March 21, 2022

Lian-Sheng Ma

Editorial Office Director and Company Editor-in-Chief

World Journal of Gastroenterology

Dear Dr. Lian-Sheng Ma:

Thank you for your letter of March 8, 2022, informing that the Manuscript NO: 74137 titled "Involvement of Met receptor pathway in the aggressive behavior of CRC cells induced by PTHrP" has been found to be potentially publishable in the journal pending appropriate revision.

We have carefully reviewed the comments of the three reviewers, the Science editor and the Company editor-in-chief and have modified the manuscript in response to their suggestions. These modifications are highlighted in red in the revised manuscript. We appreciate this criticism which has contributed to improving the presentation of our work.

Detailed in the enclosed sheet there is an enumeration of the changes made in our manuscript. We hope that it will be possible for you to find our paper fully acceptable for publication in the World Journal of Gastroenterology.

. Yours sincerely,

Dr. Claudia Gentili

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Comments to the Editorial Office

As indicated below, we revised the manuscript according to the Editorial Office's comments and indications.

Comments to the Science editor

We appreciate the opinion of the Science editor regarding our work as an interesting paper. According to the suggestion, we now summarize the abstract.

Comments to the Company editor-in-chief

Thank you for considering that the manuscript is conditionally accepted. According to the suggestion, we now include the keyword "Colorectal cancer" in the manuscript title. Therefore, the new title of the manuscript is the following: "Involvement of Met receptor pathway in the aggressive behavior of colorectal cancer cells induced by PTHrP".

Also, we provide decomposable figures that are organized into a single PowerPoint file and add tables that are according with all requirements for the revised manuscript.

We confirm that all figures are original; so, we add the copyright information on the bottom right-hand side of each picture in PowerPoint (PPT).

Comment to the Reviewers and the Editors:

We thank the Reviewers, the Science Editor and the Company editor-in-chief for taking the time to revise our work. We indicate in this letter the pages and paragraphs of the revised

manuscript where we incorporated the modifications according with the suggestions of each reviewer.

Reviewer #1:

Reviewer #1 comment 1:

Major comments: 1. In HCT116 cells, they found total Met that PTHrP treatment for 30 minutes increases Met protein levels ($p<0.01$). However, protein expression diminishes at 3 ($p<0.05$), 10 ($p<0.01$) and 60 minutes ($p<0.01$) of exposure to the peptide. They explain that PTHrP increases Met mRNA levels at 15 minutes in HCT116 cells, why did not the 60 minutes group increase mRNA expression to improve protein level？How did PTHrP continuously active PTHrP/Met axis ？

Author response 1:

According to the suggestion of the reviewer, we wanted to analyze mRNA expression of Met by RT-qPCR at longer PTHrP exposure times (60 minutes or more) but unfortunately, due to the deadlines stipulated for the submission of this revised manuscript (14 days), it will not be possible to carry out new experiments. We hope that the reviewer understands this situation.

Nevertheless, and to satisfy the reviewer´s concern, we analyzed Met protein levels by Western blot and to that end, we stripped and reproved with anti-Met antibody several membranes where the electrotransferred proteins were from HCT116 cells treated with PTHrP for 5 and 20 hours. We used membranes from three independent experiments.

As the reviewer can see in the following **Figure 1** of this letter, at 5 hours of exposure to the peptide, Met protein levels continue to decrease, as observed at 1 and 3 hours of treatment. However, PTHrP increases Met protein expression at 20 hours of treatment. This result suggests that the cytokine activates Met in a range of time between 1 to 5 hours and as a consequence of this activation there is a decrease in its protein levels due to proteasomal degradation. Then, and in order to improve levels, PTHrP (in a range of time after 5 hours

and before 20 hours) would induce Met gene expression with the consequent increase in protein levels observed at 20 hours.

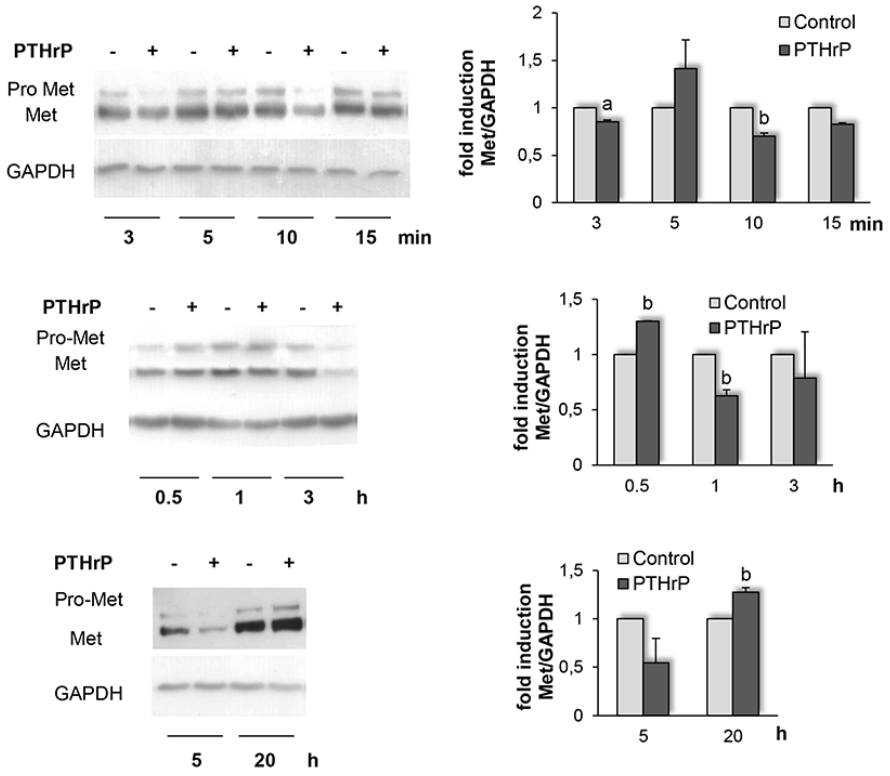

Figure 1. PTHrP modulates the protein expression of Met receptor in HCT116 cell line. Cells were treated with or without PTHrP 10^{-8} mol / L at different times. The protein levels of pro-Met (Met precursor) and Met (RTK mature form) protein levels by were analyzed by Western Blot to investigate whether the molecular mechanisms trigged by PTHR1 after binding of PTHrP are capable of modulating the expression of the mature form of the RTK Met in HCT116 cells. GAPDH protein levels were determined as control of the amount of proteins present in the membrane, since this protein is not substantially modified with the treatment by the cytokine. Graph bars represent the average of the results obtained from three independent experiments. $^{a}p < 0.05$; $^{b}p < 0.01$.

