nature portfolio

Peer Review File

Fasting boosts breast cancer therapy efficacy via glucocorticoid activation

Corresponding Author: Professor Wilbert Zwart

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
In the current work, the authors elucidate the molecular mechanism by which fasting/modified fasting (MF) potentiates the efficacy of endocrine therapy (ET) in hormone receptor (HR+) positive breast cancer. The study builds on their previous work (Caffa et al., Nature 2020) where they show that fasting potentiates ET by lowering IGF1, insulin and leptin level and by inhibiting AKT-mTOR signaling by EGR1 and PTEN upregulation. In that study, the co-administration of IGF1, insulin, and leptin reverted the beneficial effects of fasting on ET's efficacy in vivo. Here, the authors aim to fully understand the mechanism responsible for this phenotype, in order to identify alternative and long-term approaches which can mimic fasting.

The authors show that fasting causes an extensive epigenetic reprogramming which leads to activation of glucocorticoid receptor (GR), progesterone receptor (PR) and impairment of activator-protein 1 (AP-1) family activity. The authors show that GR KO reverts the beneficial effect of fasting when combined with ET (tamoxifen) in mice. In accordance with these results, GR-ligands administration phenocopies fasting in enhancing tamoxifen efficacy. In support of this proposed model, the authors provide clinical data indicating that patients undergoing MF show increase circulating level of progesterone and cortisol and that GR activation status inversely correlate with proliferation markers in tumor transcriptomic data. In conclusion, the authors propose GR-agonists as a safe and manageable alternative to fasting that can achieve similar beneficial effect as an adjuvant to ET.

Overall, the work addresses an important topic and sheds light on a novel mechanism to explain the benefits of fasting in mice with cancer.

Major comments

1) The authors mention their previous findings (Caffa et al.,Nature, 2020) can only partially explain how fasting sensitizes cancer cells to ET. However, a more thorough examination is needed to reconcile the prior and current data.
- What happens to tumor GR activity and circulating corticosterone amd progesterone levels when mice undergo fasting with the IGF-1/insulin/leptin treatment?
- GR agonists promote food intake and increases fat mass in mice and humans. Do the Dex-treated mice with MCF7 xenografts have higher insulin, IGF-1, and leptin levels?

2) The current experiments have been performed using a complete fasting regimen (ad libitum water only for 48 hours). This approach is quite severe and likely not feasible for human translation. The clinical studies use a "modified fasting" (MF) program, which is a plant-based, low-calorie, low-carb, low-protein regimen. Is there a difference in the response to MCF7 xenograft growth when comparing complete fasting and MF? Does a MF regimen enhance ET? Does GR agonist administration augment the effects of MF+ET. These experiments are crucial to discriminate if the effect observed in mice is due to pure calorie restriction, or to the macronutrient composition of the proposed MF.

3) The primary glucocorticoid in mice is corticosterone. Is cortisol or corticosterone measured in figure 3g and extended data figure 3A? Corticosterone should be measured.

4) The GR KO reverses fasting mediated sensitization to tamoxifen but not the anti-growth effect of fasting alone. Is the PR activity dominant in this setting? Would treatment with mifepristone or genetic loss of PR block the anti-growth effects of fasting in this setting?

The GR agonist treatment phenocopies the effect of fasting on tumor growth. However, the GR KO remains sensitive to fasting. How do the authors reconcile this discrepancy?

The authors should confirm that the MCF7 GR-KO xenografts do not respond to GR agonist with a GR transcriptional program? Do the MCF7 GR-KO xenografts reduce growth in response to dex?

5) The authors propose that GR agonists are a safe alternative to fasting; however, there is no long-term study performed to show a similar degree of benefit to fasting. The toxicities of chronic glucocorticoid administration are excessive on the bones, skeletal muscle, immune, and endocrine systems. Caffa et al. presented robust and durable responses with FMD+ET. The long-term effects of GR agonists should be evaluated.

6) One limit of this study is the use of only one mouse model of HR+ breast cancer (MCF7). To further confirm that GR agonists can mimic fasting beneficial effects in enhancing tamoxifen efficacy, key experiments should be carried on additional mouse models (for example T47D cell line).

7) One important side effect of tamoxifen is endometrial hyperplasia, which has been shown to be reduced by fasting and FMD (Nature, 2020). Do GR agoniss provide the same beneficial effect of fasting in protecting endometrium from hyperplasia?

8) Please provide some evidence of specificity for the GR antibody used for IHC. For example, dex-treated MCF7 tumors as a positive control and dex-treated MCF7-GR-KO tumors as a negative control.

Minor:
-supplementary figure 1a: add quantification of the Ki67 staining
-supplementary figure 1g: specify p values
- The recent presurgical window of opportunity trial MIPRA (https://pubmed.ncbi.nlm.nih.gov/36269797/) provides support for the use of mifepristone in patients with luminal breast cancer with high PRA/PRB ratios. This trial should be discussed.
- What time of day was cortisol measures in figure 3i and j?

Referee #2

(Remarks to the Author)
The manuscript entitled “Fasting boosts breast cancer therapy efficacy via glucocorticoid activation” by Professor Zwart and co-authors demonstrate that GR activation plays a key role in fasting’s ability to enhance endocrine therapy sensitivity in preclinical models and patient samples. The authors also demonstrate that glucocorticosteroid treatment can be a great treatment strategy that mimics the effects of fasting in breast cancer treatment and increases efficacy to endocrine therapy in ER+ breast cancer. This way, the need for fasting as dietary restriction is bypassed.

The study is highly clinically relevant as endocrine therapy is the mainstay for ER+ breast cancer and ways to improve it is of high clinical relevance and thus of interest to the breast cancer community. In addition, in a clinical trial fasting correlated with increase in responses in endocrine therapy but the mechanisms are largely unknown. The identification of these mechanisms may provide novel therapeutic opportunities. The manuscript is novel, well written and well-designed. Concerns remain on the mechanisms of how fasting leads to GR and PR increase of activity and how does the epigenetic landscape contribute to their activation. Thus, the study would benefit from more development on these key mechanisms.

The study will also benefit from a PDX model for in vivo studies in addition to the studies in xenografts established from MCF7 breast cancer cells. Lastly, it remains unclear why the authors only focused on GR gene signature and not PR gene signature in Fig 3 given that both GR and PR showed an increased in genome-binding upon fasting.

Minor comments include the lack of FDR (q value) for the GSEA of RNA-seq in Figure 3. In addition, the actual TF motifs should be shown in Figure 1 to really understand any overlap in motifs as nuclear hormone receptor or AP-1 TFs have similar motifs. (Fig 2).

Referee #3

(Remarks to the Author)
Summary: The authors used the hormone receptor-positive (HR+) model of breast cancer to show that fasting, in

combination with endocrine therapy, induces extensive epigenetic reprogramming. Importantly, the authors have linked the anticancer effects of fasting in this model to activation of glucocorticoid receptor (GR) and progesterone receptor (PR) signaling. They report the results of several elegant studies including: a) GR-knockout approach that resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen; b) exogenous administration of GR-ligands mimicked fasting-enhanced anti-estrogen action and tumor regression. They also show in fasted patients that systemic levels of progesterone and cortisol were elevated. Moreover, transcriptomic analysis of GR activation signatures in tumors from fasted (v unfasted) patients showed inverse correlations with proliferation markers. Their findings support the idea that GR activation may contribute importantly to fasting's ability to enhance endocrine drug activity in breast cancer. The authors also suggest that corticosteroid administration should be evaluated as an adjuvant to endocrine therapy for HR+ breast cancer.

Novelty: The link between glucocorticoid receptor signaling and fasting/dietary energy restriction in cancer is not an entirely novel concept in the field of energy balance and cancer (see a series of publications by Diane Birt and colleagues in the early 2000's, including Prevention of mouse skin tumor promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition.Stewart JW, Koehler K, Jackson W, Hawley J, Wang W, Au A, Myers R, Birt DF. Carcinogenesis. 2005 Jun;26(6):1077-84. Doi: 10.1093/carcin/bgi051. In addition, Longo and colleagues have alluded to this in prior work in the context of fasting and chemotherapy response (Di Biase, et al. PLoS Biol. 2017 May 1; 15(5): e1002603), including the concept of differential stress response in normal versus cancer cells (deGroot, et al. Nat Comm 2020; PMID: 32576828; DOI: 10.1038/s41467-020-16138-3. However, very few studies have focused on HR+ breast cancer.

Data and methodology: the reported experiments were generally well-designed and executed. My major concern is the dependence on the single in vivo model used, specifically the MCF-7 xenograft model in immunodeficient athymic nude mice. This raises several concerns. First, given the established importance of immunomodulation in the anticancer effects of fasting/fasting mimicking diets and related interventions, as well as the established interactions between glucocorticoid signaling and anticancer immune responses, the inclusion of at least one other immune-intact model (syngeneic orthotopic transplant model or genetically engineered mouse model) would have strengthened the paper. Also, the choice of the MCF-7 transplant model requiring exogenous estrogen via a 17b-estradiol pellet, which delivers a constant, high level of estrogen for the tumors to take, is also a poor mimic of a postmenopausal woman at high risk for developing HR+ breast cancer. This limitation is particularly true for a study focusing on the interactions between fasting, glucocorticoid signaling, tamoxifen and breast cancer. An additional concern is the site of tumor growth, in this case subcutaneous injection in the flank (area overlying the upper thigh and lower part of back & abdomen) instead of orthotopic transplantation into one of the mammary glands, which would have permitted assessment of tumor growth in the tissue of interest rather than subcutaneously on the back of the mouse. Given that the elegant mechanistic analyses, including their GR-knockout approach that resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen, and their assessment of the effects of exogenous administration of GR-ligands on tumor regression, extended from this limited in vivo model, I found the results interesting but lacking sufficient rigor. While the human findings are somewhat supportive, in my opinion the findings as presented would be more suited to a specialty journal (such as Breast Cancer Research) than for the broad readership of Nature.

Statistics: were appropriate and well described.

Conclusions—robustness limited to the limitations described in the data and methodology section. While the findings that GR activation may contribute importantly to fasting's ability to enhance response to endocrine therapy for breast cancer, the authors need to be cautious regarding the strength of their conclusions.

Improvements: more rigorous in vivo data are needed to strengthen the findings

Clarity and context: The introduction was generally well written, although there were a few issues regarding literature support for the premise. The WHI paper by Chlewbowski, et al was cited as one of the main clinical studies supporting the protective effects of fasting. The WHI intervention arm was a low fat, high fruit and vegetable regimen that induced minimal weight loss, so it seemed misleading to claim that study supported fasting as an anticancer approach. Another point made in the introduction that seemed confusing was the argument that insulin, IGF-1 and leptin have only a partial impact on ER+ tumor cell proliferation, and therefore other pathways must be more important. However, the paper cited focused on OSI-906, an IGF_1R inhibitor shown to have relatively modest effects on tumor cell proliferation, at least compared with other inhibitor that inhibit other receptor tyrosine kinases along this pathway besides IGF-1R. Most/all studies to date have not simultaneously manipulated multiple pathways, such as IGF-1R and leptin receptor, so the claim that fasting effects must be due to pathways other than insulin/IGF-1/leptin signaling seemed an overinterpretation of the evidence.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I have no additional concerns. Congrats on a great study.

Referee #2

(Remarks to the Author)
The authors have addressed Reviewers comments with further data and explanations.

Few minor things to consider:

Fig 19, please show tornado plots. H3K27ac meta profiles are not very strong.
Fig 20a. More information would be helpful on PDX and its genomic profile? Where is the pdx from?
Fig 20b. The effect of the combination in T47D while significant is biologically modest as tamoxifen has a good effect.

Referee #3

(Remarks to the Author)
The authors were highly responsive to the previous review, did a huge amount of additional work to strengthen their story, and their data is now quite convincing and exciting. They used 4 hormone receptor-positive (HR+) mouse models of breast cancer to more clearly establish that fasting, in combination with endocrine therapy, induces extensive epigenetic reprogramming. Importantly, the authors have linked the anticancer effects of fasting in this model to activation of glucocorticoid receptor (GR) and progesterone receptor (PR) signaling, and the have repeated many of their key experiments with additional controls to add more rigor and clarity, establishing: a) GR-knockout resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen; b) exogenous administration of GR-ligands mimicked fasting-enhanced anti-estrogen action and tumour regression. They also show in fasted patients that systemic levels of progesterone and cortisol were elevated. Moreover, transcriptomic analysis of GR activation signatures in tumours from fasted (v unfasted) patients showed inverse correlations with proliferation and immune markers. Their findings established that GR activation underlies fasting's ability to enhance endocrine drug activity in breast cancer. The findings also strongly support further interrogation of therapeutic strategies that mimic the beneficial effects of fasting on responses to endocrine therapy for HR+ breast cancer.

