in the literature, *Discussion first paragraph*.

Another point difference is much higher initial parasitemia 4-8% in combination with lower hematocrit (2%) and Albumax, which may have additionally add to the stress on parasites caused by DHA. The graphs in Fig S1A show very similar to previous studies recovery, with duration somewhat proportional to the initial parasitemia in 4 experiments.

We thank the reviewer for this comment and important suggestion. The high parasitemia could indeed contribute to the increased conversion rates, which in turn result in the apparent higher recovery rates in the bulk cultures. This comment is also in line with our recovery experiment, which stresses the importance of initial cell density for the overall outcome.

Although we did comment on the high parasitemia in our previous version, here we elaborate somewhat more in Discussion (line 447-451):

"… and possibly other experimental parameters. One of such parameter could be the higher starting parasitemia (Figure S1a), compared to the previous report that can enhance the DHA-mediated stress due to high cell density. It will be interesting to explore various experimental parameters to further improve the P. falciparum dormancy model for future studies."

This is an excellent point raised by the reviewer and could explain the high dormancy conversion rate. I have to say that the authors 'jump' on this point to provide a possible explanation for their data without convincing evidence.

To clarify this further, we now state the three main differences between ours and previous works: these include the initial parasitemia that was adjusted to 8% compared to the previous work, which used 2%; induction with mid rigs (6-12 hour post-invasion) in contrast to the previous studies using early rings (0-3 hpi); the usage of the 3D7 strain which has never been done before. The only exception is the use of the D6 strain that is genetically related but not identical. Curiously, there D6 exhibited the highest recovery rate compared to other strains (Tuecher et al (2020).

We might have sounded somewhat eager in our previous version, given that the reviewer suggested the overall parasitemia difference. We now put this into a broader context with a more measured perspective.

We discuss this in the new version of the manuscript line 466:

*"Our studies contrast all previous studies in three main aspects: (i) we used the 3D7 P. falciparum strain which was never used before (except D6 which is genetically related but not identical)[19]; (ii.) the DHA treatment was carried out with mid rings (6-12 HPI) while all previous studies used early rings (0-3HPI); (iii) here we adjusted the initial parasitemia to 8% which is substantially higher compared to the previous studies using 2% (**Figure 1** & **S1**). "*

The authors present 2 lines of evidence of all pyknotic rings being dormant: 1) the microcopy images shown in Fig 1C and 2) staining with Mitotracker Red to demonstrate that parasites are live. Unfortunately, it is not possible to clearly see if there are any dormant rings (as described in Teuscher, 2010, Tucker, 2012, Peatey, 2021 and others) with differential staining of the blue cytoplasm and the red nucleus could be seen in those images of "pyknotic" parasites in Fig 1c of the MS.

Referring to the comment above, in the new version of the manuscript we provide extensive Giemsa smear staining images (Figure S2a & b). We hope that the presence of the blue-stained cytoplasm is more clear in these. Nevertheless, to support the evidence of persisting cytoplasm in the dormant parasite, we also conducted high-resolution microscopy showing the presence of active mitochondria in essentially all dormant parasite cells (Figure 4). This strongly suggests that the dormant parasite population generated in this study on day 5 (d5MDP) represents viable cells. As such, these results support the main discovery made in this manuscript; the transcriptome of the dormant parasites in a viable state.

Same comment than above.

We assume that the reviewer meant that the MitoTracker staining is more convincing about the viability of the condensed parasites than the Giemsa smears and we fully agree with it.

Also it would help if the FACS images in Fig 4A included double stained with Hoescht and Mitotracker parasites and uninfected RBC rather than Mito vs FSC plots. Authors also state that "Third, the MitoTracker staining remains positive with reduced intensity through these stages of dormancy" in reference to Fig 4A. It is not clear from the images.

In the new version of the manuscript, we provide the double-stained FACS analysis showing that all dormancy-induced parasites are MitoTracker positive. This is supported by microscopy imaging (Figure S9) that was

analyzed quantitatively (Figure 4b). To reiterate, the reduced MitoTracker staining originates from these quantitative analyses. It is possible that this is a reflection of the dormant state of the parasites as previously suggested in stem cell research where quiescent hematopoietic stem cells (HSC) have a lower mitochondrial membrane potential compared to primed (activated) HSCs (Umemoto et al 2010; Ghaffari,2024, etc.). To support these analyses further, we carried out identical MitoTracker staining live cell imaging, this time visualized by high-resolution confocal microscopy and performed 3D volume rendering to identify the cellular compartment from where the MitoTracker signal originates (Figure 4c). We trust that this provides sufficient evidence for the presence of active mitochondria in all dormant parasites induced in this study.

