nature portfolio

Peer Review File

Spontaneously increased temperature of human pancreatic tumors promotes ferroptosis evasion and chemotherapy resistance via p38 MAPK inhibition

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author); expert in PDAC:

de Laat et al. report highly original findings relating changes in the temperature of pancreatic tumors to the activation of ferroptotic signatures and to sensitivity to gemcitabine. While the work only provides a limited mechanistic insight, the observations are interesting and potentially important. However, there are some limitations to the work that the authors need to address.

Specific comments
1. It would be important to provide a note on the robustness of the thermal measurements in patients. It would also be useful to correlate the temperature with other clinical and/or pathological parameters if possible.
2. The authors assess proliferation in very long term experiments (up to 400h) and measure proliferation as a % confluence of the flask surface. I am not questioning the validity of the latter; however, this is not an accepted proliferation parameter and it would be important to provide more direct measures of proliferation in the manuscript.
3. The fact that the cells remain stagnant for close to 10 days and then their growth takes off requires a more detailed analysis of the mechanisms involved with a time-course approach.
4. The assessment of the association with outcome in the TCGA series is inappropriate. It has been highlighted that the TCGA dataset contains only 150 bona fide PDAC whereas the remaining 35 samples are non-PDAC and therefore should be excluded from the analysis (they include endocrine tumors, pancreatitis, and non-neoplastic samples). In addition, for the p-P38 studies, it is important to determine whether the measurements refer to tumor or stromal cells - otherwise, the in vitro data cannot be extrapolated to the findings in patients tumors. This is a critical point that the authors need to address.
5. It would be useful to assess the ferroptosis signatures in other omics series to determine its association with other biological parameters.
6. I am surprised that the authors use 30 nM gemcitabine (according to the materials and methods) since this is a rather low dose of the drug and the effects are quite important. Please review and confirm.
7. Some of the experiments (e.g. Figure 2c) lack an untreated control group, which makes it impossible to assess the effects of gemcitabine alone.
8. The toxicity of the drugs administered in some experiments (specially with SB) should be noted as changes in mouse weight.

Reviewer #2 (Remarks to the Author); expert in ferroptosis, lipid metabolism:

Gemcitabine, a nucleoside analog of deoxycytidine, is widely used for the treatment of PDAC. Previous studies suggested that PDAC cells that are resistant to gemcitabine can be targeted by combination treatments (Gem plus ferroptosis inducers) that sensitize PDAC cells to ferroptosis. In this manuscript, Vincent de Laat et al., report an interesting phenomenon that the PDAC tumoral temperature is increased compared to adjacent

tissues. The authors tried to link the increased tumoral temperature to Gemcitabine resistance via lipidomic adaptation-mediated ferroptosis evasion. This working model is quite interesting; however, the authors did not provide strong experimental evidence to support their conclusions. Here are the concerns:

Major points:
1. There is not enough evidence to show that cells that cultured at 38c are more resistant to ferroptosis than the cells that cultured at 37c. Firstly, the temperature difference is so little, how can the authors ensure the culture difference can be maintained during the whole experiment? Secondly, the authors need to use more direct and reliable cell death or cell viability assays, instead of their "relative proliferation" assay to assess the ferroptosis sensitivity of the cells cultured at different temperature to different ferroptosis inducing conditions.

2. The authors tried to show that increased temperature decreases PUFA-ePC to make the cells more resistant to ferroptosis. However, it seemed PUFA-ePC only decreased significantly in PANC-1 cells, but not in BxPC3 cell, when cells were cultured at 38c (Fig 1E). More experiments are needed to support there claim and address the discrepancy between the two tested PDAC lines.

3. There is not strong evidence to support Gemcitabine directly induces ferroptosis. The conclusion that Gemcitabine first induces apoptosis, then later induces ferroptosis is questionable. Gemcitabine is known to induce oxidative stress in the cell. It cannot rule out that radical trapping anti-oxidants (RTAs), like ferrostatin and UAMC-3203, restore the growth of Gemcitabine-treated cells through reducing oxidative stress in the cells. The authors need to test whether other ferroptosis suppressing inhibitors, e.g. iron chelator and overexpression of FSP1, can rescue Gemcitabine-induced cell death.

4. The authors tried to use p38 deactivation as a link to connect gemcitabine resistance with tumoral temperature-induced adaptations in lipid metabolism. However, they didn't conduct any direct experiment to support p38 as a "link" of these events.

5. The mechanistic study in this manuscript is rather limited. It is not clear how temperature affect PUFA-ePC and whether p38 is indeed involved in this process.

Reviewer #3 (Remarks to the Author); expert in lipids, PDAC:

Title: Spontaneously increased temperature of human pancreatic tumors promotes ferroptosis evasion and chemotherapy resistance via p38 MAPK inhibition.

The manuscript by Vincent de Laat et al. started with the observation that tumors have higher temperature (T) compared to the adjacent healthy pancreas. By using 2 PDAC cell lines with in vitro experiments they provided evidence that increasing the temperature from 37C to 38C cancer cells change their lipid metabolism by lowering PUFAs that are prone to lipid peroxidation. This mechanism confers decreased sensitivity to ferroptosis induction

and accordingly, they become resistant to gemcitabine, a ferroptosis inducing drug. The observation that in pancreatic cancer the tumor T is higher than the adjacent tissue is interesting and has been previously attributed to increased vasculature and tumor metabolic activity. The technical approach/experiments for the in vitro data is adequate. However, there`re major limitations in this study at the current form.

Major comments:
1. In the entire manuscript the authors apply an increase in T from 37C to 38C. However, In Fig. 1A they show that in human patients, in the healthy pancreas the T spans from 35.5C-38C and in tumors from 36.5C-39C. Does that mean that the mechanism they described applies also for the healthy tissue? Is it the absolute increase (the max T reached) or the fact that they have increased T even if it is from 36C to 37C? Moreover, 50% of the tumors tested has a T of circa 37C and only 4/11 patients had 38 or higher.

2. The majority of the volume of the PDAC is occupied by the TME, which includes a great amount of fibrotic tissue, while cancer cells are a minority. Therefore, to understand the functional significance of the increased T it is essential to work in vivo as the TME cannot be recapitulated using cancer cells. Moreover, the authors also found that not only they could not recapitulate their finding of the T increase in mouse models of PDAC, but also the T is even decreased in tumors. This is the major limitation of the study because the manuscript lacks confirmation in an in vivo model, which recapitulates the complex TME of PDAC.

Moreover, although in the PANC1 xenograft model the T in the tumors is decreased (Figure S5), they could suppress tumor growth upon Gem + PUFA ePL and prolong the survival of mice. This result suggests that it is not the tumor T that determined this antitumor response.

3. The originality of the statement of Figure 2 is questionable. Here the authors provide evidence to show that gemcitabine kills cancer cells by lipid peroxidation-induced ferroptosis and blocking ferroptosis (by ferrostatin) leads to gemcitabine resistance. Gemcitabine has been already found to cause the production of ROS, result in downregulation of NRF2 and ferroptosis (doi: 10.1158/1535-7163.MCT-14-0420).

4. All the in vitro results have been assessed in only 2 PDAC cells lines (1 KRAS mutant and 1 wild type), which is quite limited.

Minor comments:
-Fig. 1g the graph needs to be normalized to 37C only, this is important to visualize the difference between 37C and 38C (even if there is no difference).
-Fig. 1h. The proliferation of untreated cells at 37C and 38C must be included in the graph for comparison.
-Fig. S7. The normalization of the western blots is missing.

Reviewer #4 (Remarks to the Author); expert in intra-tissue thermoregulation:

The authors present findings of a study investigating effects of endogenous (elevated) tumor temperature(s) on ferroptosis and how this contributes to gemcitibine resistance in PDAC. The findings, as presented have important implications for both research and clinical management of PDAC. While it has been recognized that human solid tumors (e.g. breast) often (if not typically) display elevated temperature when compared to surrounding normal tissues, this has previously not been reported for PDAC. Though new, the report of such in PDAC in patients is not surprising.

That said, measuring temperature of PDAC tumors is challenging as these tumors are seated very deep in the body requiring invasive thermometry with patients under general anesthesia (GA). GA typically reduces thermoregulatory control in subjects (human and other mammalian animal models), leading to mild hypothermia. This aspect, and its consequences to measured temperatures (Fig 1 a, b) ought to be discussed in the text. It ought also to be noted that the temperature data displayed shows potential (mild) hypothermia.

Notwithstanding the challenges, which are substantial, the authors have managed to obtain such. Nevertheless, confounding hypothermia induced by GA and potential uncertainties in the measurements themselves deserve more discussion in the text. One might surmise that without the GA-induced hypothermia, the actual temperature(s) in tumors may be slightly higher than measured (perhaps 39 or even 40C?)

With that as context, differences between tumor core and peripheral temperatures show elevations between 0.5 and 2.0 C (Fig 1b), the latter supporting a hypothesis of ~39C core tumor temperature. It is unclear why the in vitro studies, intending to provide more biological context for the implied effects of elevated tumor temperatures (endogenous), are limited to only two temperatures - 37 C ("normal") and 38 C (elevated). Readers would benefit from results showing a more comprehensive range of temperature that includes 39 and even 40 C. Classic hyperthermia, as treatment modality, is considered to require temperatures of at least 43 C for a duration of 30 to 60 min.

On the latter point, it is well-established that the biological effects of heat stress depend on both temperature and time-at-temperature. In the Methods, authors should provide duration of cell exposure at the elevated temperature (currently only 38 C).

One might also consider that, given time-at-temperature is an important variable, in vitro studies may be used to shed some light on the time-at-temperature dependence of the evolution of resistance. This is a minor comment, given that the studies are focused on endogenous temperature, i.e. a "permanent" condition.

Overall, the manuscript describes results obtained from important and original work that can have a meaningful impact on clinical management of PDAC. In its present form, gaps exist that ought to be addressed for benefit of readers.

REPLY TO THE REVIEWERS' COMMENTS

We thank the editor and the reviewers for their critical and constructive comments, which we address here point by point. We feel that these comments have substantially improved our manuscript and are grateful for this opportunity.

REVIEWER COMMENTS

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de Laat et al. report highly original findings relating changes in the temperature of pancreatic tumors to the activation of ferroptotic signatures and to sensitivity to gemcitabine. While the work only provides a limited mechanistic insight, the observations are interesting and potentially important. However, there are some limitations to the work that the authors need to address.

⇒ We thank the reviewer for the careful reading of our manuscript, the overall positive assessment of the work and the constructive comments.

Specific comments

1. It would be important to provide a note on the robustness of the thermal measurements in patients. It would also be useful to correlate the temperature with other clinical and/or pathological parameters if possible.

⇒ As suggested by the reviewer, a note on the robustness of the thermal measurements in patients has been included in the discussion. Specifically, we mention that "while our study successfully obtained temperature readings in patients with PDAC, potential uncertainties related to general anesthesia-induced hypothermia and depth of tumor location merit careful consideration. We therefore caution against direct inter-patient temperature comparisons."

