Manuscript WJSC 42776
“Improved guided bone regeneration by combined application of unmodified, fresh autologous adipose derived regenerative cells and plasma rich in growth factors: A first-in-human case report and literature review”
by O. Solakoglu, W. Götz, M.C. Kiessling, C. Alt, C. Schmitz and E. Alt

Point-by-point reply to the comments and suggestions of the editor and the reviewers:

Editor

Step 1: Verify the accuracy of general information for your manuscript

We have verified the accuracy of general information for our manuscript.

Step 2: Manuscript revision deadline

We informed the journal immediately after receiving notification about the need for revising our manuscript that the complexity and amount of modifications requested by the reviewers makes it impossible to submit our revised manuscript within one week. However, we were able to resubmit our revised manuscript no later than 17 days after receiving notification about the need for revising our manuscript.

Step 3: Login and download the revision-related documents

We have downloaded all revision-related documents.

Step 4: Revise the manuscript
Please update your manuscript according to the Guidelines and Requirements for Manuscript Revision and the Format for Manuscript Revision for your specific manuscript type: ‘Case Report’.

We have updated our manuscript according to the Guidelines and Requirements for Manuscript Revision and the Format for Manuscript Revision for our specific manuscript type: ‘Case Report’.

Specifically, we have (according to the instructions provided by the Editor in the edited version of our original manuscript)

- carefully and thoroughly addressed all comments and suggestions of the reviewers (outlined in detail below),
- structured the *Abstract* as requested (in track record),
- reduced the *Core Tip* to less than 100 words as requested,
- changed the Case Presentation as requested,
- reorganized the Introduction section in order to provide detailed suggestions from each expert participating in the consultation to the patient,
- changed the two parts "Case report" and "Result" into "Final diagnosis", "Treatment" and "Outcome and Follow-up" as requested,
- changed the *Conclusion* section as requested, and
- slightly modified all text that was flagged in the CrossCheck report as being identical with earlier publications (named "primary sources" in the CrossCheck report).

All minor changes are highlighted in track record in File “01-42776-Revised manuscript”; more substantial changes are highlighted in red in the latter file.

With regard to the CrossCheck report we would like to point out the following:

- The greatest similarity between our original manuscript and a primary source (Haenel et al., 2018; Reference #64 in our original manuscript) was only 3%. The senior author of the study by Haenel et al.

(2018) (Dr. Eckhard U. Alt) is also the senior author of the present manuscript. Furthermore, the second-greatest similarity between our original manuscript and a primary source (Friedmann et al., 2015; Reference #61 in our original manuscript) was only 2%. The senior author of the study by Friedmann et al. (2015) (Dr. Werner Götz) is co-author of the present manuscript. Moreover, for three primary sources (www.iti.ch, Kiessling et al., 2013; Sternecker et al., 2018; not cited in our original manuscript) the similarity to our original manuscript was 1%. First and senior authors of the study by Kiessling et al. (2013) (Dr. Maren C. Kiessling, Dr. Christoph Schmitz) are co-authors of the present manuscript; the same applies to the senior author of the study by Sternecker et al. (2018) (Dr. Christoph Schmitz). Accordingly, most probably repeated writing of very similar sentences and paragraphs (particularly in the Materials and Methods section because of the use of identical methods) by the same authors had caused the similarities flagged in the CrossCheck report.

- All other similarities between our original manuscript and primary sources in the CrossCheck report were <1%. In our view some of them should not be considered similarity that must be modified. For example, the CrossCheck report flagged the words "weeks (A,B,E,F,I,J,M,N) ... weeks (C,D,G,H,K,L,O,P)" in the legends of Figures 10 and 11 as being the only identical parts of our original manuscript compared with the website www.prolotherapy.com, and specified that 36 words would be identical. In this regard it is of note that 1) the identical parts were obviously not 36 words (rather the software found the XXX "A,B,E,F,I,J,M,N" and " C,D,G,H,K,L,O,P" in both documents), and 2) the content of the website www.prolotherapy.com is completely unrelated to the topic of the present study or the fields of research of the authors of this manuscript. Accordingly, we have not modified the legends of Figures 8 and 9 in our revised manuscript (Figures 10 and 11 in our original manuscript).

Review 03471268:

Authors wrote the report about new combination approach of guided bone regeneration. I consider that this method is meaningful for the clinical use, because UA-ADRCs are easier to use than other MSCs.

We are grateful for this comment by the reviewer. Indeed, the argument that UA-ADRCs are easier to use in the clinic than other MSCs has been our major motivation to perform this study.