These results are now incorporated in the new Figure 1 of the revised manuscript. In addition, an appropriate comment is now added in Result section (Results, Section 3.1, page 16, paragraph 3; Section 3.4, page 18-19)

Regarding how PTHrP continuously active PTHrP/Met axis, we mentioned in the manuscript that Met activation at 1 hour is mediated at least by the ERK1/2 MAPK signaling pathway (Figure 3 of the original and revised manuscript). We found effects of PTHrP

mediated by these MAPKs in HCT116 cells at longer exposure times (Martín et al. 2018). The fact that in this work we found that Met remains activated for range of time greater than 60 minutes of PTHrP treatment could be due to the participation of some pathway/s that is/are effector/s of the action of these MAPKs. More studies are needed to confirm this hypothesis. An appropriate comment is now added in Discussion section of the revised manuscript (Discussion, page 26, paragraph 5)

Reviewer #1 comment 2:

In page 5 "Currently, two of the chemotherapeutic agents approved as first and second-line adjuvants in CRC are Oxaliplatin (OXA) and Irinotecan (CPT-11)[6], [7]. However, more than half of patients with stage II and III treated with these drugs relapse and die[8]. Thus, all these facts evidence the urgent need to elucidate the molecular mechanisms associated with two key aspects of therapy: its effectiveness and resistance". Two points needs to be clarified here: 1 Patients with Stage II and III need surgical treatment, 2 the first and second lines are palliative chemotherapy, not adjuvant chemotherapy.

Author response 2:

We appreciate the comments of the reviewer to improve our work. Both points are now clarified in the revised manuscript (Introduction section, page 7 paragraph 1)

Reviewer #2:

Reviewer #2 comment 1:

The experimental methods and cell lines used in this paper are relatively single, and the amount of existing data is not enough to support the conclusions drawn. Therefore, various experimental methods are usually used to verify the results on a variety of colorectal cancer cells.

Author response 1:

We appreciate the criticism of the reviewer. So, we have now performed new experiments with the aim to support the conclusion of this manuscript regarding that Met is involved in the modulation by PTHrP of the aggressive behavior of the CRC cell model studied by us. Taking into account that PTHrP promotes cell migration in HCT116 cells (Calvo et al. 2017), we now performed wound-healing assays to evaluate if Met signaling also participates in this cell process. We observed the wound healing in culture of confluent HCT116 cells to compare the migration between untreated cells and cells treated with PTHrP in the present or absent of SU11274 (the specific inhibitor of Met). The times of PTHrP exposition were selected according with our previously published work (Calvo et al. 2017). Representative photographs, taken at time point 0, 5 and 24 hours of the identical location and the quantification of the results of two separate experiments are shown in the following Figure of this letter (**Figure 2**). A significant enhancement in wound closure was detected in cells exposed to PTHrP or PTHrP plus DMSO (the vehicle of the inhibitor) compared to control values at 5 and 24 h; however, this effect is not observed in cells pre-treated with the Met inhibitor.

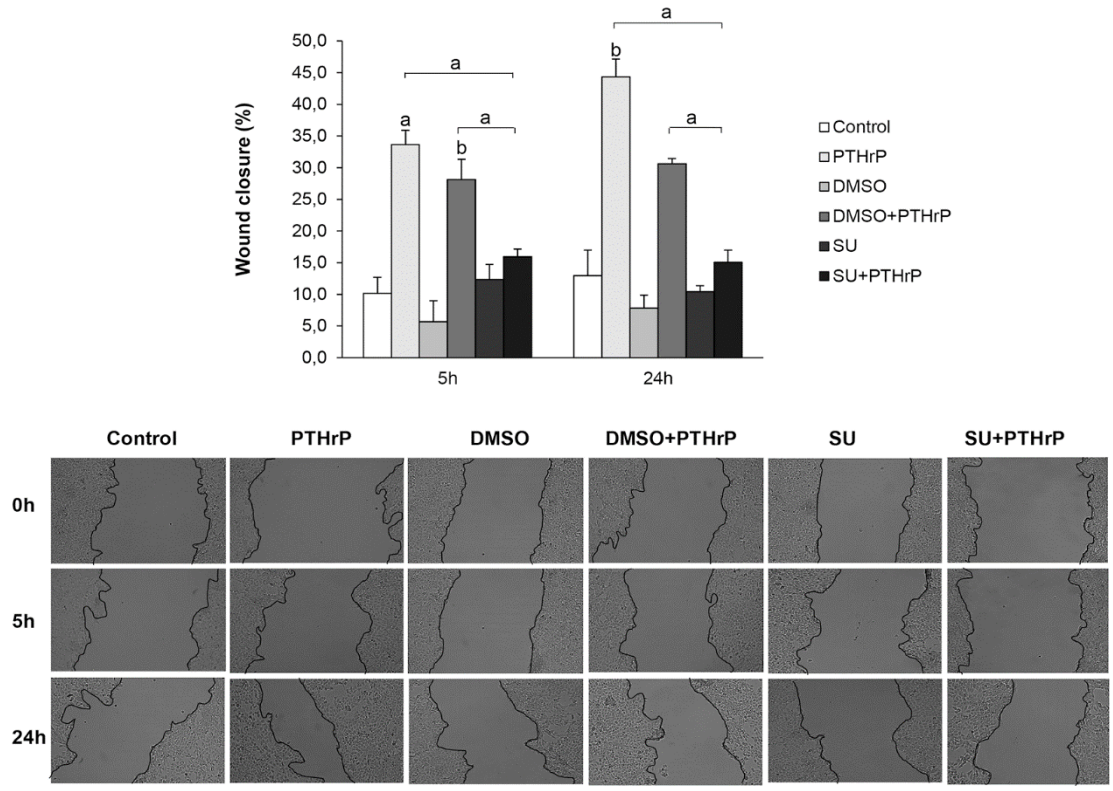

Figure 2. PTHrP promotes the wound closure in HCT116 cells through the Met signaling pathway. HCT116 cells were pre-incubated with SU11274, a specific Met inhibitor, for 30 minutes and then treated with or without PTHrP (10^{-8}M). The wound test was analyzed by Image J-NIH program. The images show that Met inhibition diminishes the wound closure promoted by PTHrP at 5 and 24h. A control with DMSO, the vehicle of the inhibitor, was performed. Graph bars represent the average of the results obtained from two independent experiments. $^{a}p < 0.05$; $^{b}p < 0.01$.

