Zonal human hepatocytes are differentially permissive to Plasmodium falciparum malaria parasites

Annie Yang, Youri van Waardenburg, Marga van de Vegte-Bolmer, Geert-Jan van Gemert, Wouter Graumans, Johannes de Wilt, and Robert Sauerwein

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Corresponding authors: Annie Yang (annie.yang@radboudumc.nl), Robert Sauerwein (robert.sauerwein@radboudumc.nl)

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Thank you for transferring your manuscript from Review Commons to The EMBO Journal. I have now read your manuscript, the reviewer comments and your revision proposal.

Based on the novelty of the study and the strong support expressed by all three reviewers, I would like to invite you to submit a revised manuscript along the lines indicated in your preliminary revision plan. While a further characterisation of the causal role of glutamine synthetase for P. falciparum intracellular growth would have been helpful, after our pre-decision discussion of the limitations of the experimental system we concluded that this point does not have to be experimentally expanded upon in the revised version.

Due to the delays to experimental work caused by the ongoing pandemic, we have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.
Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

**Estimated time to Complete Revisions (Required)**

(Decision Recommendation)

Between 1 and 3 months

2. Evidence, reproducibility and clarity:

**Evidence, reproducibility and clarity (Required)**

**Summary:** In this study, the authors set out to explore the selectivity of Pf parasites strains (NF54, NF175 and NF135) for liver cells derived from primary human hepatocytes, relating parasite liver development to the metabolic heterogeneity of fresh human hepatocytes. Hepatocytes are naturally zoned into 3 different "types". The authors show that parasites demonstrate a selective preference for the minority of zone 3 hepatocytes, which are characterized by elevated presence of glutamine synthetase (hGS) and depreciated presence of hGK. The authors also show that parasite schizont growth is significantly enhanced by hGS uptake early in development. Study of the liver stages of development in Plasmodium has been severely hampered by the lack of availability of a robust liver cell model, the low rates of infection seen with most hepatocytes cells and the complete lack of mature development (with P. falciparum) unless primary human hepatocytes are used (P. berghei murine malaria is much more robust). Immortalised lines, such as HC-04, whilst sustaining a few days of development, do not demonstrate full maturation to schizogony. This study reveals that for parasites, not all hepatocytes are the same and may therefore lay key foundations for development of a much more robust platform for exploring hepatic stages in the future. Furthermore, this study reveals the striking observation that NF54, the universally used model for parasite transmission work, may be an outlier in biology - suggesting key gains may be had simply by the community shifting parasite strain. **Major comments:** - Overall, I find the data to be sound and robust and convincing, even if it's is largely describing the phenotype. It will no doubt be of very great interest to the malaria community, and those interested in zonal hepatocyte biology/infection biology. My comments/criticisms below are largely textual suggestions to improve clarity or raise a question that could be addressed in the text. - There are some areas that require clarification (listed below) to help understanding, however I do not suggest additional experiments are required to support claims made in the paper - some image analysis might help with clarity only. **Minor comments:** - The observation of NF54's inability to make use of hGS is a clear standout from the study. Taken at face value we'd almost expect that NF54 wouldn't really develop in liver cells - yet it is the gold standard for human infections. How do the authors reconcile that NF54 is so poor at in vitro hepatocyte growth but works well in vivo? Is there any data from NF135/NF175 on in
vivo human growth? -I find the interchangeability of Zone name (Z1/Z2/Z3) and hGS and hGK nomenclature hard to follow, so I would suggest either sticking to one nomenclature or consistently reinforcing the relationship between Zone and hGS/hGK (e.g. Z1/hGK+/+/hGS-). This saves flicking back and forth between figures/text. -Can the authors spell out the counting process used? Was it FACS or by microscopy - I think it’s the latter? It may be buried in the methodology, but since it’s such a key tool for working out zone preference (and for statistical robustness) I think this should be spelled out in the main text. -Do the authors have any thoughts on levels of oxygenation? Since the Z3 hepatocytes are presumably at much lower oxygen potential than Z1? -For Figure 3, and this section in general, the presence or absence of an hGS staining pattern could do with something less subjective. Is there an imaging approach (clustering of stain/diffuseness of stain) that could be measured to give a quantitative measure of what is being seen rather than a subjective call on the phenotype? -In general, throughout I find the figures need better labelling - if only 1 parasite strain is being use have this on the figure (as well as the legend) so its clear. Sometimes the figure is one parasite (e.g. Figure 4B-D) but other times it isn’t (Figure 4A-C) this is really confusing to follow. In Figure 3 suddenly purple and green appear for NF135/NF175.... Try and stay consistent and, in my opinion, always have the label on the figure. -I do not find Figure 4E intuitive to follow - this might need more work as a schematic. E.g. what are the top and bottom row? (note legend say I rather than E). -The supplementation of AIP into cultures to ablate the hGS metabolic function is a good experiment. What about the inverse? Could the authors try addition of further hGS or its up-regulation specifically in a cell line and explore whether this boosted in vitro development? E.g. using HC-04/HepG2 cells? This could be a revolutionary tool for other researchers in the field. Even just in concept to spell this out. -The statement "Furthermore, the anti-diabetic drug metformin effectively reduces the size of Pf liver schizonts." Is intriguing but I cannot see a reference for this or data that supports this?

