Journal of Clinical and Translational Research
Peer review process file 02.201603.001

Evidence against a role for platelet-derived molecules in liver regeneration after partial hepatectomy in humans

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Review timeline:

Received: 25 April, 2016
Editorial decision: 23 May, 2016
Revision received: 23 June, 2016
Editorial decision: 31 July, 2016
Published online ahead of print: 3 August, 2016

1st editorial decision:

Date: 23-May-2016

Ref.: Ms. No. JCTRes-D-16-00012
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Dear Dr. Lisman,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.
For your guidance, reviewers' comments are appended below.
If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.
Your revision is due by Jun 06, 2016.
To submit a revision, go to http://jctres.edmgr.com/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

*****Editor comments*****

Editor-in-chief:

Dear Dr. Lisman and co-authors,

Your paper that questions the validity of some of the studies published on the role of platelets in liver regeneration has been reviewed by 5 experts. I deliberately chose to have the paper reviewed by so many different experts in the field because of the nature of your study. It is imperative that papers reporting data that deviates from putative contentions are correct in all respects to the maximum extent possible. Of the 5 recommendations we have received, 2 rendered a reject verdict, 1 rendered a major revision verdict, and 2 advised only minor revisions before your paper could be accepted.

After careful consideration with several editorial board members, we are advising major revisions in accordance to the reviewer comments provided below. We think it is of critical importance that presently published data in this field is placed under a magnifying glass and scrutinized on the basis of valid premises, in this case provided through your experimental results. Please address the reviewers' comments in a point-by-point manner, rebutting there where you deem necessary.

Lastly, I would like to draw your attention to the following individual points:

1. Some of the "Achille's heel' critiques made by the reviewers valid and reflect limitations of your study. In addition to downtuning the strong language at specific points, as suggested by some of the reviewers, I kindly ask you to include a paragraph highlighting the limitations of your study. This will bring your paper into a better balance. Readers will be made aware of the important (potential) implications of your data while concomitantly being able to contextualize the findings.

2. It is no problem that you reuse your clinical data sets, as pointed out by reviewer 2, to drive a point home. It is imperative, however, that you clearly and explicitly indicate that the data were taken from a cohort that was previously published on.

3. Reviewer 5 is requesting you to perform a sham operation or include an experimental laparotomy group in a patient cohort. I kindly point you in the direction of the Declaration of Helsinki and various literature on medical ethics. Please note that JCTR will not publish such experiments performed in human subjects.

*****Reviewer comments*****

Reviewer #1: This manuscript focuses on a clinically relevant and certainly very interesting topic, namely the relevance of platelets during liver regeneration. The author performed an

advanced and time consuming protocol with blood collections from the portal as well as the liver vein and also perioperative blood withdrawal. The authors report results that differ from previously published reports and draw the conclusion that this is evidence against a role of platelet derived molecules during liver regeneration.

Major:
Within this translational research project, the authors unfortunately used a suboptimal plasma preparation method. Indeed, it has been demonstrated that citrate anticoagulation processed at room temperature does substantially suffer from in vitro platelet activation, which results in a significant elevation of platelet stored growth factors in the measured sample.(PMID:21896999) With this preparation method the authors are unfortunately unable to measure circulating platelet derived growth factors as they are partially released in vitro during processing. This substantially reduces the relevance of the presented findings, specifically as the authors present contradicting results to published studies that found significant perioperative changes in platelet stored growth factor when using an optimized plasma preparation method. Specifically, as the authors were unable to observe a postoperative increase in the platelet stored protein TSP-1 in patients undergoing hepatectomy (now indisputable documented within several studies) suggests that there is a relevant activation of platelets during processing. Indeed reported plasma vales within this manuscript are substantially higher than reported for optimal plasma processing. Accordingly, while the time consuming work and the translational idea of this project should be appreciated, the differences observed by the authors to previously published results seems to be a result of suboptimal plasma preparation and concomitant in vitro platelet activation.

The power of the analysis seems to be fairly weak, specifically as the authors aim to compete with studies including more patients.
No clinical outcome is presented - power of the study certainly limits analyses concerning outcome.

Minor
Why are intra platelet growth factor contents not illustrated for portal and LV
A table should be included describing the basic characteristics of included patients and controls.

Reviewer #2: Authors study levels of platelet derived growth factors and other related proteins involved in liver regeneration. Human plasma samples were obtained from the portal vein and hepatic veins before and at the end of major right liver resections (n=17). Further plasma samples were analyzed at day 1, 3, 5, 7 and 1 month after surgery. Several proteins were quantified in plasma ± platelets by ELISA: VEGF (vascular endothelial (G) growth (F) factor), HGF (hepatocyte GF), FGF (fibroblast GF), PDGF (platelet derived GF), TSP1 (thrombospondin 1). Patients without surgery and with pancreaticoduodenectomy (pancreatic head resection) served as controls. Authors found comparable amount of platelet related proteins in both groups at different timepoints. Authors conclude, that compared to animals, following such liver resections in humans platelet activation and protein release has no major impact on liver regeneration.