These results are now added in the revised manuscript as new Figure 5B. An appropriate comment is also incorporated in Result and Discussion sections (Result, Section 3.5, page 19-20, paragraph 2; Discussion section, page 26-27, paragraph 7). We also incorporated the assays performed in Materials and Methods section (Materials and Methods, Section 2.7 page 11)

We recently published results revealing that PTHrP is able to induce the epithelial-mesenchymal transition (EMT) in HCT116 cells (Carriere et al. 2021). The EMT is a key program of CRC that participates in the invasion, angiogenesis, and chemoresistance associated with metastasis (Loboda et al. 2011; Tsoumas et al. 2018). Based on this previous

work, herein we analyzed if PTHrP also induces this program through Met pathway. To that end HCT116 cells were incubated with SU11274, and then treated with or without PTHrP. The cells were observed under inverted microscopy after treatment with PTHrP for 5 and 24 h to analyze morphological changes associated with EMT. These times were selected because PTHrP at 24 h of exposition induces changes related with an effective transition to the mesenchymal phenotype in HCT116 cells (Carriere et al. 2021). The arrows in the **Figure 3** of this letter point morphological changes corresponding to the transition from polygonal structure to spindle-like structure observed when the cells were treated for 24 h with PTHrP or PTHrP plus DMSO. There were no changes in both, control cells and those treated with the inhibitor of Met. To further evaluate the phenotype related with a mesenchymal shape, we measured in the photomicrographs the minor and major axis using the Image J-NIH program. The **Table 1** and the bar graph reveal that under PTHrP action, the cells lose their epithelial characteristics. The increase of the relation between the major and minor axis indicates that the exposition with PTHrP for 24 h significantly increased the degree of HCT116 cells elongation, a typical feature of the mesenchymal shape. However, when the cells were pre-incubated with SU11274, the effects of the cytokine were reverted.

It has been shown that the loss of the expression of the epithelial marker E-cadherin is associated with EMT progress in CRC (Druzhkova et al. 2019; Gurzu et al. 2016). In our recent work (Carriere et al. 2021) we showed that in HCT116 cells PTHrP at 5 hours modulates E-cadherin and others epithelial markers as well as certain mesenchymal markers. Based on these morphological changes related to EMT and mediated through Met, we then explored by Western blot analysis if Met is involved in the modulation of E-cadherin protein expression by the cytokine. As the reviewer can see in the **Figure 3**, when the cells were pre-incubated with SU11274, the effects of the cytokine on E-cadherin protein levels were reverted.

Taken together, these findings suggest that PTHrP induces the EMT program in HCT116 cells through Met signaling pathway.

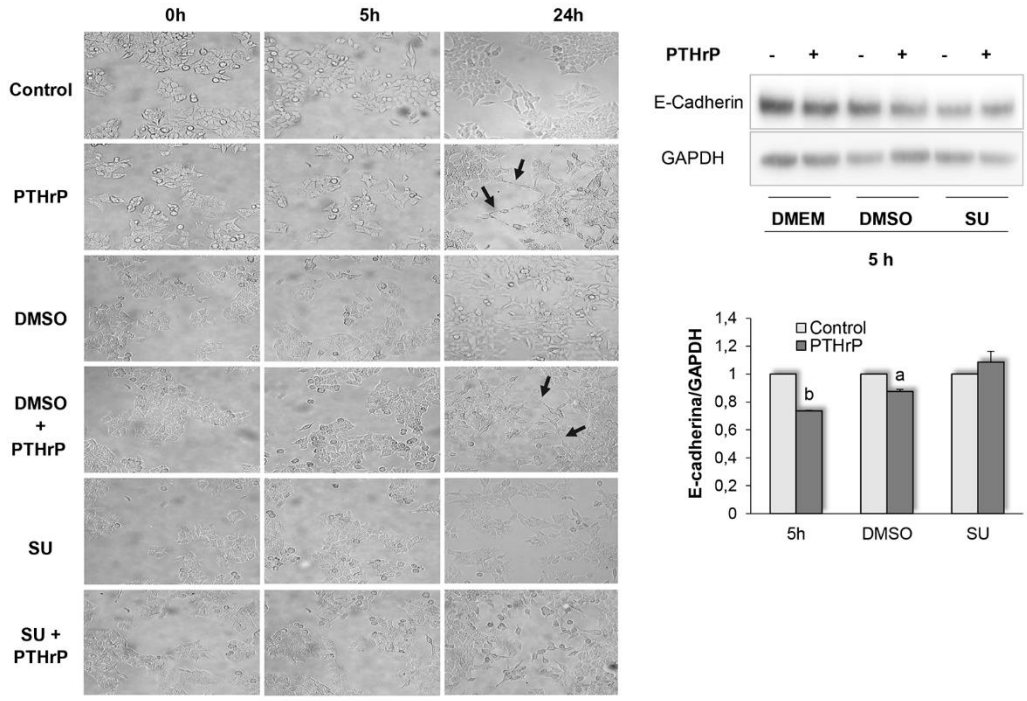

Figure 3. PTHrP promotes molecular and morphological changes in HCT116 cells through the Met signaling pathway. HCT116 cells were pre-incubated with SU11274, a specific Met inhibitor, for 30 minutes and then treated with or without PTHrP (10^{-8}M). E-cadherin protein levels were analyzed by Western Blot to investigate if Met is involved in the decrease of this protein induced by PTHrP in HCT116 cells. By the Image J-NIH program we performed the analysis of the parameters related to cell morphology. The arrows indicate the morphological changes corresponding to the transition from polygonal structure to spindle-like structure observed when the cells were treated for 24 h with PTHrP or PTHrP plus DMSO. In each experiment a control with DMSO, the vehicle of the inhibitor, was performed. $^{a}p < 0.05$; $^{b}p < 0.01$.