3. Significance:

Significance (Required)

The study is of major significance for the field as the first to truly address the very challenging question of why Plasmodium falciparum is so poor at in vitro liver cell development, relating the question in part to cell type and also parasite strain. This is very important, though it also raises many questions. Indeed, what is wrong with NF54? Looking to the future it would be extremely valuable if someone could do a cross between these lines and look at progeny preferences. This may reveal the genetic basis for NF54 inability to utilise hGS to boost schizont development, though clearly beyond the possibility for this study. The study provides a major conceptual advance - pushing the community to think beyond one parasite and to think about liver cells as a heterogeneous population, not a monoculture (and certainly not an immortalised hepatocyte like HepG2). Indeed, it makes me wonder whether a lot of the work to date on P. berghei may well be almost entirely irrelevant when it comes to understanding P. falciparum biology! This is particularly important given the dominance of these models on understanding the liver stage. I am a Plasmodium cell biologist with experience across the lifecycle. As someone who has tried in vitro hepatocyte infections with P. falciparum sporozoites I am acutely aware of the lack of understanding in this area, and the potential this study has to illuminate understanding moving forwards! (and
hopefully help to develop more robust models for liver stage development moving forwards). REFEREES CROSS-COMMENTING I concur with the broad support from the other reviewers and the suggestions made for improving the manuscript, which I maintain will be received very positively by the malaria and liver-infective pathogen community.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The first obligate replication phase of malarial parasites occurs in the liver of the mammalian host and constitutes a prime target for urgently needed anti-malarial strategies, such as vaccines and prophylactic drugs. Increased recognition and interest in liver stage development has led to several systematic descriptive (transcriptome, proteome,...) and mechanistic (experimental genetics) studies. Yet, virtually all rest on the simplified assumption of infection of and development within a 'host hepatocyte'. In this study, the authors study the, hepatic lobule'. The three distinctive metabolic zones of the hepatic lobules are the periportal zone I, which is, of course, rich in oxygen and performs oxidative functions, including gluconeogenesis and beta-oxidation of fatty acids, the somewhat ill-defined transition zone II, and the zone III, which is close to the central vein, poorly oxygenated, and active in glycolysis and lipogenesis. How do these distinctive regions affect Plasmodium falciparum liver stage maturation, and is there a preference for one metabolic pattern as opposed to adaptability? This important question is studied for the first time, and it is far from trivial. Plasmodium berghei is a useless model for this question, since it is very promiscuous in its host cell choice. The human parasite Plasmodium falciparum is the perfect choice, but the strict host specificity makes it a tricky task due to the lack of in vivo models. The authors cultured surgical liver material for two days followed by the addition of Pf sporozoites. There are perhaps many reasons to believe that two-dimensional cell cultures, as exemplified in Fig. 1B, do not properly reflect the 3-D architecture in the organ, oxygenation status, and accessibility of sporozoites that enter through the periportal zone, to name a few. Yet, this is certainly as far as we can possibly get to approach this critical research question. Accordingly, my assessment is based on the feasibility of this proxy of hepatic lobules. -Figure 1: While one can come up with additional markers for zones I to III I
find the scheme, selection of GK and GS as markers for metabolic activity and the data from four donors very convincing. I am not sure that the diagram in the upper right labeled 'permissiveness' is necessary, because that's what the study is all about, and I would hope every reader can grasp this without the graphic. -Figure 2: The authors show that two out of three Pf strains display higher infection rates and slightly larger liver schizonts in cultured primary hepatocytes. The most important finding is a remarkable high infection of GK- cells with NF135 and NF175 Pf parasites. My main points here are: i)Please provide more precise information on the experimental design and comparability: How was sporozoite fitness assessed? Same mosquito batches for all three lines? Were infections done in parallel with same patient material? ii)Statistics: Double check significance. n=3 (I safely assume this also means from three different donors), so the mean values of average values (infected cells, schizont size....) should be used for statistics as opposed to making every single well or schizont a value. There is considerable doubt that, while in D-F (and likely also in A,B) the mean values are shown, statistics are done with every value. No worries; the effects are nice, so very ok if p values are not as minimal. iii)Where is GS in the panels? It's mentioned in the text, but no reference to GS+ or GS- stains is made in the figure. Maybe it's a color problem (GS and Pf would both be in green), but then it would have to be adjusted in the main text. Just curious: since panel 2E is so striking, is there a representative IFA as in 1C to show multiple Pf infections of GS+ cells? If yes, that would make an awesome cover image. -Figure 3: minor changes. I suggest to include size bars in panels A to C and move E to I to Supplement. All in all, an interesting and unexpected observation. -Figure 4: Very high drug concentrations, that is greater than 100 micromolar (this is getting towards the mM range), are always suspicious. Can the authors think of alternative methods? If viability of host cells were impaired at these high concentrations it'll be a rather trivial outcome. **Minor points:** -Line 30: 'and may delineate novel pathways for intervention strategies' is not really clear from this study, particularly since all three Pf lines develop to some extent in zone I hepatocytes as well. I don't think it necessary to make this statement. -References: improve citations. In general, I was rather disappointed with the References. Many unrelated, weird, or outdated citations are shown. Ref. 1: update to 2019 report; Ref. 2 is weird. Refs. 17-19 don't make sense, Ref. 17 is SRB1 in HCV infection, Ref. 18 has been proven false; 19 should probably be: Silvie et al. 2003 (https://doi.org/10.1038/nm808). These are just examples. The authors should carefully go through and reconsider their selection of citations. -Line 73: 'freshly isolated zonal hepatocytes'. Well, yes and no. They obviously stem from hepatic lobes, but over 48 hours 2-D culture and uniform oxygen they retain some features (incl. GK/GS distribution), but may have changed some properties. This is not meant to belittle this really important work, but I feel this should be stated more precisely in the introduction. -Line 87: the little 'test-the-reviewers' error: Z3 (instead of Z2). -Lines 268-279. The speculation on anti-cell death functions makes many reference to murine models, while this study successfully distances itself from this work. I would just omit this small part entirely.