Though the impact of platelets and serotonin on liver regeneration has been studied extensively

in animals, their role in human liver regeneration is currently under debate.
Authors analyze levels of platelet-related proteins, which were previously shown to be involved in liver regeneration in human plasma the context of major right hepatectomies. Though this is an interesting approach, some aspects should be clarified:

1) Authors perform ELISA from plasma samples at the end of surgery and at day 1, though this might be appropriate time points in mice, maybe in humans authors miss the significant protein drop in the first hours after hepatectomy. This should be discussed.

2) Surgical approaches, are done completely different in mice, standard hepatectomies for example do not involve tissue transection, since the lobulated mouse liver is just ligated at the pedicle of each resected lobe. Authors should also discuss such technical aspects as possible causes for having different results in human and animals.

3) To provide more data to support the conclusion, measurement of plasma serotonin would be of interest for the reader.

4) I would also suggest to modify the title of the manuscript, since at least two publications already exist with almost the same wording of the title.

5) Did authors perform liver biopsies after hepatectomy and perform histology (i.e. staining for platelets) or consider measurment of such platelet derived substances on mRNA level?
6) I would be interested in the level of platelets in both groups. The amount of circulating platelet derived proteins is related to the number of platelets and should be demonstrated.

7) How was liver function after hepatectomy and did all resected livers regenerate well enough? I would be interested in outcome of patients.

8) Why did authors add 2 patients with extended hepatectomies, while majority of included patients underwent standard right hepatetcomy? Did authors observe an impact on results?

9) The figures could be improved by replacement of columns by box plots, to improve visibility of each single values, since the IQR seems quite big.

10) The ability of the pancreas to regenerate has been shown in the past in mice. The relation to serotonin has also been shon frequently. Authors should provide alternative explanations why both groups have the same amount of those proteins in their discussion.

Reviewer #3: 1. Whereas the Introduction is well written, the Abstract is virtually too long for this paper.
2. Reading from the graphs, the data would thought to be normally distributed and are presented in a mean±SD fashion. However in the text (first sentence in the statistics chapter), it is written that 'values are expressed as medians (with interquartile ranges)'.
3. It might be interesting if authors would present the number of the platelets from both afferent and efferent liver veins in the result.

Reviewer #4: The authors present a very interesting study complementing their previous publication "Evidence against a role of serotonin in liver regeneration in humans".

I have a few comments:

- If the authors stored their samples, it would be of interest to assess IGF-1, EGF and SDF-1a concentrations.
- MAJOR COMMENT: The authors did not use PRP but platelet lysate. This should be corrected throughout the manuscript and in figures.
- Many grammatical and orthographic errors remain throughout the text (f.ex. "Transaction" instead of "Transection")

P3L5-7 : First 2 sentences have to be reformulated
P3L46: Please describe the outcomes
P4L12-14: Many in vitro studies demonstrated that platelet-derived growth factors stimulate hepatocyte proliferation (f.ex. PMID:17688880). This assertion is therefore not accurate.
P4L22: Please give precisions about "lack of appropriate controls"
P6, section "Patients": Please define "extended right hepatectomy"
P7L1: How was the platelet count determined?
P7L5-7: How was the platelet-poor plasma prepared? By double centrifugation of what component? MAJOR COMMENT: How did the authors prevent platelets to be activated and release their content by the centrifugation speed applied?
P9, section "Patient characteristics": MAJOR COMMENT: Please provide a "Patients Demographics" Table as well as an inclusion flowchart
P9, L21-39: Please define "just after the completion of the parenchymal transection"
P10: MAJOR COMMENT: The finding that plasma levels of endostatin are lower in patients undergoing PH than PPPD is of importance as endostatin has anti-angiogenic properties (publication discussing the interactions between platelets and LSEC: PMID: 26169159). This finding should be discussed in the Discussion section.
P11L3: Please replace "no evidence of consumption" by "no evidence of release"
P11L3: Please replace "vital" by a more appropriate term
P11L8-13: There were differences (f.ex. endostatin)
P11L23-25: MAJOR COMMENT: Serotonin was NOT assessed in the present study, please reformulate the sentence to remove any mention to serotonin
P11: The discussion about serotonin refers to a previous publication; this should not be done in the present paper
P12L3-7: MAJOR COMMENT: Platelet activation (expression of CD62P, release of PF4, etc.) was not assessed in the present study.

Reviewer #5: In the article, "Evidence against a role for platelet-derived molecules in liver regeneration after partial hepatectomy in humans", the authors compared the levels of platelet-derived growth factors at various time points between the post-hepatectomy and PPPD patients. They first measured the levels from portal and hepatic vein prior and just after completion of parenchymal transection, and could not find any difference at any time points between the post-hepatectomy and PPPD patients. Further, they measured sequential levels of the growths factors

in PRP, PPP and inside platelets up to POD 30, and they also could not find significant differences between the two groups. Based on these results, they insisted against a role of release of growth factors in stimulation of liver regeneration. This is well-written and interesting, however several concerning points should be addressed to the authors.