Rebuttal letter - Fasting boosts breast cancer therapy efficacy via glucocorticoid receptor activation

Referee #1

In the current work, the authors elucidate the molecular mechanism by which fasting/modified fasting (MF) potentiates the efficacy of endocrine therapy (ET) in hormone receptor (HR+) positive breast cancer. The study builds on their previous work (Caffa et al., Nature 2020) where they show that fasting potentiates ET by lowering IGF1, insulin and leptin level and by inhibiting AKT-mTOR signaling by EGR1 and PTEN upregulation. In that study, the co-administration of IGF1, insulin, and leptin reverted the beneficial effects of fasting on ET's efficacy in vivo. Here, the authors aim to fully understand the mechanism responsible for this phenotype, in order to identify alternative and long-term approaches which can mimic fasting.

The authors show that fasting causes an extensive epigenetic reprogramming which leads to activation of glucocorticoid receptor (GR), progesterone receptor (PR) and impairment of activator-protein 1 (AP-1) family activity. The authors show that GR KO reverts the beneficial effect of fasting when combined with ET (tamoxifen) in mice. In accordance with these results, GR-ligands administration phenocopies fasting in enhancing tamoxifen efficacy. In support of this proposed model, the authors provide clinical data indicating that patients undergoing MF show increase circulating level of progesterone and cortisol and that GR activation status inversely correlate with proliferation markers in tumour transcriptomic data. In conclusion, the authors propose GR-agonists as a safe and manageable alternative to fasting that can achieve similar beneficial effect as an adjuvant to ET.

Overall, the work addresses an important topic and sheds light on a novel mechanism to explain the benefits of fasting in mice with cancer.

We thank the reviewer for the time invested, their insightful recommendations and for critically evaluating our manuscript. We are delighted to read that the reviewer finds our work interesting and unravels unknown mechanisms that can benefit breast cancer patients.

As can be appreciated in our point-by-point response to reviewers below, we attempted to address all points raised in full, to the best of our capacities. With this,

we hope the reviewer finds the study sufficiently improved to render it acceptable for publication.

Major comments

1) The authors mention their previous findings (Caffa et al.,Nature, 2020) can only partially explain how fasting sensitizes cancer cells to ET. However, a more thorough examination is needed to reconcile the prior and current data.

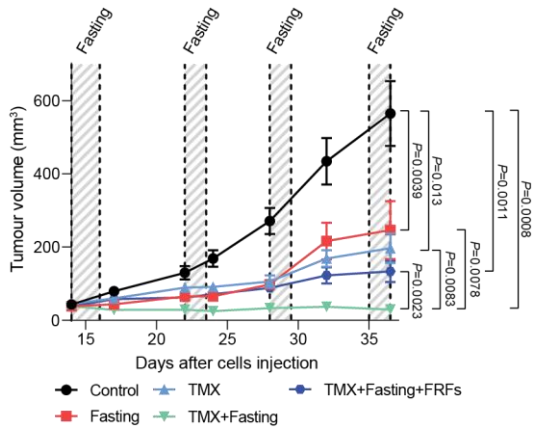
The reviewer raises an important topic. Please find below our responses to the specific questions in which we reconcile the prior study with the current findings.

- What happens to tumour GR activity and circulating corticosterone and progesterone levels when mice undergo fasting with the IGF-1/insulin/leptin treatment?

We thank the reviewer for raising this important point.
To address this intriguing question, we re-performed the animal studies as described in the first submitted manuscript version (original Fig. 1A and B), but now with the addition of a 5th treatment arm where mice were treated with a combined Fasting+TMX regimen, along with a combination of IGF-1 + insulin + leptin (Fasting-reducing factors, FRFs) (see **Extended Data Fig. 3b** in the revised manuscript, **Figure 1 for reviewers**). Importantly, we observed in these analyses, that the addition of FRFs rescues the growth that was impaired by the Fasting+TMX combination. This is in line with the previous published results where the addition of the same growth factors is able to restore the tumour growth that was impaired by fasting (Caffa et. al. Nature 2020; Fig. 1c). To further expand on these observations, we performed GR ChIP-seq and RNA-seq analyses on these tumours, to understand if and how FRFs treatment and GR activity are connected, biologically. Of note, to further strengthen the physiological implications of our studies, in part in response to a request from Reviewer 3, all these experiments were performed using cells that were transplanted orthotopically, into the mammary fat pad.

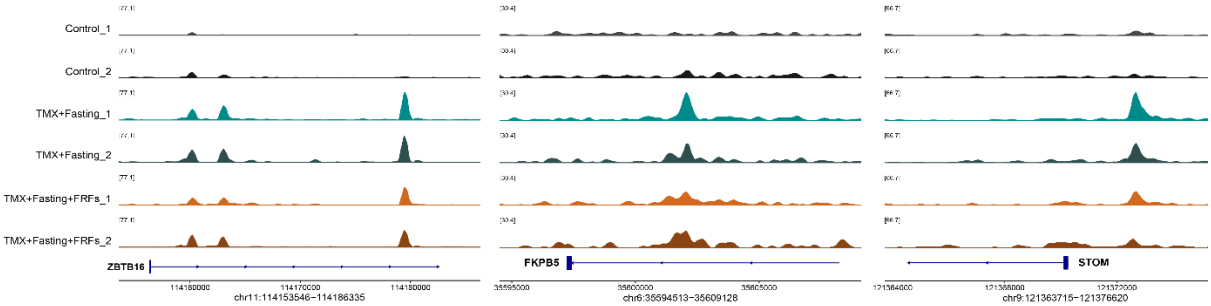
FRFs treatment decreased GR chromatin interactions for classical GR-dependent genes loci, (*ZBTB16*, *FKBP5* and *STOM)* in xenografted tumours (see **Figure for Reviewers 2**). In line with these observations, RNA-seq in the same tumour samples show reduced mRNA signal for these hallmark GR-responsive genes following FRFs treatment (see **Figure for Reviewers 3**) Cumulatively, these results imply that FRFs treatment selectively decreases GR activity, and consequently enhances tumour growth capacity.

Fig. 1



MCF7 xenograft tumour growth in six/eight-week-old female athymic nude mice in the different treatment arms. Data are shown as mean ± SEM and analysed by linear mixed-effects model (*P*-value of last day is shown).

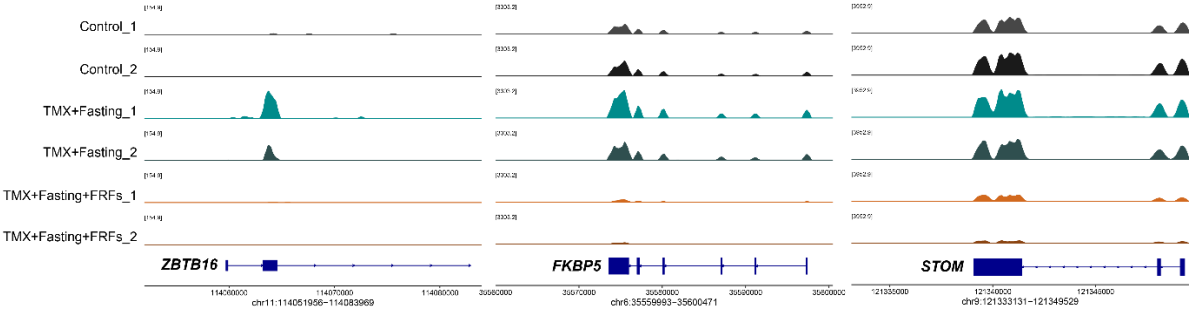
Fig. 2



Snapshots of GR ChIP-seq signal at the *ZBTB16, FKBP5* and *STOM* gene locus between Control, TMX+Fasting and TMX+Fasting+FRFs treated xenografts. 2 technical replicates each.

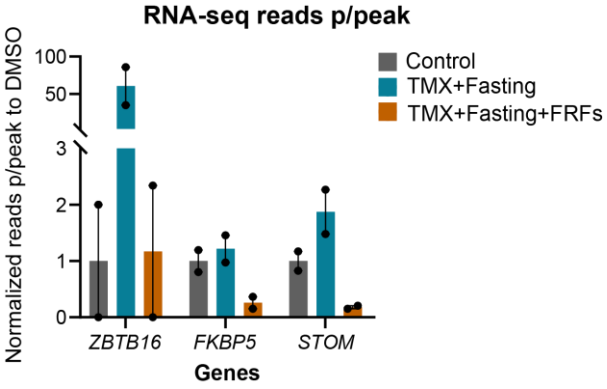
Fig. 3

a)



Snapshots of RNA-seq signal at the *ZBTB16, FKBP5* and *STOM* gene locus between Control, TMX+Fasting and TMX+Fasting+FRFs treated xenografts. 2 technical replicates each.

b)

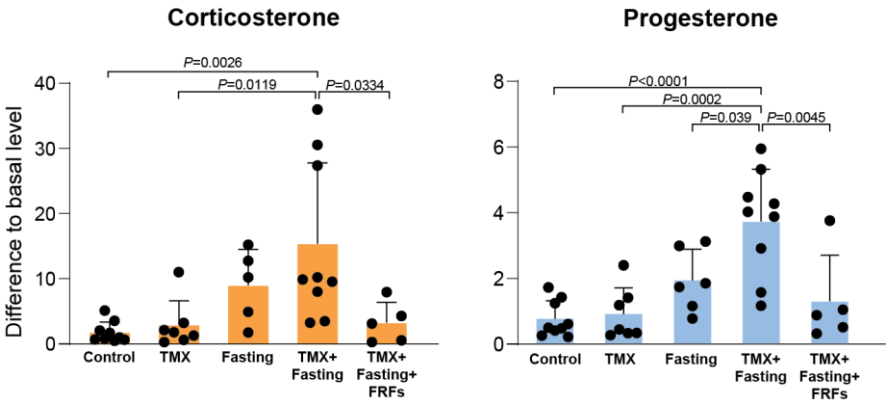


Normalized RNA reads per peak at the promoter of each depicted gene from tumours treated with the different conditions.

While circulating corticosterone and progesterone levels in the blood of these mice were increased upon TMX+Fasting exposure, this effect was prevented by FRFs co-treatment in mice (**Extended Data Fig. 3a** and **Figure for Reviewers 4** below). Jointly, these results support a conclusion that FRFs treatment impairs GR activity in these tumours due to a systemic reduction of corticosterone levels. We have updated the manuscript accordingly - *Lines 116-120: "Fasting and FMDs were previously found to reduce blood IGF-1, insulin and leptin levels, here referred to as fasting-reducing factors (FRFs), in mice and humans with BC[3,6,21,22]. The re-introduction of these FRFs prevented the beneficial effects of fasting on tumour growth3 (Extended Data Fig. 3b) and the elevation of circulating corticosterone and progesterone levels in mice treated with TMX+Fasting (Extended Data Fig. 3a)."*

These new results have all been incorporated into the revised version of our manuscript, and we thank the reviewer once more for the thoughtful suggestions that motivated us to explore this interesting question.

Fig. 4

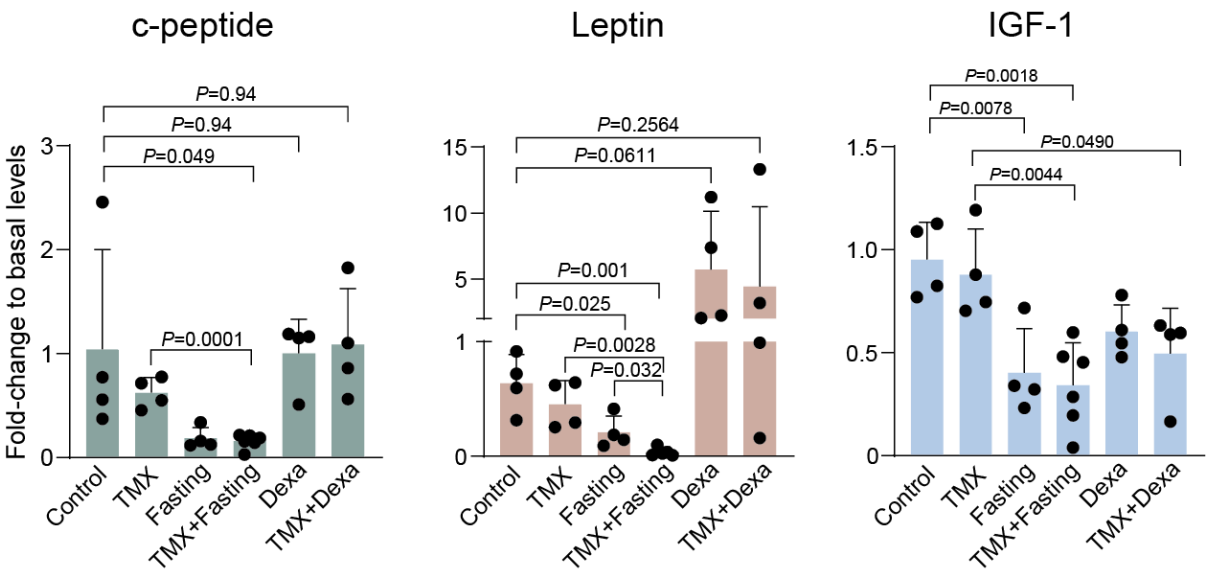


Corticosterone and progesterone levels, in relation to basal levels, on mice blood after 4 cycles of all treatment conditions in relation to basal levels (pre-treatment). Data are shown as mean ± SD and analysed by one-way ANOVA followed by Tukey multiple comparison test.

