We thank the editors and reviewers for your useful suggestions that helped our manuscript improve.

RESPONSE TO REVIEWER #1

Comment 1: Authors needs to revise the Abstract to be more concise about the background of study, describing the importance research gap and finding which directs the reader to understand the general idea about this manuscript in a more precise way.

Response 1: Thank you for your comment. We modified the abstract so it is more concise and straightforward.

- In ABSTRACT-Background

We changed: "Resistance to chemotherapy complicates the evolution of these patients. Several authors have highlighted the participation of nicotinic acetylcholine receptors (nAChR) in the modulation of conventional chemotherapy treatment in lung, head and neck, oral and nasal cavity, and pancreatic cancers. However, in smoking cancer patients, the action of nicotine on nAChR expressed in the breast or other organs near the tumor during chemotherapy treatment is less known"

by:" Resistance to chemotherapy complicates the course of patients' treatment. Several authors have highlighted the participation of nicotinic acetylcholine receptors (nAChR) in the modulation of conventional chemotherapy treatment in cancers of the airways. However, in breast cancer, less is known about the effect of nAChR activation by nicotine on chemotherapy treatment in smoking patients."

Comment 2: The effect of the addition of nicotine is investigated in a concentration similar to that found in smokers' blood ? Pleased double check the concentration.

Response 2: Thank you for your accurate comment. Truly, the concentration of nicotine is **similar** to the one observed in the blood stream of **passive** smokers, which is between 10^{-8}M and 10^{-9}M according to different authors (see the following list). It has been corrected in the R1 manuscript (see below).

• Zil-a-Rubab, Mohammad Ata-ur-Rahman. Estimation of serum nicotine by gas chromatography in smokers, passive smokers and never smokers. Comparative Study J Pak Med Assoc. 2012;62(8):790-3. PMID: 23862251.

• Pokorski TL, Chen WW, Bertholf RL. Use of urine cotinine to validate smoking self-reports in U.S. Navy recruits. Addict Behav. 1994;19:451–454.

• Moyer TP, Charlson JR, Enger RJ, Dale LC, Ebbert JO, Schroeder DR, Hurt RD. Simultaneous analysis of nicotine, nicotine metabolites, and tobacco alkaloids in serum or urine by tandem mass spectrometry, with clinically relevant metabolic profiles. Clin Chem. 2002;48(9):1460-1471. PMID: 1219492.

• Perkins KA, Fonte C, Sanders M, Meeker J, Wilson A. Threshold doses for nicotine discrimination in smokers and non-smokers. Psychopharmacology (Berl). 2001;155(2):163-170. doi: 10.1007/s002130000660. PMID: 11401005 Clinical Trial.

• García Calzado M, J.F. García Rojas, A. Mangas , D. Martínez Izquierdo, M. Repetto, J. Millán. Tobacco and arterial pressure (I.). The hormonal changes in a model of acute nicotine overload. An Med Interna 1990;7: 340-344. PMID: 1966467

• Lindell G, Farnebo LO, Chen D, Nexø E, Rask Madsen J, Bukhave K, Graffner H. Acute effects of smoking during modified sham feeding in duodenal ulcer patients. An analysis of nicotine, acid secretion, gastrin, catecholamines, epidermal growth factor, prostaglandin E2, and bile acids. Scand J Gastroenterol. 1993; 28: 487-494. PMID: 8322024 DOI: 10.3109/00365529309098254.

- In ABSTRACT-Methods

We changed: "(in a concentration similar to that found in smokers' blood)"

By: "(at a concentration similar to that found in passive smokers' blood)"

- In RESULTS- Paclitaxel treatment of MDA-MB-231 cells in the presence of nicotine

We changed: "It has been documented that 10^{-10}M can be considered the concentration of NIC present in the blood stream of smoking patients"

By: "It has been documented that 10^{-10}M can be considered a concentration of NIC similar to that present in the blood stream of passive smoking patients"

- In DISCUSSION

We changed: "The presence of NIC in a concentration similar to that present in the blood of smokers"

By: "The presence of NIC at a concentration similar to that present in the blood of passive smokers"

Comment 3: It is suggested to do pretreatment of Nicotinic antagonists in the context of PX and nicotine treatment. It would double check whether the NIC-reduced PX effectiveness coursed by NIC.