I agree that these data provide sufficient evidence for the presence of active mitochondria in artemisinin-induced dormant parasites, but I am sceptical that all parasites exposed to artemisinin are MitoTracker positive and therefore dormant parasites.

The reviewer's skepticism is understandable. Due to that, we provide more detailed descriptions of the MitoTracker staining experiment.: In the first step we used live-cell imaging such that for each sample (Rings on Day0 and Condensed form of Day 1 and 5) 100 cells with DAPI (nuclear) signal (regardless MitoTracker) were unbiasedly recorded for an automated image analysis (using Image J). The MitoTracker signal intensity was subsequently measured along with the DAPI staining. We observed that with the exception of two or three cells, for each set of 100 cell sets, significant, albeit variant intensity of MitoTracker staining can be observed. The MitoTracker intensity plots are now included in **Figure 4B** and **Figure S9B,** along with the micrographs of the live-cell imaging. Nevertheless, we also provide a more conservative estimate of the MitoTracker signal that can be detected in at least 74.7% d5MDP by FACS (**Figure 4A**). However, this is a highly conservative estimate given the lack of a clear division between MitoTracker positive and negative cells, similar to previous studies such as Connelly et al 2021.

In contrast to the majority of observed pyknotic (dead) forms, dormant rings were only a small fraction ~2% of all DHA-treated rings, with only about half of that recovered, which was actually quantified by Tuescher, 2010 using limited dilution cloning and removing emerging live parasites by using magnetic columns. This is much more accurate quantification that the formula used by the authors to arrive at 60.2% of nearly 100% presumed dormant parasites. Authors acknowledge, that they observe live growing trophozoites as early as after day 3 in minute quantities. With exponential nature of parasite amplification (4-8-fold every 48 h or even faster in cultures), without removing live parasites by magnetic columns it is impossible to differentiate parasites, which have just recovered from the progenies of the recovered earlier parasites.

I believe, it would be very important for the MS to be convinced of what was the composition of parasite populations taken for RNA-seq. If the majority of these cells are "pyknotic" (dying) cells, it is plausible that the transcripts (at least in part, for days 1-5) may correspond to the transcriptome associated with pyknosis caused by DHA rather than dormancy.

In agreement with the reviewer, we also believe that it is important to demonstrate the nature of the parasite cell population that was taken for the RNA-Seq analysis. We would like to reiterate that we performed MACS on 3 consecuative days to remove any asexual parasites that survived the DHA treatment and then used multiple strategies to confirm the viability of the dormant parasites. One cannot rule out that the emergence of parasites on day 3 could be due to the stochastic nature of parasite recovery. Furthermore, previous studies in bacterial cells indeed established that upon antibiotic exposure, bacterial cells can enter varying degree of dormancy referred to as "dormancy depth" (Pu et al, 2019, Mol. Cell) which has a direct relation to their ability to recover. Based on all results collectively we can say that an overwhelming proportion (possibly all) of dormant cells analyzed by RNA-Seq are NOT in the state of pyknosis but instead are viable. This is based on the presence of the blue-stained cytoplasm by Giemsa staining (Figure S2a & b) but also active mitochondria in essentially all induced dormant parasites shown by FACS analysis (Figure 4a), live cell imaging (Figure 4b & Figure S9) and finally high-resolution confocal microscopy (Figure 4 c). Although, in this manuscript, we did not establish the exact recovery rate of these parasites, we provide a close approximation by our limited dilution experiment that in their number resembles the previous studies. We however also demonstrate that the apparent recovery rate is strongly dependent on growth conditions, particularly on cell density.

With all due respect, we wish to argue that establishing such recovery rates in in vitro cultures can be highly precarious and as such do not necessarily reflect the overall viability of the dormant parasite population. Here we

opted for a more direct approach, showing the presence of active mitochondria as the key measure of cell viability. Nevertheless, we agree that developing more assays for estimation of the potential of dormant parasite recovery will be important in the future. This will be particularly important for studies of the potential if recrudesce of malaria infection in vivo. We believe that our manuscript provides a good foundation for such work.

I agree with the authors' response. Even if their data are not completely convincing, the manuscript presents some very interesting data that may open new perspectives on a new mechanism for survival (and not resistance) of Plasmodium falciparum to antimalarial drugs.