- GR agonists promote food intake and increases fat mass in mice and humans. Do the Dex-treated mice with MCF7 xenografts have higher insulin, IGF-1, and leptin levels?

We thank the reviewer for raising this interesting question. We observe that Dexamethasone treatment (alone or combined with TMX) does not impact c-peptide levels relative to control *ad libitum* fed animals and increases (although not significantly) circulating leptin levels (**Extended Data Fig. 7b** and **Figure for the Reviewers 5** below). This is in contrast to fasting, which decreases levels of both c-peptide and leptin. Unexpectedly, although slightly higher than fasted treated animals, circulating IGF-1 levels are lower when animals are treated with Dexamethasone -with or without TMX- compared to *ad libitum* fed or TMX treated mice. Thus, mice after dexamethasone treatment have higher leptin and c-peptide levels compared to fasting, while IGF-1 levels are comparable between fasting and Dexamethasone. We updated the manuscript to include these new observations - *lines 180-183: "We previously reported that fasting and FMDs lower insulin, IGF-1 and leptin plasma levels in mice and humans with BC[3,6,21,22]. Thus, we assessed whether the ability of Dexa to phenocopy the antitumor activity of fasting would reflect similar effects on these FRFs. Leptin and c-peptide levels were not affected by Dexa, whereas serum IGF-1 decreased in response to Dexa (Extended Data Fig. 7b)."*

Fig. 5



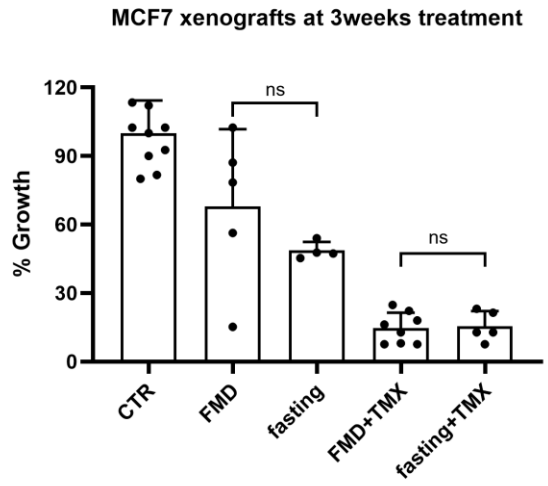
Fold-change differences to basal conditions of circulating c-peptide, leptin and IGF-1 levels in MCF7 xenografts growth in six/eight-week-old female athymic nude mice treated with the respective depicted conditions. Data are shown as mean ± SD and analysed by Student *t*-test.

2) The current experiments have been performed using a complete fasting regimen (ad libitum water only for 48 hours). This approach is quite severe and likely not feasible for human translation. The clinical studies use a "modified fasting" (MF) program, which is a plant-based, low-calorie, low-carb, low-protein regimen. Is there

a difference in the response to MCF7 xenograft growth when comparing complete fasting and MF? Does a MF regimen enhance ET? Does GR agonist administration augment the effects of MF+ET. These experiments are crucial to discriminate if the effect observed in mice is due to pure calorie restriction, or to the macronutrient composition of the proposed MF.

We thank the reviewer for raising these highly relevant points. The reviewer is absolutely correct that the fasting regime is quite severe, and difficult for patients to adhere to. We do want to emphasize that the goal of our study is not to translate fasting-interventions towards clinical implementation (as there is extensive research ongoing on this topic already), but rather to replace a fasting program with corticosteroid use. We updated our introduction and discussion sections, to better communicate this argumentation. The previous study from members of our team (Caffa et. al, Nature 2020) performed a head-to-head comparison between *ad libitum* water only and a FMD-program, in which no differences were observed in MCF7 xenograft growth as well in enhancement of endocrine therapeutics (See **Figure for Reviewers 6** below). Experiments on whether GR agonist treatment augments the FMD+ET effect, were not included in the study, as -with the elevated serum levels of corticosterone in animals after FMD- an impact of even further elevating corticosteroid levels by exogenous administration, would be challenging to interpret biologically as -based on our observations- both interventions effectively activate the same cascades.

Fig. 6



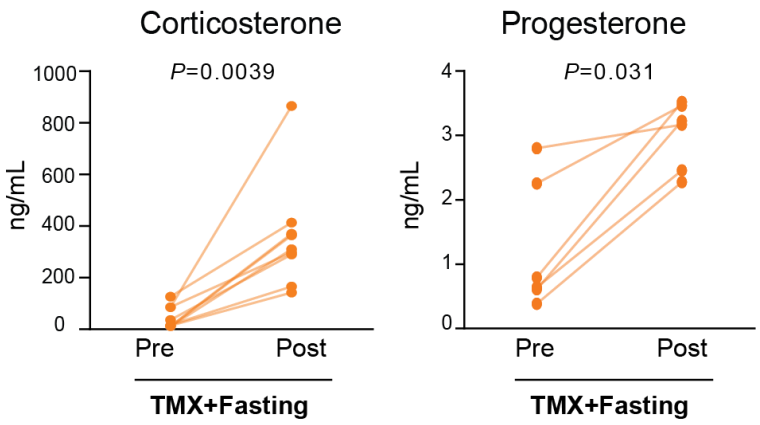
Percentage of tumour growth compared to mean control for all the depicted conditions.

3) The primary glucocorticoid in mice is corticosterone. Is cortisol or corticosterone measured in figure 3g and extended data figure 3A? Corticosterone should be measured.

We thank the reviewer for spotting this design error. We were not aware of the dominant role of corticosterone instead of cortisol in mice, and we are grateful to the reviewer bringing this to our attention. For the revised manuscript, we re-analysed all

the mouse blood samples throughout the study, and measured the corticosterone levels. As can be appreciated in the updated **Fig. 2g, Extended Data Fig. 3a**, and displayed in **Figure for Reviewers 4** (above) and **Figure for Reviewers 7** (below), the main conclusions remained fully consistent with our original observations, with corticosterone levels being elevated in blood of the animals after fasting. The results section has now been corrected, referring to corticosterone levels for all the animal studies.

Fig. 7

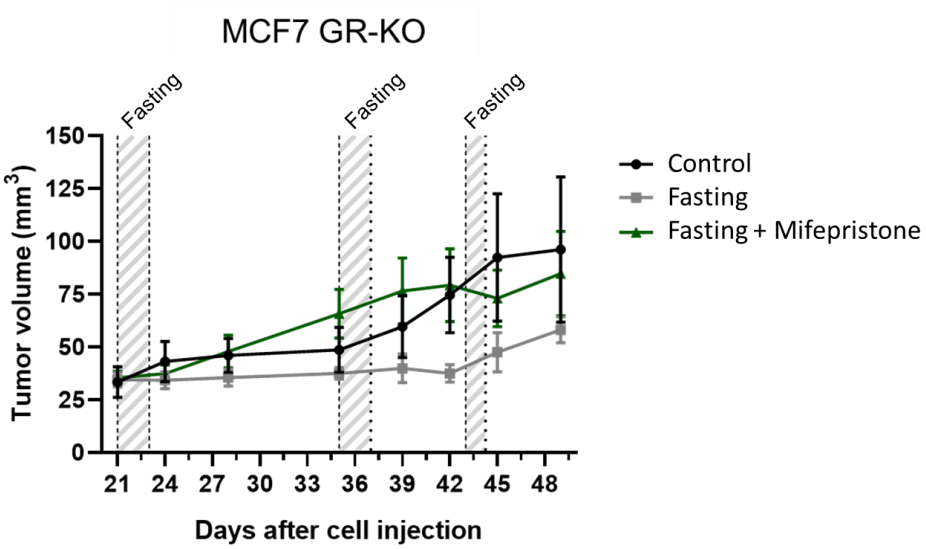


Corticosterone and progesterone levels in blood of mice before (Pre) and after (Post) 4 cycles of TMX+Fasting Fasting (*n*=6). Data were analysed by two-tailed Wilcoxon signed rank test.

4) The GR KO reverses fasting mediated sensitization to tamoxifen but not the anti-growth effect of fasting alone. Is the PR activity dominant in this setting? Would treatment with mifepristone or genetic loss of PR block the anti-growth effects of fasting in this setting?

We thank the reviewer for this highly interesting question. Fully following the reviewers' recommendation, we xenografted GR-KO cells and treated the animals with PR antagonist mifepristone. While tumour growth in the GR-KO animals was diminished by fasting, in agreement with our original observations, tumour growth was accelerated by mifepristone treatment (see below, **Figure for Reviewers 8**). These results support an argumentation that joint activation of both PR and GR contribute to the beneficial effects of fasting. Sadly, secondary effects of mifepristone in these mice, such as uterus hyperplasia and oedema, led to premature death of many animals, which may be attributed to the long duration of the treatment (one month *vs* one week) and/or high concentration (10.3892/etm.2015.2203; 10.3109/15376516.2015.1118715). Therefore, we are cautious in making statements about these results at later time points and we therefore would prefer to not include these results in the revised version of the manuscript.

Fig. 8



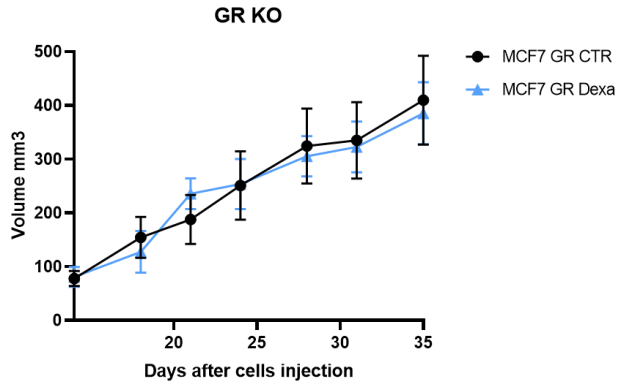
MCF7 GR-KO xenograft tumour growth in six/eight-week-old female athymic nude mice treated with Control (ad libitum diet; black), fasting (48h water-only; grey) or Fasting + Mifepristone (60 mg/kg; green). Data are shown as mean ± SEM.

The GR agonist treatment phenocopies the effect of fasting on tumour growth. However, the GR KO remains sensitive to fasting. How do the authors reconcile this discrepancy?

We thank the reviewer for pointing out this discrepancy. While the synergistic effect of fasting and endocrine treatment was abrogated in the GR-KO tumours, we indeed observed that the GR-KO still remained sensitive to fasting alone. In our newly added analyses, we now show that GR-KO xenograft growth is completely unresponsive to dexamethasone (**Extended Data Fig. 5c** and **Figure for reviewers 9**). In agreement with these observations, we lose GR signature activity in the GR-KOs (**Extended Data Fig. 5e-f** and **Figure for reviewers 10**). These data illustrate that the response to dexamethasone treatment and GR-action in relation to tumour growth reduction, is a tumour-intrinsic feature and not imposed by the microenvironment. In line with this, we see that GR activation diminishes tumour growth, both in immuno-deficient (**Fig. 4f from original submission (now Fig. 4d) and Figure for Reviewers 11**) as well as immuno-competent animals (**Extended Data Fig. 7d and Figure 12 for Reviewers**). As PR is also activated by fasting (**Fig. 2b**) and serum levels of progesterone are elevated in mice (**Fig. 2g**) and patients (**Fig. 2i**) after fasting, we hypothesize the observed tumour-suppressive phenotype of fasting in the GR-KO animals is mediated by elevated PR activity. PR activation is well-established as a tumour suppressive cascade in HR+ BC (10.1038/nature14583), further solidifying these observations. Our

newly performed mifepristone experiments (**Figure for reviewers 8**), as suggested by the reviewer, confirm this hypothesis.

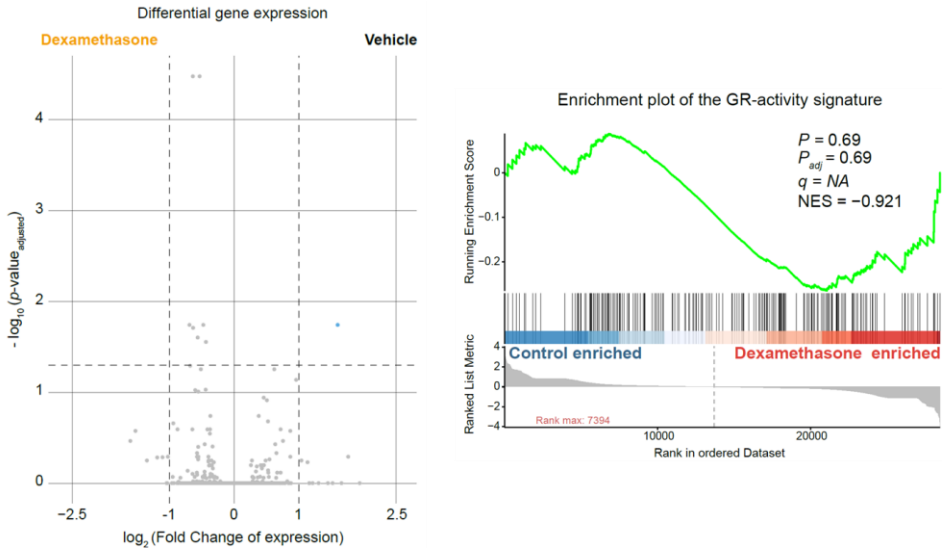
Fig. 9



Xenograft MCF7 GR-KO tumour growth in six/eight-week-old female athymic nude mice treated with vehicle or dexamethasone. Data are shown as mean ± SEM.