3. Significance:

Significance (Required)

This is a novel and very interesting finding that should inspire the infectious disease community. Its implications are certainly beyond malaria research and extend to other
liver pathogens, most notably hepatitis viruses. The study also raises the critical awareness of how distinct metabolic profiles of superficially similar host cells modulate pathogen maturation (and by extension host cell selection). The findings of different infectivity of Pf strains are both striking and puzzling, and certainly worthy of follow-up analyses as well. All in all, I really like the work. Yes, there are limitations and open questions, but that’s what ground-breaking work is all about. I anticipate that this study will inspire many follow-up experiments, and I consider it a big step forward towards a better molecular understanding of the enigmatic long Pf liver stage maturation process. I don’t want to embark on details, but I also believe there are many important translational implications, maybe not so much in terms of anti-infectives development, but in revisiting data from the field. REFEREEES CROSS-COMMENTING I liked the paper and believe that only clarifications on the reproducibility and a much better work on the references (they were very poorly put together) are needed. There were a total of four donor tissues from surgery (which is a major task every time), and I simply can’t envisage yet how the biological replicates from the donors were synchronized with the three Pf sporozoite strains so they were all done in parallel (the only way of comparing infectivity). Even more so, since GS IFAs (to show uptake by parasites) and assays with GS inhibitors were done, which to me sounds like they were done consecutively. Maybe a scheme showing the work flow can also clarify this.

**Review #3**

1. **How much time do you estimate the authors will need to complete the suggested revisions:**

*Estimated time to Complete Revisions (Required)*

(Decision Recommendation)

Less than 1 month

2. **Evidence, reproducibility and clarity:**

*Evidence, reproducibility and clarity (Required)*

Most of the experiments are well designed, performed and analysed, although proper statistical analysis should be used for many of the figures. **Specific comments**

1. Line 41: merozoites are not release directly into the circulation (Sturm et al, Science, 2006). This should be modified. 2. Ref 17 is not appropriate since it relates to HCV. Silvie et al, (Nat Med, 2013), Yalaoui et al, (Cell Host Microbe, 2008; Rodrigues et al, Cell Host Microbe, 2008) should be used instead. 3. Figure 1: Mann-Whitney test is not adequate, since they compare 3 sets of dat in the same graph. Authors should use Kruskal Wallis test, followed by a post-test (both tests should be indicated). 4. Figures 2A, B, C, D and E. Unless the authors have demonstrated that schizont distribution follow a normal distribution (and this should be stated in the text and/or figure legends, they should use
Kruskal Wallis test, followed by a post-test. 5. Figure 2: To get a better idea on the infectivity of the different sporozoites, the authors should mention in the figure legend, the mean schizont numbers +/- SD per well. This will help to compare with other previously published study. 6. Figure 4 and many of the supplemental figures: same as above for the statistical analysis. 7. The authors should discuss the possibility to ameliorate culture conditions by either enriching on hGS hepatocytes, or engineering hGS cells

3. Significance:

Significance (Required)

Yang et al have studied the effect of hepatic zonality on P. falciparum sporozoite infectivity. This is a timely and important study which uncovers an important aspect of sporozoite biology. It will be of interest for scientists working in malaia but more generally in the field of infectious diseases, metabolism and hepatology. REFEREES CROSS-COMMENTING I had nothing to add to more. This is an important work and the improvements will be mainly textual
Reviewer #1 (Evidence, reproducibility and clarity (Required)):

**Summary:**
In this study, the authors set out to explore the selectivity of Pf parasites strains (NF54, NF175 and NF135) for liver cells derived from primary human hepatocytes, relating parasite liver development to the metabolic heterogeneity of fresh human hepatocytes. Hepatocytes are naturally zoned into 3 different “types”. The authors show that parasites demonstrate a selective preference for the minority of zone 3 hepatocytes, which are characterized by elevated presence of glutamine synthetase (hGS) and depreciated presence of hGK. The authors also show that parasite schizont growth is significantly enhanced by hGS uptake early in development.

Study of the liver stages of development in Plasmodium has been severely hampered by the lack of availability of a robust liver cell model, the low rates of infection seen with most hepatocytes cells and the complete lack of mature development (with P. falciparum) unless primary human hepatocytes are used (P. berghei murine malaria is much more robust). Immortalised lines, such as HC-04, whilst sustaining a few days of development, do not demonstrate full maturation to schizogony. This study reveals that for parasites, not all hepatocytes are the same and may therefore lay key foundations for development of a much more robust platform for exploring hepatic stages in the future. Furthermore, this study reveals the striking observation that NF54, the universally used model for parasite transmission work, may be an outlier in biology - suggesting key gains may be had simply by the community shifting parasite strain.

**Major comments:**
- Overall, I find the data to be sound and robust and convincing, even if it's is largely describing the phenotype. It will no doubt be of very great interest to the malaria community, and those interested in zonal hepatocyte biology/infection biology. My comments/criticisms below are largely textual suggestions to improve clarity or raise a question that could be addressed in the text.

