We would be grateful if you could consider this revised version of the manuscript entitled: "Highly active antiretroviral therapy (HAART) dysregulates proliferation and differentiation of human preadipocytes" (manuscript #: 33083) for publication in *The World Journal of Virology.*

The manuscript has been revised taking into account the reviewers' comments and suggestions. All the changes to the text are reported in yellow highlighting and have been described in details in the reply to the reviewers' specific comments. In the revision process we noticed that the grants previously acknowledged in the 'supportive foundations' section were erroneously reported and were therefore removed in the revised version.

Thank you for your consideration.
Sincerely,

Giuseppe Caso, MD, PhD

# REPLY TO REVIEWERS' COMMENTS

**Manuscript # 33083**

**Title: Highly active antiretroviral therapy (HAART) dysregulates proliferation and differentiation of human pre-adipocytes**

Reviewer #1:

1) *In "Patients and study design" section, more detailed information regarding the usage of the clinical samples is required. Did authors use the mixed samples of 10 donors or did they use each sample individually? Please describe precise information.*

**Fat samples from each donor were processed and each of the test conditions (drug combinations) were repeated with each donor sample. Data represent the mean ± SEM of the response of preadipocytes isolated from each individual donor. ~~combined for the analyses.~~ This was specified in the revised version of the manuscript (p5 ln 28-29).**

2) *In "Pre-adipocyte isolation and culture" section, more detailed information about "albumin" is required (in page 6, 2nd line from the bottom). Did authors use human serum albumin, bovine serum albumin or recombinant human albumin? Please describe precise information.*

**Bovine serum albumin (Sigma, St. Louis, MO) was used in all experiments. This has been added to the revised version of the manuscript (p7 ln 1).**

3) *Although the findings are solid and convincing, the results consist of only digital data. To enhance the credibility of the findings, it is strongly*

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*recommended that authors show some analog data as well; for example, photos of the Oil Red O staining of the typical finding in Fig. 2.*

**Photos of the Oil Red O staining of each treatment group are not available for this specific experiment. We have previously compared assessment of human preadipocyte differentiation obtained with GDPH assay and Oil Red O staining as used in this study with direct count of the number and percentage of differentiated cells from pictures of cultures stained with Oil Red O. We found that both techniques are comparable to direct count of differentiated cells obtained from pictures in detecting changes in preadypocyte differentiation resulting from treatment with antiretroviral drugs (Caso et al. Effect of ritonavir and atazanavir on human subcutaneous preadipocyte proliferation and differentiation. *Antiviral Res* 2010; 86: 137-143) or from aging (Caso G et al. Peripheral fat loss and decline in adipogenesis in older humans *Metabolism* 2013; 62:337-40).**

4) *In Fig. 1, authors performed MTT assay to evaluate cell proliferation. However, this assay also reflects cell viability. Therefore, it is strongly suggested that the term "proliferation" would be replaced by other words such as "proliferation/viability" and "MTT-reducing activity" and so on.*

**The reviewer is quite correct that the MTT assay measures viable cells. In our assays, all cultures were plated at the same initial cell number; therefore, MTT quantitation at 48 and 72 hours represents an increase or decrease in viable cells. ~~We have added a statement to the text (page line) to indicate this.~~ Since these are rapidly growing cells, the assumption is made that an increase or decrease in viable cells represents a change in the potential of cells to increase in number, i.e. proliferate. We have added a statement to the text ~~(page line)~~ to indicate this (p7 ln 9-11). This convention of using an increase or decrease in the number of viable cells as a measure of cell proliferation**

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**is widespread across many disciplines. For example, a PubMed search locates a very large number of publications, in a wide range of disciplines and journals where MTT assay is used as a measure of cell proliferation.**

5) *Grammatical concerns:*

- *In page 6, 4th line from the bottom, the phrase "… or same medium ….." should be corrected as "… or the same medium …..".*
- *In page 7, line 15, the word "500nm" should be corrected as "500 nm".*

**Both errors were corrected in revised version of the manuscript (p6– ln 30 and p7– ln 22).**

Reviewer #2:

1) *The study only concentrate on preadipocytes to adipocyte differentiation. It should include some other cell types, ie. fibroblast, as a control to show the specificity of those antiviral agents.*

**This experiment focused on predipocytes to address more directly the clinical problem of HAART-associated lipodystrophy. However, we agree with the reviewer that it would be very interesting and informative investigating the effect of antiviral agents on other cell types as well, including fibroblasts or other rapidly proliferating cells, which could also be affected by the drugs. Hopefully this point will be addressed in future studies.**

2) *The experimental design to test the hypothesis on those anti-viral agents on preadipocytes is reasonable, however lack some of the details. All data were presented in relative scale, so it is hard to judge the successfulness of their*

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*approach. It would be convincing if some Oil Red O stain picture for the differentiation of preadipocytes to adipocytes were presented.*

**This point was addressed in the reply to comment #3 of Reviewer 1.**
**In addition, we would like to clarify that both the GDPH assay and Oil Red O staining assays yield quantitative data. Results are ~~presented in the figures~~pictured in a relative scale (Fig. 1 and 2) to simplify comparison among groups and interpretation. Since the control (i.e. untreated cells) had the capacity to increase (proliferate) and differentiate the data from treated cells were normalized to this changing value for displaying purposes. Pictures of Oil Red O staining with different treatments would not quantify the changes in differentiation and thus would not facilitate statistical analysis of the data.**

3) *The authors should perform some RT-PCR analysis on the gene expression pattern in the cells to analyze the genes that are involved in adipogenesis.*

**RT-PCR analysis was not performed in this experiment. We have previously shown that expression of PPARγ, C/EBP1α, and aP2 genes progressively increases during differentiation of preadipocytes and that the presence of antiretrovirals (i.e. ATV and RTV) at concentrations inhibiting preadipocyte differentiation is associated with an inhibition of the increase in expression of these genes (Caso et al. *Antiviral Res* 2010; 86: 137-143).**

4) *There are some difference in their presentation of the nomenclature. The HSLS were referred to as HAART-lipodystrophy in Page 4, and HAART-associated lipodystrophy in Page 10 and 11.*
*It need to be consistent.*

**The term HAART-lipodystrophy was replaced with the term**

**HAART-associated lipodystrophy (p4 ln 2 and p11 ln 23).**

5) *The choice of wording is a little off. It would be better to describe the effect of the anti-viral agents to the differentiation to be suppression or inhibitory rather than depression.*

**The term depression was replaced in the revised manuscript.**

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