Fig. 10

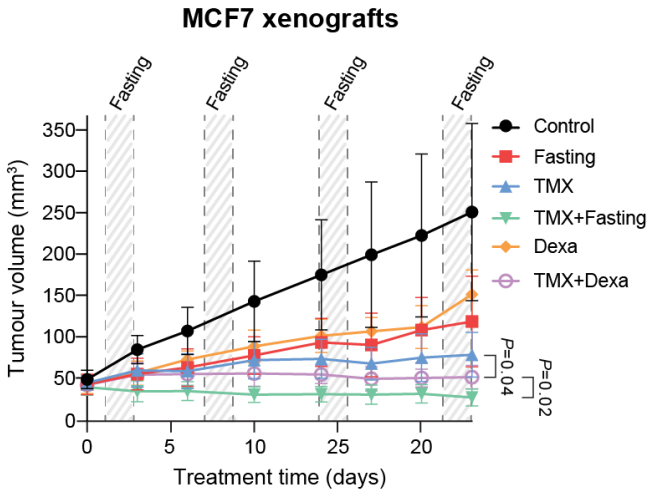
a) b)



a) Volcano plot of the differentially expressed transcripts between vehicle and dexamethasone treated MCF7 GR-KO xenografts.

b) Enrichment plot of a pan-cancer GR-activity signature for the depicted conditions. NES (Normal Enrichment Score) and *P*-value are shown.

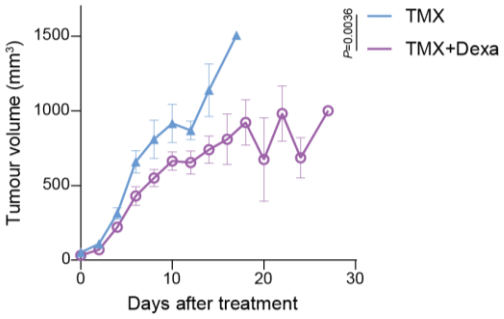
Fig.11

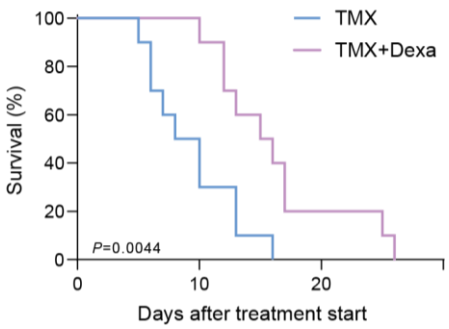


MCF7 xenograft outgrowth in six/eight-week-old female athymic nude mice in the different treatment arms (Control *n*=8; TMX *n*=9; Dexa *n*=6; Fasting *n*=8; TMX+Fasting *n*=11 and TMX+Dexa *n*=7) . Data are shown as mean ± SEM and analysed by linear mixed-effects model (*P*-value of last day is shown).

Fig. 12

a) ***b)***





a) TSAE1 syngeneic tumour growth in immunocompetent mice. Data are shown as mean ± SEM and analysed by linear mixed-effects model.
b) Survival curves of mice engrafted with TSAE1 cells and treated with either TMX or TMX+Dexa.

The authors should confirm that the MCF7 GR-KO xenografts do not respond to GR agonist with a GR transcriptional program? Do the MCF7 GR-KO xenografts reduce growth in response to dex?

We want to thank the reviewer for suggesting this valuable control experiment. To address this question, we xenografted MCF7 GR-KO cells and treated the mice with either dexamethasone or vehicle (**Extended Data Fig. 5a-c** and **Figure for Reviewers 9**). In agreement with our other observations and in line with our hypothesis, GR-KO MCF7 tumours do not experience growth differences when treated with dexamethasone, compared to vehicle control. To further solidify these results, in

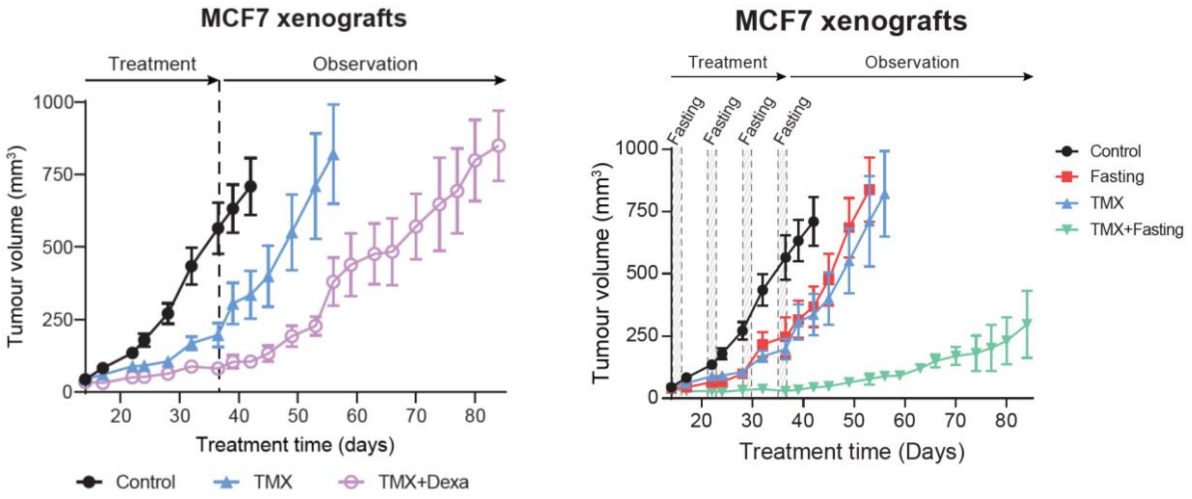
full concordance with the reviewer's suggestion, we also performed RNA-seq in these tumours and found no significantly differentially expressed genes between dexamethasone treated animals and vehicle control (**Extended Data Fig. 5e-f** and **Figure for Reviewers 10**). In agreement with this, the GR-activity transcriptional program showed no significant alteration after dexamethasone exposure in the GR-KO cells (**Extended Data Fig. 5f** and **Figure for Reviewers 10**). Altogether, we show that our GR-KO MCF7 cells do not respond to dexamethasone treatment, implying that the anti-tumour effect of fasting is a tumour cell-intrinsic phenomenon. We would like to express our gratitude to the reviewer for suggesting these valuable control experiments.

5) The authors propose that GR agonists are a safe alternative to fasting; however, there is no long-term study performed to show a similar degree of benefit to fasting. The toxicities of chronic glucocorticoid administration are excessive on the bones, skeletal muscle, immune, and endocrine systems. Caffa et al. presented robust and durable responses with FMD+ET. The long-term effects of GR agonists should be evaluated.

We fully agree with this important point raised by the reviewer, for which we are grateful. Caffa et.al. showed that animals treated with fasting combined with TMX for a limited period of time, experienced a durable response on delaying tumour outgrowth when treatment was stopped. Following the reviewer's suggestion, we performed an analogous short-term exposure experiment, followed by a long-term observation period in which tumour outgrowth was monitored. Strikingly, tumour outgrowth in Dexa+TMX treated mice was delayed with a factor of 2, as compared to TMX alone (**Fig. 4f** and **Figure for Reviewers 13a**). These results illustrate the long-term beneficial effects of Dexa treatment mimicking, in part, the effects of fasting. Interestingly, the effect of Dexa in delaying the tumour is not as potent as fasting (**Extended Data Fig. 7a** and **Figure for Reviewers 13b**). Since GR-KO does perturb the synergistic effects of fasting (**Fig. 4b**), we conclude that endogenous corticosteroid effects may be more durable as compared to exogenous administration. These new findings were added to the results section - *Lines 180-183: "Consistent with the ability of combined fasting plus ET to also exert carry-over antitumor activity, we found that, as compared to fasting or TMX alone, a one-month treatment of MCF7-xenograft-bearing mice with Dexa plus TMX delayed tumour growth by a factor of 2, after treatment withdrawal (Fig. 4f; Extended Data Fig. 7a)."*

Fig. 13

a) *b)*



a) MCF7 xenograft outgrowth in six/eight-week-old female athymic nude mice in the different treatment arms (Control *n*=7; TMX *n*=7; Dexa *n*=5 and TMX+Dexa *n*=6). After 4 weeks, all treatments were stopped and tumours were allowed to re-grow. Data are shown as mean ± SEM

a) MCF7 xenografts tumours volume in six/eight-week-old female athymic nude mice treated with the depicted conditions (Control *n*=7; TMX *n*=7, Fasting *n*=8; TMX+Fasting *n*=8). After one month, all treatments were stopped and tumours were allowed to grow. Data are shown as mean ± SEM.

Additionally, as well pointed-out by the reviewer, it is well established that dexamethasone treatment has systemic effects including immune modulation, that may impact the interpretation of our results. To address these concerns, we added additional in vivo experiments to the revised manuscript, in which we made use of ERα+ mouse BC cells (TSAE1 cells; 10.1038/s43018-023-00525-y), which we syngeneic implanted in immunocompetent mice to evaluate the impact of GR activation tumour-related survival and the impact in the immune system. In full concordance with our xenograft studies in our primary submission, also immunocompetent mice treated with dexamethasone plus TMX have significantly longer survival compared to mice treated with TMX alone (**Fig. 4g and Figure for Reviewers 12b**).

Furthermore, to provide a more complete overview of the immunological impact of GR activation, TSAE1-tumour bearing mice were treated with TMX, Dexa, TMX+Dexa or vehicle control for 7 days, after which blood was analysed by FACS (**Extended Data Fig. 7f and Figure for Reviewers 14**).

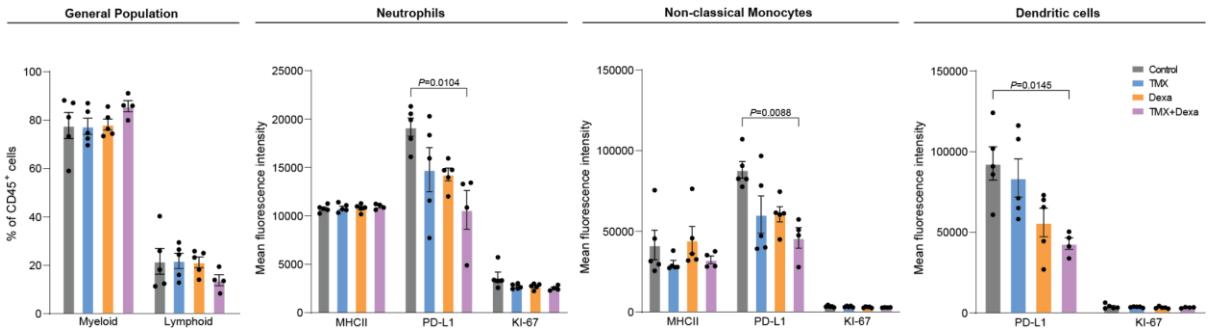
Overall, no significant alterations in immune cell populations were observed following 7 days of treatment with 4 mg/kg dexamethasone, as assessed by Ki-67 expression, suggesting that dexamethasone does not substantially impair immune cell proliferation under these conditions. Furthermore, the antigen-presenting capacity of neutrophils and monocytes, as indicated by MHC-II expression, appears to be preserved,

supporting the notion that dexamethasone does not compromise their potential anti-tumour function, for this treatment period.