We thank this reviewer for recognizing the importance of our results and the opportunities this manuscript opens for future studies of multiple aspects of malaria infections, including the mechanism of the parasite's survival in its natural host and, thus, disease outcome after treatment. Like all studies, this manuscript cannot address all aspects of plasmodium dormancy, and many questions remain open (see below). Nevertheless, we are glad that this reviewer believes that our manuscript is worthy of publication pending the resolution of these open questions.

I think the authors should insist on the limitations of their work in the discussion to put into perspective the work they have done, but also the potential technical biases raised by reviewer 2.

Perhaps the main technical bias of this study is the induction protocol that was carried out differently compared to previous works, including higher parasitemia, mid-ring induction time, and the usage of the 3D7 strain. It is feasible to suggest that other experimental setups generate somewhat different dynamics of the dormancy parasite populations or the dormancy physiology altogether, albeit unlikely. The main limitation of this study is the growth recovery rate, which remains somewhat elusive. We believe that such studies are beyond the scope of this work but can be addressed in the future. It will be interesting to compare recovery rates between different strains but also induction stimuli that go beyond artemisinin compounds, such as antimalarial drugs and other external stimuli that were previously reported. It is also feasible to suggest that the recovery rate of dormant parasites could be stimulated by various induction conditions and, as such, could be much higher than the presented regular growth conditions.

We acknowledge this in the first two paragraphs of the discussion section. Line 474

"Hence, currently, it is unclear whether the dormancy parasites induced in these studies are identical to those induced earlier. However, it is feasible to speculate on their analogy given the overall similarities in the culture dynamics and the distribution of the morphology distribution over the experimental time course. In future studies, it will be interesting to explore further the nature of the variability in the inductions of dormancy mediated by artemisinins but possibly other conditions, including other antimalarial drugs or other external perturbations, some of which were reported previously[40,41]."

I have several comments, which I list below:

Abstract.

Introduction.
Lines 47-49. ACT should be replaced by artemisinin. I suggest rewording the sentence as follows: "Unfortunately, the reduced susceptibility of P. falciparum to artemisinins, which emerged in the eastern part of the Greater Mekong Subregion as early as 20092,3 and is now emerging in other parts of the world, including sub-Saharan Africa, is a major concern for the future.

Line 51. ACTs should be replaced by artemisinins.

Corrected

Line 76. I suggest adding 'ring stages' to the sentence, which could read: 'Indeed, exposure of P. falciparum ring stages to artemisinins in vitro induces dormancy-like forms that may represent this residual parasite (sub)population.

Corrected

Results

Fig1. Please reconcile figure legends and fig1 (i.e. B -> C and C-> B, there is no panel E in fig1).

Corrected

Lines 366_367. This is very surprising that d5MDPs appear resistant to high IC50 concentrations of atovaquone and gibberellic acid (GA) did not affect the recrudescence and delay of d5MDPs.

We agree that this is highly unexpected for atovaquone, given that the functional mitochondria in the d5MDPs. The most logical explanation is that while the mitochondrion is functional, the parasites do not rely on its respiratory chain, which is the atovaquone target.

We mention this in the text.

Discussion

Line 449. Stress not strass

Corrected

Additional question. Why did the authors use the smart-seq2 protocol and perform bulk transcriptome analysis rather than single cell transcriptome analysis? This would have allowed to deconvolute the dormant and ring stage populations.

In this manuscript, we opted to use the “few-cell” approach in which 500 cells were FACS sorted for RNA-Seq instead of a single cell. The main reason is that the overall gene coverage achieved allows for the full characterization of the entire transcriptome. Even the best single-cell RNA sequencing technologies only cover about 20-25% of the transcriptome. Our previous study showed that input of as few as 100 cells will reproduce a bulk transcriptome with as many as 4000 (80%) covered genes, while a single cell will cover only 20% (Tripathi et al, 2022). Hence, while single-cell analyses are suitable for studying cell subpopulations and transcriptional heterogeneity, they are not suitable for full transcriptome coverage, which was the main focus of this study. Single-cell transcriptomics of d5MDPs will be highly interesting in order to provide a full evaluation of the dormant parasite developmental trajectory studies in future studies.

Reviewers' Comments:

Reviewer #4:
Remarks to the Author:
I have no further comments. The authors have responded to all the comments and the paper can be considered ready for publication. The additional data provided by the authors removes a number of grey areas from the original paper.