Table 1. Analysis of the morphological characteristics of the cells

Time	Image J Analysis of cells aspect radio (AR)	Area	Perimeter	Major	Minor	AR	Round
0h	Control	371608,35	83700,44	29714,67	15072,84	2,06	0,51
	PTHrP	445243,58	95072,71	32963,65	16999,08	2,12	0,55
	DMSO	401221,98	90350,81	31896,07	16001,69	2,19	0,53
	DMSO + PTHrP	507695,83	102479,62	35812,26	18038,25	2,02	0,53
	SU	540780,15	104863,69	33247,98	17729,93	1,87	0,53
	SU+ PTHrP	502373,55	104550,15	35027,30	18751,45	2,10	0,57
5h	Control	549152,49	114728,86	40994,62	20538,01	2,00	0,50
	PTHrP	572497,91	134398,89	48988,78	15197,87	3,47	0,34
	DMSO	459528,84	104492,96	36189,49	16991,24	2,51	0,53
	DMSO + PTHrP	540958,38	132183,13	47145,72	15112,32	3,45	0,36
	SU	433503,43	91961,62	32499,24	17279,31	2,00	0,56
	SU+ PTHrP	474989,59	107135,04	37739,83	16617,10	2,51	0,48
24h	Control	487973,50	108293,98	37998,97	16948,69	2,55	0,48
	PTHrP	440312,67	134882,49	44247,08	13044,18	3,70	0,32
	DMSO	453802,96	117009,26	37199,63	16191,98	2,57	0,47
	DMSO + PTHrP	492995,40	132117,10	44167,20	13864,80	3,20	0,31
	SU	453382,04	118630,81	37624,58	15650,04	2,50	0,45
	SU+ PTHrP	508934,37	124074,22	41166,22	16517,61	2,80	0,44

DMSO, Dimethylsulfoxide; PTHrP, Parathyroid hormone-related peptide; SU, SU11274.

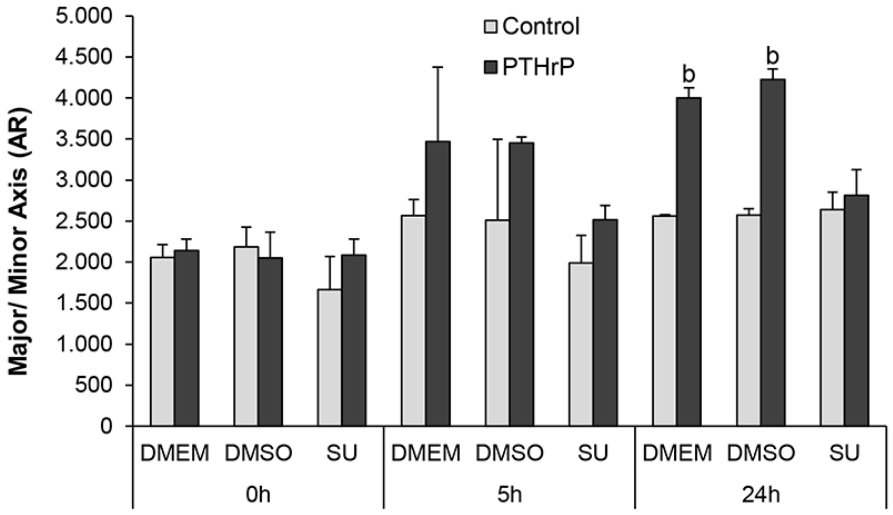

Table 1. Analysis of the aspect ratio (major / minor axis) obtained using the Image J-NIH program. The Table shows values of parameters associated to cell morphology: area, perimeter, round, major axis, minor axis and aspect radio, major axis / minor axis (AR). The graph bars show the AR for each condition. The increase of the degree of cell elongation at 24h of exposure to PTHrP is significantly reversed in the presence of the Met inhibitor. $^{b}p < 0.01$.

In the revised manuscript we now added these results in the new Figure 5C and in the Table 1 with its corresponding bar graphs. An appropriate comment is also incorporated in Result and Discussion sections (Results, Section 3.5, page 20, paragraph 3; Discussion section, pages 26-27, paragraph 7). We also incorporated the assays performed in Materials and Methods section (Materials and Methods, Section 2.7 page 11)

The modulation of cell proliferation, migration and EMT program through Met showed in this manuscript strongly suggest that the Met signaling pathway induced by PTHrP is involved in cell events related to tumor aggressive behavior in the HCT116 cells.

According with these new results, a paragraph in the Results section was changed (Results, Section 3.6, pages 20-21, paragraph 2)

Regarding the use of a different cell lines from CRC, we now replicate our initial observations in the Caco-2 cells which have less aggressiveness than HCT116 cells and were selected because previously we verified that these cells express the receptor PTHR1 and their cell responses are mediated exclusively by this receptor (Calvo et al. 2009; Martín et al. 2018). As the reviewer can see in the following **Figure 4,** no changes with statistical significance were found between Caco-2 cells untreated (control cells) and Caco-2 cells treated with PTHrP over a wide interval of exposure times (15 to 180 minutes and 5 to 24 hours).

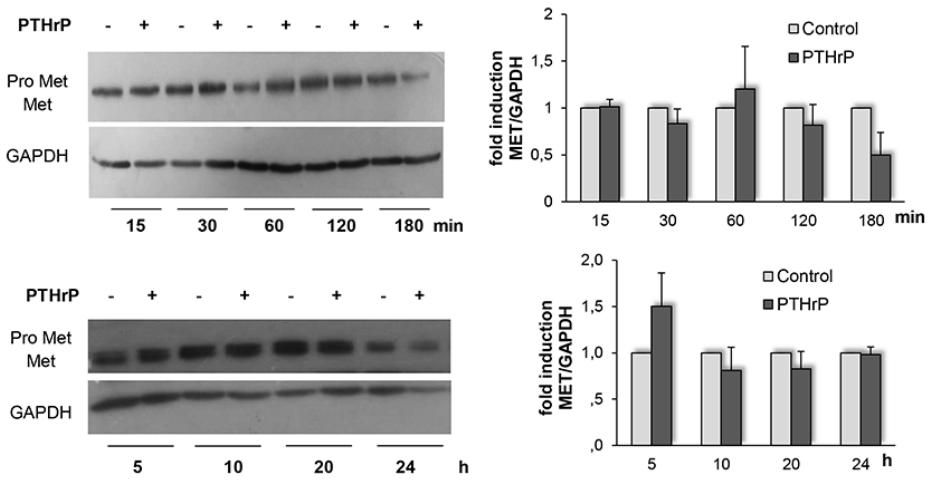

Figure 4. Protein expression of Met receptor in Caco-2 cell line exposed to PTHrP. Cells were treated with or without PTHrP 10^{-8} mol / L at different times. The protein levels of pro-Met (Met precursor) and Met (RTK mature form) protein levels were analyzed by Western Blot to investigate whether the molecular mechanisms trigged by PTHR1 after binding of PTHrP are capable of modulating the expression of the mature form of the RTK Met in Caco-2 cells. GAPDH protein levels were determined as control of the amount of proteins present in the membrane, since this protein is not substantially modified with the treatment by the cytokine. Graph bars represent the average of the results obtained from three independent experiments.