⇒ We agree with the usefulness of a potential correlation with other clinical and/or pathological parameters, but this was not included in the scope of this non-interventional clinical study, mainly because the sample size is low, given the observation-based nature of the original research question.

2. The authors assess proliferation in very long term experiments (up to 400h) and measure proliferation as a % confluence of the flask surface. I am not questioning the validity of the latter; however, this is not an accepted proliferation parameter and it would be important to provide more direct measures of proliferation in the manuscript.

⇒ The long-term nature of our experiments is justified by our very discovery that PDAC cells adapt to temperature in a time-dependent manner (Fig. 1g, Supplementary Fig. 1), and that gemcitabine induces ferroptotic cell death in a time-dependent manner (Fig. 2b,c, see also response to comment 3).

⇒ We thank the reviewer for correctly pointing out that proliferation is not a synonym of cell growth. We now include the correct usage of the words proliferation and cell growth across the manuscript. Measuring confluence is indeed a common method to assess cell culture growth, including in the field of ferroptosis (https://doi.org/10.1038/s41467-021-22336-4; https://doi.org/10.1038/s41586-020-2732-8; https://doi.org/10.1038/s41419-023-06063-w)

⇒ We agree with the usefulness of providing more direct measures of proliferation, and we have now included these data in the manuscript (Fig. 1h, Supplementary Fig. 2b)

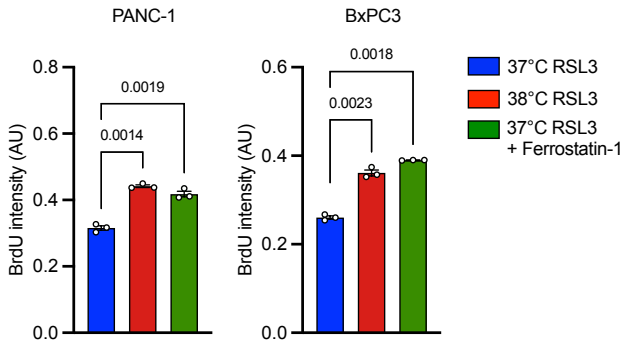
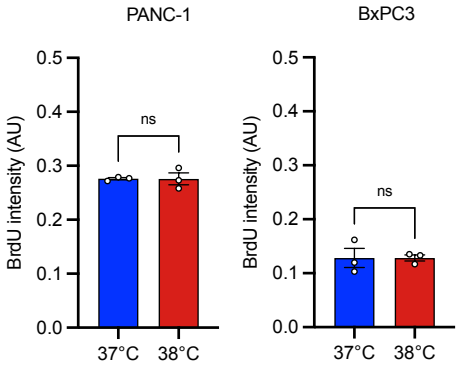


Fig. 1h Proliferation as measured by BrdU incorporation of PANC-1 or BxPC3 cells treated with RSL3 for 10 days, cultured at 37°C or 38°C (n = 3). One-way ANOVA with Šídák's multiple comparisons test.

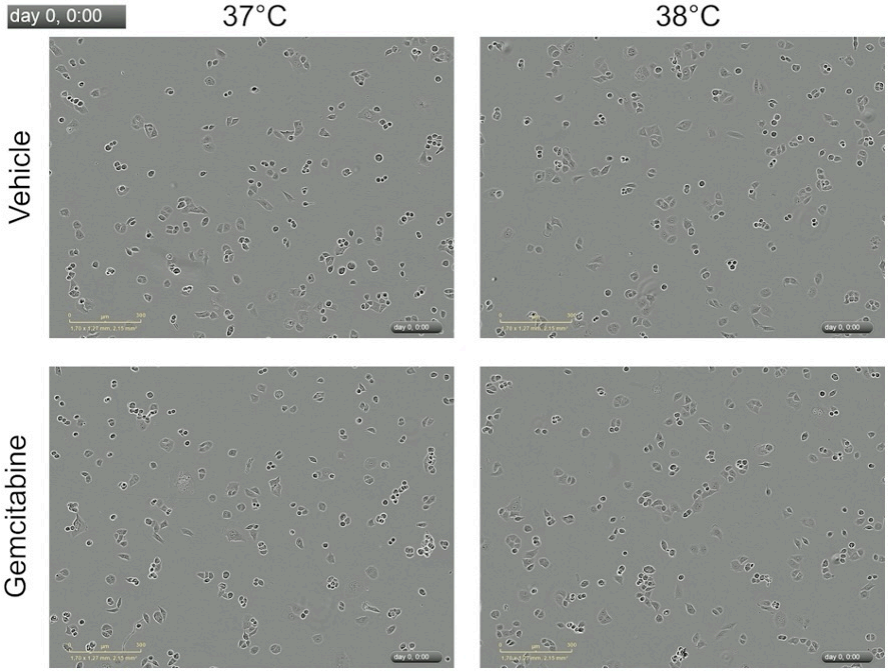


Supplementary Fig. 2b Proliferation as measured by BrdU incorporation of PANC-1 or BxPC3 cells, cultured at 37°C or 38°C for 10 days (n = 3). Unpaired two-sided Student's t-test (ns: not significant).

3. The fact that the cells remain stagnant for close to 10 days and then their growth takes off requires a more detailed analysis of the mechanisms involved with a time-course approach.

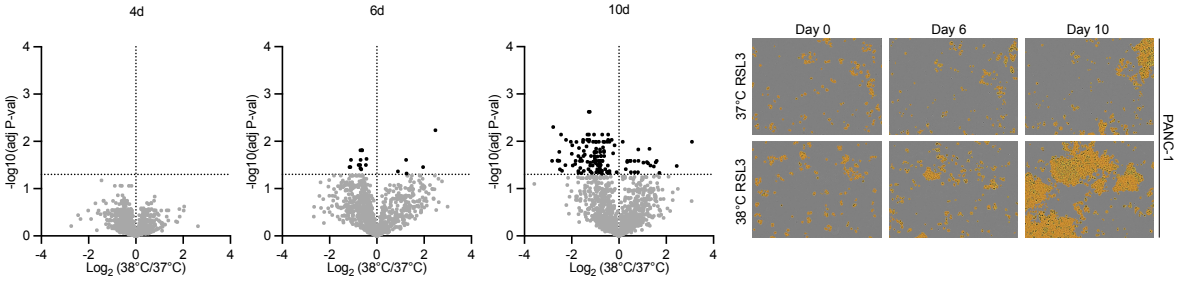
⇒ We now include more detailed analyses in a time-course approach:

- We have incorporated a movie (Supplemental Movie) that shows a time-course overview of tumoral-temperature induced resistance to gemcitabine.



Supplementary Movie (Still screenshot)

- We now provide time-course lipidomics data in combination with ferroptosis-resistance data (Supplementary Fig. 1).



Supplementary Fig. 1. Tumoral temperature drives ferroptosis evasion in PDAC cell lines (Left) Volcano plot of the lipidomic analysis of PANC-1 cells upon culturing at 38°C compared to 37°C for 4, 6 or 10 days (n = 3). Two-tailed Student's t-tests with Benjamini–Hochber's multiple comparisons test. (Right) Marked confluence at 0, 6 and 10 days of PANC-1 cells treated with RSL3, cultured at 37°C or 38°C.

- We provide detailed and time-course analysis of cell death mechanisms involved in response to gemcitabine (Fig 2b,c).

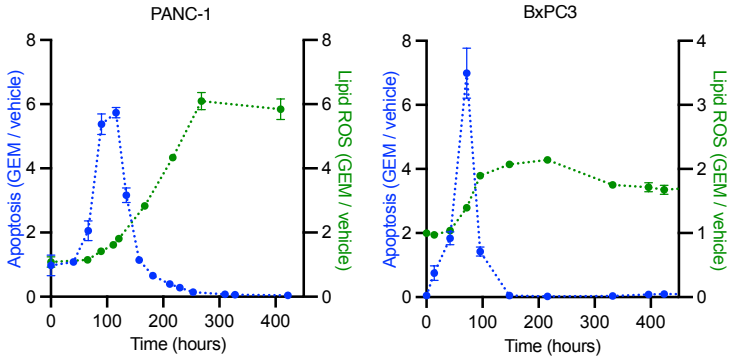


Fig. 2b Relative apoptosis and lipid peroxidation of PANC-1 cells (n = 4; n =3, respectively) or BxPC3 cells (n = 6; n =5, respectively) treated with gemcitabine (GEM) compared to vehicle.

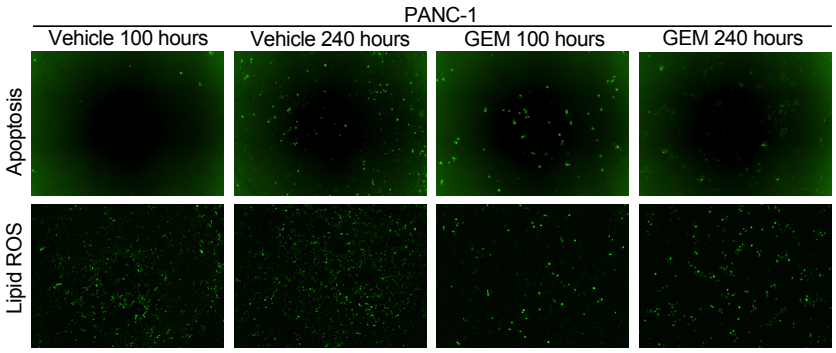
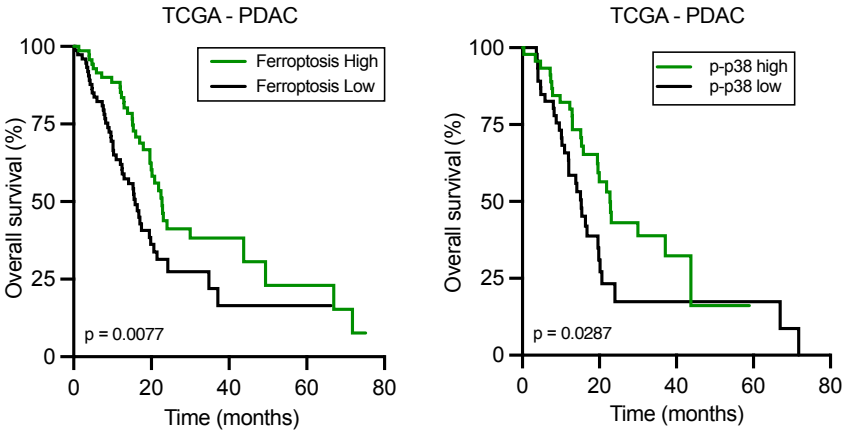


Fig. 2c Gemcitabine-induced apoptosis and lipid ROS after 100h and 240h in PANC-1 cells.

4. The assessment of the association with outcome in the TCGA series is inappropriate. It has been highlighted that the TCGA dataset contains only 150 bona fide PDAC whereas the remaining 35 samples are non-PDAC and therefore should be excluded from the analysis (they include endocrine tumors, pancreatitis, and non-neoplastic samples). In addition, for the p-P38 studies, it is important to determine whether the measurements refer to tumor or stromal cells - otherwise, the in vitro data cannot be extrapolated to the findings in patients tumors. This is a critical point that the authors need to address.