Major points

1. It is not clear what the authors want to say in this article. Are they against "platelet is a promoter of liver regeneration" or against "the mechanism of release of platelet-derived molecule at the liver after hepatectomy"? They have to keep consistency from the title, abstract, to the conclusion in the main text.
2. It is difficult to lead their conclusion only from these "indirect" evidences.

First, we disagree with setting PPPD patients as a control group. The role of platelets after PD is not clear. There might be consumption of platelets also in the pancreas (whatever reason is) after PPPD, and this might be the reason that the levels were the same at any time points between the two groups. Thus, comparing the levels of platelet-derived growth factors between the two groups (Figure 2, 3, and 4) does not make any sense. We consider the control should be sham-operation group (or experimental laparotomy).

Second, there should be difference in the number of platelets inside the liver before and after hepatectomy (more platelets are floating inside the liver after hepatectomy), and we disagree with simply comparing the pre- and post PV and HV levels (Figure 1) support their conclusion. Moreover, they also have to consider the levels of hepatic artery (30% of blood flow in the liver). To be consistent with their conclusion, we would recommend them to show "direct" evidence that platelets are not activated by immunostainings from the biopsy specimens prior and post transection.

However, I personally have an interest in Figure 1 if they did the same approach in figure 2-4, measure growth factor levels in PRP and PPP, and calculate levels inside the platelets (including hepatic artery).

3 . HGF is not contained in human platelets (Nakamura T, Nature. 1989).

Minor points.

1. Introduction is too long and redundant.
2. Results section, third paragraph, "Levels of growth factor and angiogenetic molecules in plasma and---." This paragraph should be divided properly.
3. Conclusion section, "Selective sequestration of angiogenic proteins" needs to be explained more.

Authors' rebuttal:

Response to the comments of the editorial team

We appreciate the interest of editors and reviewers in our manuscript on platelet-derived growth factors and liver regeneration. We realize that our data are controversial, and that there are issues with our dataset in terms of sample size and our blood processing protocol. Nevertheless, given that firm data on a role of platelet-derived proteins in platelet-mediated liver regeneration is lacking (reviewed by us in

http://www.bloodjournal.org/content/early/2016/06/13/blood-2016-04-692665), we appreciate the opportunity to submit a revised version of our manuscript.

Questions and comments of editors and reviewers are listed below, and our responses are indicated as 'bullets'. Within the revised manuscript file, adjusted text is highlighted using red font.

1. Some of the "Achille's heel' critiques made by the reviewers valid and reflect limitations of your study. In addition to downtuning the strong language at specific points, as suggested by some of the reviewers, I kindly ask you to include a paragraph highlighting the limitations of your study. This will bring your paper into a better balance. Readers will be made aware of the important (potential) implications of your data while concomitantly being able to contextualize the findings.

- This point is well taken. We have significantly adjusted the tone of the manuscript and have added a limitations paragraph in the discussion section.

2. It is no problem that you reuse your clinical data sets, as pointed out by reviewer 2, to drive a point home. It is imperative, however, that you clearly and explicitly indicate that the data were taken from a cohort that was previously published on.

- We don't think we have reused a clinical dataset (we only report levels of analytes that have not been reported previously), but we did report on this cohort of patients previously. We have altered the manuscript accordingly. We have chosen not to insert a table with patient characteristics as requested by reviewer 1, as these data have already been published, but would be happy to insert such as table would the editorial team would prefer this.

3. Reviewer 5 is requesting you to perform a sham operation or include an experimental laparotomy group in a patient cohort. I kindly point you in the direction of the Declaration of Helsinki and various literature on medical ethics. Please note that JCTR will not publish such experiments performed in human subjects.

Response to the comments of reviewer 1

Reviewer #1: This manuscript focuses on a clinically relevant and certainly very interesting topic, namely the relevance of platelets during liver regeneration. The author performed an advanced and time consuming protocol with blood collections from the portal as well as the liver vein and also perioperative blood withdrawal. The authors report results that differ from previously published reports and draw the conclusion that this is evidence against a role of platelet derived molecules during liver regeneration.

- We thank the reviewer for an insightful review and important technical comments. Although we do not fully agree with the reviewers' conclusion that technical issues prevent us from drawing meaningful conclusions, we do agree that procedures to process blood have been largely ignored in studies on platelet-derived molecules, and have therefore entered comments on this in the discussion section of the manuscript.

Major:
Within this translational research project, the authors unfortunately used a suboptimal plasma preparation method. Indeed, it has been demonstrated that citrate anticoagulation processed at room temperature does substantially suffer from in vitro platelet activation, which results in a significant elevation of platelet stored growth factors in the measured sample.(PMID:21896999)

- We are aware of the data the reviewer refers to that indicate substantial differences in plasma concentration of platelet-derived molecules with different protocols to separate plasma from whole blood. We unfortunately were not yet aware of this at the time this study was designed (patient inclusion started in January 2012).