In our previous work (Carriere et al. 2021) we reported that PTHrP modulates the protein levels of SPARC (an invasion marker) and E-cadherin (a marker related with EMT program) in HCT116 cells but not in Caco-2 cells. The fact that the cytokine can modulate the expression of markers associated with the malignant behavior in a more aggressive cell line (HCT116) but not in a less aggressive cell line (Caco-2) may be because these CRC cells have different mutations (Ahmed et al. 2013; Ilyas et al. 1997) and perhaps several events triggered by PTHrP in these two cell lines may be dependent of these mutations explaining the different response between Caco-2 and HCT116 cells.

An appropriate comment is now added in the Result section (Results, Section 3.1, page 16 paragraph 3) and in the Discussion section (Discussion, page 25, paragraph 3). Also, we

incorporated the Caco-2 cell line in Materials and Methods section (Materials and Methods, Section 2.2, page 9).

For the reviewer information: we wanted to verify the results from HCT116 cell in other colorectal cancer cells, due to the results obtained in Caco-2 cells. Because the deadlines stipulated for the submission of this revised manuscript (14 days), it will not be possible to acquire other CRC cell lines, verify that they express PTHR1 (and if so, confirm that the cell responses dependent exclusively of PTHR1 activation) and also perform new experiments to replicate the findings from HCT116 cells both *in vitro* and *in vivo*. We hope that the reviewer understands this situation.

In view of the concern of the reviewer and as this manuscript is focused in the effects of PTHrP on HCT116 cells both *in vitro* as *in vivo*, we now consider more appropriate the following six titles in Result section:

1- 3.1-PTHrP modulates the expression of the RTK Met in human HCT116 cells (page 15)

Instead of:

3.1-PTHrP modulates the expression of the RTK Met in human CRC-derived cells

2- 3.2-PTHrP promotes Met phosphorylation and its activation through Src kinase in human HCT116 cells (page 17)

Instead of:

3.2-PTHrP promotes Met phosphorylation and its activation through Src kinase in human CRC-derived cells

3- 3.3-PTHrP promotes the phosphorylation / activation of Met through the MAPK signaling pathway in human HCT116 cells (page 18)

Instead of:

3.3-PTHrP promotes the phosphorylation / activation of Met through the MAPK signaling pathway in human CRC derived cells

4- 3.4 PTHrP increases Met gene expression in human HCT 116 cells (page 18)

Instead of:

3.4 PTHrP increases Met gene expression in human CRC derived cells

5- 3.5- MET signaling pathway induced by PTHrP participates in cell events related to the aggressive behavior of human HCT 116 cells (page 19)

Instead of:

3.5- MET signaling pathway induced by PTHrP participates in cell events related to the aggressive behavior of human CRC derived cells.

6- 3.6-PTHrP attenuates the cytotoxic effect of CPT-11, OXA and DOXO in human HCT 116 cells through the Met signaling pathway (page 21)

Instead of:

3.6-PTHrP attenuates the cytotoxic effect of CPT-11, OXA and DOXO in human CRC derived cells through the Met signaling pathway

Reviewer #2 comment 2:

The number of clinical tissue samples used for immunohistochemistry is also too small, and large clinical samples are usually required to support such a conclusion

Author response 2:

Currently, our purpose is to use human samples to validate our previous findings *in vitro* and *in vivo*; we expect that the results observed in these human tissues from CRC patients (which in part are showed in this manuscript) would support those obtained in these last 10 years by our research group in both, cell and animal models of CRC. Therefore, our goal now

is to obtain, by means of cell models, animals and human samples, the knowledge of the molecular mechanisms associated with PTHrP-induced malignant behavior; this information will then guide us for future retrospective studies with the aim to identify markers as potential candidates to be used in the detection of CRC at early stages, in the prediction of the response to certain therapies and/or in the detection of CRC recurrence.

We agree with the reviewer regarding that to provide a clinical conclusion, we should use a larger number of samples. In this sense, when we will perform the retrospective studies, we plan to analyze 300 to 400 samples to consider the data as statistically significant. These samples are from the Hospital Provincial de Neuquén (Province of Neuquén, Argentina) and from the Hospital Dr. José Penna, Bahia Blanca (Province of Buenos Aires, Argentina), which are two hospitals with a high attendance of patients, and therefore we consider that our work will have a regional scope because we will evaluate biopsies of CRC patients from two different provinces of our country.

According to the above criteria, we have now incorporated the following sentences:

1- In the Introduction section of the revised manuscript (Introduction, page 7, paragraph 3)

Despite these contributions, relevant aspects from the action of this peptide are still unknown, specifically whether PTHrP could promote resistance to other forms of chemotherapy and the validation of our previous observations using human samples.
Instead of:

Despite these contributions, relevant aspects from the action of this peptide are still unknown, specifically whether PTHrP could promote resistance to other forms of chemotherapy and the clinical relevance of our observations.

2- In Result section of the revised manuscript (Results, Section 3.8, page 23, paragraph 1):

"In this context, and taking into account the findings obtained by *in vitro* and *in vivo* assays, we decided to validate our observations…"

Instead of:

"In this context, and taking into account the findings obtained by *in vitro* and *in vivo* assays, we decided to evaluate the clinical relevance of our observations…"

Reviewer #2 comment 3:

To support this conclusion, animal experiments are also necessary.

Author response 3:

We agree with the suggestion of the reviewer that it is necessary more experiments in the xenografts to replicate our *in vitro* assays. We planner administrate PTHrP joint with the chemotherapeutic drugs employed in this work and also perform experiments to elucidate if Met is involvement in the events studied in the cell model (by Met knockdown with siRNA and/or inhibiting its activity by specific inhibitors). The execution of these trials is conditioned to the approval of the new protocols by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE-UNS) that we estimate it will be in 2023.

However, and taking into account that the present work is aimed to elucidate the relationship between PTHR1, PTHrP and Met, therefore we now performed new immunohistochemical analysis using anti- PTHR1 antibody in HCT116 cells xenografts of nude mice to evaluate the relation between both receptors (Met and PTHR1) in this CRC animal model under PTHrP action. Interestingly, PTHrP modulates the protein expression of its receptor in this CRC model, as seen in the following **Figure 5.**