- There are some areas that require clarification (listed below) to help understanding, however I do not suggest additional experiments are required to support claims made in the paper - some image analysis might help with clarity only.
The observation of NF54's inability to make use of hGS is a clear standout from the study. Taken at face value we’d almost expect that NF54 wouldn’t really develop in liver cells - yet it is the gold standard for human infections. How do the authors reconcile that NF54 is so poor at in vitro hepatocyte growth but works well in vivo? Is there any data from NF135/NF175 on in vivo human growth? NF54 has been the unprecedented reference for successful in vitro- and clinical studies for decades without comparator. Apparently NF54 is able to develop in the absence of hGS as illustrated by the presence of schizonts in zone 1 and 2. Other factors appear to suffice for schizont formation albeit hGS (present only in zone 3 hepatocytes) is a strong host factor for schizont growth.

Only recently we pioneered studies with the other parasite isolates which showed improved performances as for liver-stage development. It is well established that in vitro growth and in vivo infections show only modest correlations. We have (un)published data showing that the relatively high performance in liver culture actually translates into a high release of parasites into the circulation in clinical studies [1, 2]. We do not have in vivo human data for NF175 (given its recent patient isolation) but for NF135, parasitemia (blood stage) arising from sporozoite infection is higher than NF54 which either points to higher infection rate of the liver and/or larger liver schizont size [2]. We expect that NF175 development in vivo may be quite similar to that of NF135.

I find the interchangeability of Zone name (Z1/Z2/Z3) and hGS and hGK nomenclature hard to follow, so I would suggest either sticking to one nomenclature or consistently reinforcing the relationship between Zone and hGS/hGK (e.g. Z1/hGK++/hGS-). This saves flicking back and forth between figures/text. We have now addressed the different types of host cells as Z1/hGK++/hGS-, Z2/hGK+/hGS-, and Z3/hGK-/hGS+. This is highlighted in blue in the main text.

Can the authors spell out the counting process used? Was it FACS or by microscopy - I think it's the latter? It may be buried in the methodology, but since it's such a key tool for working out zone preference (and for statistical robustness) I think this should be spelled out in the main text. We have addressed this in the main text see lines 116-117.

Do the authors have any thoughts on levels of oxygenation? Since the Z3 hepatocytes are presumably at much lower oxygen potential than Z1?

Yes, an oxygen gradient is indeed one of the parameters present in liver zonation. However, creating an oxygen gradient in vitro on a hepatic monolayer is difficult. While liver models have been developed [3, 4], this needs to be optimized and adapted for P. falciparum studies and is as such beyond the scope of this study. Indeed, we can assume that Z3 hepatocytes are exposed to lower oxygen levels compared to Z1: Plasmodium parasites have been previously shown to grow to a larger size in low oxygen conditions [5].

For Figure 3, and this section in general, the presence or absence of an hGS staining pattern could do with something less subjective. Is there an imaging approach (clustering of stain/diffuseness of stain) that could be measured to give a quantitative measure of what is being seen rather than a subjective call on the phenotype?

Figure 3D was generated based on the distinct GS staining pattern within parasites (as shown in 3B and 3C) i.e. subjective but 3E, F (the now expanded view 3 panels A and B) the actual GS levels within any liver schizont show a more objective representation.

In general, throughout I find the figures need better labelling - if only 1 parasite strain is being use have this on the figure (as well as the legend) so its clear. Sometimes the figure is one parasite (e.g. Figure 4B-D) but...
other times it isn’t (Figure 4A-C) this really confusing to follow. In Figure 3 suddenly purple and green appear for NF135/NF175…. Try and stay consistent and, in my opinion, always have the label on the figure. We have checked and modified the figures making NF54 blue, NF135 green and NF175 purple.

-I do not find Figure 4E intuitive to follow - this might need more work as a schematic. E.g. what are the top and bottom row? (note legend say I rather than E).
We have modified Figure 4E: the first row refers to parasite invasion of hepatocytes while parasite development in the different hepatic cell types is shown in the second row.

-The supplementation of AIP into cultures to ablate the hGS metabolic function is a good experiment. What about the inverse? Could the authors try addition of further hGS or its up-regulation specifically in a cell line and explore whether this boosted in vitro development? E.g. using HC-04/HepG2 cells? This could be a revolutionary tool for other researchers in the field. Even just in concept to spell this out.
This is indeed conceptually an attractive suggestion, but likely hard to achieve. Although a subunit of eukaryotic GS is approximately 42 kDa, it exist naturally in a octameric complex (i.e. 336 kDa or 100 Angstrom in height)[6] and its cellular uptake will require receptor mediated endocytosis. In the current absence of known receptors for glutamine synthetase, it seems unlikely that simple incubation with recombinant hGS will result in host cell uptake.
Alternatively, intracellular upregulation of exclusively hGS will be difficult to achieve –an established way is by activation of the wnt- signaling which not only upregulates hGS expression but also increases expression of other genes including B-catenin, OAT and GLT-1[7-9]. This may have confounding effects: B-catenin anchors actin cytoskeleton to cell junctions i.e. makes the cells more rigid which likely impacts on schizont development as the growing schizont will presumably stretch the hepatocyte.

-The statement "Furthermore, the anti-diabetic drug metformin effectively reduces the size of Pf liver schizonts." Is intriguing but I cannot see a reference for this or data that supports this?
Apologies for this omission, we now inserted the reference (Vera et al 2019 JCI: insight) as reference 32.