With this preparation method the authors are unfortunately unable to measure circulating platelet derived growth factors as they are partially released in vitro during processing.

- Although it is obvious from the paper cited above that plasma levels of platelet-derived proteins are much higher when blood is processed by our protocol as compared to the CTAD protocol described by Starlinger and coworkers, the level of 'artifactual' platelet granule content release using our methodology is still limited. For instance, in experiments performed in our lab and in data presented by Starlinger and coworkers, thrombospondin levels in plasma are around 10-fold higher when blood is processed according to our protocol. However, the thrombospondin levels in plasma (~50 ng/ml using the Starlinger protocol, ~500 ng/ml using our protocol) are only a fraction of the levels in serum (~20.000 ng/ml, as reported by Starlinger, ~4000 ng/ml in our study). In other words, using the Starlinger protocol, thrombospondin levels in plasma are ~0.15-1.25% of the levels found in serum, while using our protocol this is ~1.5-12.5%. Thus, using our protocol, at least 87.5% of the thrombospondin in circulation is within platelets. Thrombospondin levels in platelets thus are not very different between the blood processing methods (the difference approaches the analytical variation of the test) regardless of the processing method used. As the primary outcome of our study is levels of various proteins within platelets, and as we have treated all samples identically, our conclusions would not have been different when using the Starlinger protocol to process blood. We do appreciate that the plasma levels we report are erroneously high. However, as we have processed all blood samples identically, it is not unconceivable that all samples are affected by artifactual platelet activation to the same extent. We have entered comments on methodology in the discussion section of the manuscript.

This substantially reduces the relevance of the presented findings, specifically as the authors present contradicting results to published studies that found significant perioperative changes in platelet stored growth factor when using an optimized plasma preparation method.

- We object to the phrase 'optimised plasma preparation method'. It has been well established that platelets are immediately activated upon venipuncture, and it is likely that any blood processing method is confounded to some extent by ex-vivo platelet activation. Starlinger and coworkers did not 'optimise' plasma preparation, but rather tested several methods and measured levels of selected proteins in plasma. It may very well be that 'true' in vivo plasma levels of proteins examined are even lower than those reported by Starlinger using CTAD/4 degrees processing of blood.

Specifically, as the authors were unable to observe a postoperative increase in the platelet stored protein TSP-1 in patients undergoing hepatectomy (now indisputable documented within several studies) suggests that there is a relevant activation of platelets during processing.

- Again, we concur there is slight platelet activation with substantial increases in plasma levels of platelet derived proteins, but do note that this activation affects all samples tested. To our knowledge the increase in plasma thrombospondin following partial hepatectomy in humans has only been described by 2 studies of a single research group. The fact that we do not find an increase in our study may be related to many factors other than 'suboptimal sample processing'

including differences in case-mix, surgical and anesthesiological techniques, or may be related to the relatively low numbers in our study.

Indeed reported plasma vales within this manuscript are substantially higher than reported for optimal plasma processing. Accordingly, while the time consuming work and the translational idea of this project should be appreciated, the differences observed by the authors to previously published results seems to be a result of suboptimal plasma preparation and concomitant in vitro platelet activation.

The power of the analysis seems to be fairly weak, specifically as the authors aim to compete with studies including more patients.
No clinical outcome is presented - power of the study certainly limits analyses concerning outcome.

- Our study was designed to test the hypothesis that specific consumption of platelet-derived molecules would occur following liver resection. The study was specifically designed as proof-of-concept study without the aim to link levels of platelet-derived proteins to clinical outcome. We acknowledge that the size of our study is limited, and have added comments on this to the results section of the manuscript, but do feel our results justify the conclusion that in our cohort of patients little evidence for liver resection-specific alterations in platelet-derived molecules occur.

Minor
Why are intra platelet growth factor contents not illustrated for portal and LV A table should be included describing the basic characteristics of included patients and controls.

- We were only able to take small volumes of blood from the portal vein and liver vein, and were simply only able to process blood to PRP, and did not have sufficient blood to also generate and examine PPP. A comment on this has been inserted in the results section. As details of this cohort have been described previously, we chose not to insert a table with subject characteristics but rather refer to published data. However, if reviewer and/or editorial team would prefer to duplicate the table with subject characteristics, we will be happy to insert.

Response to the comments of reviewer 2

Reviewer #2: Authors study levels of platelet derived growth factors and other related proteins involved in liver regeneration. Human plasma samples were obtained from the portal vein and hepatic veins before and at the end of major right liver resections (n=17). Further plasma samples were analyzed at day 1, 3, 5, 7 and 1 month after surgery. Several proteins were quantified in plasma ± platelets by ELISA: VEGF (vascular endothelial (G) growth (F) factor), HGF (hepatocyte GF), FGF (fibroblast GF), PDGF (platelet derived GF), TSP1 (thrombospondin 1). Patients without surgery and with pancreaticoduodenectomy (pancreatic head resection) served as controls. Authors found comparable amount of platelet related proteins in both groups at different timepoints. Authors conclude, that compared to animals, following such liver resections in humans platelet activation and protein release has no major impact on liver regeneration.