Response 3: Thank you very much for your suggestion. We have confirmed by the pretreatment with the nicotinic antagonists MM, Lut and MLA that the effect of the treatment with nicotine alone is mediated by the activation of the nicotinic receptors α7 and α9. On the other hand, when we studied the treatment with PX alone, we determined that it exerts its effect by a pathway that is independent of the nicotinic receptors, since the pretreatment with the antagonists did not modify it. We added a new paragraph in the R1 of the manuscript indicating these results.

- In RESULTS- Paclitaxel treatment of MDA-MB-231 cells in the presence of nicotine

We changed: "This effect of NIC on PX action was partially reduced by the pre-treatment of cells with nicotinic antagonists (MM, MLA or Lut) added at 10-6M ($P<0.001$ vs PX+NIC) (Figure 5A)".

By: "This effect of NIC on PX action was partially reduced by the pre-treatment of cells with nicotinic antagonists (MM, MLA or Lut) added at 10-6M (P<0.001 vs PX+NIC) (Figure 5A) in a manner similar to that of NIC treatment alone (NIC: 138.16±6.08; NIC+MM: 116.52±0.12; NIC+Lut: 126.16±5.96; NIC+MLA: 119.69±4.26; P<0.001; P<0.05 and P<0.001 vs NIC respectively). We confirmed that PX effect was independent of nAChR activation since pre-treatment with nicotinic antagonists did not modify the effect of PX treatment alone (PX+MM: 52.12±6.65; PX+Lut: 49.97±5.88; PX+MLA: 54.6±4.01)."

Comment 4: Not only ABCG2, but also other drug resistance gene and protein should be evaluated in this study.

Response 4: Although the participation of different genes and proteins has been found to be linked to the acquired resistance to drugs, in this investigation we only considered to study ABCG2 transporter because it is strongly related to the extrusion of paclitaxel and it was reported that it plays an important role in the generation of resistance to PX treatment in breast adenocarcinomas.

Here we present bibliography that highlights the importance of the expression levels of ABCG2 in the resistance to paclitaxel treatment in different types of cancer.

• Chung WM, Ho YP, Chang WC, Dai YC, Chen L et al. Increase Paclitaxel Sensitivity to Better Suppress Serous Epithelial Ovarian Cancer via Ablating Androgen Receptor/Aryl Hydrocarbon Receptor-ABCG2 Axis. Cancers (Basel). 2019;11(4):463.

• Němcová-Fürstová V, Kopperová D, Balušíková K, Ehrlichová M, Brynychová V et al. Characterization of acquired paclitaxel resistance of breast cancer cells and involvement of ABC transporters. Toxicol Appl Pharmacol 2016; 310: 215-228.

• Español AJ, Salem A, Di Bari M, Cristofaro I, Sanchez Y et al. The metronomic combination of paclitaxel with cholinergic agonists inhibits triple negative breast tumor progression. Participation of M2 receptor subtype. PLoS One. 2020;15(9):e0226450.

• Kim SH, Kim MJ, Cho YJ, Jeong YY, Kim HC et al. Clinical Significance of ABCG2 Haplotype-tagging Single Nucleotide Polymorphisms in Patients With Unresectable Non-Small Cell Lung Cancer Treated With First-line Platinum-based Chemotherapy.Am J Clin Oncol. 2015;38(3):294-299.

• Yang C, Xiong F, Dou J, Xue J, Zhan X et al. Target therapy of multiple myeloma by PTX-NPs and ABCG2 antibody in a mouse xenograft model.Oncotarget. 2015;6(29):27714-27724.

• Salem AR, Martínez Pulido P, Sanchez F, Sanchez Y, Español AJ et al. Effect of low dose metronomic therapy on MCF-7 tumor cells growth and angiogenesis. Role of muscarinic acetylcholine receptors. Int Immunopharmacol. 2020;84:106514.

• Fung KL, Kapoor K, Pixley JN, Talbert DJ, Kwit AD et al. Using the BacMam Baculovirus System to Study Expression and Function of Recombinant Efflux Drug Transporters in Polarized Epithelial Cell Monolayers.Drug Metab Dispos. 2016;44(2):180-188.

• Yang JP, Liu Y, Zhong W, Yu D, Wen LJ et al. Chemoresistance of CD133+ cancer stem cells in laryngeal carcinoma.Chin Med J (Engl). 2011;124(7):1055-1060.