Although it is a single case report, they compare the 2 method by using left side and right side. I suggest some points needed to be corrected.
<Major points>

- Authors mentioned that previous method need to be improved. However, its reason is not written clearly. Previous method would be not so much satisfactory for clinical use because of strength or durability. It is also needed to be mentioned which kind of adverse event occur after the conventional approach. And if possible, it is needed to be mentioned whether this new approach reaches satisfactory level or not.

To address this comment by the reviewer we have added the following paragraphs in the *Introduction* section of our revised manuscript (pages 6f, starting on page 6, line 10:

"*However, for the following reasons there is a need for developing novel strategies for improving GBR-MSA.*

First, autologous bone is considered to be the „gold standard" in GBR-MSA due to its osteogenic, osteoinductive and osteoconductive properties including lack of immunogeneicity[7,8]*. However, autologous bone grafts may show a number of disadvantages, such as increased operation time, donor site morbidity, post-operative discomfort, limitations in bone quantity and volume, unpredictable bone quality, reduced volume stability and fast resorption rate*[9-13]*. It may also be only effective under good recipient conditions. A further disadvantage is the missing possibility to create individual transplants being defect customized by using e.g. CAD-CAM technology. Furthermore, the intraoral amount of autologous bone is limited and therefore extraoral donor sites are needed for larger defects. Extraoral donor sites like the*

iliac crest may lead to further discomfort for the patient and an even higher morbidity rate compared to intraoral donor sites.

Second, while allografts have osteogenic properties, their probable osteoinductive and osteoconductive functions are still discussed contradictorily[7,11,14-16]*. Especially the osteoconductive property of bone allografts leads to a significant higher volume stability compared to autologous bone, although it is still resorbable and will be degraded into autologous bone. Demineralized freeze-dried bone allografts (FDBAs) were shown to be osteoinductive and osteoconductive due to the release of bone morphogenetic proteins*[17]*, although clinical outcomes comparing mineralized and demineralized FDBAs were reported to be similar*[18]*. Studies in vitro and animal investigations revealed osteoinductive functions of demineralized FDBAs by recruiting cells and ectopic bone formation*[19]*. Disadvantages of allogeneic materials may be a protracted vascularization, slow remodeling and resorption or longer time for osseointegration and the risk of immunogenic reactions*[15-18]*. Furthermore, the risk of graft infection may be higher compared to autologous bone*."

- All of Fig. 6, 7, and 8, are hematoxylin and eosin stain of biopsies. I couldn't understand why authors divided to 3 parts. Especially about fig.7, there is no detail description in main manuscript. If authors don't need to mention anything about fig.7, it may be not needed.

To address this comment by the reviewer we have collapsed Figs 6 and 8 into a single figure in our revised manuscript, and have removed Fig. 7 from our revised manuscript.

- In the result they wrote that osteoclast were increased in the sample with UA-ADRCs 6 weeks after the procedure. This effect is seemingly opposite reaction in the aspect of bone formation. Therefore, reason or estimated mechanism for this phenomenon is needed.

To address this comment by the reviewer we have added the following sentences in the *Discussion* section of our revised manuscript (pages 23f, starting on page 23, line 22:

"*The finding of middle-sized osteoclasts on the surface of newly formed and allogenic bone and on debris accumulations at six weeks after GBR-MSA, with a higher osteoclast density after the application of UA-ADRCs than without UA-ADRCs (Figs 6EF and 8A-D), was in line with earlier reports in the literature*[78,79] *about osteoclasts involved in bone remodeling (which was increased after application of UA-ADRCs compared to the situation without UA-ADRCs). This phenomenon must not be confused with foreign material resorption by multinucleated giant cells*[78] *that was not observed in the present study.*"

<Minor points>

- At line 1 on page 21, "osteblasts'4" would be a spelling mistake.

We have corrected this typo in our revised manuscript:

Review 03671529:

The article is devoted to the burning problem: developing an approach to improve guided bone regeneration (GBR) in oral surgery. Despite the fact that it was case report study design, histochemitry, justification for the use of the cell type is at a high level. But there are a few comments:

1) The article does not describe in detail the shortcomings of the "old" methods of restoring bone volume. Maybe the newly formed bone tissue was absorbed, the implants fell out or staggered, causing discomfort in patients?

This has been addressed in detail in our reply to Review 03471268 above.