Though the impact of platelets and serotonin on liver regeneration has been studied extensively in animals, their role in human liver regeneration is currently under debate. Authors analyze levels of platelet-related proteins, which were previously shown to be involved in liver regeneration in human plasma the context of major right hepatectomies. Though this is an interesting approach, some aspects should be clarified:

- We thank the reviewer for a thoughtful review and useful comments

1) Authors perform ELISA from plasma samples at the end of surgery and at day 1, though this might be appropriate time points in mice, maybe in humans authors miss the significant protein drop in the first hours after hepatectomy. This should be discussed.

- We agree and have added comments on this in a new paragraph in the discussion section indicating limitations of the study.

2) Surgical approaches, are done completely different in mice, standard hepatectomies for example do not involve tissue transection, since the lobulated mouse liver is just ligated at the pedicle of each resected lobe. Authors should also discuss such technical aspects as possible causes for having different results in human and animals.

- We apologise for giving the reviewer the impression that we think our results in humans are not in line with data from experiments in mice. This is not at all the case. To our knowledge, there is no direct evidence showing that platelet-derived factors drive platelet-mediated liver regeneration in mice. It is certainly proposed by many that release of proteins from platelets in the liver remnant drive liver regeneration, but experimental evidence supporting either release of these proteins or involvement of platelet-derived molecules in directly stimulating liver regeneration is lacking. We have adjusted the text in the introduction to better clarify this, and we kindly refer the reviewer to a recent review by our group on this topic: http://www.bloodjournal.org/content/early/2016/06/13/blood-2016-04-692665

3) To provide more data to support the conclusion, measurement of plasma serotonin would be of interest for the reader.

- We have previously reported on serotonin levels in this cohort, and have adjusted the text to better clarify this.

4) I would also suggest to modify the title of the manuscript, since at least two publications already exist with almost the same wording of the title.

- We are only aware of one paper with a similar title, which is a letter to the editor from our group on serotonin. As the title of the paper fully reflects the message we aim to convey, we prefer to keep the title as it is despite overlap with the serotonin letter.

5) Did authors perform liver biopsies after hepatectomy and perform histology (i.e. staining for platelets) or consider measurment of such platelet derived substances on mRNA level?

- We unfortunately did not take liver biopsies, but did isolate RNA from platelets in these samples. Results from our studies on RNA will be reported elsewhere.

6) I would be interested in the level of platelets in both groups. The amount of circulating platelet derived proteins is related to the number of platelets and should be demonstrated.

- This point is well taken, and we have added these data to the revised version of the manuscript.

7) How was liver function after hepatectomy and did all resected livers regenerate well enough? I would be interested in outcome of patients.

- None of the patients had clinical evidence of post-hepatectomy liver failure.
  The extent of regeneration has not been assessed routinely (this would have required imaging studies, for which there was no clinical indication). Given the limited size of our study, we prefer not to perform analyses linking our findings to outcome, particularly since outcome of partial hepatectomy is influenced by multiple factors.

8) Why did authors add 2 patients with extended hepatectomies, while majority of included patients underwent standard right hepatetcomy? Did authors observe an impact on results?

- In order to obtain sufficient patient recruitment, inclusion criteria of the study were standard and extended right hepatectomies. We did not observe clear differences between the 15 patients with standard and the 2 with extended right hepatectomies, but do note the numbers are too small for meaningful analyses.

9) The figures could be improved by replacement of columns by box plots, to improve visibility of each single values, since the IQR seems quite big.

- This point is well taken – we have adjusted all figures accordingly.

10) The ability of the pancreas to regenerate has been shown in the past in mice. The relation to serotonin has also been shon frequently. Authors should provide alternative explanations why both groups have the same amount of those proteins in their discussion.

- We are aware of the data showing pancreas regeneration following pancreatetcomy. However, in humans at least two studies have reported a lack of pancreas regeneration after pancreas resection (Diabetes 2008 Jan; 57(1): 142-149, Pancreas. 1999 Oct;19(3):310-3). We have commented briefly on this in the discussion section.

Response to the comments of reviewer 3

1. Whereas the Introduction is well written, the Abstract is virtually too long for this paper.

- We have shortened the abstract slightly, but fail to see how we can shorten it even further without compromising on the message.

2. Reading from the graphs, the data would thought to be normally distributed and are presented in a mean±SD fashion. However in the text (first sentence in the statistics chapter), it is written that 'values are expressed as medians (with interquartile ranges)'.

- In our initial report, we showed medians with interquartile ranges (and only the IQR above the median). It is not possible to predict distribution from such graphs, so we are slightly confused by the reviewers' comment. Upon request of another reviewer, we have changed all graphs into box plots, and the reviewer will now be able to appreciate the requirement for reporting and statistically analyzing medians rather than means.