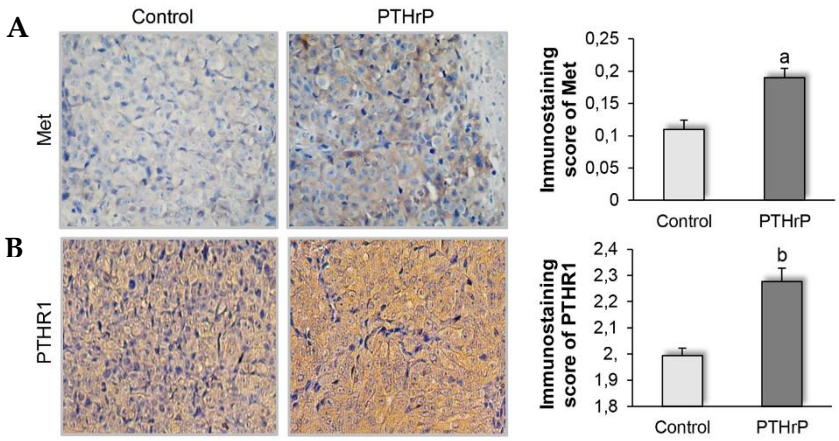

Figure 5. PTHrP increases the protein expression of Met and PTHR1 in tumor xenografts. (A) Met and (B) PTHR1 protein levels were evaluated by the Immunohistochemistry technique in the HCT116 cells tumor-bearing nude mouse model. Tumor sections were stained with A) an anti-Met antibody or B) with an anti-PTHR1 antibody. Images (400X) are from the tumor treated with saline solution (left) or with PTHrP (right). The immunostaining was quantified with the Fiji image processing package of the Image J-NIH program. [a]$p<0.05$; [b]$P<0.01$.

Perhaps the cytokine modulates the expression of its own receptor in this animal model in order to amplify its signaling within the cell and thus make its effects more effective in CRC. More studies are necessary to confirm this hypothesis.

The images in **Figure 5** with the corresponding quantifications are now added in the revised manuscript in the new Figure 7. An appropriate comment is also incorporated in Result section (Results, Section 3.7, page 22-23) and in Discussion section (Discussion, page 28, paragraph 12).

Reviewer #3:

Thank you very much for your valuable suggestions to improve our study. As indicated below, appropriate changes have been made to meet the comments of the reviewer.

Reviewer #3 comment 1:

In this paper, based on the results of previous studies, the authors hypothesized that the pTHRP-triggered signaling pathway may be involved in the retrotranscriptional activation of Met, and thus participate in the invasive behavior of CRC cells. A series of experiments

and immunohistochemical tests of clinical specimens were carried out to verify the results. The content of the article is relatively smooth, but there are still the following problems:

About Figure1 in this article, the author should clarify what is "pro-Met" and the relationship between "pro-Met" and "Met". It is better to explain the intention of doing Western Blot and the implications of the experimental results in the annotation of the picture. By the way, please provide clearer pictures of western blot results.

Author response 1:

According to the reviewer suggestion, we clarify the meaning of "pro-Met" and the relationship between "pro-Met" (Met precursor) and "Met" (RTK mature form). Pro-Met and Met are shown together to visualize the changes in the protein levels of both, the receptor and its precursor form, in response to PTHrP. An appropriate comment is now added in the legend of Figure 1 of the revised manuscript (Figure legends, page 49) Furthermore, we carefully improved the immunoblots.

Reviewer #3, comment 2.

In the Result 3.2 "PTHrP promotes Met phosphorylation and its activation through Src kinase in human CRC-derived cells", the second last sentence--"As shown in Figure 2B, we observed that PP1 decreases PTHrP-induced phosphorylation of Met at Tyr1234/1235." The oxidative phosphorylation site of Met cannot be known from figure 2 alone. Please provide an explanation.

Author response 2:

According to the reviewer's suggestion, we now added in the blots of the Figure 2 the Met phosphorylation sites, which constitute activating domains of the receptor. An appropriate comment is now added in Result section (Results, Section 3.2, page 17, paragraph 1) and in the legend of Figure 2 of the revised manuscript (Figure and legends, page 49).

Reviewer #3, comment 3:

In the description of Figure 3, the authors need to indicate the meaning of p-Met in the annotation of the picture. A brief explanation of the results can be given in the annotation.

Author response 3:

To satisfy the suggestion of the reviewer, we indicated the meaning of p-Met and the Met phosphorylation sites in Figure 3 of the revised manuscript and explained the results in the legend of this figure (Figure and legends, page 50)

Reviewer #3, comment 4:

On page nine, paragraph two, the format of the sentence describing the primer sequence needs to be adjusted.

Author response 4:

According to the reviewer's suggestion, we adjusted the format of the sentence describing the primer sequence in the Materials and Methods (Materials and Methods, Section 2.6, page 11).

´´The primers used were the following:

5′-GGAAACACCCATCCAGAATGTCATT-3′ (forward) and 5′-TGATATCGAATGCAATGGATGATCT-3′ (reverse) for Met; 5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse) for GAPDH…´´

Instead of:

´´The primers used were the following:

5′-GGAAACACCCATCCAGAATGTCATT-3′ (forward) and 5′-TGATATCGAATGCAATGGATGATCT-3′ (reverse) for Met; 5′-ACCACAGTCCATGCCATCA-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse) for GAPDH...

Reviewer #3, comment 5:

In result 3.5, The authors conclude that "MET Signaling Pathway induced by PTHrP participates in cell events related to the aggressive behavior of human CRC derived cells", but there is just one experiment, other phenotypic tests were lacking, for example, Transwell experiment and tumor sphere invasion experiment. I think the author needs to conduct more experiments to support his conclusions. The same problem appears in the following conclusions for instance, the result 3.6 and 3.7. The author always uses a single experiment to infer the conclusion.

Author response 5:

We appreciate the criticism of the reviewer. The experiments explained below were performed to support the conclusion regarding the participation of Met in the modulation of events associated with the aggressive behavior of CRC cells induced by PTHrP. These experiments were commented previously in this letter to the Reviewer #2.

Taking into account that PTHrP promotes cell migration in HCT116 cells (Calvo et al. 2017), we now performed wound-healing assays to evaluate if Met signaling also participates in this cell process. We observed the wound healing in culture of confluent HCT116 cells to compare the migration between untreated cells and cells treated with PTHrP in the present or absent of SU11274 (the specific inhibitor of Met). The times of PTHrP exposition were selected according with our previously published work (Calvo et al. 2017). Representative photographs, taken at time point 0, 5 and 24 hours of the identical location and the quantification of the results of two separate experiments are shown in **Figure 2** of this letter. A significant enhancement in wound closure was detected in cells exposed to PTHrP or PTHrP plus DMSO (the vehicle of the inhibitor) compared to control values at 5 and 24 h; however, this effect is not observed in cells pre-treated with the Met inhibitor.