⇒ We thank the reviewer for rightfully pointing out that the TCGA data only contains 150 bona fide PDAC samples (https://doi.org/10.1016/j.ccell.2017.07.007). We have now excluded the remaining 35 samples from the analysis (Fig. 2a, Fig. 4b). It is important to note that these adjustments do not impact the conclusions drawn from the data analysis.



(Left) Fig. 2a Overall survival of patients with PDAC with either high (n = 75 patients, Ferroptosis Low) or low (n = 75 patients, Ferroptosis High) expression of ferroptosis suppressor genes as determined by gene-expression signature (gene list provided in Supplementary Data 3). Log-rank (Mantel-Cox) test.
(Right) Fig. 4b Overall survival of patients with PDAC with either high (n = 47) or low (n = 48) p-p38 expression. Log-rank (Mantel-Cox) test.

⇒ We have now determined tumor-cell specific p-p38 MAPK protein expression in clinical samples (Fig. 4c), allowing the extrapolation of our *in vitro* data to the findings in patient tumors.

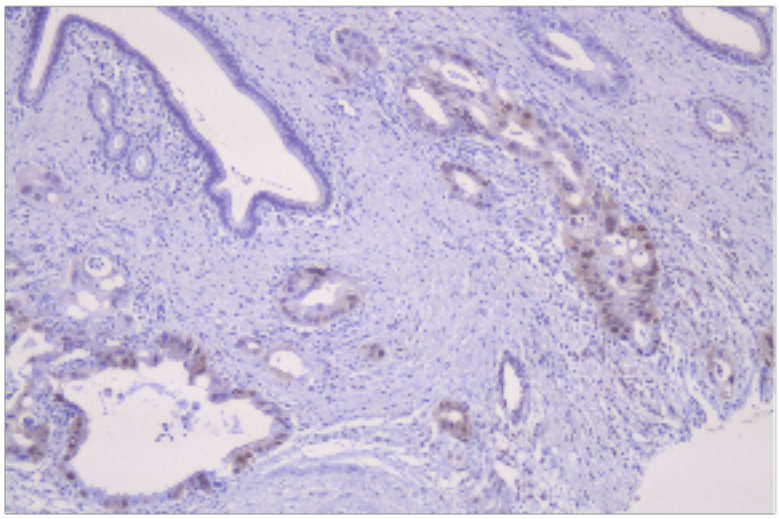
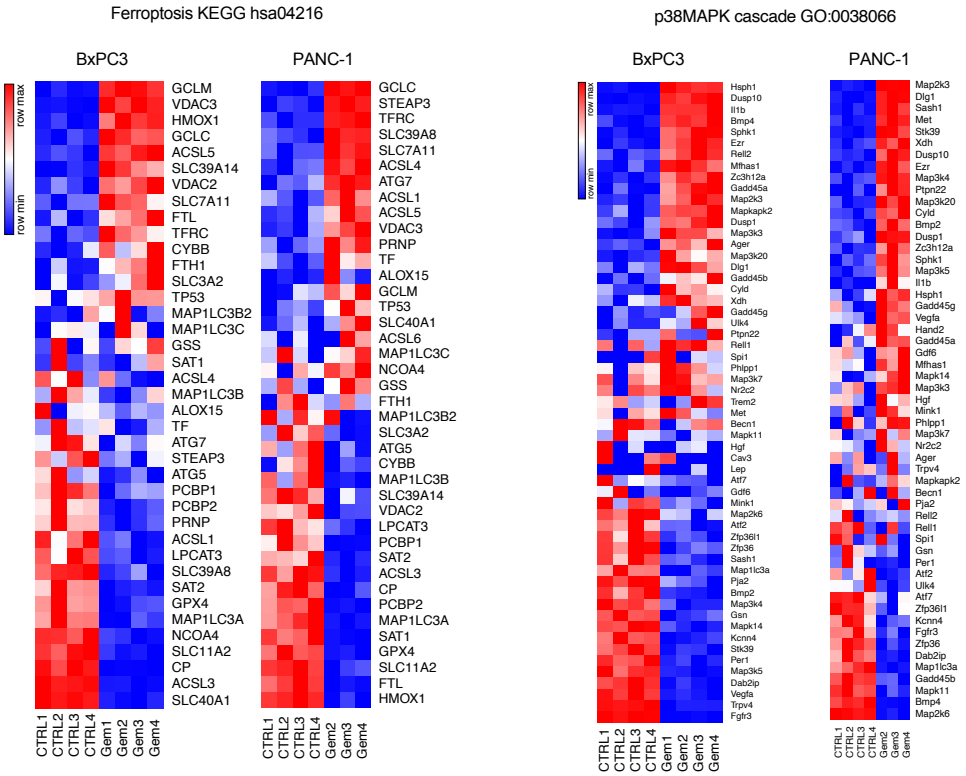


Fig. 4c Representative image of IHC staining for p-p38 in human PDAC

5. It would be useful to assess the ferroptosis signatures in other omics series to determine its association with other biological parameters.

⇒ We now have performed RNAseq on PANC-1 and BxPC3 cells upon treatment with gemcitabine. Analysis of these transcriptomic data confirms that gemcitabine affects ferroptosis and p38 MAPK pathway (Fig. 2d, Supplementary Fig. 4c, Fig. 4e, Supplementary Fig. 7a).



(Left) Fig. 2d and Supplementary Fig. 4c Expression of ferroptosis-related genes as measured by RNA-Seq in PANC-1 or BxPC3 cells treated for 240h with vehicle or gemcitabine.
(Right) Fig. 4e, Supplementary Fig. 7a Expression of p38 MAPK-related genes as measured by RNA-Seq in PANC-1 or BxPC3 cells treated for 240h with vehicle or gemcitabine.

6. I am surprised that the authors use 30 nM gemcitabine (according to the materials and methods) since this is a rather low dose of the drug and the effects are quite important. Please review and confirm.

⇒ We appreciate the reviewer's attention to the concentration of gemcitabine used in our study. We have reviewed and confirmed this concentration in our experiments. Additionally, this concentration (and lower) has been employed in other well-regarded studies as well (https://doi.org/10.1016/j.cmet.2019.02.001; https://doi.org/10.1016/j.ccell.2017.11.007; https://doi.org/10.1016/j.ccell.2023.04.003; https://doi.org/10.1038/s41586-022-04638-9). These references provide additional context for the chosen concentration in our study.

7. Some of the experiments (e.g. Figure 2c) lack an untreated control group, which makes it impossible to assess the effects of gemcitabine alone.

⇒ We thank the reviewer for bringing attention to the need for untreated control groups in certain experiments, particularly in Figure 2c. In response to this concern, we have incorporated additional untreated control groups in Figure 2c to facilitate a comprehensive assessment.

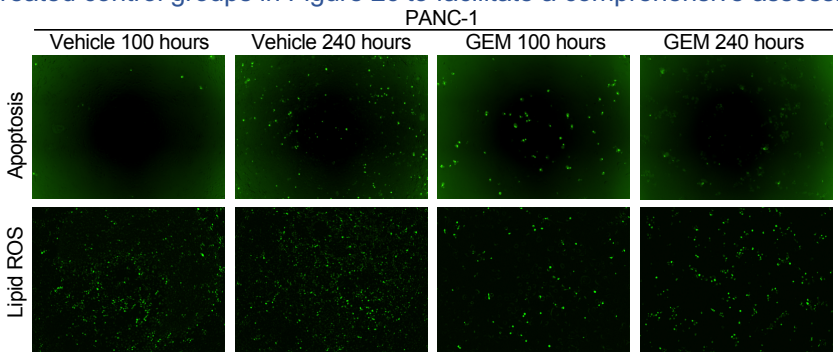
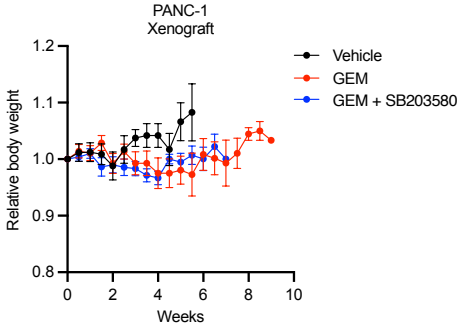


Fig. 2c Gemcitabine-induced apoptosis and lipid ROS after 100h and 240h in PANC-1 cells.

⇒ To address the specific dynamics observed in our study, it's important to note that our investigation revealed an increase in GEM-induced lipid peroxidation between 100h and 240h, juxtaposed with a decrease in GEM-induced apoptosis over the same time period (Fig. 2b-c). For clarity, we have included the relevant control group for GEM at 240h, using GEM at 100h as a demonstrative control in Figure 2c. The quantification of this effect is further elucidated in Figure 2b, while a direct comparison with the untreated control group is presented in Figure 3c.

8. The toxicity of the drugs administered in some experiments (specially with SB) should be noted as changes in mouse weight.

⇒ We thank the reviewer for highlighting the concern regarding the potential toxicity of SB-203580. While it is important to note that SB-203580 is not intended as a therapeutic drug due to its role in inducing resistance, we acknowledge the significance of assessing potential adverse effects. In response to this feedback, we have now incorporated relevant toxicity data in Supplementary Fig. 7e.



Supplementary Fig. 7e Body weight changes in PANC-1 xenograft bearing NMRInu/nu mice.

Reviewer #2 (Remarks to the Author); expert in ferroptosis, lipid metabolism:

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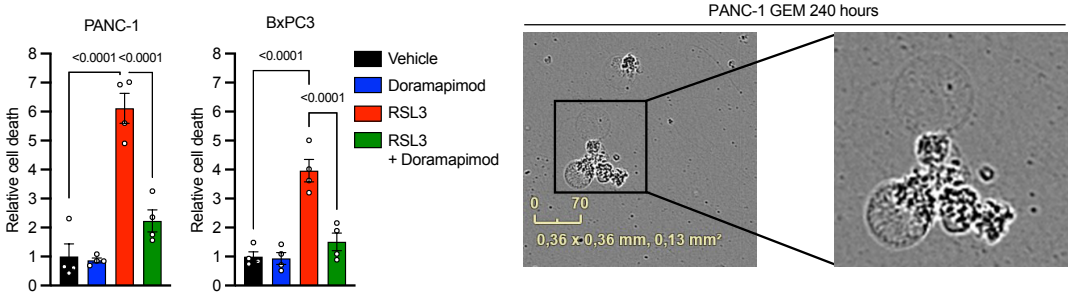
⇒ We express our gratitude to the reviewer for the meticulous review of our manuscript, the favorable evaluation of the work, and the insightful feedback provided.