• Zhou Q, Ye M, Lu Y, Zhang H, Chen Q et al. Curcumin Improves the Tumoricidal Effect of Mitomycin C by Suppressing ABCG2 Expression in Stem Cell-Like Breast Cancer Cells.PLoS One. 2015;10(8):e0136694.

• Yang C, He X, Song L, Zhan X, Zhang Y et al. Gamma-Fe2O3 nanoparticles increase therapeutic efficacy of combination with paclitaxel and anti-ABCG2 monoclonal antibody on multiple myeloma cancer stem cells in mouse model.J Biomed Nanotechnol. 2014;10(2):336-44.

• Yang C, Xiong F, Wang J, Dou J, Chen J et al. Anti-ABCG2 monoclonal antibody in combination with paclitaxel nanoparticles against cancer stem-like cell activity in multiple myeloma. Nanomedicine (Lond). 2014;9(1):45-60.

• Xie ZY, Lv K, Xiong Y, Guo WH. ABCG2-meditated multidrug resistance and tumor-initiating capacity of side population cells from colon cancer. Oncol Res Treat. 2014;37(11):666-8, 670-2.

• Ricci F, Bernasconi S, Perego P, Ganzinelli M, Russo G et al. Ovarian carcinoma tumor-initiating cells have a mesenchymal phenotype.Cell Cycle. 2012;11(10):1966-76.

• Huang Y, Wang Y, Li Y, Guo K, He Y. Role of sorafenib and sunitinib in the induction of expressions of NKG2D ligands in nasopharyngeal carcinoma with high expression of ABCG2.J Cancer Res Clin Oncol. 2011;137(5):829-37.

• Huisman MT, Chhatta AA, van Tellingen O, Beijnen JH, Schinkel AH. MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. Int J Cancer. 2005;116(5):824-9.

Comment 5: There is only one TNBC cell lines used in this study. Author could consider to use another TNBC or BC cell line to verify whether the NIC effect is cell line bias.

Response 5: Your suggestion is very useful. We also conducted cell viability assays in the breast cancer cell lines MDA-MB468 (TN) and MCF-7 (luminal A) treating them with PX and/or NIC in the absence or presence of MM. We observed similar results as the ones obtained with the MDA-MB231 cell line, which could indicate that the modulatory effect of nicotine on PX occurs also in other human breast cancer cell lines. The manuscript was modified and these results were added in the R1 version.

- In RESULTS- Paclitaxel treatment of MDA-MB-231 cells in the presence of nicotine

We added: "To confirm that nicotinic agonists can modulate the action of PX in reducing viability in other breast cancer cells, we tested the effect of PX (10^{-7}M) in the absence or presence of NIC (10^{-10}M) on MDA-MB-468 and MCF-7 cell viability. We determined that the presence of NIC reduced the effect of PX in MDA-MB-468 and MCF-7 cells, and that these effects were prevented by pre-incubating cells with 10^{-6}M of MM (Table 1)."

Table 1: Effect of nicotine on paclitaxel treatment in MDA-MB-468 and MCF-7 cell viability.

treatment	MDA-MB-468 Cell viability (% of control)	MCF-7 Cell viability (% of control)
PX	61.01±3.79	65.36±4.86
NIC	137.79±3.69[c]	141.94±4.07[c]
PX+NIC	79.15±6.94a	117.99±10.06[c]
PX+NIC+MM	62.37±4.71	69.13±7.22

Cells were treated with paclitaxel (PX) (10^{-7}M) alone or combined with nicotine (NIC) (10^{-10}M) for 48 h in the absence or presence of mecamylamine (MM) (10^{-6}M). Results are expressed as cell viability respect to control (cells without treatment). Values are the mean ± S.D. of three experiments performed in duplicate. ^{a}P<0.05; ^{c}P<0.001 vs control considered as 100%.

- In MATERIALS AND METHODS-Cell culture

We changed: "The human breast adenocarcinoma cell line MDA-MB-231 (CRM-HTB-26) obtained…"

By: "The human breast adenocarcinoma cell lines MDA-MB-231 (TN, CRM-HTB-26), MDA-MB-468 (TN, HTB-132) and MCF-7 (luminal A, HTB-22) were obtained…"

- In DISCUSSION

We changed: "reduced the potency of PX in more than one order of magnitude. In a human gastric cancer model, Tu et al.[64] observed a reduction in…"

By: "reduced the potency of PX in more than one order of magnitude. It is also important to highlight that the administration of NIC is effective in reducing PX effect in other TN tumor cells such as MDA-MB-468, and in luminal A MCF-7 tumor cells. In a human gastric cancer model, Tu et al.[64] observed a reduction in…"

Comment 6: In Fig5, legend： aP<0.05; bP<0.01 vs PX. cP<0.05; dP<0.01; eP<0.001 vs PX+NIC. But only "a,b, f, g" in fig.