Interestingly, for neutrophils, non-classical monocytes and dendritic cells, a decrease in PD-L1 expression was observed, most strikingly in the combined TMX+Dexa treatment group (**Extended Data Fig. 7f** and **Figure for Reviewers 14**), suggesting enhanced anti-tumour immune action for this treatment group. These results are now included in the results section - *Lines 191-204: "Dexamethasone is a potent immunomodulator, with significant adverse effects when chronically administered[26]. Thus, we aimed at ruling out that adding Dexa to TMX could be less effective or even detrimental in an immunocompetent HR+ BC model (by affecting anti-tumour immunity). In allografts of the ERα+ TSAE1 murine BC cell line[27,28] in BALB/c mice, combined treatment of Dexa with TMX, significantly reduced tumour growth (Extended Data Fig. 7d-e) and increased the survival of these mice, compared to TMX treatment alone (Fig. 4g).*

To better understand whether and how treatment with Dexa would modulate the peripheral immune landscape, particularly with respect to the effects that were specific for TMX, we performed an immune-profiling of BALB/c mice bearing TSAE1 tumours from the different treatment groups (i.e., Control, TMX, Dexa and TMX+Dexa). We found no overt changes in leukocyte populations and in their proliferative capacities in response to the different treatments. However, the combination of Dexa plus TMX led to a significant reduction in PD-L1 expression in neutrophils, non-classical monocytes and dendritic cells as compared to control (Extended Data Fig. 7f)." and further discussed in the conclusions - Lines 239-249*: "Chronic treatment with corticosteroids may exert undesirable effects on the immune system, bones, muscles and endocrine system[26]. Our in vivo studies using an immuno-competent mice model revealed that Dexa significantly delayed tumour growth and extended survival of mice bearing an HR+ tumour, compared to TMX alone (Fig. 4g). Moreover, immune profiling of these mice indicated a balanced immune state, with no signs of pronounced activation or suppression. We observed a significant reduction in PD-L1 expression in some immune cell populations, when Dexa was combined with TMX (Extended Data Fig. 7f). Since low PD-L1 expression is considered to be a marker of immune cell activation and increased anticancer activity of the immune system[34], it is possible that the strong antitumor effects we observed with TMX plus Dexa in this immunocompetent mouse BC model also reflected favourable systemic effects of Dexa on antitumor immunity, although this mechanism needs to be confirmed through further studies."*

Fig. 14



Barplots show the mean fluorescence intensity for selected markers for systemic myeloid cells and lymphoid cells ($CD45^+$ $CD11b^+$ and $CD45^+$ $CD11b^-$ respectively), neutrophils ($CD45^+$ $CD11b^+$ $Ly6G^+$), non-classical monocytes ($CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^-$) and dendritic cells ($CD45^+$ $F4/80^-$ $CD11c^{high}$ $MHCII^{high}$) from TSAE1-bearing mice treated with three rounds of control treatment, tamoxifen, dexamethasone, or tamoxifen + dexamethasone. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test.

6) One limit of this study is the use of only one mouse model of HR+ breast cancer (MCF7). To further confirm that GR agonists can mimic fasting beneficial effects in enhancing tamoxifen efficacy, key experiments should be carried on additional mouse models (for example T47D cell line).

We thank the reviewer for raising this important point regarding the limited number of *in vivo* models used in our study. To address this point as thoroughly as possible, we extensively increased the number of mouse models, beyond MCF7 alone, to now also study the impact of GR activation to phenotype fasting in:

1. Orthotopically-implanted T47D cells (**Extended Data Fig. 6f-g** and **Figure for Reviewers 15a**)
2. Orthotopically-implanted ERα+ BC patient-derived xenografts (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 15b**)
3. Orthotopically-implanted TSAE1 cells in immune-competent animals (**Fig. 4f; Extended Data Fig. 7d-e** and **Figure for Reviewers 12**)

Below, we present a summary of each added *in vivo* experiment and respective conclusions:

1. *In vivo* T47D experiments were added to the study, as a second ERα+ BC model in which the tumour cells were implanted orthotopically through intraductal injections (Mouse Intraductal (MIND) model; 10.1186/bcr2358). These studies fully confirmed our original observations that GR activation boosted the effects of TMX treatment. These results have now been incorporated in the manuscript, as new **Extended Data Fig. 6f-g; Figure for**

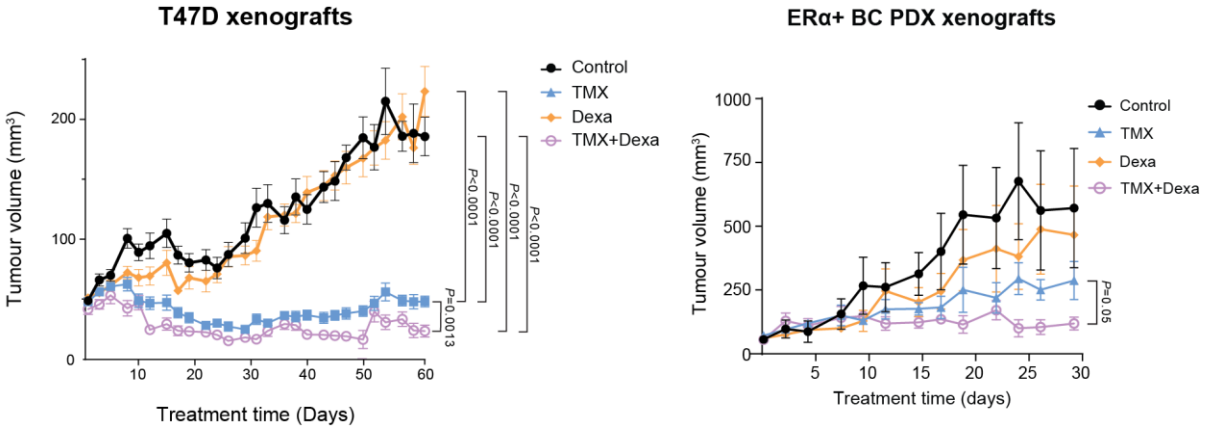
Reviewers 15a). With this, we addressed the validation in additional cell line models.

2. To further substantiate our findings in more clinically relevant models, ERα+ BC patient-derived xenografts were established, grafted through intraductal injections, and tested for response. With these studies we further confirmed that TMX combined with Dexa show a strong impairment of ERα+ BC PDX tumour growth, as compared to either treatment alone. These results further support the impact of GR activation to boost endocrine therapy potential in patient-derived model systems (**Fig. 4e; Extended Data Fig. 6h-l; Figure for Reviewers 15b**) .

3. Finally, to address the important comment regarding the impact of dexamethasone treatment in immunomodulation and anti-cancer effects, we made use of a recently-developed ERα+ mouse BC cell line (TSAE1; 10.1038/s43018-023-00525-y) and expanded our studies to immunocompetent mice. Interestingly, and in line with our results in immunodeficient mice, we observe an impairment on tumour growth when mice are treated with the combination TMX+Dexa, versus TMX alone (**Fig. 4g; Extended Data Fig. 7d-e;** and **Figure for Reviewers 12**). Of note, these experiments were also performed using the MIND intraductal injection model, without the use of exogenous estrogens. Moreover, taking advantage of the immune proficient mice model, we performed systemic immune-profiling to better understand the impact of Dexa treatment in the anti-tumour response of the immune system.

Fig. 15

a) ***b)***



a) T47D xenograft tumour outgrowth in six-eight-week old female NSG mice in the different treatment arms (Control n=8; TMX n=7; Dexa n=8 and TMX+Dexa n=7) . Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

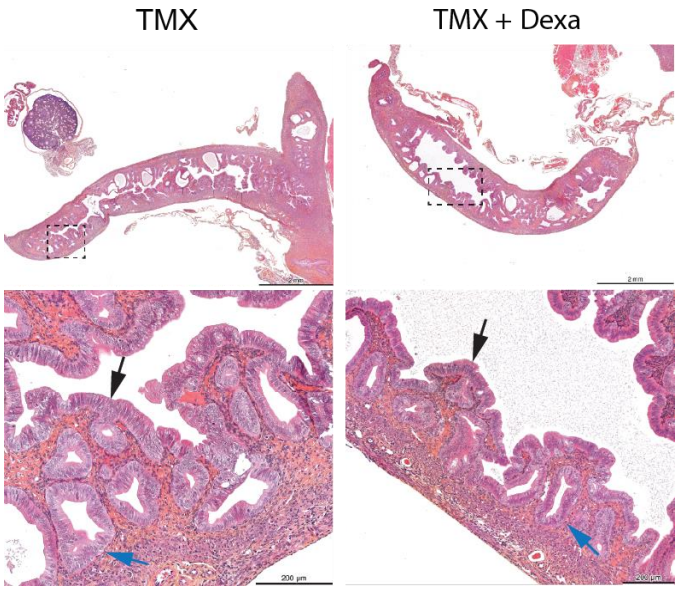
b) HR+ BC Patient-derived xenograft (PDX) outgrowth in six/eight-week-old female NSG mice in the different treatment arms (Control n=4; TMX n=4; Dexa n=4; and TMX+Dexa n=5). Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

Cumulatively, all these data consistently showed that GR-activation acted synergistically with TMX exposure in tumour inhibition, phenocopying the beneficial effects of fasting.

7) One important side effect of tamoxifen is endometrial hyperplasia, which has been shown to be reduced by fasting and FMD (Nature, 2020). Do GR agonists provide the same beneficial effect of fasting in protecting endometrium from hyperplasia?

We would like to thank the reviewer for highlighting this important comparison to the previous work. To address this issue, we performed additional intervention studies, specifically focussing on endometrial hyperplasia. For this, 6-8 week-old NSG mice were treated with either TMX alone or combined with dexamethasone for 4 weeks. Subsequently, mice were sacrificed and the uteri were sent for pathological evaluation by a trained animal pathologist. In these analyses, mice treated with TMX combined with Dexa showed decreased endometrial thickness with less granular structures (**Extended Data Fig. 7c** and **Figure for Reviewers 16**). Based on these results, we concluded that dexamethasone treatment, analogous to fasting, protects endometrial tissue from TMX-induced hyperplasia. These results are now included in the revised manuscript - *Lines 188-190: "Finally, in mice that were treated with TMX, Dexa attenuated TMX-induced uterus hyperplasia, a relatively common side effect of TMX which we previously reported to be effectively prevented by fasting[3] (Extended Data Fig. 7c).*"

Fig. 16



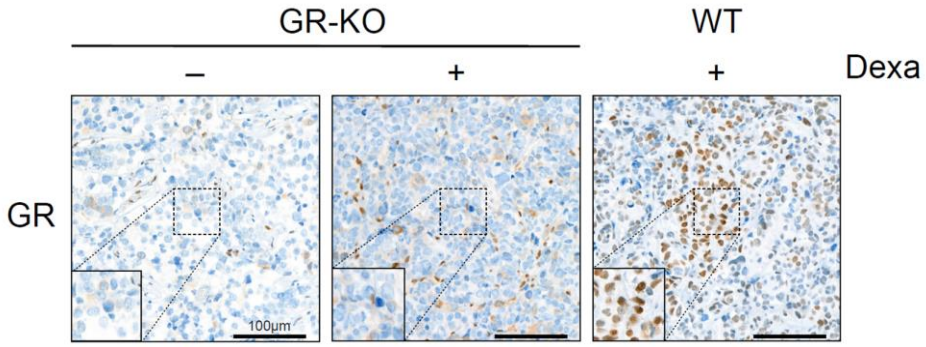
Microphotographs of H&E staining of uterus after TMX (left panel) and TMX + Dexa (right panel) treatments. Black arrows indicate luminal epithelia and blue arrows indicate glandular epithelia of the endometrium.

8) Please provide some evidence of specificity for the GR antibody used for IHC. For

example, dex-treated MCF7 tumours as a positive control and dex-treated MCF7-GR-KO tumours as a negative control.

We thank the reviewer for recommending this important control experiment. To address this point, we made use of xenografted GR-KO MCF7 tumours, subjected to either dexamethasone or vehicle control. As control, dexamethasone-treated wildtype tumours were included in these analyses. Tumours were analysed by IHC for GR, applying the same antibody used throughout the manuscript. Confirming the specificity of our antibody, GR-KO MCF7 cells do not stain for GR, irrespective of Dexamethasone exposure. Of note, in contrast to the GR-KO tumour cells, mouse-derived infiltrating stromal cells (smaller cell size) still stained positively for GR, serving as internal positive control **(Extended Data Fig. 5d** and **Figure for Reviewers 17**). Altogether, these results confirm the specificity of our IHC antibody.

Fig. 17



Representative IHC images for GR in MCF7 GR-KO or WT xenografts treated with vehicle or dexamethasone.

Minor:

1) supplementary figure 1a: add quantification of the Ki67 staining

The ki-67 quantification of Extended Data Fig. 1a has now been added to the revised manuscript (**Extended Data Fig. 1b**)

2) supplementary figure 1g: specify p values

P-values for Extended Data Fig. 1g are now added.