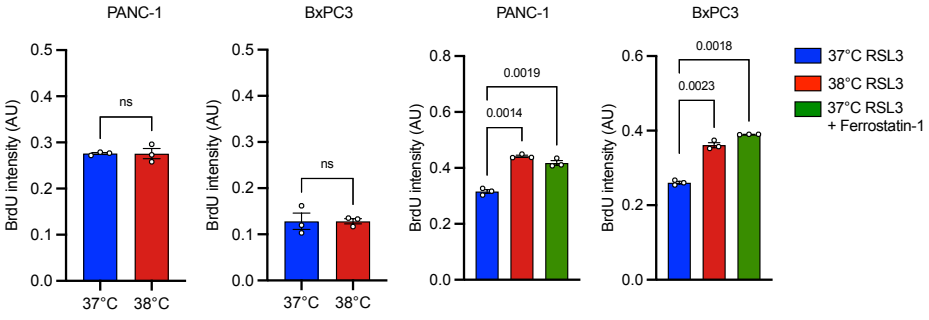
Major points:
1. There is not enough evidence to show that cells that cultured at 38c are more resistant to ferroptosis than the cells that cultured at 37c. Firstly, the temperature difference is so little, how can the authors ensure the culture difference can be maintained during the whole experiment? Secondly, the authors need to use more direct and reliable cell death or cell viability assays, instead of their “relative proliferation” assay to assess the ferroptosis sensitivity of the cells cultured at different temperature to different ferroptosis inducing conditions.

⇒ We thank the reviewer for raising the technical question on maintaining culture differences throughout the experiment. We want to assure the reviewer that we have employed calibrated incubators to address this issue, as mentioned in the methods section. To provide further clarity and emphasis, we have now explicitly highlighted the use of calibrated incubators in the main text as well.

⇒ We appreciate the reviewer's feedback regarding the need for more direct and reliable cell death or cell viability assays, particularly in evaluating ferroptosis sensitivity under different conditions. In response to this suggestion, we have incorporated a range of additional assays to enhance the robustness of our findings. Specifically, we have included cell death dye assays (Figure 4a), BrdU incorporation for proliferation assessment (Fig. 1h, Supplementary Fig. 2b), and microscopy to confirm ferroptotic cell death morphology (Fig. 2e). The manuscript also includes caspase 3/7 activity assays for apoptotic cell death evaluation (Fig. 2b-c, Supplementary Fig. 4a) and BODIPY™ 581/591 C11 staining to assess ferroptotic cell death (Fig. 1i, Fig. 2b-c, Supplementary Fig. 4b).



(Left) Fig. 4a Cell death of PANC-1 or BxPC3 cells treated with RSL3 and Doramapimod for 24h (n = 4). One-way ANOVA with Šídák's multiple comparisons test.
(Right) Fig. 2e Phase contrast image showing PANC-1 cells treated with gemcitabine undergoing cell death.



(Left) Supplementary Fig. 2b Proliferation as measured by BrdU incorporation of PANC-1 or BxPC3 cells, cultured at 37°C or 38°C for 10 days (n = 3). Unpaired two-sided Student's t-test (ns: not significant).
(Right) Fig. 1h Proliferation as measured by BrdU incorporation of PANC-1 or BxPC3 cells treated with RSL3 for 10 days, cultured at 37°C or 38°C (n = 3). One-way ANOVA with Šídák's multiple comparisons test.

2. The authors tried to show that increased temperature decreases PUFA-ePC to make the cells more resistant to ferroptosis. However, it seemed PUFA-ePC only decreased significantly in PANC-1 cells, but not in BxPC3 cell, when cells were cultured at 38c (Fig 1E). More experiments are needed to support there claim and address the discrepancy between the two tested PDAC lines.

⇒ We thank the reviewer for the feedback regarding Fig. 1e. We would like to emphasize that in both PANC-1 and BxPC3 cell lines, a statistically significant reduction in total PUFA ether lipids was observed when cultured at 38°C, as depicted in Fig. 1e. This consistent finding aligns with our main conclusion of reduced sensitivity to ferroptosis in these cells (Fig. 1g-j, Supplementary Fig. 1a-b). Importantly, these results are in line with the established role of total PUFA ether lipids in ferroptosis sensitivity, as documented recently (https://doi.org/10.1038/s41586-020-2732-8).

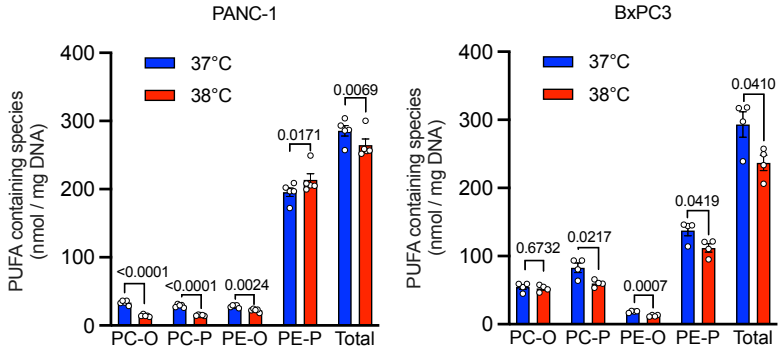


Fig. 1e Total abundance of PUFA containing ether lipid species (PC-O: 1-alkyl,2-acylphosphatidylcholine; PC-P: 1-alkenyl,2-acylphosphatidylcholine; PE-O: 1-alkyl,2-acylphosphatidylethanolamine; PE-P: 1-alkenyl,2-acylphosphatidylethanolamine) in PANC-1 (n = 5) or BxPC3 (n = 4) cells cultured at 37°C or 38°C for 10 days. Unpaired two-sided Student's t-test.

3. There is not strong evidence to support Gemcitabine directly induces ferroptosis.
The conclusion that Gemcitabine first induces apoptosis, then later induces ferroptosis is questionable. Gemcitabine is known to induce oxidative stress in the cell. It cannot rule out that radical trapping anti-oxidants (RTAs), like ferrostatin and UAMC-3203, restore the growth of Gemcitabine-treated cells through reducing oxidative stress in the cells. The authors need to test whether other ferroptosis suppressing inhibitors, e.g. iron chelator and overexpression of FSP1, can rescue Gemcitabine-induced cell death.

⇒ We thank the reviewer for the thoughtful consideration and suggestions. We have taken into account this feedback regarding the evidence supporting the direct induction of ferroptosis by gemcitabine, and we appreciate the opportunity to provide more conclusive data. In addition to our use of the established method of measuring lipid peroxidation as a marker for ferroptotic cell death (Fig. 1g, Fig. 2b, Fig 2c, Supplementary Fig. 4b), and the observation that well-established ferroptosis inhibitors Ferrostatin-1 and UAMC-3203 induce gemcitabine resistance *in vitro and in vivo*, we have incorporated additional analyses. These include the following:

- Microscopy to confirm ferroptotic cell death morphology (Fig. 2e)

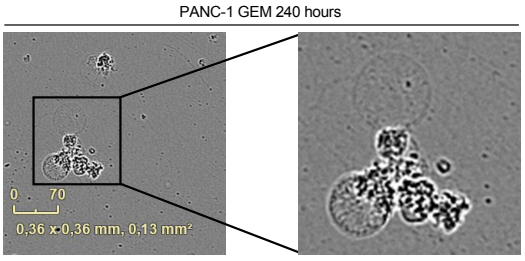


Fig. 2e Phase contrast image showing PANC-1 cells treated with gemcitabine undergoing cell death.

- Significant alterations in the expression of the ferroptotic gene signature (Fig. 2d, Supplementary Fig. 4c).

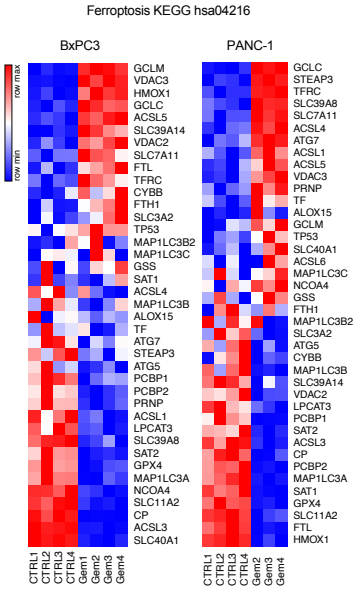
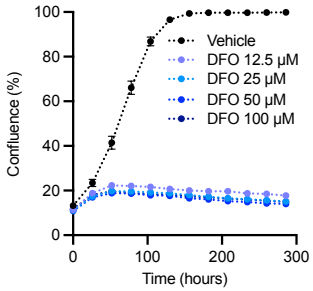


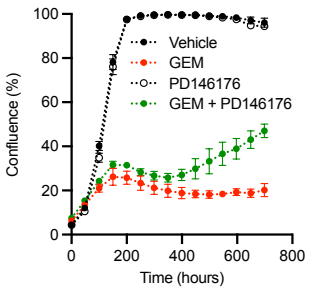
Fig. 2d and Supplementary Fig. 4c Expression of ferroptosis-related genes as measured by RNA-Seq in PANC-1 or BxPC3 cells treated for 240h with vehicle or gemcitabine.

- In response to the suggestion, we attempted experiments with an iron-chelator; however, due to the long-term toxicity associated with established concentrations (https://doi.org/10.1038/nature24297) of DFO (Supplementary Fig. 4d), these experiments were not feasible.



Supplementary Fig. 4d Time-course confluence of PANC-1 cells treated with DFO (n = 3).

- Finally, to further support our findings, we now demonstrate that PD146176, a non-radical trapping anti-oxidant-ferroptosis inhibitor (https://doi.org/10.1038/nature24297 ; https://doi.org/10.1038/nature23007), induces resistance to gemcitabine (Supplementary Fig. 4e).



Supplementary Fig. 4e Time-course confluence of PANC-1 cells treated with gemcitabine and supplemented with PD146176 (n = 3).

We believe these additional experiments and analyses strengthen the evidence for Gemcitabine-induced ferroptosis and its association with drug resistance.

4. The authors tried to use p38 deactivation as a link to connect gemcitabine resistance with tumoral temperature-induced adaptations in lipid metabolism. However, they didn't conduct any direct experiment to support p38 as a “link” of these events.

⇒ We thank the reviewer for the thorough evaluation and the suggestion for additional experiments related to p38 deactivation as a link between gemcitabine resistance and tumoral temperature-induced adaptations in lipid metabolism. In the original version of the paper, we demonstrated that gemcitabine-induced p38 activation is prevented by culturing at tumoral temperature or Ferrostatin-1 supplementation (Supplementary Fig. 7b-c in the revised version). Additionally, we found that direct p38 inhibition phenocopies tumoral temperature-induced or Ferrostatin-1-induced gemcitabine resistance (Fig. 4g in the revised version). Furthermore, we established that p38 inhibition acts downstream of tumoral-temperature induced lipid adaptations in the context of gemcitabine resistance (Fig. 4h-i in the revised version).

⇒ To provide even more mechanistic insight, we have expanded our analysis. Specifically, we now show that p38 inhibition by Doramapimod directly prevents ferroptotic cell death in PDAC cells (Fig. 4a).