Reviewer #1 (Significance (Required)):
The study is of major significance for the field as the first to truly address the very challenging question of why Plasmodium falciparum is so poor at in vitro liver cell development, relating the question in part to cell type and also parasite strain. This is very important, though it also raises many questions. Indeed, what is wrong with NF54? Looking to the future it would be extremely valuable if someone could do a cross between these lines and look at progeny preferences. This may reveal the genetic basis for NF54 inability to utilise hGS to boost schizont development, though clearly beyond the possibility for this study.
The study provides a major conceptual advance - pushing the community to think beyond one parasite and to think about liver cells as a heterogeneous population, not a monoculture (and certainly not an immortalised hepatocyte like HepG2). Indeed, it makes me wonder whether a lot of the work to date on P.berghei may well be almost entirely irrelevant when it comes to understanding P. falciparum biology! This is particularly important given the dominance of these models on understanding the liver stage.
I am a Plasmodium cell biologist with experience across the lifecycle. As someone who has tried in vitro hepatocyte infections with P. falciparum sporozoites I am acutely aware of the lack of understanding in this area, and the potential this study has to illuminate understanding moving forwards! (and hopefully help to develop more robust models for liver stage development moving forwards).

REFEREES CROSS-COMMENTING
I concur with the broad support from the other reviewers and the suggestions made for improving the manuscript, which I maintain will be received very positively by the malaria and liver-infective pathogen community.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):
The first obligate replication phase of malarial parasites occurs in the liver of the mammalian host and constitutes a prime target for urgently needed anti-malarial strategies, such as vaccines and prophylactic drugs. Increased recognition and interest in liver stage development has led to several systematic descriptive (transcriptome, proteome,...) and mechanistic (experimental genetics) studies. Yet, virtually all rest on the simplified assumption of infection of and development within a 'host hepatocyte'. In this study, the authors study the hepatic lobule. The three distinctive metabolic zones of the hepatic lobules are the periportal zone I, which is, of course, rich in oxygen and performs oxidative functions, including gluconeogenesis and beta-oxidation of fatty acids, the somewhat ill-defined transition zone II, and the zone III, which is close to the central vein, poorly oxygenated, and active in glycolysis and lipogenesis. How do these distinctive regions affect Plasmodium falciparum liver stage maturation, and is there a preference for one metabolic pattern as opposed to adaptability? This important question is studied for the first time, and it is far from trivial.

Plasmodium berghei is a useless model for this question, since it is very promiscuous in its host cell choice. The human parasite Plasmodium falciparum is the perfect choice, but the strict host specificity makes it a tricky task due to the lack of in vivo models. The authors cultured surgical liver material for two days followed by the addition of Pf sporozoites. There are perhaps many reasons to believe that two-dimensional cell cultures, as exemplified in Fig. 1B, do not properly reflect the 3-D architecture in the organ, oxygenation status, and accessibility of sporozoites that enter through the periportal zone, to name a few. Yet, this is certainly as far as we can possibly get to approach this critical research question. Accordingly, my assessment is based on the feasibility of this proxy of hepatic lobules.

-Figure 1: While one can come up with additional markers for zones I to III I find the scheme, selection of GK and GS as markers for metabolic activity and the data from four donors very convincing. I am not sure that the diagram in the upper right labeled ‘permissiveness’ is necessary, because that's what the study is all about, and I would hope every reader can grasp this without the graphic. We made an effort for the non-malaria readers to visualize and quickly understand the premise; this figure may be removed if the editor wishes.

-Figure 2: The authors show that two out of three Pf strains display higher infection rates and slightly larger liver schizonts in cultured primary hepatocytes. The most important finding is a remarkable high infection of GK- cells with NF135 and NF175 Pf parasites. My main points here are:

i)Please provide more precise information on the experimental design and comparability: How was sporozoite fitness assessed? Same mosquito batches for all three lines? Were infections done in parallel with same patient material?
We have created Table 2 in the Methods section (after the IFA reagent table) which shows the assays as conducted per donor/liver segment. The sporozoite fitness was not specifically assessed (there is no established standard assay) but sporozoites isolated from mosquitoes at 16-20 days post blood meal are accepted to be infectious for hepatocytes. Sporozoites of the same age were used from the different Pf strains for the same liver donor i.e. the same mosquito batch was used per biological replicate. Between the biological replicates, the sporozoite ages were different but within the established infectious range of day 16-20. This is added in the text in green on page 19.

ii)Statistics: Double check significance. n=3 (I safely assume this also means from three different donors), so the mean values of average values (infected cells, schizont size,...) should be used for statistics as opposed to
making every single well or schizont a value. There is considerable doubt that, while in D-F (and likely also in A,B) the mean values are shown, statistics are done with every value. No worries; the effects are nice, so very ok if p values are not as minimal.

To also accommodate a similar request from third reviewer, we have both modified Figure legends for more clarity and provided an Excel sheet containing the statistical tests done per Figure panel so P values (along with other parameters can be viewed for full transparency).

iii) Where is GS in the panels? It's mentioned in the text, but no reference to GS+ or GS- stains is made in the figure. Maybe it's a color problem (GS and Pf would both be in green), but then it would have to be adjusted in the main text.

Just curious: since panel 2E is so striking, is there a representative IFA as in 1C to show multiple Pf infections of GS+ cells? If yes, that would make an awesome cover image.