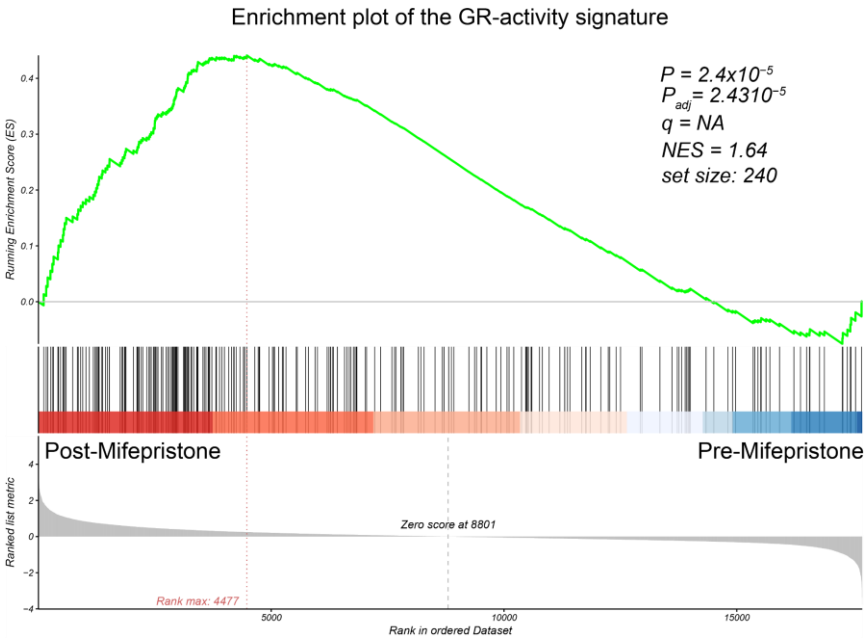
3) The recent presurgical window of opportunity trial MIPRA (https://pubmed.ncbi.nlm.nih.gov/36269797/) provides support for the use of mifepristone in patients with luminal breast cancer with high PRA/PRB ratios. This trial should be discussed.

We thank the reviewer for bringing this trial to our attention. We have updated the discussion section accordingly, mentioning the MIPRA trial, in relation to other clinical trial activities on PR agonists.

Interestingly, mifepristone treatment is known to increase circulating cortisol levels (10.1210/jc.2016-2405; 10.1007/s11064-006-9055-5; 10.1038/sj.jp.7211127). Although mifepristone does compete with cortisol to bind GR, the pharmacodynamics of the drug might not be sufficient to keep a stable inhibition of the hormone-receptor. Moreover, high doses of mifepristone have been reported to exert agonist effect on GR (10.1210/en.2004-0411; 10.1111/bph.13477) and another study reported that treatment of patients with 200mg/day of mifepristone (the same dose used in the mentioned clinical trial) does activate GR, when the levels of circulating cortisol are low (e.g. at the end of the day/night; doi.org/10.1096/fj.13-237958) or when GR levels are low (10.1016/j.steroids.2007.03.012).

Based on these previous observations, we decided to perform GSEA analysis using our GR-activity signature on the differential transcripts between pre and post mifepristone treatment of BC patients included in the mentioned clinical trial. Strikingly, we find a highly significant enrichment of GR-responsive genes in patients after treatment with mifepristone (see below **Fig. 18**). These results reinforce that the dose given to these patients may be activating GR and, based on our results, lead to tumour suppression, low Ki-67 and cell proliferation gene transcription observed by the authors of the study.

Fig. 18



Enrichment plot of a pan-cancer GR-activity signature for the depicted conditions on samples of the MIPRA trial. NES (Normal Enrichment Score) and *P*-value are shown.

As requested, we updated our results and discussion sections to include a summary of the above mentioned points and better inform the reader on the different results of both clinical trials, in relation to our study - *Lines 230-235: "PR agonist treatment is currently evaluated in combination with letrozole, in a phase II randomized clinical trial in ERα+ BC patients (NCT03306472). However, the MIPRA clinical trial showed that PR inhibition by mifepristone also reduced tumour cell proliferation in HR+ BC patients (NCT02651844)[30]. These contradictory results can be partially explained by the use of mifepristone, which is a potent PR inhibitor but, when used at high doses or depending on the time of the day when the drug is taken (night vs morning), also acts as a GR-agonist[31-33]."*

4) What time of day was cortisol measures in figure 3i and j?

Considering the dynamics of cortisol levels throughout the day, we collected all patient's blood samples at the same time of the day, during the morning between 8:30h and 10:00h, pre and post the FMD dietary intervention. This is now mentioned on *lines 447-452*: "*Enrolled patients initiated the FMD 12–15 days before surgery, and underwent blood sampling after at least 8-hour complete fasting on the morning (8:30h-10:00h) of FMD initiation ("Pre-FMD"), and on the morning of FMD completion ("Post-FMD"). Tumour samples for RNA-seq analyses were obtained diagnostic core biopsies performed at baseline (Pre-) and from matched surgical specimens (Post-)".*

Referee #2

The manuscript entitled “Fasting boosts breast cancer therapy efficacy via glucocorticoid activation” by Professor Zwart and co-authors demonstrate that GR activation plays a key role in fasting’s ability to enhance endocrine therapy sensitivity in preclinical models and patient samples. The authors also demonstrate that glucocorticosteroid treatment can be a great treatment strategy that mimics the effects of fasting in breast cancer treatment and increases efficacy to endocrine therapy in ER+ breast cancer. This way, the need for fasting as dietary restriction is bypassed.

The study is highly clinically relevant as endocrine therapy is the mainstay for ER+ breast cancer and ways to improve it is of high clinical relevance and thus of interest to the breast cancer community. In addition, in a clinical trial fasting correlated with increase in responses in endocrine therapy but the mechanisms are largely unknown. The identification of these mechanisms may provide novel therapeutic opportunities. The manuscript is novel, well written and well-designed.

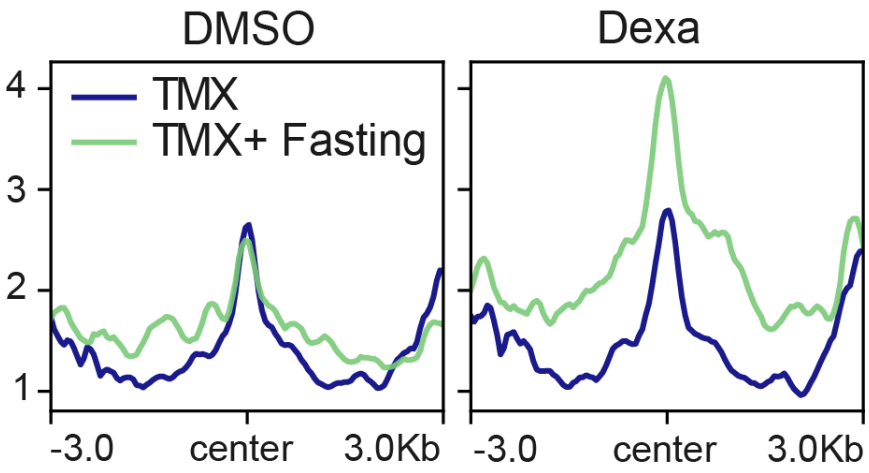
1) Concerns remain on the mechanisms of how fasting leads to GR and PR increase of activity and how does the epigenetic landscape contribute to their activation. Thus, the study would benefit from more development on these key mechanisms.

We want to thank the reviewer for the time invested in critically evaluating our work. We were delighted to learn that the reviewer finds our manuscript highly clinically relevant, novel, well-written and well-designed.

We are grateful to the reviewer for sharing the expressed concerns regarding mechanistic interpretation of our results, on which we gladly further expand below. Prior studies have reported an elevation of circulating cortisol levels following fasting or FMD through activation of the hypothalamic-pituitary-adrenal (HPA) axis (10.1152/ajpendo.2001.280.1.E50; 10.1210/jcem.81.2.8636290; 10.1530/eje.0.1500185), since fasting is systemically perceived as a form of metabolic stress. The same HPA axis activation is responsible for regulating the levels of circulating progesterone in women (10.3389/fendo.2023.1295261). We confirmed these observations in our mice and patient samples, and observed strong increases of progesterone and cortisol (patients) or corticosterone (mice) upon fasting. In agreement with the elevated levels of these hormones in circulation, we see a striking intra-tumour activation of both GR and PR, leading to DNA binding of these transcription factors and consequent altered expression of genes that enhance endocrine therapy efficacy in breast cancer cells, including the tumour suppressor ZBTB16.

In strengthening the mechanistic aspects of our study, we tested the hypothesis, whether GR activation by itself would be responsible for the observed alterations in H3K27ac profiles. Such observations would be in line with previous reports from the Gordon Hager lab, who reported that GR -when activated- can actively induce active enhancer states (10.1101/gr.212175.116; 10.1093/nar/gkx1044). To test this hypothesis, we re-analysed a H3K27ac ChIP-seq dataset in MCF7 cells from our own team (10.15252/emmm.202317737), in which cells were treated for 7 days with dexamethasone. Interestingly, we observed that in vitro dexamethasone exposure sufficed to elevante H3K27ac signal at our fasting-induced H3K27ac sites (**Figure for Reviewers 19**). Cumulatively, these results support the following mechanistic model: fasting increased circulating levels of progesterone and cortisol. These hormones activate the intratumoural PR and GR, leading to increased H3K27ac, indicative of activated enhancers and promoters, at our fasting-induced H3K27ac sites. Activated GR and PR subsequently result in an increased activity of tumour suppressors to block tumour growth.

Fig. 19



Average H3K27ac ChIP-seq signal in MCF7 cells treated with 7 days of Dexa or DMSO at TMX-induced (blue) or TMX+Fasting induced (green) H3K27ac sites from Figure 1c of the manuscript.

2) The study will also benefit from a PDX model for in vivo studies in addition to the studies in xenografts established from MCF7 breast cancer cells.

We thank the reviewer for raising this important point regarding the limited number of *in vivo* models used in our study. To address this point as thoroughly as possible, in addition to the requested ERα+ BC PDX model (see below) , we extensively increased the number of mouse models to now also study the impact of GR activation to phenocopy fasting in:

1. Orthotopically-implanted ERα+ BC patient-derived xenografts (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 20a**)

2. Orthotopically-implanted T47D cells (**Extended Data Fig. 6f-g** and **Figure for Reviewers 20b**)
3. Orthotopically-implanted TSAE1 cells in immune-competent animals (**Fig. 4f; Extended Data Fig. 7d-f** and **Figure for Reviewers 20c**)

Below, we present a summary of each added *in vivo* experiment and respective conclusion:

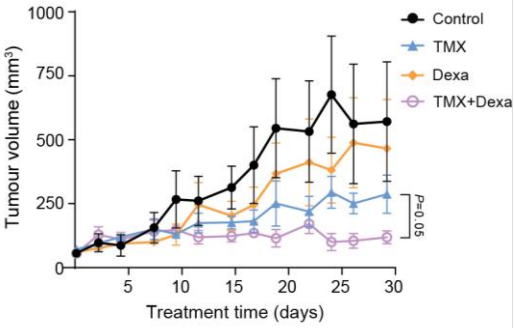
1. To further substantiate our findings in more clinically relevant models, ERα+ BC patient-derived xenografts were established, grafted through intraductal injections, and tested for response. With these studies we further confirmed that TMX combined with Dexa show a strong impairment of ERα+ BC PDX tumour growth, as compared to either treatment alone. These results further support the impact of GR activation to boost endocrine therapy potential in patient-derived model systems (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 20a**).

2. *In vivo* T47D experiments were added to the study, as a second ERα+ BC model in which the tumour cells were implanted orthotopically through intraductal injections (Mouse Intraductal (MIND) model; 10.1186/bcr2358). These studies fully confirmed our original observations that GR activation boosted the effects of tamoxifen treatment. These results have now been incorporated in the manuscript, as new **Extended Data Fig. 6f-g; Figure for reviewers 20b**. With this, we addressed the validation in additional cell line models.

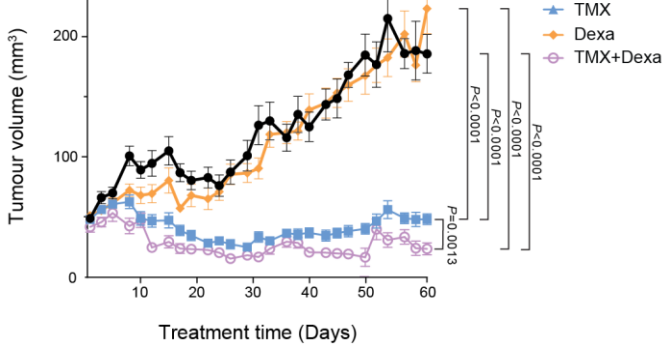
3. Finally, to address the important comment regarding the impact of dexamethasone treatment in immunomodulation and anti-cancer effects, we made use of a recently-developed ERα+ mouse BC cell line (TSAE1; 10.1038/s43018-023-00525-y) and expanded our studies to immunocompetent mice. Interestingly, and in line with our results in immunodeficient mice, we observe an impairment on tumour growth when mice are treated with the combination TMX+Dexa, versus tamoxifen alone (**Fig. 4f; Extended Data Fig. 7d-e;** and **Figure for Reviewers 20c-d**). Of note, these experiments were also performed using the MIND intraductal injection model, without the use of exogenous estrogens. Moreover, taking advantage of the immune proficient mice model, we performed systemic immune-profiling to better understand the impact of Dexa treatment in the anti-tumour response of the immune system

Fig. 20

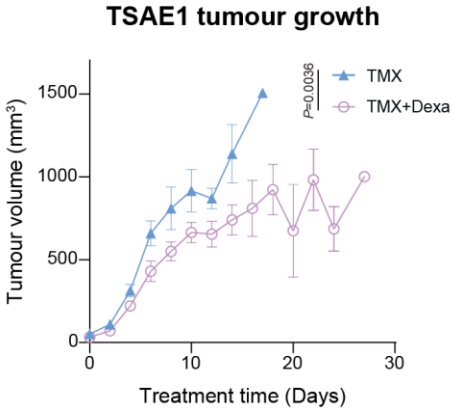
a)



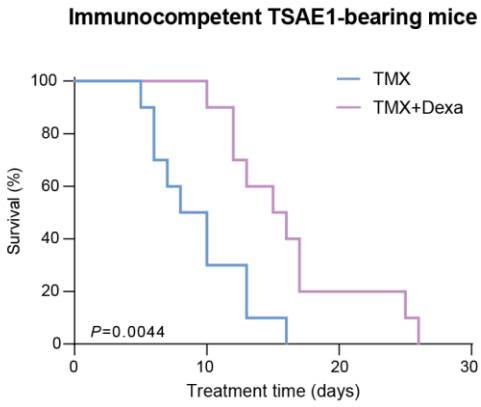
b)



c)



d)



a) HR+ BC Patient-derived xenograft (PDX) outgrowth in six/eight-week-old female NSG mice in the different treatment arms (Control n=4; TMX n=4; Dexa n=4; and TMX+Dexa n=5). Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

b) T47D xenograft tumour outgrowth in six-eight-week old female NSG mice in the different treatment arms (Control n=8; TMX n=7; Dexa n=8 and TMX+Dexa n=7) . Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

c) TSAE1 syngeneic tumour growth in immunocompetent mice. Data are shown as mean ± SEM and analysed by linear mixed-effects model.

d) Survival curves of mice engrafted with TSAE1 cells and treated with either TMX or TMX+Dexa.