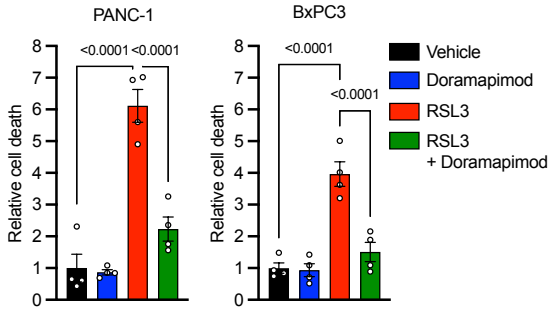


Fig. 4a Cell death of PANC-1 or BxPC3 cells treated with RSL3 and Doramapimod for 24h (n = 4). One-way ANOVA with Šídák's multiple comparisons test.

⇒ Moreover, we demonstrate that gemcitabine induces dramatic alterations in the p38 MAPK-gene signature as measured by RNA seq (Fig. 4g).

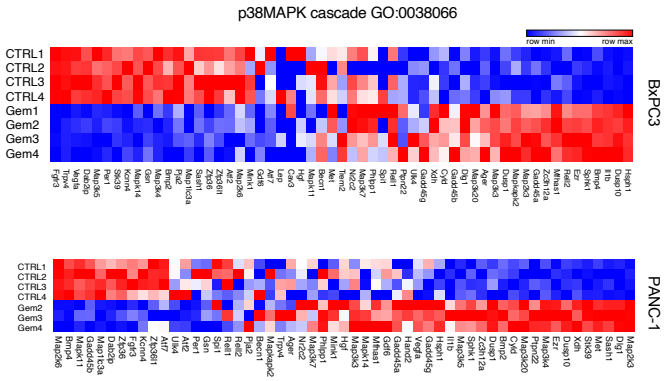
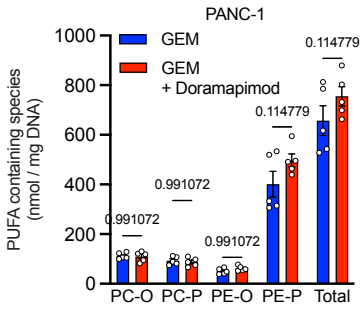


Fig. 4e, Supplementary Fig. 7a Expression of p38 MAPK-related genes as measured by RNA-Seq in PANC-1 or BxPC3 cells treated for 240h with vehicle or gemcitabine.

⇒ Regarding the suggestion for an experiment to force p38 activation upon temperature-induced p38 inhibition and concomitant chemoresistance, we want to acknowledge the limitation that, to the best of our knowledge, no specific p38 activator is currently available.

5. The mechanistic study in this manuscript is rather limited. It is not clear how temperature affect PUFA-ePC and whether p38 is indeed involved in this process.

⇒ We thank the reviewer for the question whether p38 is involved in the observed temperature-induced lipid adaptations. In response to this question, we have conducted lipidomics analysis of cells treated with gemcitabine (GEM) and p38 inhibitor over a 10-day period (Supplementary Fig. 7d). This data demonstrates that the observed p38-inhibition in response to temperature (Fig. 4f, Supplementary Fig. 7b) does not act upstream of the temperature-induced changes in PUFA-ePL as observed in Fig. 1e.



Supplementary Fig. 7d Total abundance of PUFA containing ether lipid species (PC-O: 1-alkyl,2-acylphosphatidylcholine; PC-P: 1-alkenyl,2-acylphosphatidylcholine; PE-O: 1-alkyl,2-acylphosphatidylethanolamine; PE-P: 1-alkenyl,2-acylphosphatidylethanol-amine) in PANC-1 (n = 5) cells treated with gemcitabine (GEM) and Doramapimod for 10 days. Unpaired two-sided Student's t-test.

Reviewer #3 (Remarks to the Author); expert in lipids, PDAC:

Title: Spontaneously increased temperature of human pancreatic tumors promotes ferroptosis evasion and chemotherapy resistance via p38 MAPK inhibition.

The manuscript by Vincent de Laat et al. started with the observation that tumors have higher temperature (T) compared to the adjacent healthy pancreas. By using 2 PDAC cell lines with in vitro experiments they provided evidence that increasing the temperature from 37C to 38C cancer cells change their lipid metabolism by lowering PUFAs that are prone to lipid peroxidation. This mechanism confers decreased sensitivity to ferroptosis induction and accordingly, they become resistant to gemcitabine, a ferroptosis inducing drug. The observation that in pancreatic cancer the tumor T is higher than the adjacent tissue is interesting and has been previously attributed to increased vasculature and tumor metabolic activity. The technical approach/experiments for the in vitro data is adequate. However, there`re major limitations in this study at the current form.

⇒ We thank the reviewer for the careful reading of our manuscript and the constructive comments and questions.

Major comments:
1. In the entire manuscript the authors apply an increase in T from 37C to 38C. However, In Fig. 1A they show that in human patients, in the healthy pancreas the T spans from 35.5C-38C and in tumors from 36.5C-39C. Does that mean that the mechanism they described applies also for the healthy tissue? Is it the absolute increase (the max T reached) or the fact that they have increased T even if it is from 36C to 37C? Moreover, 50% of the tumors tested has a T of circa 37C and only 4/11 patients had 38 or higher.

⇒ We thank the reviewer for raising the points of temperature variations observed in both healthy pancreas and tumors, as well as the potential implications of the temperature increase in the context of the described mechanisms.

⇒ We acknowledge the variability in temperature spans in both healthy pancreas and tumors in human patients, as presented in Figure 1a. The increase in temperature applied in our experimental model from 37°C to 38°C is intended to study the impact of a relatively modest temperature change. We recognize the complexities introduced by the diverse temperature ranges observed in patient tissues. To address this concern, we have included a cautionary note in the discussion section, highlighting potential uncertainties related to general anesthesia-induced hypothermia and the depth of tumor location. We emphasize the importance of considering these factors and caution against direct inter-patient temperature comparisons. This perspective is also shared by Reviewer 4 (expert in intra-tissue thermoregulation).

2. The majority of the volume of the PDAC is occupied by the TME, which includes a great amount of fibrotic tissue, while cancer cells are a minority. Therefore, to understand the functional significance of the increased T it is essential to work in vivo as the TME cannot be recapitulated using cancer cells.

Moreover, the authors also found that not only they could not recapitulate their finding of the T increase in mouse models of PDAC, but also the T is even decreased in tumors. This is the major limitation of the study because the manuscript lacks confirmation in an in vivo model, which recapitulates the complex TME of PDAC.

⇒ We thank the reviewer for the insightful comments regarding the relevance of *in vivo* models and the observed temperature variations in the tumor microenvironment (TME). We appreciate the opportunity to address these concerns and provide additional context.

⇒ In this study, our focus is on unveiling the increased temperature as an element within the human tumor microenvironment and its impact on pancreatic cancer cell sensitivity to standard chemotherapy, particularly through the regulation of ferroptosis sensitivity. We acknowledge the complexity of the TME, including the significant contribution of non-malignant cells and fibrotic tissue. While we agree with the reviewer that non-malignant cells within the TME may also be influenced by tumoral temperature, the primary emphasis of our investigation centers on the effects observed in cancer cells.

To further validate our findings in a more complex *in vivo* setting, we have extensively confirmed the role of ferroptosis modulation in response to gemcitabine in an *in vivo* model (Fig 2g-j). This inclusion aims to bridge the gap between our *in vitro* observations and potential *in vivo* implications within the complex TME.

Additionally, we appreciate the reviewer's astute observation that murine PDAC models are natively hypothermic. We address this finding in the discussion section, acknowledging the novel insight into the potential shortcomings of mouse models in studying key aspects of cancer biology, including temperature-related effects.

Moreover, although in the PANC1 xenograft model the T in the tumors is decreased (Figure S5), they could suppress tumor growth upon Gem + PUFA ePL and prolong the survival of mice. This result suggests that it is not the tumor T that determined this antitumor response.

⇒ It is understandable that the observation that PANC-1 xenograft tumors exhibit decreased temperature, yet respond to the combination of Gem + PUFA ePL with suppressed tumor growth and prolonged survival, raises a question about the role of tumor temperature in this antitumor response. Therefore, we appreciate the opportunity to provide further clarification.

In light of the limitations posed by mouse models regarding differences in thermoregulation, it is essential to clarify that the primary objective of this experiment is not to establish a direct link with temperature. Instead, the focus is on serving as an *in vivo* extension of our *in vitro* data, specifically concerning the role of ferroptosis, and its impact on pancreatic cancer cell sensitivity to standard chemotherapy within the human tumor microenvironment.

Importantly, *in vivo* administration of ferroptosis-modulating agents affects lipid peroxidation (Fig. 2g), even in hypothermic tumors (Supplementary Fig. 5a-b). Thus, our finding that the modulation of lipid peroxidation *in vivo* directly impacts the response to gemcitabine, even in natively hypothermic murine tumors, is consistent with our broader observation that alterations in lipid peroxidation serve as a downstream effector of human tumoral temperature, driving resistance to gemcitabine.

3. The originality of the statement of Figure 2 is questionable. Here the authors provide evidence to show that gemcitabine kills cancer cells by lipid peroxidation-induced ferroptosis and blocking ferroptosis (by ferrostatin) leads to gemcitabine resistance. Gemcitabine has been already found to cause the production of ROS, result in downregulation of NRF2 and ferroptosis (doi: 10.1158/1535-7163.MCT-14-0420).

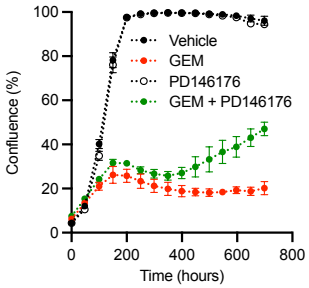
⇒ We appreciate the opportunity to further clarify the originality of our findings in Figure 2. In this section, we provide evidence showing that gemcitabine induces cancer cell death through lipid peroxidation-induced ferroptosis and that blocking ferroptosis with Ferrostatin-1 and UAMC-3203 leads to gemcitabine resistance *in vitro* and *in vivo*.

While it is established that chemotherapies, including gemcitabine, induce reactive oxygen species (ROS), the reference provided (doi: 10.1158/1535-7163.MCT-14-0420) does not specifically demonstrate that gemcitabine induces ferroptosis. We acknowledge the induction of ROS by gemcitabine in existing literature; however, our study aims to contribute novel insights by linking gemcitabine-induced ROS to lipid peroxidation-induced ferroptosis, specifically within the context of pancreatic cancer.

Furthermore, as described in the manuscript, we acknowledge that resistance to gemcitabine can be targeted by combination treatments that sensitize pancreatic ductal adenocarcinoma (PDAC) cells to ferroptosis. However, the question of whether gemcitabine itself induces ferroptosis has not been definitively addressed in the existing literature (doi: 10.3390/ijms222010944).

Of note, in response to feedback from Reviewer 2, we have conducted additional experiments that further support the specificity of our observations to lipid ROS, emphasizing the connection between gemcitabine, lipid peroxidation, and ferroptosis.