We have adjusted it in the text Line 110-112 – yes hGS and Pf are both mouse antibodies whereas hGK is rabbit. We are limited as actin and DAPI are required to stain up the border and the nuclei of the host cell respectively. This does mean that we cannot use hGS in the same staining.

Apologies – as these pictures are taken on the high content microscope (not known for their high resolution), we do not feel comfortable supplying it as cover image. However, to improve transparency and better understanding of how distribution is calculated (request of reviewer 1), we have created supplementary figures 2 and 3 (NF135 and NF175 respectively) where schizonts in zone 3 hepatocytes are enclosed in yellow squares. In many of these images, you can see double infections in Z3/hGK-/hGS+ cells.

-Figure 3: minor changes. I suggest to include size bars in panels A to C and move E to I to Supplement. All in all, an interesting and unexpected observation.

We have improved the scale bars in panels A to C so it is more obvious.

-Figure 4: Very high drug concentrations, that is greater than 100 micromolar (this is getting towards the mM range), are always suspicious. Can the authors think of alternative methods? If viability of host cells were impaired at these high concentrations it'll be a rather trivial outcome.

Indeed, the applied high drug concentrations are based on maximal efficacy against Mycobacterium tuberculosis (bacterial) GS; there is currently no specific inhibitor against eukaryotic/human GS. Furthermore, as mentioned in the discussion, Z3 hepatocytes are involved in drug metabolism so the GS inhibitors may be degraded/metabolized possibly requiring a higher concentration for efficacy.

The number of host cells in a monolayer can be used as a proxy for the viability of host cells. As such there is no evidence for toxicity in the culture at the concentration tested; if this would have been the case, one may expect a reduction in number of schizonts which is indeed not observed (Figure 4C). We have uploaded the total number of host cells for the control and treated conditions (new Figure 4D) to show that grossly, host cell viability is not impaired. Alternative approaches to reduce hGS function are exploratory and may include 1) RNA silencing methods and 2) generation of a hGS gene KO in hepatic organoid/cell line. However, both approaches have not been established in Pf liver stage models.

**Minor points:**

-Line 30: .. ‘and may delineate novel pathways for intervention strategies’ is not really clear from this study, particularly since all three Pf lines develop to some extent in zone I hepatocytes as well. I don’t think it necessary to make this statement.

With all due respect to the reviewer, we disagree. Given the differences in zonal hepatocytes, it may be intuitive to assume that parasite interaction with hepatocytes may be different depending on the zone and therefore requiring different therapeutic strategies.
-References: improve citations. In general, I was rather disappointed with the References. Many unrelated, weird, or outdated citations are shown.
Ref. 1: update to 2019 report; Ref. 2 is weird. Refs. 17-19 don't make sense, Ref. 17 is SRB1 in HCV infection, Ref. 18 has been proven false; 19 should probably be: Silvie et al. 2003 (https://doi.org/10.1038/nm808). These are just examples. The authors should carefully go through and reconsider their selection of citations.
We have carefully gone through the citations and they are now up to date. Although respectfully we could not find published evidence of Kaushansky et al's EphA2 to be proven false.

-Line 73: ‘freshly isolated zonal hepatocytes’. Well, yes and no. They obviously stem from hepatic lobes, but over 48 hours 2-D culture and uniform oxygen they retain some features (incl. GK/GS distribution), but may have changed some properties. This is not meant to belittle this really important work, but I feel this should be stated more precisely in the introduction.
Thanks for the comment — we agree and have amended by removing the word “zonal” from the sentence.

-Line 87: the little ‘test-the-reviewers’ error: Z3 (instead of Z2).
We have amended.

-Lines 268-279. The speculation on anti-cell death functions makes many reference to murine models, while this study successfully distances itself from this work. I would just omit this small part entirely.
We have amended the paragraph to omit the murine models i.e. the following text has been deleted “Upon detection of the invader, host cells are programmed to create an “anti-microbial defense state” in their cytoplasm. Recently, it has been shown that rodent malaria parasites upregulate cellular inhibitors of apoptosis proteins (cIAPs) in infected hepatocytes. While prevention of cell death by the presence of intracellular parasites has been established in rodent malaria models, it remains unknown for Pf where the development period in hepatocytes takes much longer”.
However, with all due respect, we do prefer to speculate on the possible effects of parasite hGS sequestration for the host hepatocyte, given the recent literature by Villar and colleague that abnormal glutamine intracellular concentrations can lead to cell death.

Reviewer #2 (Significance (Required)):
This is a novel and very interesting finding that should inspire the infectious disease community. Its implications are certainly beyond malaria research and extend to other liver pathogens, most notably hepatitis viruses. The study also raises the critical awareness of how distinct metabolic profiles of superficially similar host cells modulate pathogen maturation (and by extension host cell selection). The findings of different infectivity of Pf strains are both striking and puzzling, and certainly worthy of follow-up analyses as well. All in all, I really like the work. Yes, there are limitations and open questions, but that's what ground-breaking work is all about. I anticipate that this study will inspire many follow-up experiments, and I consider it a big step forward towards a better molecular understanding of the enigmatic long Pf liver stage maturation process. I don't want to embark on details, but I also believe there are many important translational implications, maybe not so much in terms of anti-infectives development, but in revisiting data from the field.