3. It might be interesting if authors would present the number of the platelets from both afferent and efferent liver veins in the result.

- We agree, and have added these data to the revised version of the manuscript.

Response to the comments of reviewer 4

Reviewer #4: The authors present a very interesting study complementing their previous publication "Evidence against a role of serotonin in liver regeneration in humans".

- We appreciate the thoughtful review and thank the reviewer for useful comments.

I have a few comments:

- If the authors stored their samples, it would be of interest to assess IGF-1, EGF and SDF-1a concentrations.

- We fully agree that additional analyses would be of interest. However, unfortunately, too little plasma sample is left for additional analyses, as we have used plasma samples not only for this study, but also for two other studies on hemostasis following liver resection (Br J Surg. 2016 Mar 23. doi: 10.1002/bjs.10107 and Aliment Pharmacol Ther. 2015 Jan;41(2):189-98), and a study on serotonin (Hepatology. 2015 Sep;62(3):983).

- MAJOR COMMENT: The authors did not use PRP but platelet lysate. This should be corrected throughout the manuscript and in figures.

- We did use PRP, and adjusted the manuscript to make this more clear.

- Many grammatical and orthographic errors remain throughout the text (f.ex. "Transaction" instead of "Transection")

- Checked and modified.

P3L5-7 : First 2 sentences have to be reformulated
- Done

P3L46: Please describe the outcomes
- Sentence adjusted

P4L12-14: Many in vitro studies demonstrated that platelet-derived growth factors stimulate hepatocyte proliferation (f.ex. PMID:17688880). This assertion is therefore not accurate.
- We respectfully disagree. The fact that platelet-derived growth factors stimulate hepatocyte proliferation has indeed been well established, but studies showing that this mechanism contributes to platelet-mediated liver regeneration in vivo are lacking. We have substantially rewritten the introduction to be clearer on this.

P4L22: Please give precisions about "lack of appropriate controls"
- Adjusted

P6, section "Patients": Please define "extended right hepatectomy"
- Adjusted

P7L1: How was the platelet count determined?

- Adjusted

P7L5-7: How was the platelet-poor plasma prepared? By double centrifugation of what component?

- Adjusted

MAJOR COMMENT: How did the authors prevent platelets to be activated and release their content by the centrifugation speed applied?

- Please see our response to comments of reviewer 1 and the extensive comments inserted on this issue in the discussion section..

P9, section "Patient characteristics": MAJOR COMMENT: Please provide a "Patients Demographics" Table as well as an inclusion flowchart P9, L21-39: Please define "just after the completion of the parenchymal transection"

- We have chosen not to insert a table with patient characteristics, as these data have already been published, but would be happy to insert such as table would the reviewer and editorial team would prefer this. Instead we have now referred to our published papers.

P10: MAJOR COMMENT: The finding that plasma levels of endostatin are lower in patients undergoing PH than PPPD is of importance as endostatin has anti-angiogenic properties (publication discussing the interactions between platelets and LSEC: PMID: 26169159). This finding should be discussed in the Discussion section.

- Agree and adjusted.

P11L3: Please replace "no evidence of consumption" by "no evidence of release"

- Adjusted

P11L3: Please replace "vital" by a more appropriate term

- Adjusted

P11L8-13: There were differences (f.ex. endostatin)

- Adjusted to clarify this

P11L23-25: MAJOR COMMENT: Serotonin was NOT assessed in the present study, please reformulate the sentence to remove any mention to serotonin

- Adjusted

P11: The discussion about serotonin refers to a previous publication; this should not be done in the present paper

- The serotonin data were reported in a letter to the editor. We feel we do need to discuss these data here to some extent.

P12L3-7: MAJOR COMMENT: Platelet activation (expression of CD62P, release of PF4, etc.) was not assessed in the present study.

- Agreed and adjusted.

Response to the comments of reviewer 5

Reviewer #5: In the article, "Evidence against a role for platelet-derived molecules in liver regeneration after partial hepatectomy in humans", the authors compared the levels of platelet-derived growth factors at various time points between the post-hepatectomy and PPPD patients. They first measured the levels from portal and hepatic vein prior and just after completion of parenchymal transection, and could not find any difference at any time points between the post-hepatectomy and PPPD patients. Further, they measured sequential levels of the growths factors in PRP, PPP and inside platelets up to POD 30, and they also could not find significant differences between the two groups. Based on these results, they insisted against a role of release of growth factors in stimulation of liver regeneration. This is well-written and interesting, however several concerning points should be addressed to the authors.

- We thank the reviewer for a thoughtful review and appreciate comments and suggestions

Major points
1. It is not clear what the authors want to say in this article. Are they against "platelet is a promoter of liver regeneration" or against "the mechanism of release of platelet-derived molecule at the liver after hepatectomy"? They have to keep consistency from the title, abstract, to the conclusion in the main text.