- PD146176, a non-radical trapping anti-oxidant-ferroptosis inhibitor (https://doi.org/10.1038/nature24297 ; https://doi.org/10.1038/nature23007), induces resistance to gemcitabine (Supplementary Fig. 4e).



Supplementary Fig. 4e Time-course confluence of PANC-1 cells treated with gemcitabine and supplemented with PD146176 (n = 3).

- Microscopy confirms gemcitabine-induced ferroptotic cell death morphology (Fig. 2e)

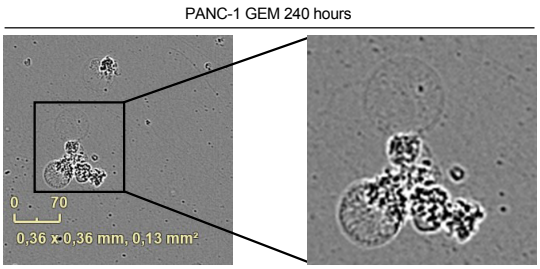


Fig. 2e Phase contrast image showing PANC-1 cells treated with gemcitabine undergoing cell death.

- Gemcitabine-induced alterations in the expression of the ferroptotic gene signature (Fig. 2d, Supplementary Fig. 4c).

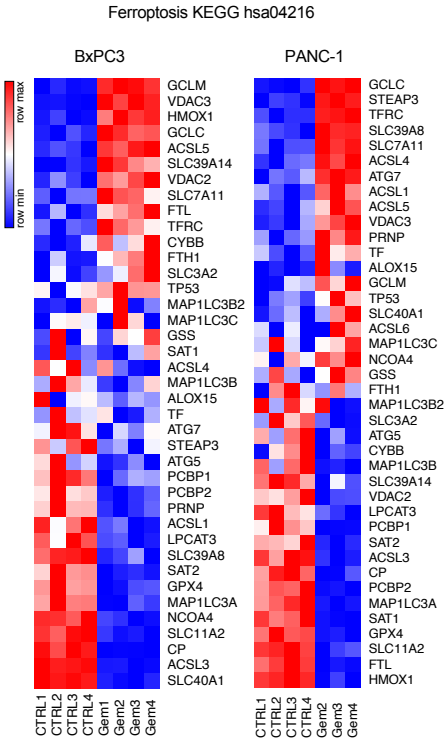
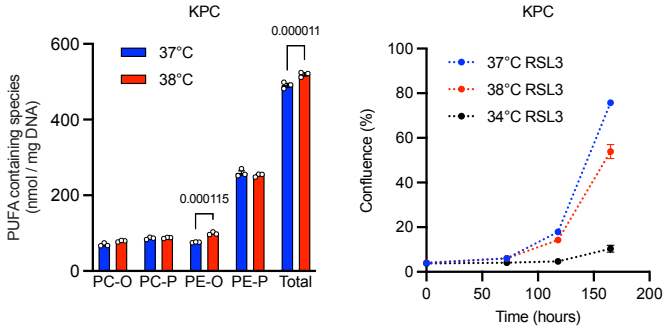


Fig. 2d and Supplementary Fig. 4c Expression of ferroptosis-related genes as measured by RNA-Seq in PANC-1 or BxPC3 cells treated for 240h with vehicle or gemcitabine.

4. All the in vitro results have been assessed in only 2 PDAC cells lines (1 KRAS mutant and 1 wild type), which is quite limited.

⇒ We thank the reviewer for pointing out the limitation in our initial *in vitro* experiments. In response to this feedback, we have now included *in vitro* experiments using murine PDAC KPC cells. In these experiments, we observed that total PUFA ether lipids are upregulated upon culture at 38°C (Supplementary Fig. 5g). The divergence in effects observed in murine PDAC cells compared to human PDAC cells adds to the earlier insight into the potential shortcomings of mouse models in studying key aspects of cancer biology, including temperature-related effects. Importantly however, this finding aligns with our observation of increased sensitivity to ferroptosis in these cells (Supplementary Fig. 5h). These results are consistent with the established role of PUFA ether lipids in ferroptosis sensitivity, as documented in the literature (https://doi.org/10.1038/s41586-020-2732-8).



(Left) Supplementary Fig. 5g Total abundance of PUFA containing ether lipid species (PC-O: 1-alkyl,2-acylphosphatidylcholine; PC-P: 1-alkenyl,2-acylphosphatidylcholine; PE-O: 1-alkyl,2-acylphosphatidylethanolamine; PE-P: 1-alkenyl,2-acylphosphatidylethanol-amine) in KPC (n = 3) cells cultured at 37°C or 38°C for 10 days. Unpaired two-sided Student's t-test.
(Right) Supplementary Fig. 5h Time-course confluence of KPC cells treated with RSL3, cultured at 34°C, 37°C or 38°C (n = 4).

⇒ Thus, the observation of increased PUFA ether lipids in these cells, along with heightened sensitivity to ferroptosis, aligns with our overarching finding that changes in PUFA ether lipids function as a downstream effector of human tumoral temperature, affecting sensitivity to ferroptosis.

Minor comments:
Fig. 1g the graph needs to be normalized to 37C only, this is important to visualize the difference between 37C and 38C (even if there is no difference).

⇒ We agree with the reviewer's comment. For this reason, there has been no double normalization to 38°C in this graph. As detailed in the methods, cells with a FITC/PE fluorescence ratio greater than 98% of the untreated cells are defined as lipid ROS positive. This is an established way to plot such data (https://doi.org/10.1038/s41556-021-00818-3).

Fig. 1h. The proliferation of untreated cells at 37C and 38C must be included in the graph for comparison.

⇒ We have now included the proliferation data of untreated cells at both 37°C and 38°C in the graph for comparison in Fig. 1g. This addition should provide a more comprehensive view and aid in the interpretation of the result.

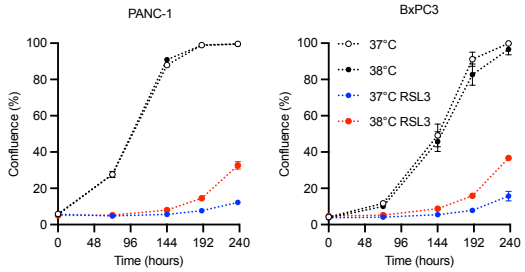
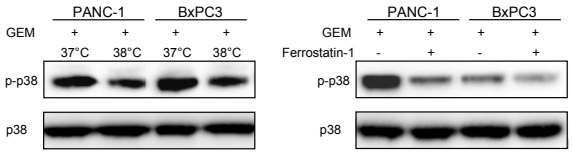


Fig. 1g Time-course confluence of PANC-1 or BxPC3 cells treated with RSL3, cultured at 37°C or 38°C (n = 6).

Fig. S7. The normalization of the western blots is missing.

⇒ We acknowledge the importance of normalization in Western blot analysis. We have carefully addressed this concern by including the normalization of the western blots in Supplementary Fig. 7b,c.



Supplementary Fig. 7b,c (b) p-p38 expression in PANC-1 and BxPC3 cells treated with gemcitabine (GEM), cultured at 37°C or 38°C. (c) p-p38 expression in PANC-1 and BxPC3 cells treated with gemcitabine (GEM), supplemented with Ferrostatin-1.

Reviewer #4 (Remarks to the Author); expert in intra-tissue thermoregulation:

The authors present findings of a study investigating effects of endogenous (elevated) tumor temperature(s) on ferroptosis and how this contributes to gemcitibine resistance in PDAC. The findings, as presented have important implications for both research and clinical management of PDAC.

⇒ We thank the reviewer for the favorable overall assessment, and the constructive insights provided.

While it has been recognized that human solid tumors (e.g. breast) often (if not typically) display elevated temperature when compared to surrounding normal tissues, this has previously not been reported for PDAC. Though new, the report of such in PDAC in patients is not surprising.

That said, measuring temperature of PDAC tumors is challenging as these tumors are seated very deep in the body requiring invasive thermometry with patients under general anesthesia (GA). GA typically reduces thermoregulatory control in subjects (human and other mammalian animal models), leading to mild hypothermia. This aspect, and its consequences to measured temperatures (Fig 1 a, b) ought to be discussed in the text. It ought also to be noted that the temperature data displayed shows potential (mild) hypothermia.

Notwithstanding the challenges, which are substantial, the authors have managed to obtain such. Nevertheless, confounding hypothermia induced by GA and potential uncertainties in the measurements themselves deserve more discussion in the text. One might surmise that without the GA-induced hypothermia, the actual temperature(s) in tumors may be slightly higher than measured (perhaps 39 or even 40C?)

⇒ We thank the reviewer for raising the important points of temperature variations observed in both healthy pancreas and tumors. To address this aspect, we have included a cautionary note in the discussion section, highlighting potential uncertainties related to general anesthesia-induced hypothermia and the depth of tumor location. We emphasize the importance of considering these factors and caution against direct inter-patient temperature comparisons.

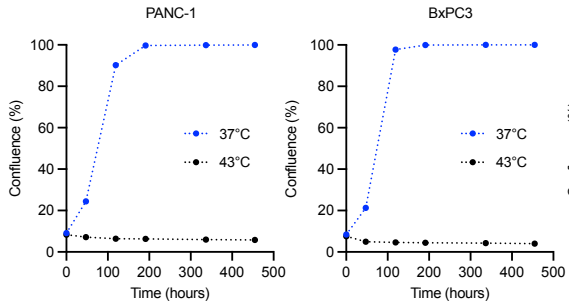
> "*Of note, while our study successfully obtained temperature readings in patients with PDAC, potential uncertainties related to general anesthesia-induced hypothermia and depth of tumor location merit careful consideration. We therefore caution against direct inter-patient temperature comparisons.*"

With that as context, differences between tumor core and peripheral temperatures show elevations between 0.5 and 2.0 C (Fig 1b), the latter supporting a hypothesis of ~39C core tumor temperature. It is unclear why the in vitro studies, intending to provide more biological context for the implied effects of elevated tumor temperatures (endogenous), are limited to only two temperatures - 37 C ("normal") and 38 C (elevated). Readers would benefit from results showing a more comprehensive range of temperature that includes 39 and even 40 C. Classic hyperthermia, as treatment modality, is considered to require temperatures of at least 43 C for a duration of 30 to 60 min.

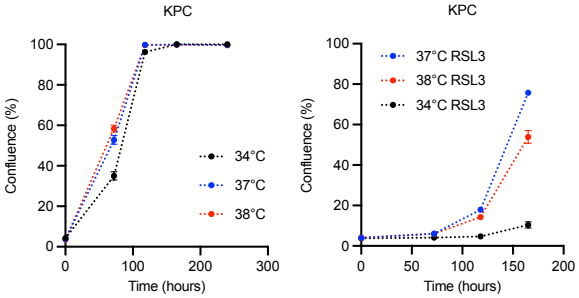
On the latter point, it is well-established that the biological effects of heat stress depend on both temperature and time-at-temperature. In the Methods, authors should provide duration of cell exposure at the elevated temperature (currently only 38 C).