Response 6: Thank you for your precise observation. It was a mistake we made in the manuscript, the legend of the figure is correct but we presented a 5B figure (new figure 6B) that shows the results of a viability assay using nicotine treatment alone. In the R1 version of the manuscript we changed this figure for the correct one that describes the results of a viability assay with nicotine and paclitaxel treatment combined.

The description and discussion of this figure in the manuscript is correctly developed since its original version.

Comment 7*:* In the section :"Apoptosis is a cell death .....". "The presence of NIC reduced the effect of PX (9.3±0.6%, P<0.05 vs control or P<0.001 vs PX) (Figure 6)." Propose modifying. "The presence of NIC reduced the effect of PX. The percentage of apoptotic cells was....."

Response 7: Thank you very much for your comment. We made the correct modifications in the R1 manuscript.

- In RESULTS- Signal transduction pathways and mechanism involved in the effect of paclitaxel on nicotine-treated tumor cells

We changed: "The presence of NIC reduced the effect of PX (9.3±0.6%, P<0.05 vs control or P<0.001 vs PX)…"

By: "The presence of NIC reduced the effect of PX. The percentage of apoptotic cells was 9.3±0.6 (P<0.05 vs control or P<0.001 vs PX)…"

Comment 8: Author should take care of the reference list, make sure the information is presented in consistent.

Response 8: Thank you for your observation. We checked and modified the reference list according to the journal's indications.

Comment 9: Author should take care of grammatical errors and typos. More importantly, a professional editing service should be asked for assistance.

Response 9: Thank you for your comment. The R1 version of the manuscript has been revised by a professional editing service and a new professional English certification has been attached.

RESPONSE TO REVIEWER #2

Comment 1: I really appreciated the effort made by the authors in addressing such an important and novel topic in Nicotinic receptors modulate antitumor therapy response in triple negative breast cancer cells . I've found the work is well written and informative. However the following comments is required: In introduction section, activation of nAChRs induce an increase in intracellular calcium levels[14], which may in turn activate different signaling pathways. What is the applied clinical pathophysiology in tumorogenesis which was proved in literature in induction of cancer in different organs by disruption of this pathway?

Response 1: Thank you for your precise comments. We modified in the introduction section lines 113/117 according to your comment.

- In INTRODUCTION

We changed: "The activation of nAChRs can induce an increase in intracellular calcium levels[14], which may in turn activate different signaling pathways, including those involving kinases[15-16], which regulate different parameters of tumor biology such as proliferation, migration and invasion[17-19]."

By: "The activation of nAChRs can induce an increase in the levels of intracellular calcium [14], which has been related to tumorigenesis in the lungs[15], liver[16], pancreas[17] and brain[18]. This increase in intracellular calcium can in turn activate kinase signaling

pathways[19-20], which regulate different parameters of tumor biology such as proliferation, migration and invasion”

Comment 2: An illustration of cell culture, viability assay and uses of western blot by a figure or a diagram is recommended

Response 2: Thank you for your comment. A diagram of the schedule of administration of the drugs was added in the Materials and Methods section (Figure 1).

- In MATERIALS AND METHODS- Cell viability assay

We added: “A diagram of the administration schedule for the determination of cell viability or cell sensitivity to chemotherapy is shown in figure 1.”

RESPONSE TO REVIEWER #3

Comment 1: I only have one comment: -Please check this sentence: "After treatment, the medium was removed and 100 µL of MTT solution (500 mg/L medium free of phenol red and FBS).". It sounds incomplete.

Response 1: Thank you very much for your comment. In effect, that sentence was incomplete. We have completed it in the R1 version of the manuscript.

- In MATERIALS AND METHODS- Cell viability assay

We changed: “After treatment, the medium was removed and 100 µL of MTT solution (500 mg/L medium free of phenol red and FBS).”

By: “After treatment, the medium was removed and 100 µL of MTT solution (500 mg/L medium free of phenol red and FBS) was added.”