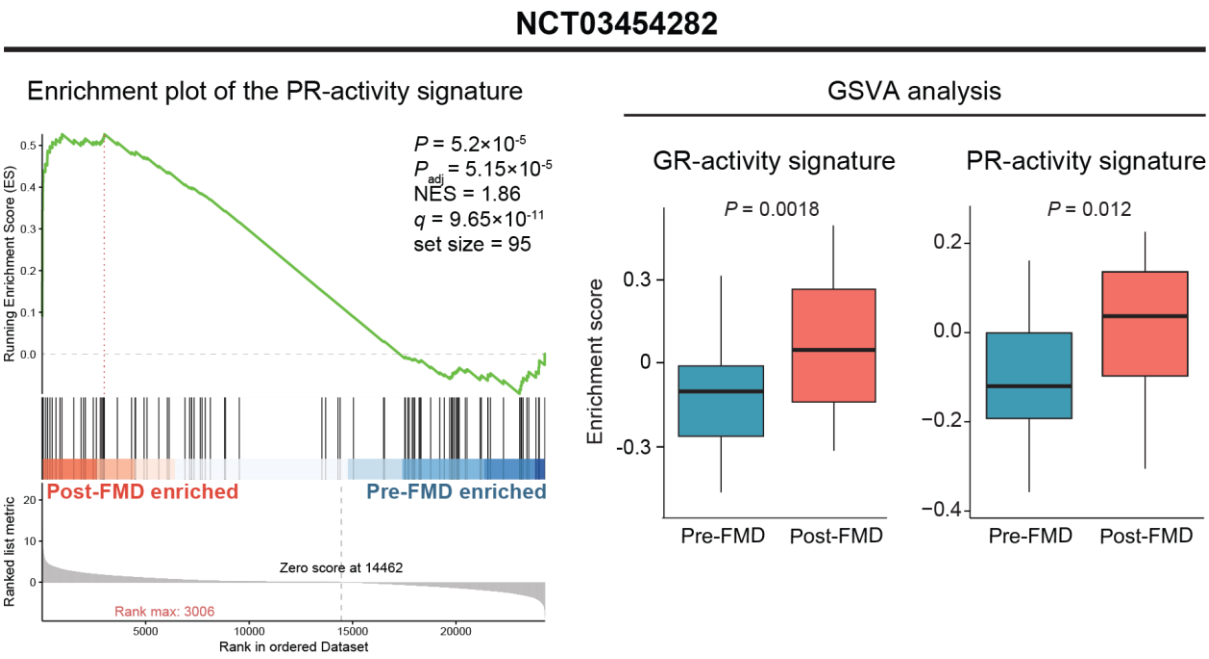
Cumulatively, all these data consistently showed that GR-activation acted synergistically with tamoxifen exposure in tumour inhibition, phenocopying the beneficial effects of fasting.

3) Lastly, it remains unclear why the authors only focused on GR gene signature and not PR gene signature in Fig 3 given that both GR and PR showed an increased in genome-binding upon fasting.

We thank the reviewer highlighting this apparent inconsistency. Indeed, we see both increased chromatin binding of both PR and GR after fasting treatment (**Figure 2b-c**), which was consistent with the elevated cortisol/corticosterone and progesterone levels in plasma upon fasting (**Figure 2g, i**). The main focus of our manuscript is centered elucidating the contribution of GR in this context, along with its activation for potential clinical application, based on the following rationale:

(A) The role of PR activation as an inhibitor of ERα+ BC tumour growth has already been extensively studied previously, including work by the team of Jason Carroll (10.1038/nature14583). Based on these observations, a window-of-opportunity clinical trial has been initiated in which patients are treated with megestrol, a PR agonist, in combination with letrozole (NCT03306472l; 10.1158/1538-7445.SABCS23-PS15-06). To further strengthen our observations regarding the impact of PR in the phenotype we observed, we expanded our analyses by compiling a new PR-responsive gene set of 95 genes (based on ChIP-seq and RNA-seq derived from 10.1038/nature14583) and performed GSEA in BC patients treated with 5 days of FMD (**Extended Data Fig. 4b-c** and **Figure for Reviewers 21**). In line with our observations on increased progesterone levels in plasma (**Fig. 2g, i**) and increased PR chromatin binding in xenografts (**Fig. 2b-c**), we find a significant enrichment of PR-responsive genes in patients fasted for 5 days compared to pre-fasting.

Fig. 21



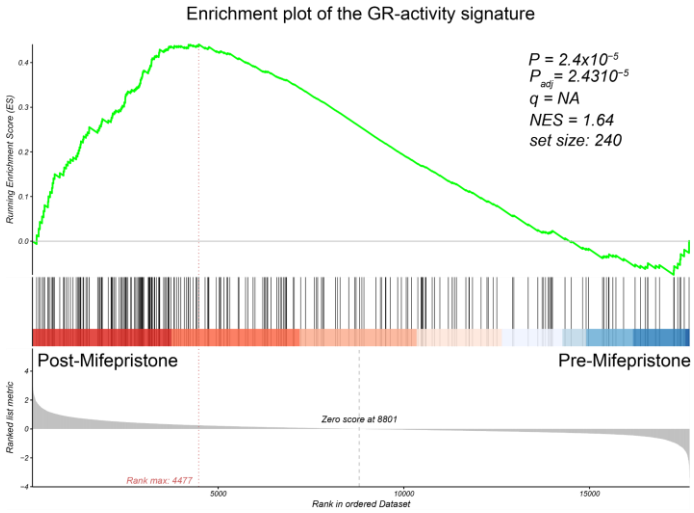
(Left) Enrichment plot of a PR-activity signature for patients treated with 5 days FMD vs before the treatment. NES (Normal Enrichment Score) and *P*-value are shown.

(Right) Gene-set variation analysis (GSVA) for the PR-activity signature in patients treated with 5 days FMD versus before treatment. *P*-value represents paired Wilcoxon testing.

These new observations have now been incorporated into the revised manuscript, and we are grateful for the reviewer comments that motivated us to explore into this direction. *Lines 151-154:* "*In line with the increased PR chromatin binding (Fig. 2b-c; Extended Data Fig. 2a-b; Extended Data Table 1) and the elevated progesterone levels (Fig. 2g, i; Extended Data Fig. 3a) PR transcriptional activity was also increased in patients after the FMD (Extended Data Fig. 4b-c; Extended Data Table 3).*"

Of note, a second window-of-opportunity trial on PR antagonism was recently published (https://pubmed.ncbi.nlm.nih.gov/36269797/), which reported the benefit of PR antagonist Mifepristone in this setting. Interestingly, mifepristone treatment is known to increase circulating cortisol levels (10.1210/jc.2016-2405; 10.1007/s11064-006-9055-5; 10.1038/sj.jp.7211127). Although mifepristone does compete with cortisol to bind GR, the pharmacodynamics of the drug might not be sufficient to keep a stable inhibition of the hormone-receptor. Moreover, high doses of mifepristone have been reported to exert agonist effect on GR (10.1210/en.2004-0411; 10.1111/bph.13477) and another study reported that treatment of patients with 200mg/day of mifepristone (the same dose used in the mentioned clinical trial) does activate GR, when the levels of circulating cortisol are low (e.g. at the end of the day/night; doi.org/10.1096/fj.13-237958) or when GR levels are low (10.1016/j.steroids.2007.03.012). Based on these previous observations, we decided to perform GSEA analysis using our GR-activity signature on the differential transcripts between pre and post mifepristone treatment of breast cancer patients included in the mentioned clinical trial (MIPRA). Strikingly, we find a highly significant enrichment of GR-responsive genes in patients after treatment with mifepristone (see below **Fig. 22**). These results reinforce that the dose given to these patients may be activating GR and, based on our results, lead to tumour suppression, low Ki-67 and cell proliferation gene transcription observed by the authors of the study.

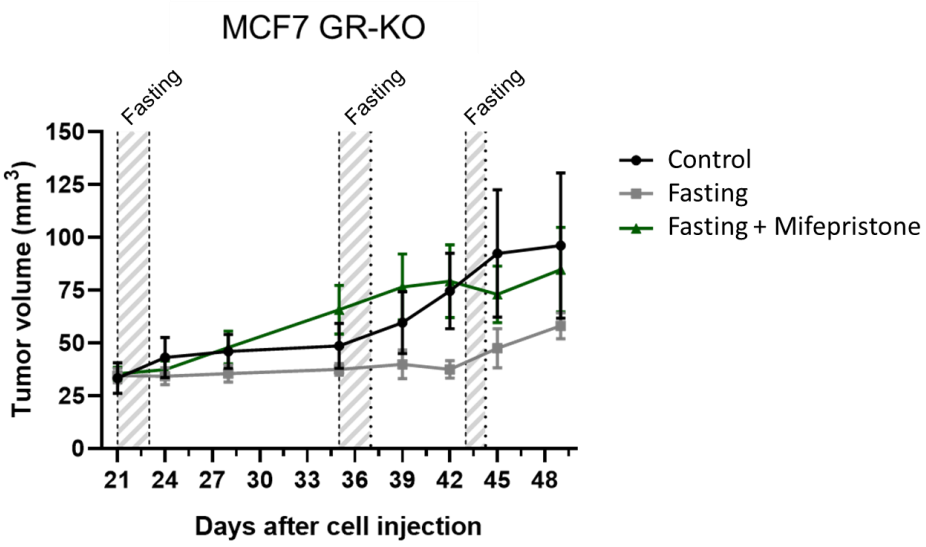
Fig. 22



Enrichment plot of a pan-cancer GR-activity signature for the depicted conditions on samples of the MIPRA trial. NES (Normal Enrichment Score) and *P*-value are shown.

(B) By request of reviewer #1, we treated MCF7 GR-KO mice with mifepristone to evaluate whether the combined inhibition of both PR and GR action would even more strongly inhibit the beneficial effects of fasting, restoring tumour growth. We observe an almost complete restoration of tumour growth in fasted-mice xenografted with MCF7 GR-KO and treated with mifepristone (**Figure for Reviewers 23**). However, prolonged mifepristone exposure led to adverse side effects in these mice and consequent death of many animals. This can be attributed to the long duration of the treatment (one month vs one week) and/or the high concentration that was used (10.3892/etm.2015.2203; 10.3109/15376516.2015.1118715).

Fig. 23



MCF7 GR-KO xenograft tumour growth in six/eight-week-old female athymic nude mice treated with Control (ad libitum diet), fasting (48h water-only) or Fasting combined with Mifepristone (60 mg/kg). Data are shown as mean ± SEM.

4) Minor comments include the lack of FDR (q value) for the GSEA of RNA-seq in Figure 3. In addition, the actual TF motifs should be shown in Figure 1 to really understand any overlap in motifs as nuclear hormone receptor or AP-1 TFs have similar motifs. (Fig 2).

We thank the reviewer for raising these important points. The GSEA analysis in the original submission indeed had a *p*-value depicted and no *q*-value, as the application of FDR (*q*-value) is to correct for multiple testing. In this case, a singular hypothesis was tested (enrichment of our GR activity signature), and therefore *p*-value equals FDR corrected *q*-value. Nonetheless, to address the reviewer concern we now included FDR values, and incorporated both GR and PR signature gene sets in the Hallmarks GSEA to obtain FDR values. Based on these new analyses, all the GSEA analyses of the manuscript have been updated, now including FDR values.