2) In my opinion, it is necessary to characterize transplanted cells, since the proliferative potential can vary greatly from person to person and depends on age. And for subsequent studies to

obtain a comparable result, it is necessary to know the number of MSCs in samples, their ability to differentiate precisely in the osteogenic direction. The authors decided not to characterize the transplanted cells referring to the article, which characterized porcine UA-ADRCs, which is not entirely correct.

In general we agree with this reviewer. To address this comment by the reviewer we have added the following paragraphs in the *Discussion* section of our revised manuscript (pages 24f, starting on page 24, line 6):

"We did not characterize the UA-ADRCs isolated from lipoaspirate with the Transpose RT system and Matrase Reagent (both from InGeneron) in the present study. However, a very recent study compared cell suspensions that were obtained by isolating cells from lipoaspirate from 12 healthy donors using the Transpose RT system and Matrase Reagent (thereafter: "TRT-MR cell suspensions") with cell suspensions that were obtained by isolating cells from lipoaspirate from the same donors just mechanically (i.e., using the Transpose RT system but without Matrase Reagent) (thereafter: "TRT cell suspensions")[53]. It was found that the mean cell yield (numbers of cells/gram of processed lipoaspirate) was approximately twelve times higher in TRT-MR cell suspensions than in TRT cell suspensions ($p<0.001$), and cells in TRT-MR cell suspensions formed on average 16 times more colony forming units (considered to be an indicator of stemness) per g lipoaspirate than cells in TRT cell suspensions ($p<0.001$)[53]. Of note, the mean relative number of viable cells in TRT-MR cell suspensions (85.9% ± 1.1%; mean ± standard error of the mean) exceeded the proposed minimum threshold for the viability of cells in the SVF of 70 % established by the International Federation for Adipose Therapeutics and Science (IFATS)[80], whereas the mean relative number of viable cells in TRT suspensions (61.7% ± 2.6%) did not ($p<0.001$)[53]. On the other hand, cells in TRT-MR cell suspensions exhibited no statistically significant differences in the expression of regenerative cell-associated genes such as Oct4, Hes1 and Klf4 compared to cells in TRT cell suspensions[53].

The same study demonstrated that upon stimulation with specific differentiation media cells in TRT-MR and TRT cell suspensions were independently able to differentiate into cells of all three germ layers (i.e., into the adipogenic, osteogenic, hepatogenic and neurogenic lineages)[53]. The latter is in line with earlier findings that adult stem cells may obtain any of the three lineages but depend on constant induction of differentiation and re-confirmation by signals released and communicated from the local microenvironment (c.f., e.g.,[81-83]). If this information and confirmation is missing or ceases, adult stem cells stop differentiating[84,85]. In fact, only true stem cells are able to continue their expected differentiation pathway as supported by the local microenvironment (e.g.,[86-88]). This is one of several reasons why adult stem cell therapy with UA-ARDCs is very safe (e.g.,[89-91]). In contrast, safety concerns have been raised regarding stem cell therapy with cultured adult stem cells since with higher passages an increased rate of potential malignant transformation may occur[92-94].

A study on fresh, uncultured cells that were isolated from adipose tissue of pigs using the Transpose RT system and Matrase Reagent showed that approximately 40% of cells in the SVF were immunopositive for CD29 (thereafter: "$CD29^+$") and $CD44^+$[95], which are markers of adipose tissue-derived stem cells[50,96]. Furthermore, on average only 9% of the cells were $CD45^+$ (a marker of blood derived cells[50]), and on average only 11% of the cells were $CD31^+$ (a marker of endothelial cells[50]). Another study on fresh, uncultured cells that were isolated from adipose tissue of horses using a predecessor of the Transpose RT system (ARC system; InGeneron) and Matrase Reagent found highest relative gene expression of osteocalcin (a gene associated with the osteogenic lineage[97]) when investigating these cells for the relative gene expression of CD44, CD73, CD90, CD105, CD146 and CD166 (MSC surface markers), CD34 and CD45 (hematopoietic markers), CD31 (endothelial cell marker), type-1 collagen, PPARG2 (a gene associated with the adipogenic lineage) and osteocalcin[98].

Collectively, these data underline the osteogenic potential of the UA-ADRCs used in the present study."

On the other hand, we slightly disagree with this reviewer regarding the age-dependent proliferative potential of adipose-derived regenerative and stem cells. This issue was addressed in detail on page 22 of

our original manuscript. In fact, according to the latest research the patient's age may not have a significant impact on the cell yield, ADRC subpopulation composition, proliferation rates of ASCs and the capability of tri-lineage differentiation of the cells (c.f. Kokai et al., Aesthet Surg J 2017; 37: 454-463).