These results are now added in the revised manuscript as new Figure 5B. An appropriate comment is also incorporated in Result and Discussion sections (Result, Section 3.5, page 19-20, paragraph 2; Discussion section, page 26-27, paragraph 7). We also incorporated the assays performed in Materials and Methods section (Section 2.7 page 11)

We recently published results revealing that PTHrP is able to induce the epithelial-mesenchymal transition (EMT) in HCT116 cells (Carriere et al. 2021). The EMT is a key program of CRC that participates in the invasion, angiogenesis, and chemoresistance associated with metastasis (Loboda et al. 2011; Tsoumas et al. 2018). Based on this previous work, herein we analyzed if PTHrP also induces this program through Met pathway. To that end HCT116 cells were incubated with SU11274, and then treated with or without PTHrP. The cells were observed under inverted microscopy after treatment with PTHrP for 5 and 24 h to analyze morphological changes associated with EMT. These times were selected because PTHrP at 24 h of exposition induces changes related with an effective transition to the mesenchymal phenotype in HCT116 cells (Carriere et al. 2021). The arrows in the **Figure 3** of this letter point morphological changes corresponding to the transition from polygonal structure to spindle-like structure observed when the cells were treated for 24 h with PTHrP or PTHrP plus DMSO. There were no changes in both, control cells and those treated with the inhibitor of Met. To further evaluate the phenotype related with a mesenchymal shape, we measured in the photomicrographs the minor and major axis using the Image J-NIH program. The **Table 1** of this letter and the bar graph reveal that under PTHrP action, the cells lose their epithelial characteristics. The increase of the relation between the major and minor axis indicates that the exposition with PTHrP for 24 h significantly increased the degree of HCT116 cells elongation, a typical feature of the mesenchymal shape. However, when the cells were pre-incubated with SU11274, the effects of the cytokine were reverted. Taken together, these findings suggest that PTHrP induced the EMT program in HCT116 cells through Met signaling pathway.

It has been shown that the loss of the expression of the epithelial marker E-cadherin is associated with EMT progress in CRC (Druzhkova et al. 2019; Gurzu et al. 2016). In our recent work (Carriere et al. 2021) we showed that in HCT116 cells PTHrP at 5 hours modulates E-cadherin and others epithelial markers as well as certain mesenchymal markers. Based on these morphological changes related to EMT and mediated through Met, we then explored by Western blot analysis if Met is involved in the modulation of E-cadherin protein expression by the cytokine. As the reviewer can see in the **Figure 3** of this letter, when the

cells were pre-incubated with SU11274, the effects of the cytokine on E-cadherin protein levels were reverted.

Taken together, these findings suggest that PTHrP induces the EMT program in HCT116 cells through Met signaling pathway.

In the revised manuscript we now added these results in the new Figure 5C and in the Table 1 with its corresponding bar graphs. An appropriate comment is also incorporated in Result and Discussion sections (Results, Section 3.5, page 20, paragraph 3; Discussion section, pages 26-27, paragraph 7). We also incorporated the assays performed in Materials and Methods section (Section 2.7 page 11)

The modulation of cell proliferation, migration and EMT program through Met showed in this manuscript strongly suggest that the Met signaling pathway induced by PTHrP is involved in cell events related to tumor aggressive behavior in the HCT116 cells.

According with these new results, a paragraph...in Results section (3.6) was changed.

Regarding the concern of the Reviewer in the Section 3.6 of the original manuscript, the use of the Trypan Blue staining technique as a method to evaluate cell viability is widely used by other researchers (Strober 2015). Furthermore, our research group has employed this method in previous works to investigate the citotoxyc effects of several drugs (5-FU, OXA, CPT-11) and the results were evaluated by peer reviewers and published in prestigious scientific journals(Calvo et al. 2017; Martín et al. 2018; Novoa Díaz et al. 2020)

Regarding point 3.7, we agree with the suggestions of the reviewer 2 and 3 that it is necessary more experiments in the xenografts to replicate our *in vitro* assays. As we comment earlier to reviewer 2, we planner administrate PTHrP joint with the chemotherapeutic drugs employed in this work and also perform experiments to elucidate if Met is involvement in the events studied in the cell model (by Met knockdown with siRNA and/or inhibiting its activity by specific inhibitors). The execution of these trials is conditioned to the approval of the new

protocols by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE-UNS) that we estimate it will be in 2023.

However, and considering that the present work is aimed to elucidate the relationship between PTHR1, PTHrP and Met, therefore we now performed new immunohistochemical analysis using anti- PTHR1 antibody in HCT116 cells xenografts of nude mice to evaluate the relation between both receptors (Met and PTHR1) in this CRC animal model under PTHrP action. Interestingly, PTHrP modulates the protein expression of its receptor in this CRC model, as seen in **Figure 5** of this letter. Perhaps the cytokine modulates the expression of its own receptor in this animal model in order to amplify its signaling within the cell and thus make its effects more effective in CRC. More studies are necessary to confirm this hypothesis.

The images in **Figure 5** with the corresponding quantifications are now added in the revised manuscript in the new Figure 7. An appropriate comment is also incorporated in Result section (Results, Section 3.7, page 22-23) and in Discussion section (Discussion, page 28, paragraph 12).

Reviewer #3, comment 6:

In result 3.6, the original text said there was figure A, figureB and figureC, but in Figure6, only Figure AB was seen.

Author response 6:

We regret that Figure 6 C is not displayed. Now the reviewer can observe that this figure is located at the bottom of Figure 6 (figure file PPT)

Reviewer #3, comment 7:

P16 result3.6, the phrase “is a major barrier for achieving effective treatment' needs to be reformatted”.

Author response 7:

According to the reviewer's suggestion, we change the phrase " is a major barrier for achieving effective treatment" to " constitutes a great challenge to the effective treatment." (Results, Section 3.6, page 21, paragraph 1).

Reviewer #3 comment 8:

In result 3.7, there were no photos of tumor formation in nude mice. There's only one iHC image. Immunohistochemistry shows increased expression of Met. Does the over-expression of Met affect tumorability, tumor size, and other phenotypes in nude mice? The author is expected to give an explanation.

Author response 8:

The following explanation was also provided to the referees that revised our previous work published in 2018 (Martín et al. 2018).

As shown in the **Table 2** and **Figure 6** from this letter, at the end of the trial the differences between the volumes and weights of tumors from untreated and treated animals were not significant but we are sure that if we had continued the assay the size of the tumors in the mice treated with PTHrP would be higher than that observed in control mice. However, we forced us to finish the assay in order to preserve the welfare of the animal for two reasons: 1- On the last day of the treatment the relation between the size of the tumor and the weight of

Tumor volume (cm^3)		Tumor weight (g)	
C	PTHrP	C	PTHrP
0,14	0,20	0,19	0,28
0,23	0,38	0,33	0,54
0,11	0,25	0,19	0,34
0,27	0,35	0,38	0,49
0,32	0,20	0,45	0,34
0,17	0,22	0,24	0,40

Table 2. Values of tumor volume (cm^3) and weight from control and PTHrP-treated mice. Mean and St deviation for each group are shown.

the animal was near the limit value tolerated for this type of animal (according to data from Basic Surgery Guidelines for Animal Research. Institutional Animal Care and Use Committee. Howard University, 58 pp 2007). 2- Most important, the irritability and greater sensitivity of the animals due to the daily manipulation of them when they were injected with the vehicle or PTHrP.