REFEREES CROSS-COMMENTING
I liked the paper and believe that only clarifications on the reproducibility and a much better work on the references (they were very poorly put together) are needed. There were a total of four donor tissues from surgery (which is a major task every time), and I simply can’t envisage yet how the biological replicates from the donors were synchronized with the three Pf sporozoite strains so they were all done in parallel (the only way of comparing infectivity). Even more so, since GS IFAs
(to show uptake by parasites) and assays with GS inhibitors were done, which to me sounds like they were done consecutively. Maybe a scheme showing the work flow can also clarify this.

**Reviewer #3 (Evidence, reproducibility and clarity (Required)):
Most of the experiments are well designed, performed and analysed, although proper statistical analysis should be used for many of the figures.

**Specific comments**

1. Line 41: merozoites are not release directly into the circulation (Sturm et al, Science, 2006). This should be modified.
   Although it has indeed be shown for the rodent malaria Pb that merozoites are released into circulation via merosomes (referenced in Sturm et al), this has not (yet) been shown in case of P. falciparum. There are many biological differences between the Pb model and Pf. We therefore prefer not to follow the reviewer’s comment and do include this statement until formerly proven.

2. Ref 17 is not appropriate since it relates to HCV. Silvie et al, (Nat Med, 2013), Yalaoui et al, (Cell Host Microbe, 2008; Rodrigues et al, Cell Host Microbe, 2008) should be used instead.
   We thank the reviewer for pointing out our omission: Ref 17 is now Silvie et al (Nat Med, 2013), 18 is Kaushansky (Science 2015), 19 is Yalaoui (Cell Host Microbe 2008) and 20 is Rodrigues (Cell Host Microbe 2008) on line 72.

3. Figure 1: Mann-Whitney test is not adequate, since they compare 3 sets of dat in the same graph. Authors should use Kruskal Wallis test, followed by a post-test (both tests should be indicated).
   Yes, we have performed the Kruskal Wallis test followed by a Dunn’s multiple comparisons test (see Fig 1D tab in the Statistical Excel Sheet).

4. Figures 2A, B, C, D and E. Unless the authors have demonstrated that schizont distribution follow a normal distribution (and this should be stated in the text and/or figure legends, they should use Kruskal Wallis test, followed by a post-test.
   We have performed a two-way RM ANOVA followed by a Tukey’s multiple comparisons test. Please see the Statistical excel sheet.

5. Figure 2: To get a better idea on the infectivity of the different sporozoites, the authors should mention in the figure legend, the mean schizont numbers +/- SD per well. This will help to compare with other previously published study.
   Mean schizont numbers are included for each line +/- SD per 96 well (see lines 136-138).

6. Figure 4 and many of the supplemental figures: same as above for the statistical analysis.
   We have performed the relevant tests shown in the Excel Sheet for Statistical Tests.

7. The authors should discuss the possibility to ameliorate culture conditions by either enriching on hGS hepatocytes, or engineering hGS cells.
   We have addressed this in comments to reviewer 1 (enriching hGS via activation of wnt signalling) and 2 (overexpression or knocking out hGS in organoid cells). Unfortunately, while both methods will most likely increase the expression of hGS, it will also affect the expression of other genes and involved technologies are quite exploratory and preliminary.

**Reviewer #3 (Significance (Required)):
Yang et al have studied the effect of hepatic zonality on P. falciparum sporozoite infectivity. This is a timely and important study which uncovers an important aspect of sporozoite biology. It will be of interest for scientists working in malaria but more generally in the field of infectious diseases, metabolism and hepatology.

REFEREES CROSS-COMMENTING

I had nothing to add to more. This is an important work and the improvements will be mainly textual

Thank you for your invitation and consideration of our revised manuscript. We look forward to hearing back from you.

Yours sincerely

References

1. Langenberg, M.C.C., et al., Controlled Human Malaria Infection with Graded Numbers of Plasmodium falciparum NF135.C10- or NF166.C8-Infected Mosquitoes. Am J Trop Med Hyg, 2018. 99(3): p. 709-712.
2. McCall, M.B.B., et al., Infectivity of Plasmodium falciparum sporozoites determines emerging parasitemia in infected volunteers. Sci Transl Med, 2017. 9(395).
3. Tonon, F., et al., In vitro metabolic zonation through oxygen gradient on a chip. Sci Rep, 2019. 9(1): p. 13557.
4. Allen, J.W. and S.N. Bhatia, Formation of steady-state oxygen gradients in vitro: application to liver zonation. Biotechnol Bioeng, 2003. 82(3): p. 253-62.
5. Ng, S., et al., Hypoxia promotes liver-stage malaria infection in primary human hepatocytes in vitro. Dis Model Mech, 2014. 7(2): p. 215-24.
6. Llorca, O., et al., The three-dimensional structure of an eukaryotic glutamine synthetase: functional implications of its oligomeric structure. J Struct Biol, 2006. 156(3): p. 469-79.
7. Monga, S.P., Role of Wnt/beta-catenin signaling in liver metabolism and cancer. Int J Biochem Cell Biol, 2011. 43(7): p. 1021-9.
8. Monga, S.P., Role and regulation of beta-catenin signaling during physiological liver growth. Gene Expr, 2014. 16(2): p. 51-62.
9. Cadoret, A., et al., New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. Oncogene, 2002. 21(54): p. 8293-301.
Thank you for submitting a revised version of your Review Commons manuscript. It has now been evaluated by two of the original referees, who find that their main concerns have been addressed and are now in favour of publication of the manuscript. There now remain only a few editorial issues that have to be addressed before I can extend formal acceptance of the manuscript.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

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Referee #1:

I have reviewed the changes made and feel the manuscript is much improved. The authors have addressed my own comments, and those from the other referees, very well and I have no further edits to suggest. I congratulate the team on a terrific and important piece of work.