- We apologise our message was unclear. We have substantially rewritten introduction and discussion (also based on comments from other reviewers) to better explain that we do think platelets are drivers of liver regeneration, but that evidence for a role of platelet-derived proteins in this process is uncertain at present.

2. It is difficult to lead their conclusion only from these "indirect" evidences.
First, we disagree with setting PPPD patients as a control group. The role of platelets after PD is not clear. There might be consumption of platelets also in the pancreas (whatever reason is) after PPPD, and this might be the reason that the levels were the same at any time points between the two groups. Thus, comparing the levels of platelet-derived growth factors between the two groups (Figure 2, 3, and 4) does not make any sense. We consider the control should be sham-operation group (or experimental laparotomy).

- We respectfully disagree. We chose PPPD as a control group as we could then compare two groups both with 1) cancer, 2) major abdominal surgery, 3) comparable age. The difference between the groups is a lack of liver regeneration in the PPPD group, so differences between the groups would likely be attributable to liver regeneration. There is a decrease in platelet count after any major surgical procedure, so the remark that there may be platelet consumption in the pancreas, although valid, holds true for any major procedure. A sham operation for the purpose of scientific hypothesis testing is unethical (and it is also unclear in which patients the reviewer is suggesting to perform such a sham operation), and we genuinely believe that our comparator is much more informative than a comparison between a hepatectomy and a sham operation would be. Nevertheless, based on the comment of another reviewer we acknowledge that the PPPD control group has theoretical issues and we have inserted comments on this in the revised version of the manuscript.

Second, there should be difference in the number of platelets inside the liver before and after hepatectomy (more platelets are floating inside the liver after hepatectomy)

- We are only aware of one small human study examining this (Hepatology. 2016 May;63(5):1675-88). We thus feel there is little evidence to support the notion that there ‘should be a difference’.

, and we disagree with simply comparing the pre- and post PV and HV levels (Figure 1) support their conclusion.

- We do not understand why the reviewer disagrees. We have tested the hypothesis that there is consumption of the proteins examined by the liver remnant (and found no evidence for this). To investigate gradients of proteins over a solid organ to look at consumption or production of proteins by the organ has been previously reported in literature (e.g., Am J Transplant. 2009 Jul;9(7):1574-84).

Moreover, they also have to consider the levels of hepatic artery (30% of blood flow in the liver). To be consistent with their conclusion, we would recommend them to show "direct" evidence that platelets are not activated by immunostainings from the biopsy specimens prior and post transection.

- We think that it may theoretically be possible that there are differences in platelet protein composition between the hepatic artery and portal vein, but we are unsure what would cause such a difference. We did not take these samples, so we will not be able to examine this. We appreciate the suggestion to look at liver biopsy specimens, but also these were unfortunately not taken in this study.

However, I personally have an interest in Figure 1 if they did the same approach in figure 2-4, measure growth factor levels in PRP and PPP, and calculate levels inside the platelets (including hepatic artery).

- We were only able to take small volumes of blood from the portal vein and liver vein, and were only able to process blood to PRP, and did not have sufficient blood to also generate and examine PPP. We thus do not have these data.

3. HGF is not contained in human platelets (Nakamura T, Nature. 1989).

- The reference provided does not state or show that HGF is not present in human platelets. It only states (without showing data!) that HGF levels in human platelets are much lower than in rat platelets. In fact, a study from the same group has reported HGF in human platelets (Proc. Nati. Acad. Sci. USA Vol. 83, pp. 6489-6493, September 1986).

Minor points.

1. Introduction is too long and redundant.

- The introduction has been substantially rewritten.

2. Results section, third paragraph, "Levels of growth factor and angiogenetic molecules in plasma and---." This paragraph should be divided properly.

- Adjusted

3. Conclusion section, "Selective sequestration of angiogenic proteins" needs to be explained more.

- Adjusted

2nd editorial decision:

Date: 31-Jul-2016

Ref.: Ms. No. JCTRes-D-16-00012R1
Evidence against a role for platelet-derived molecules in liver regeneration after partial hepatectomy in humans
Journal of Clinical and Translational Research

Dear Dr. Lisman,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

*****Editor comments*****

Dear authors,

All the reviewer reports about your article have been received by the JCTR editorial board. Because of the nature of your clinical results, which contradict putative dogma in liver regeneration mechanisms, we have invited 5 reviewers with different subspecialties to critically appraise your work. Of the 5, 3 reviewers find your work acceptable for publication in current form, while 2 reviewers argue that the paper should be rejected on the basis of technical flaws in methodology.

After careful deliberation with one of my associate editors and juxtaposition of the modifications made to those that had been requested, we decided to accept the paper for publication.

This decision does not come lightly, as we are going against the expert opinion of 2 equally prestigious scientists in the field. Nevertheless, we rendered a positive verdict for several reasons. First, we felt that you very carefully balanced the issues at hand in mainly the Discussion section. The main critiques that the reviewers used as basis for their negative decision are explicitly addressed. The readers will therefore be able to contextualize your results and determine for themselves which arm of the scale weighs more and proceed accordingly.