⇒ We acknowledge the variability in temperature spans in both healthy pancreas and tumors in human patients, as presented in Figure 1a. The increase in temperature applied in our experimental model from 37°C to 38°C is intended to study the impact of a relatively modest temperature change.

⇒ In an effort to provide additional data and explore a broader temperature range, we now include data across a range from 34°C to 43°C (Supplementary Fig. 2c, Supplementary Fig. 5f,h). This inclusion aims to capture a more comprehensive understanding of the potential temperature-related effects.



Supplementary Fig. 2c Time-course confluence of PANC-1 or BxPC3 cells cultured at 37°C or 43°C (n = 4). Error bars represent s.e.m. from mean of biologically independent samples, but are not depicted when they are shorter than the symbol size.



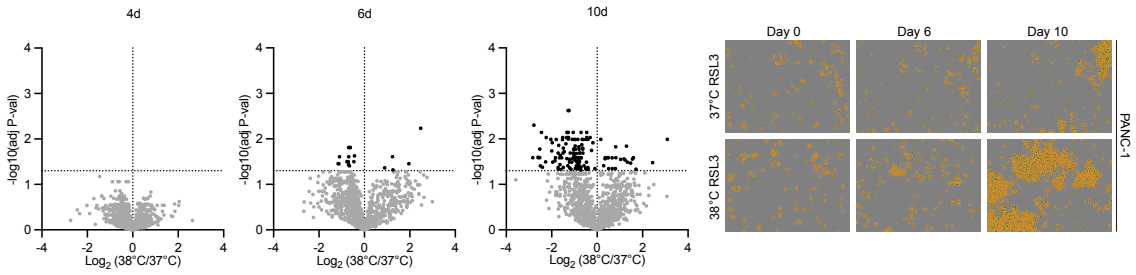
(Left) Supplementary Fig. 5f Time-course confluence of KPC cells cultured at 34°C, 37°C or 38°C (n = 4).
(Right) Supplementary Fig. 5h Time-course confluence of KPC cells treated with RSL3, cultured at 34°C, 37°C or 38°C (n = 4).

⇒ The representation of time at temperature is indeed indicated in all figures to provide a comprehensive overview of our experimental conditions.

One might also consider that, given time-at-temperature is an important variable, in vitro studies may be used to shed some light on the time-at-temperature dependence of the evolution of resistance. This is a minor comment, given that the studies are focused on endogenous temperature, i.e. a "permanent" condition.

⇒ We thank the reviewer for the insightful suggestion regarding the consideration of time-at-temperature as an important variable in shedding light on the evolution of resistance. We agree that understanding the time-dependent aspects of resistance can provide valuable insights. While our primary focus is indeed on the endogenous temperature as a "permanent" condition, we recognize the merit in exploring the time-course dynamics.

To address this, we have now incorporated time-course lipidomics data in combination with ferroptosis-resistance data. This new information is presented in Supplemental Figure 1, offering a more comprehensive view of the effects of tumoral temperature on lipid metabolism and sensitivity to ferroptosis at different time points. We believe this addition enhances the temporal dimension of our study and provides a more nuanced understanding of the interplay between temperature and resistance.



Supplementary Fig. 1. Tumoral temperature drives ferroptosis evasion in PDAC cell lines (Left) Volcano plot of the lipidomic analysis of PANC-1 cells upon culturing at 38°C compared to 37°C for 4, 6 or 10 days (n = 3). Two-tailed Student's t-tests with Benjamini–Hochber's multiple comparisons test. (Right) Marked confluence at 0, 6 and 10 days of PANC-1 cells treated with RSL3, cultured at 37°C or 38°C.

Overall, the manuscript describes results obtained from important and original work that can have a meaningful impact on clinical management of PDAC. In its present form, gaps exist that ought to be addressed for benefit of readers.

⇒ We thank the reviewer for recognizing the potential impact of our work on the clinical management of PDAC. We appreciate the valuable input regarding the identified gaps, and we believe to have addressed them, enhancing the overall quality and benefit for readers.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

While the authors have not responded to all my requests, my major concerns are satisfied with their response.

Reviewer #2 (Remarks to the Author):

The authors made notable improvement and the revised manuscript is in better shape. However, there are still multiple significant issues. For example,

1. The causative role of p38 is still not established. The authors did pharmacological experiments using p38 inhibitor Doramapimod. However, based on the structure of the compound, it is very likely to have radical trapping activity (this possibility can be tested by performing FENIX assay or by testing if the compound can inhibit ferroptosis in another setting that is independent of p38). The clean experiments to test the causative role of p38 should be genetic loss-of-function experiments.

2. Related to point-1, in their supplemental Fig. 7c, they showed ferrostain-1 can inhibit p38 phosphorylation. This observation is interesting, but it appears to argue against their hypothesis of an upstream role of p38 in ferroptosis, because ferrostain-1 inhibits lipid peroxidation, a rather late and committing event of ferroptosis.

3. The evidence that what they are looking at is ferroptosis instead of an ROS-stimulated non-ferroptotic necrosis phenomenon is still weak and is even further weakened by their data showing that iron chelator DFO cannot block the death. The morphological feature they use to support ferroptosis is not valid, because this morphology is shared by various other necrotic death processes. PD146176 does not help either, because it is an anti-oxidant and hence can mitigate ROS effect, be it ferroptosis or not.

Reviewer #3 (Remarks to the Author):

The part of the manuscript proving that gemcitabine induces cell death by ferroptosis has greatly improved and I don`t have any comments. Moreover, the findings that even a small increase in temperature in vitro may result in a big difference in the sensitivity to gem is very interesting. However, with the data provided I am even less convinced that the temperature changes have been adequately addressed to draw meaningful, for the clinic and readers, conclusions. Specifically, it fails to show the role of the observed temperature changes (fig. 1a) from 36-39 C in the impact of gem response. This was also part of my major concern #1, which has not been adequately addressed. The lack of a comprehensive characterization of the different temperatures (not only 38C) on the anti-proliferative response of gem makes the study limited in this point of view. The new experiments included a temperature of only 34 and 43, but did not include the temperatures observed in human patients (Fig. 1a). As already mentioned in my previous comments, only 2 patients

actually showed a temperature of 38 C and it is still not clear if temperatures of 36, 39 or 40 C confer resistance to gem.

Moreover, in the major concern #4 the authors did not expand their experiments to more human PDAC cell lines, despite the fact that they even observed that mouse KPC cells do not agree with the human cells. It was crucial also because the BxPC3 cell line is a wild type KRAS and therefore, quite an exception for PDAC.

Suggestion #1: The authors state in the manuscript “tumoral temperature” to refer to 38C. However, in Fig. 1a, they show that there are is also healthy tissue that can have a temperature close to 38C, thus, it is not specific for the tumor. Therefore, they need to replace this sentence from “tumoral temperature” to “38C” temperature as it is confusing.

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REPLY TO THE REVIEWERS' COMMENTS

We thank the editor and the reviewers for their critical and constructive comments, which we address here point by point. We feel that these comments have substantially improved our manuscript and are grateful for this opportunity.

REVIEWER COMMENTS

Reviewer #2 :

The authors made notable improvement and the revised manuscript is in better shape. However, there are still multiple significant issues.

We thank the reviewer for the careful reading of our manuscript, the overall positive assessment of the work and the constructive comments.

Specific comments:

1. The causative role of p38 is still not established. The authors did pharmacological experiments using p38 inhibitor Doramapimod. However, based on the structure of the compound, it is very likely to have radical trapping activity (this possibility can be tested by performing FENIX assay or by testing if the compound can inhibit ferroptosis in another setting that is independent of p38). The clean experiments to test the causative role of p38 should be genetic loss-of-function experiments.

We thank the reviewer for the suggestion of the genetic loss-of-function experiments. In line with previous work demonstrating that genetic loss-of-function of p38 leads to ferroptosis inhibition (DOI: 10.1080/23723556.2015.1054549) we now demonstrate that genetic loss-of-function of p38 indeed attenuates RSL3-induced ferroptotic cell death (new Fig 4b and c) and induces gemcitabine-resistance (new Fig 4j).

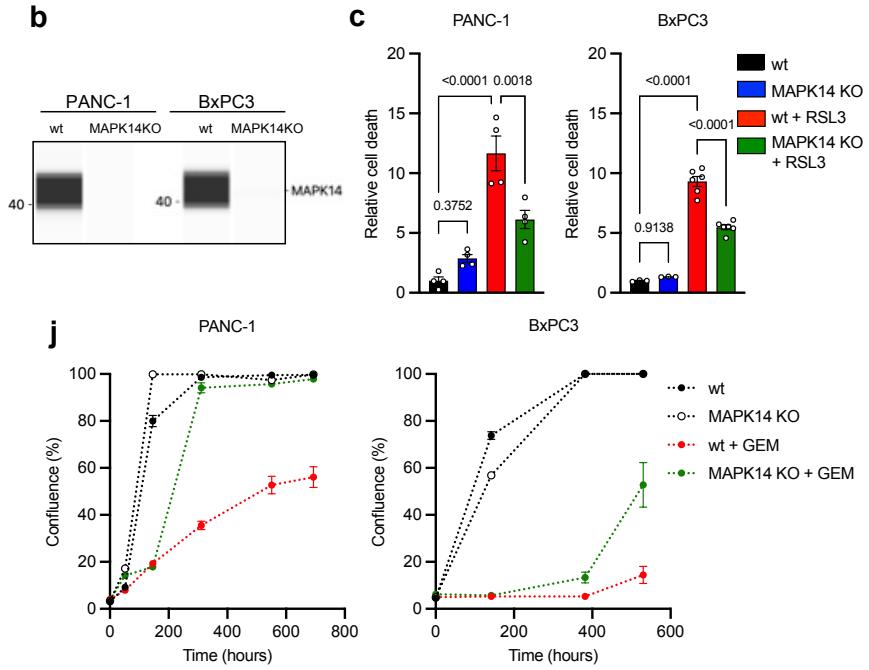


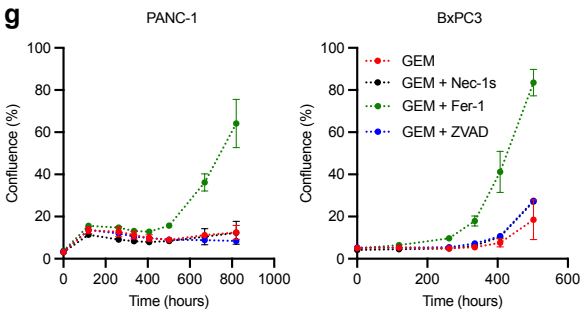
Fig. 4 (b, c and j)
(b) p-p38 expression in wild-type or MAPK14 KO PANC-1 and BxPC3 cells. **(c)** Cell death of wild-type or MAPK14 KO PANC-1 and BxPC3 cells treated with RSL3 for 24h (n = 4). One-way ANOVA with Šídák's multiple comparisons test. **(j)** Time-course confluence of wild-type or MAPK14 KO PANC-1 or BxPC3 cells treated with GEM (n = 6).