Referee #2:

In the revised version the authors have convincingly amended the manuscript with important information, including a description of the experiments performed per clinical isolate. My points have been adequately addressed, with a very minor exception:
- For some unknown reason the author appear not to follow the recent literature re: Ref.18. While the paper was already dubious (one day delay in patency as claim for an important role of a host cell receptor) the claims have now been convincingly rejected by Langlois et al., 2018 (doi: 10.1371/journal.pone.0200032). This is arguably a minor point, but since the present paper is likely to be influential to the field, it should also be up to date and avoid citing erroneous publications. All in all, this is a major step forward towards a better understanding of the host contributions to successful human colonization after transmission of P.falciparum sporozoites
Please find enclosed our revised manuscript titled “Zonal human hepatocytes are differentially permissive to *Plasmodium falciparum* malaria parasites” by Yang et al submitted as Research Article to the EMBO Journal.

We have addressed your queries. Briefly:

1. **Reviewer 2’s comment**: We have critically assessed Reference 18 (Kaushansky et al 2015) and the new reference (Langlois et al 2018): Langlois et al was intentionally not referenced as studies were only performed in rodent malaria models whereas the Kaushansky study also included human *Pf* parasites. Langlois and colleagues indeed convincingly disprove Kaushansky’s claims in rodents, but *Pf* findings may formally still be valid as the corresponding *Pf* experiment was not conducted. Notwithstanding, we agree with the reviewer that Langlois’ findings do raise doubts about on the validity of the *Pf* findings. We have therefore chosen to remove the
Editor accepted the manuscript.
### Reporting Checklist for Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal’s authorship guidelines in preparing your manuscript.

#### A- Figures

1. **Data**
   - The data shown in figures should satisfy the following conditions:
     - the data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner;
     - figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way;
     - graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates;
     - if n<5, the individual data points from each experiment should be plotted and any statistical test employed should be justified;
     - Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. **Captions**
   - Each figure caption should contain the following information, for each panel where they are relevant:
     - a specification of the experimental system investigated (e.g. cell line, species name);
     - the analysis and methods used to carry out the reported observations and measurements;
     - an explicit mention of the biological and chemical entity(ies) that are being measured;
     - an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner;
     - the exact sample size (n) for each experimental group/condition, given as a number, not a range;
     - a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.);
     - a statement of how many times the experiment shown was independently replicated in the laboratory;
     - definitions of statistical methods and measures:
       - common tests, such as t test (please specify whether paired vs. unpaired), simple $\chi^2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
       - are two-sided or two-tailed;
       - are there adjustments for multiple comparisons?
       - exact statistical test results, e.g., $P$ value = x but not $P$ value < x;
     - the exact sample size (n) for each experimental group/condition, given as a number, not a range;
     - the assay(s) and method(s) used to carry out the reported observations and measurements;
     - an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

### B- Statistics and general methods

| Question | Answer |
|----------|--------|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | The sample size per experiment is n=3, each with 2-3 technical replicates (depending on the amount of hepatocytes isolated) per condition. Although this is less than n>5, it is in accordance to the malaria field’s practice where three independent experiments showing the same phenotype is the best we could have done. |
| 2. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 3. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
| 4. Were any steps taken to minimise the effects of subjective bias when allocating animals/samples to treatment (e.g. randomisation procedure)? If yes, please describe. | NA |
| 5. For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 6. Were any steps taken to minimise the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 7. For animal studies, include a statement about blinding even if no blinding was done. | NA |
| 8. For every figure, are statistical tests justified as appropriate? | Yes |
| 9. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | D’Agostino and Pearson Normality test was used to predict if the population measured is normally distributed (p=0.05 for the schizont data). The test result is in the excel sheet titled “statistical test” |
| 10. Is there an estimate of variation within each group of data? | Yes, this is provided in the Excel sheet containing all the statistical tests. |
C- Reagents

2. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., proteomics data: [PRIDE PXD000208, 1]. We recommend consulting the ARRIVE guidelines [see link list at top right] and depositing their model in a public database such as Biomodels (see link list at top right).

4. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., proteomics data: [PRIDE PXD000208, 1]. We recommend consulting the ARRIVE guidelines [see link list at top right] and depositing their model in a public database such as Biomodels (see link list at top right).

5. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., proteomics data: [PRIDE PXD000208, 1]. We recommend consulting the ARRIVE guidelines [see link list at top right] and depositing their model in a public database such as Biomodels (see link list at top right).

D- Animal Models

6. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

D- Animal Models

6. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

7. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

F- Data Accessibility

14. Provide a "data availability" statement at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PHOEBE PXID00820 etc.). Please refer to our author guidelines for 'Data Deposition'.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines [see link list at top right] and submit the CONSORT checklist [see link list at top right] with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

17. For data accessibility, please ensure that all data is properly cited and deposited in a public repository. Please refer to our author guidelines for 'Data Deposition'.

G- Dual use research of concern

18. Could your study fall under dual use research restrictions? Please check biosecurity documents [see link list at top right] and list of select agents and toxins (APHIS/CDC) [see link list at top right].