Moreover, the reviewer comments and your rebuttal will be published as metadata on the JCTR website, which will provide additional resolution and depth to the philosophical disagreement. Second, your paragraph on the limitations of the study is very frank and extensive, which adds fuel to the entire discussion while dampening the hardness of the conclusions. Lastly, Reviewer 1 argues that the data are “dangerous” and “confusing” to the uneducated reader, which we certainly sympathize with but do not necessarily agree with. It is good that this work and your other publications in very reputable journals caution against a one-sided mechanistic paradigm. We believe that your data broaden the perspective rather than narrow it and pave the way for more focused research and additional validation of the results, preferably by other groups. The path to truth is a very dynamic process that is often forged by opposing forces. In this light, we embrace the contrasting results and look forward to others corroborating or refuting your data. Publishing your study therefore serves as an impetus for the acquisition of more robust evidence.

Congratulations with your study and kindest regards,

Michal.

*****Reviewer comments*****

Reviewer #1: We thank the authors for their specific reply:

Unfortunately, as we believe, the authors illustrate the perioperative time course of growth factors in a descriptive manner, while they ultimately used an inappropriate plasma preparation. Despite the fact that the authors aimed to give some explanations how their processing might still be appropriate, we still believe that these data on platelet poor plasma are of minor relevance (explanations given below), specifically as they aim to question previously published results (as already stated clearly in the titel of the manuscript) with an optimized preparation method that might ultimately allow the observation of biologically relevant processes.

With this suboptimal preparation the authors do not reflect in vivo plasma values of growth factors released by platelets. In fact the values the authors report are presumably largely dependent on absolute platelet counts. The association of for example TSP-1 levels with platelet counts is nicely illustrated when combining Figure 1 and Figure 4- when you observe the only significant increase of TSP-1 in PPPDP patients at the day you find platelet counts substantially higher in the PPPDP group.
Further, it is well known that platelets decrease after hepatectomy as well s other types of surgery. This activation of platelets during processing will mask biological processes. Specifically when you aim at reflecting very slight changes as expected prior as compared to after the liver. This is further supported by the fact that the authors are unable to detect previously observed changes of growth factors that are presumably masked by in vitro activation of platelets during suboptimal processing. Furthermore the DuoSet ELISA by R&D Systems is indeed less sensitive that the commercially available precoated plates. While we understand that these are more costly, measuring very slight changes of growth factors should be performed by

the most sensitive test. Certainly, even if all samples are processed the identical way, small biologically relevant effect will not be observed as in vitro activation will by far exceed the absolute in vivo secreted values.

What however is certainly interesting, is that PRP and platelet adjusted TSP-1 levels are significantly lower after surgery in patients undergoing liver resection - this however would argue for a specific release from platelets - and therefore against the hypothesis the authors are trying to make. The authors comment this with: Thrombospondin 1 levels within platelets were consistently higher in the PPPD group compared to the partial hepatectomy group, but no changes in platelet thrombospondin 1 levels occurred over time. - We do not agree with this statement. When looking closely at figure 3 and 5 one can observe that TSP-1 levels seem to increase after surgery in PPPDP patients while they decrease after partial hepatectomy - finally reaching statistical significance on postoperative day 1.

Also the authors do not present clinical outcome measures but only the time course of growth factor levels so that a clear association with postoperative liver function recovery cannot be determined.

Taken together, still the author used an inappropriate plasma preparation to specifically question observations from a study that used appropriate plasma preparation. Moreover, the data concerning TSP-1 in PRP and platelet adjusted PRP rather supports the hypothesis of platelet granule release after partical hepatectomy and provides evidence against this hypothesis.

Concerning the reply of the authors on processing issues: "We unfortunately were not yet aware of this at the time this study was designed" - this does certainly not justify to publish results that are likely to be in part affected by in vitro artifacts and this is specifically true if these results are in clear contrast to previously published results using optimized plasma preparation methods.

Reviewer #2: This is the first revision of a manuscript on evidence against a role of platelets-derived molecules in liver regeneration after hepatectomy in human livers.
Authors provide now further details, requested by the reviewers. The impact of platelets and serotonin on liver regeneration has been studied and authors discuss here their role in human liver regeneration. Several points, raised by the reviewers have been included in the manuscript. Authors have modified their discussion and significantly improved the overall message.

Reviewer #3: Dear Editor(s),
I have no further questions concerning the revised paper.

Reviewer #5: In the article, "Evidence against a role for platelet-derived molecules in liver regeneration after partial hepatectomy in humans", the authors did their best to revise the article. However, we still consider their results are too poor to reach the scientific evidence.
Number of the cases is too small to reach the negative conclusion. Evidences the author presented are indirect. In order to conclude that platelets are not activated in the liver, the authors should take biopsy samples and show direct evidences that platelets are not activated.

Furthermore, the authors doesn't take importance of considering arterial flow, but theoretically 30% of flows are coming from the artery, and it might add significant amount of growth factors.

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