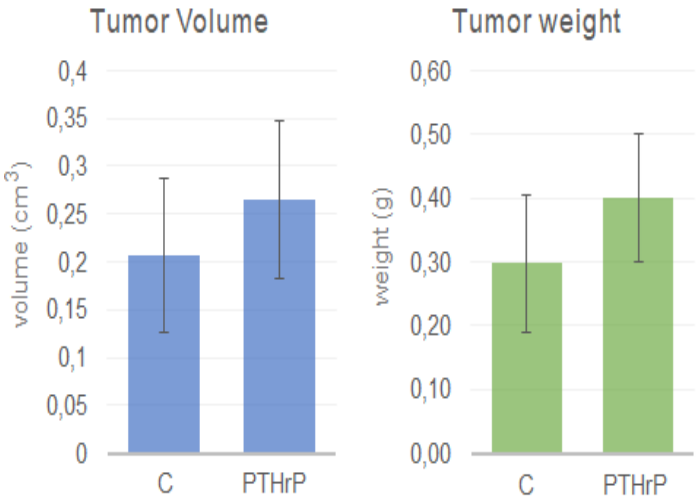

Figure 6. Tumor volume (cm^3) and weight (g) from control and PTHrP-treated mice.

As we mentioned in the original manuscript (Introduction Section, page 7, paragraph 3) and in the revised manuscript (Introduction Section, page 7 line 3) in this CRC animal model we found that PTHrP modulates the levels of markers linked to tumor progression and these findings support our idea that PTHrP affect the tumor phenotype in HCT116 xenografts of nude mice. Specifically, the cytokine regulates the protein expression of ERK1/2 MAPK, cyclin D1, CREB/ATF-1, RSK (markers associated with proliferation and migration), VEGF (angiogenesis marker), E-cadherin (epithelial marker associated with the epithelial-mesenchymal transition) and SPARC (invasion marker) (Calvo et al. 2017, 2019; Carriere et al. 2021; Martín et al. 2018).

An additional and interesting result published by us (Martín et al. 2018) evidences the role of PTHrP *in vivo* as a regulator of the expression of tumor proliferation markers since the

cytokine is able to increase the expression of Ki67, which is a nuclear protein present in proliferating cells and is a marker for cell proliferation of solid tumors, including CRC (Li et al. 2016). By immunohistochemistry analysis using anti-Ki67 antibody and based on the immunostained cells, we determined the Ki67 index in untreated and treated tumors. To that end, positive and total nuclei were quantified in five random microscopic areas at 40× by two independent observers with 100% agreement in their observations. Weak and strong nuclear labeling was considered positive. Both indices were expressed as a percentage of stained cells to total cells. As shown in **Figure 7A**, PTHrP increased the protein levels of Ki67 whereas the proliferation index was significantly greater in treated tumors than in non-treated tumors (**Figure 7C**). As at the same time, the levels of this cell proliferation marker were analyzed by IHC in breast cancer tissue as a positive control **(Figure 7B).**

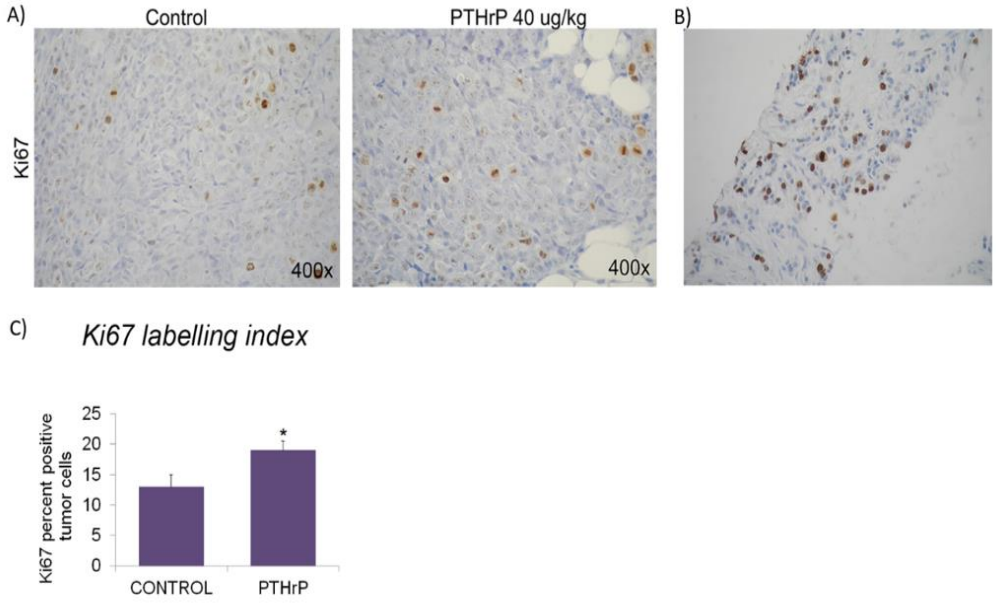

Figure 7. (A) Representative images of Ki67 expression in tumor xenografts. Magnification 400x. (B) Ki67 expression in breast cancer tissue as a positive control. Magnification 400x. (C) Ki67 labelling index was significantly greater in tumor treated with PTHrP (40 µg/kg).

Our previous data (Calvo et al. 2017, 2019; Carriere et al. 2021; Martín et al. 2018) together with the findings showed in the manuscript suggest that PTHrP modulates the expression

not only of markers linked to tumor progression (including Met) but also of its own receptor and these events could lead to favor the tumor phenotype in the CRC animal model employed by us. As we comment earlier to reviewer 3, to confirm the involvement of Met in the tumor phenotype we planner perform new experiments silencing Met (by knockdown with siRNA) and/or inhibiting its activity (by specific inhibitors) in the xenografts but the execution of these trials is conditioned to the approval of the new protocols by the CICUAE-UNS.

According to the reviewer suggestion, we now included xenograft tumor size (as volume) and tumor weight data in Materials and Methods Section (Materials and Methods, Section 2.9, page 13)

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