2. Related to point-1, in their supplemental Fig. 7c, they showed ferrostain-1 can inhibit p38 phosphorylation. This observation is interesting, but it appears to argue against their hypothesis of an upstream role of p38 in ferroptosis, because ferrostain-1 inhibits lipid peroxidation, a rather late and committing event of ferroptosis.

We appreciate the reviewer's careful assessment and the opportunity to clarify our model. We would like to point out that our evidence indicates that p38 MAPK functions downstream, rather than upstream of the ferroptosis pathway (see also response to point 1). This is now clearly indicated in our text where we discuss the signaling cascade involved in ferroptosis regulation. To avoid any confusion, we have reiterated this point in the revised manuscript to ensure clarity. Moreover, our observations (Fig. 4, Supplementary Fig. 7) corroborate the substantial evidence in the literature that supports the downstream role of p38 MAPK in ferroptosis (eg DOI: 10.1080/23723556.2015.1054549, DOI: 10.15252/embr.201744228).

3. The evidence that what they are looking at is ferroptosis instead of an ROS-stimulated non-ferroptotic necrosis phenomenon is still weak and is even further weakened by their data showing that iron chelator DFO cannot block the death. The morphological feature they use to support ferroptosis is not valid, because this morphology is shared by various other necrotic death processes. PD146176 does not help either, because it is an anti-oxidant and hence can mitigate ROS effect, be it ferroptosis or not.

We appreciate the reviewer's detailed critique and the opportunity to further improve our study. Although it was previously evidenced that Ferrostatin-1 only inhibits lipid ROS-induced cell death and not other forms of ROS-induced cell death (DOI: 10.1016/j.cell.2012.03.042), establishing Ferrostatin-1 is an established specific inhibitor of ferroptosis (eg DOI: 10.1038/s41586-020-2732-8), we now also used the necroptosis inhibitor Nec-1s. Our findings (new Supplementary Fig 4g) demonstrate that only ferrostatin-1 and not Nec-1s and ZVAD are able to restore growth under gemcitabine treatment.



Supplementary Fig. 4g
Time-course confluence of PANC-1 or BxPC3 cells treated with GEM and supplemented with Nec-1s, Fer-1 or ZVAD (n = 3).

Finally, as phospholipid peroxidation is specific to ferroptotic cell death (DOI: 10.1038/s41419-020-03118-0), we now directly measured oxidized phospholipids and found these species to be dramatically increased upon treatment with gemcitabine (new Figure 2f). Taken together, our findings now more accurately reflect the specific role of lipid peroxidation and ferroptosis induced by gemcitabine. Furthermore, to address the reviewer's point even more comprehensively, the manuscript was adapted to ensure clarity in the use of specific terminology.

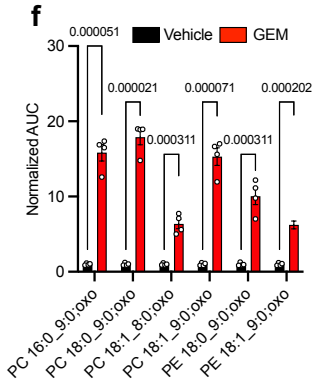


Fig. 2f
Oxidized lipid species in PANC-1 cells treated for 240h with vehicle or gemcitabine (n=4). Unpaired two-sided Student's t-test with Holm-Šídák's multiple comparisons test.

Reviewer #3:

The part of the manuscript proving that gemcitabine induces cell death by ferroptosis has greatly improved and I don`t have any comments. Moreover, the findings that even a small increase in temperature in vitro may result in a big difference in the sensitivity to gem is very interesting.

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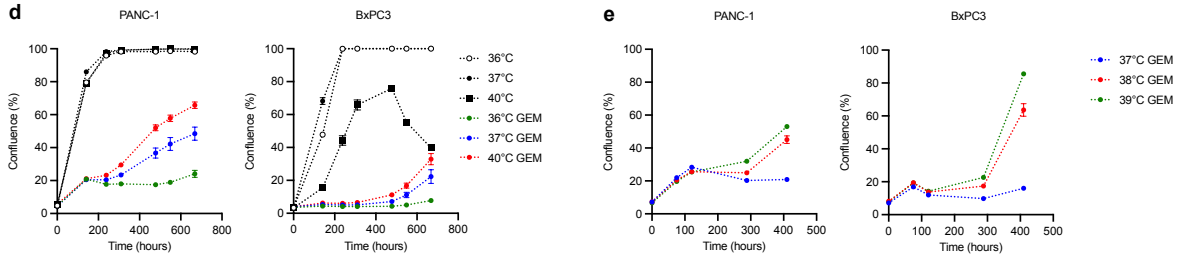
Specific comments:
However, with the data provided I am even less convinced that the temperature changes have been adequately addressed to draw meaningful, for the clinic and readers, conclusions. Specifically, it fails to show the role of the observed temperature changes (fig. 1a) from 36-39 C in the impact of gem response. This was also part of my major concern #1, which has not been adequately addressed. The lack of a comprehensive characterization of the different temperatures (not only 38C) on the anti-proliferative response of gem makes the study limited in this point of view. The new experiments included a temperature of only 34 and 43, but did not include the temperatures observed in human patients (Fig. 1a). As already mentioned in my previous comments, only 2 patients actually showed a temperature of 38 C and it is still not clear if temperatures of 36, 39 or 40 C confer resistance to gem.

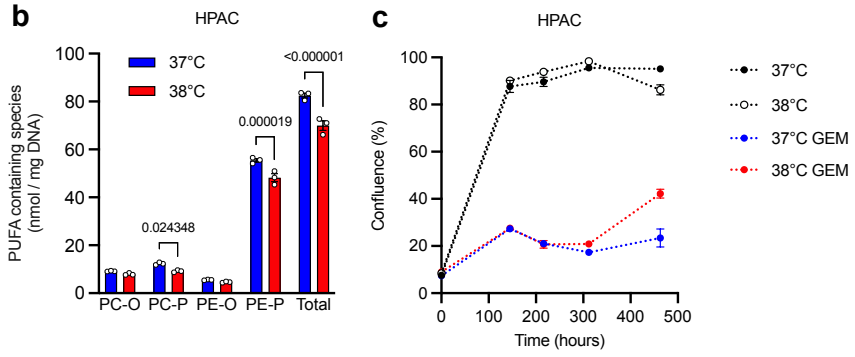
We have now included an even more comprehensive characterisation of different temperatures on the response to gemcitabine by including the 36°C - 37°C - 38°C - 39°C - 40°C range (in addition to the previously included data on 34°C, 37°C, 38°C and 43°C) (new supplementary Fig 6 d and e). This expanded analysis reveals that resistance to gemcitabine is influenced by a spectrum of temperature variations rather than a single point.



Supplementary Fig. 6 (d and e)
(d) Time-course confluence of PANC-1 or BxPC3 cells treated with vehicle or GEM, cultured at 36°C, 37°C or 40°C (n = 6). **(e)** Time-course confluence of PANC-1 or BxPC3 cells treated with GEM, cultured at 37°C, 38°C or 39°C (n = 6).

Moreover, in the major concern #4 the authors did not expand their experiments to more human PDAC cell lines, despite the fact that they even observed that mouse KPC cells do not agree with the human cells. It was crucial also because the BxPC3 cell line is a wild type KRAS and therefore, quite an exception for PDAC.

We have now included new experiments with the human PDAC cell line HPAC, which is KRAS mutant and a commonly used cell line model (New Supplementary Fig 6 b and c). In this additional cell line, we confirm our key findings that culturing at 38°C for 10 days reduces PUFA ether lipids and induces resistance to gemcitabine, further reinforcing the relevance of these results.



Supplementary Fig. 6 (b and c)
(b) Total abundance of PUFA containing ether lipid species (PC-O: 1-alkyl,2-acylphosphatidylcholine; PC-P: 1-alkenyl,2-acylphosphatidylcholine; PE-O: 1-alkyl,2-acylphosphatidylethanolamine; PE-P: 1-alkenyl,2-acylphosphatidylethanol-amine) in HPAC (n = 3) cells cultured at 37°C or 38°C for 10 days. Unpaired two-sided Student's t-test. **(c)** Time-course confluence of HPAC cells treated with vehicle or GEM, cultured at 37°C or 38°C (n = 6).

Suggestion #1: The authors state in the manuscript "tumoral temperature" to refer to 38C. However, in Fig. 1a, they show that there are is also healthy tissue that can have a temperature close to 38C, thus, it is not specific for the tumor. Therefore, they need to replace this sentence from "tumoral temperature" to "38C" temperature as it is confusing.

We agree with and appreciate the reviewer's suggestion. The manuscript has been accordingly adapted to clarify the terminology used when referring to temperature.

Suggestion #2: The membrane fluidity exp (suppl Fig.3) methods and legend do not provide information when was this experiment performed (how many hours after temperature change). Ideally, this has to be done with a time course to reflect the proliferation curves because measuring membrane fluidity at only one time point may not reflect the real situation.

The manuscript has been updated to provide information on the timing of the membrane fluidity experiments, detailing it matches the time point where lipid changes are observed, as now clearly indicated in the revised manuscript.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The remaining issue is still about how p38 can function to mediate ferroptosis in the downstream of lipid peroxidation, which is considered as the end stage of ferroptosis. The authors should include a thorough discussion about this in their final manuscript. Other than that, this reviewer does not have further comments.

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The authors have adequately answered all my concerns and as the manuscript has greatly improved. I don't have any other comments.

REPLY TO THE REVIEWERS' COMMENTS

We thank the editor and the reviewers for their critical and constructive comments, which we address here point by point. We feel that these comments have substantially improved our manuscript and are grateful for this opportunity.

REVIEWER COMMENTS

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The remaining issue is still about how p38 can function to mediate ferroptosis in the downstream of lipid peroxidation, which is considered as the end stage of ferroptosis. The authors should include a thorough discussion about this in their final manuscript. Other than that, this reviewer does not have further comments.

We agree with the reviewer's insightful observation regarding the role of p38 in mediating ferroptosis downstream of lipid peroxidation. The role of lipid peroxidation in ferroptosis has been well-documented, with the accumulation of lipid peroxides being involved in disrupting membrane integrity, propagation of ROS and the generation of degradation products that crosslink to DNA and proteins. With its established role in stress response pathways, the activation of p38 in response to lipid peroxidation indicates an additional regulatory mechanism in the ferroptotic cell death process. Our findings add to previous studies that suggest a mediating role for p38 downstream of lipid peroxidation, although further research is needed to delineate the exact molecular mechanisms by which p38 contributes to the ferroptotic process relative to the established and contextual roles of lipid peroxides.

We have included this topic in the discussion section of our final manuscript.

Reviewer #3 :

The authors have adequately answered all my concerns and as the manuscript has greatly improved. I don't have any other comments.

We thank the reviewer for the positive assessment of the additional work.