3) The authors indicate the advantage of using UA-ADRCs is that you do not need to wait and spend time on cultivation. But with such long periods of treatment and rehabilitation (34 weeks, 32 months), the time for cultivation does not play a significant role. Especially often before the bone grafting is necessary to treat the teeth, this time can be spent on standardization of the transplanted cells.

We did not indicate in our original manuscript that "*the advantage of using UA-ADRCs is that you do not need to wait and spend time on cultivation*". Rather, we pointed out the following:

Introduction section, page 7, lines 14ff of our original manuscript:

"*Compared to cultured and potentially modified ASCs, freshly isolated, unmodified ADRCs have the advantage of lower safety requirements because no culturing and no modification is involved*."

Discussion section, page 19, lines 6ff of our original manuscript:

"*…our decision to use UA-ADRCs rather than other types of cells (including ASCs, BMSCs, PDSCs, allogenic and/or modified ASCs/BMSCs, dental-derived mesenchymal stem cell-like cells … or induced pluripotent stem cells) was based on the fact that UA-ADRCs are the only type of cell that allows immediate usage at point of care, with the lowest safety concerns in cell-based therapy as no culturing or modification is required. This is fundamentally different from all other types of cells that have been considered for cell-based therapies in dentistry …*".

In order to further highlight the use of freshly isolated UA-ADRCs over the use of cultivated ASCs we added the following paragraph in the Discussion section of our revised manuscript (pages 24f, starting on page 24, line 23 (also mentioned above):

"The same study demonstrated that upon stimulation with specific differentiation media cells in TRT-MR and TRT cell suspensions were independently able to differentiate into cells of all three germ layers (i.e., into the adipogenic, osteogenic, hepatogenic and neurogenic lineages)[53]. The latter is in line with earlier findings that adult stem cells may obtain any of the three lineages but depend on constant induction of differentiation and re-confirmation by signals released and communicated from the local microenvironment (c.f., e.g.,[81-83]). If this information and confirmation is missing or ceases, adult stem cells stop differentiating[84,85]. In fact, only true stem cells are able to continue their expected differentiation pathway as supported by the local microenvironment (e.g.,[86-88]). This is one of several reasons why adult stem cell therapy with UA-ARDCs is very safe (e.g.,[89-91]). In contrast, safety concerns have been raised regarding stem cell therapy with cultured adult stem cells since with higher passages an increased rate of potential malignant transformation may occur[92-94]."

Accordingly, we have not modified our revised manuscript according to this comment by the reviewer.

Review 03671529:

The manuscript by Solakoglu Ö et al. describes a case report and literature review. In the case, a 79-year old patient was treated with a bilateral external sinus lift procedure as well as a bilateral lateral alveolar ridge augmentation. Bone healing of GBR-MSA/LRA is superior to that of MCBPA/PRGF-2/saline, and also no inflammation was observed. So guiding bone regeneration in maxillary sinus augmentation with a combination of UA-ADRCs, PRGF-2 and an OIS as performed shows effectiveness without adverse effects. Overall, it is an interesting story. There are some minor questions to be improved.

1. There are lack of sections about the methods and materials in the manuscript.

We assume that this comment by the reviewer is related to the other issues addressed by her/him (below). In fact, our original manuscript comprised a very detailed, 2400-word-long description of Materials and Methods, plus two tables and four figures showing details of the used methods.

Accordingly, we have not modified our revised manuscript according to this comment by the reviewer.

2. The components such as growth factors of plasma fraction 2 should be confirmed.

To address this comment by the reviewer we have added the following sentences in the *Discussion* section of our revised manuscript (pages 25f, starting on page 25, line 23:

"The content of PRGF-2 prepared using the PRGF-Endoret technology (BTI) was investigated in several studies in the literature. Most relevant to the results of the present study was the demontration of high amounts of growth factors in PRGF-2[98], i.e. on average approximately 14,000 pg/ml platelet derived growth factor AB (PDGF-AB), 46,000 pg/ml transforming growth factor β1 (TGF-β1), 220 pg/ml VEGF, 400 pg/ml hepatocyte growth factor (HGF), 83,000 pg/ml IGF-I and 600 pg/ml endothelial growth factor (EGF) (note that what was named "PRGF F3" in[98] is nowadays named "Fraction 2 of PRGF" according to BTI, and the latter terminology was used in the present study).

3. The isolated UA-ADRCs should be analyzed.

This has been addressed in detail in our reply to Review 03471268 above.