SCIENCE EDITOR COMMENTS AND SUGGESTIONS

Comment 1: In sentence “Resistance to chemotherapy complicates the evolution of these patients” – is the “evolution” not mistakenly put instead of “evaluation”? Or maybe it is better to say that “resistance to chemotherapy complicates the course of patients’ treatment”?

Response 1: we modify that sentence

- In ABSTRACT-Background

We changed: “Resistance to chemotherapy complicates the evolution of these patients.”

By: “Resistance to chemotherapy complicates the course of patients’ treatment.”

Comment 2: Throughout the entire paper, add the second hyphen in “MDA-MB231”, between “MB” and “231”

Response 2: we modify it according to your indications.

Comment 3: Remove excessive space marks in the text, example in Abstract: “Moreover, we observed that the presence of nicotine reduced” (two space marks are before this sentence). There are also other examples in the text (e.g. “cytotoxic / apoptotic ”), please double-check the paper.

Response 2: we modify it according to your indications.

Comment 4: I would change the sentence “To inhibit the action of the nicotinic agonist, cells were previously treated with mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) (which are nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists respectively) at 10-6M” to: “To inhibit the action of the nicotinic agonist, cells were previously treated with mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) at 10-6M; these three are respectively nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists”

Response 4: we modify that sentence

- In MATERIALS AND METHODS- Cell viability assay

We changed: “To inhibit the action of the nicotinic agonist, cells were previously treated with mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) (which are nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists respectively) at 10-6M.”

By: “To inhibit the action of the nicotinic agonist, cells were previously treated with the antagonists mecamylamine (MM), methyllycaconitine (MLA) or luteolin (Lut) at 10-6M; these three are respectively nAChR non-selective, α7 nAChR selective and α9 nAChR selective antagonists.”

Comment 5: The sentence “After treatment, the medium was removed and 100 µL of MTT solution” should have some ending like “was added” maybe?

Response 5: Thank you very much for your comment. In effect, that sentence was incomplete. We have completed it in the R1 version of the manuscript

- In MATERIALS AND METHODS- Cell viability assay

We changed: “After treatment, the medium was removed and 100 µL of MTT solution (500 mg/L medium free of phenol red and FBS).”

By: "After treatment, the medium was removed and 100 µL of MTT solution (500 mg/L medium free of phenol red and FBS) was added."

Comment 6: To avoid "and" word repetition in this sentence: "Samples were incubated on ice for 1 h and centrifuged at 800 G for 20 min at 4˚C, and supernatants were collected and saved at -80˚C. Protein concentrations were determined by the Bradford assay", you can use change the second one to e.g. "afterwards".

Response 6: Thank you very much for your instructions. We change that sentence

- In MATERIALS AND METHODS- Detection of nicotinic receptors by Western blot

We changed: Samples were incubated on ice for 1 h and centrifuged at 800 G for 20 min at 4˚C and supernatants were collected and saved at -80˚C

By: Samples were incubated on ice for 1 h and centrifuged at 800 G for 20 min at 4˚C, afterwards supernatants were collected and saved at -80˚C

Comment 7: Please standardize the referencing to figures, sometimes they are put abbreviated e.g. Fig 1A, while sometimes full word is provided e.g. Figure 1A

Response 7: we modify it according to your indications

Comment 8: Moreover, please double-check that the subfigures of a specific figure are provided as single graph/file – I noticed that they are separately put in the Word manuscript file.

Response 8: we modify figures according to your indications

Comment 9: Please provide original blots from Figure 1B and 7A.

Response 9: We provide file with original figures with the name: "74097-Raw images Fig2B and Fig8A"

Comment 10: Correct the obvious typo "Staub" in section "Signal transduction pathways and mechanism involved in the effect of paclitaxel on nicotine-treated tumor cells" (should be "Stau")

Response 10: we modify it according to your indications

COMPANY EDITOR-IN-CHIEF COMMENTS AND SUGGESTIONS

Comment 1: Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, “Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...”.

Response 1: we modify figure legends according to your indications

Comment 2: Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response 2: we modify figure according to your indications

Comment 3: If the picture is ‘original’, the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Response 3: we modify original file figures according to your indications

Comment 4: Please upload the approved grant application form(s) or funding agency copy of any approval document(s)

Response 4: We provide file with the approved grant application form with the name: “74097-Approved grant application form (s) or funding agency copy of any approval document (s)”