As requested by the reviewer, all motifs for the TFs listed in Figure 1f are now included in the panel.

Referee #3

Summary: The authors used the hormone receptor-positive (HR+) model of breast cancer to show that fasting, in combination with endocrine therapy, induces extensive epigenetic reprogramming. Importantly, the authors have linked the anticancer effects of fasting in this model to activation of glucocorticoid receptor (GR) and progesterone receptor (PR) signaling. They report the results of several elegant studies including: a) GR-knockout approach that resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen; b) exogenous administration of GR-ligands mimicked fasting-enhanced anti-estrogen action and tumour regression. They also show in fasted patients that systemic levels of progesterone and cortisol were elevated. Moreover, transcriptomic analysis of GR activation signatures in tumours from fasted (v unfasted) patients showed inverse correlations with proliferation markers. Their findings support the idea that GR activation may contribute importantly to fasting's ability to enhance endocrine drug activity in breast cancer. The authors also suggest that corticosteroid administration should be evaluated as an adjuvant to endocrine therapy for HR+ breast cancer.

We thank the reviewer for the time invested in evaluating our manuscript. We are grateful for the valuable suggestions and recommendations that clearly aided us in further improving the quality of our work. As can be appreciated below, we have done our utmost best in addressing all concerns that were raised, in full.

1) Novelty: The link between glucocorticoid receptor signaling and fasting/dietary energy restriction in cancer is not an entirely novel concept in the field of energy balance and cancer (see a series of publications by Diane Birt and colleagues in the early 2000's, including Prevention of mouse skin tumour promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition.Stewart JW, Koehler K, Jackson W, Hawley J, Wang W, Au A, Myers R, Birt DF. Carcinogenesis. 2005 Jun;26(6):1077-84. Doi: 10.1093/carcin/bgi051. In addition, Longo and colleagues have alluded to this in prior work in the context of fasting and chemotherapy response (Di Biase, et al. PLoS Biol. 2017 May 1; 15(5): e1002603), including the concept of differential stress response in normal versus cancer cells (deGroot, et al. Nat Comm 2020; PMID: 32576828; DOI: 10.1038/s41467-020-16138-3. However, very few studies have focused on HR+ breast cancer.

We thank the reviewer for highlighting the unique positioning of our study, in which we indeed focus or study in HR+ breast cancer. While this may seem as a nuance at first,

as we explain further below, the context dependency of our findings is of high scientific and clinical relevance for a broad readership. First, we report that fasting and GR activation are associated with favourable features and enhance the effect of endocrine therapy in breast cancer treatment, in which we provide conclusive evidence (through tumour-specific CRISPR-KO of GR) that tumour intrinsic GR signalling is the main driver of this effect. Importantly, in hormone receptor negative breast, the effects of GR activation status is quite the opposite, a study by the Bentires-Alj lab reported in Nature that GR stimulation induces metastasis formation and tumour outgrowth (10.1038/s41586-019-1019-4). While these results seem to completely contradict our observations, it is of relevance to stress that this report exclusively focussed on triple negative breast cancer; a subpopulation of 15% of breast cancer patients, while we study luminal hormone receptor positive cases; 75-80% of all patients with the disease. In absolute numbers, these patients with luminal disease represent, by far, the largest group of cases who succumb to the consequences of the disease.

The response to chemotherapeutics, further adds to the observed complexity of GR action in oncology, as GR activation is reported in ovarian cancer to diminish response to chemotherapy, as was recently reported in a Phase II study (2010.1200/JCO.22.02624) and confirmed in the follow-up Phase 3 ROSELLA clinical trial on the combination of a GR antagonist with chemotherapy response (https://ir.corcept.com/news-releases/news-release-details/primary-endpoint-met-corcepts-pivotal-phase-3-rosella-trial; manuscript yet to be published).

Importantly, we believe that our mechanistic studies, revealing causality of GR activation status to drive the beneficial responses of fasting, strongly underscores the novelty of our findings. These results also communicate a clear warning to the general readership of Nature, as fasting -and consequent GR activation- in the wrong clinical context (e.g. triple negative breast cancer, but also ovarian cancer or advanced metastatic castrate resistant prostate cancer (2010.1016/j.cell.2013.11.012)) would likely have detrimental consequences by activating GR. The timely nature, and implications on the general readership of Nature, is further highlighted by a recent follow-up study on the manuscript the reviewer highlighted in TNBC (10.1038/s41467-020-16138-3). A study that is currently under consideration for publication from the same team, on the long-term breast cancer-specific survival of the TNBC patients in their original patient population undergoing fasting in the neoadjuvant setting, showed in a small cohort that TNBC patients actually had a worse breast cancer-specific survival after the fasting intervention, versus those TNBC patients who were on a regular diet (personal communication with Dr. Judith Kroep; the senior author of the study the reviewer highlights (10.1038/s41467-020-16138-3)). Therefore, our study is not only timely, we believe it is also well positioned in explaining why explicitly in particular contexts, cancer types or tumour subtypes, fasting has a favourable impact on patient outcome, and how we can therapeutically mimic this with corticosteroid treatment.

2) Data and methodology: the reported experiments were generally well-designed and executed. My major concern is the dependence on the single in vivo model used, specifically the MCF-7 xenograft model in immunodeficient athymic nude mice. This raises several concerns. First, given the established importance of immunomodulation in the anticancer effects of fasting/fasting mimicking diets and related interventions, as well as the established interactions between glucocorticoid signaling and anticancer immune responses, the inclusion of at least one other immune-intact model (syngeneic orthotopic transplant model or genetically engineered mouse model) would have strengthened the paper. Also, the choice of the MCF-7 transplant model requiring exogenous estrogen via a 17b-estradiol pellet, which delivers a constant, high level of estrogen for the tumours to take, is also a poor mimic of a postmenopausal woman at high risk for developing HR+ breast cancer. This limitation is particularly true for a study focusing on the interactions between fasting, glucocorticoid signaling, tamoxifen and breast cancer. An additional concern is the site of tumour growth, in this case subcutaneous injection in the flank (area overlying the upper thigh and lower part of back & abdomen) instead of orthotopic transplantation into one of the mammary glands, which would have permitted assessment of tumour growth in the tissue of interest rather than subcutaneously on the back of the mouse. Given that the elegant mechanistic analyses, including their GR-knockout approach that resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen, and their assessment of the effects of exogenous administration of GR-ligands on tumour regression, extended from this limited in vivo model, I found the results interesting but lacking sufficient rigor. While the human findings are somewhat supportive, in my opinion the findings as presented would be more suited to a specialty journal (such as Breast Cancer Research) than for the broad readership of Nature.

We thank the reviewer for this thorough and detailed assessment of both the strengths and weaknesses of our original submission. Based on these highly valuable reviewer comments, we decided to extensively expand all *in vivo* aspects of our entire paper, which includes four additional independent in vivo data streams to strengthen our study:

1. Orthotopically-implanted T47D cells (**revised manuscript Extended Data Fig. 6f-g** and **Figure for Reviewers 24b**)
2. Orthotopically-implanted TSAE1 cells in immune-competent animals (**Fig. 4f; Extended Data Fig. 7d-e** and **Figure for Reviewers 24c**)
3. Orthotopically-implanted ERα+ BC patient-derived xenografts (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 24d**)

Cumulatively, all these data consistently showed that GR-activation acted synergistically with tamoxifen exposure in tumour inhibition, phenocopying the beneficial effects of fasting.

Below, we present an extensive series of additional experiments, addressing all these points that were raised:

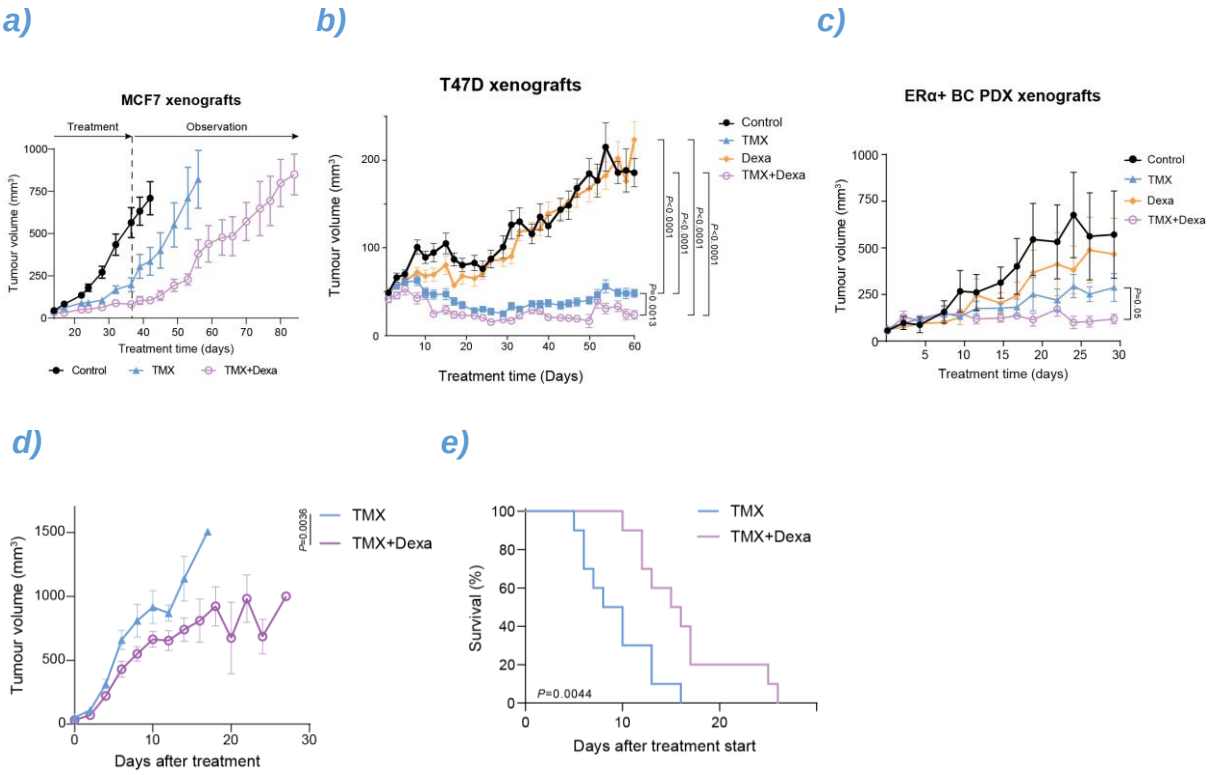
1. MCF-7 cells were xenografted orthotopically in the mouse mammary fat-pad and treated (Dexa, TMX, Dexa + TMX, fasting, TMX + fasting, or vehicle control), for a month. Subsequently, all therapies were stopped and tumours were allowed to re-growth to evaluate the tumour growth delay impacted by the different treatments. Tumour outgrowth in Dexa+TMX treated mice was delayed with a factor of 2, as compared to TMX alone (**Fig. 4f; Extended Data Fig. 7a** and **Figure for Reviewers 24a**)

2. *In vivo* T47D experiments were added to the study, as a second ERα+ BC model in which the tumour cells were implanted orthotopically through intraductal injections (Mouse Intraductal (MIND) model; 10.1186/bcr2358). These studies fully confirmed our original observations that GR activation boosted the effects of tamoxifen treatment. These results have now been incorporated in the manuscript, as new **Extended Data Fig. 6f-g** and **Figure for Reviewers 24b**. With this, we addressed both the concern on orthotopic grafting and validation in additional cell line models.

3. To further substantiate our findings in more clinically relevant models, ERα+ BC patient-derived xenografts were established, grafted through intraductal injections, and tested for response. With these studies we further confirmed that TMX combined with Dexa show a strong impairment of ERα+ BC PDX tumour growth, as compared to either treatment alone. These results further support the impact of GR activation to boost endocrine therapy potential in patient-derived model systems (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 24c**) .

4. Finally, to address the important comment regarding the impact of dexamethasone treatment in immunomodulation and anti-cancer effects, we made use of a recently-developed ERα+ mouse BC cell line (TSAE1; 10.1038/s43018-023-00525-y) and expanded our studies to immunocompetent mice. Interestingly, and in line with our results in immunodeficient mice, we observe an impairment on tumour growth when mice are treated with the combination TMX+Dexa, versus tamoxifen alone (**Fig. 4g; Extended Data Fig. 7d-f** and **Figure for Reviewers 24d**). Of note, these experiments were also performed using the MIND intraductal injection model, without the use of exogenous oestrogens, addressing both of these concerns.

Fig. 24



a) MCF7 xenograft outgrowth in six/eight-week-old female athymic nude mice in the different treatment arms (Control n=7; TMX n=7; Dexa n=5 and TMX+Dexa n=6). After 4 weeks, all treatments were stopped and tumours were allowed to re-grow. Data are shown as mean ± SEM.

b) T47D xenograft tumour outgrowth in six-eight-week old female NSG mice in the different treatment arms (Control n=8; TMX n=7; Dexa n=8 and TMX+Dexa n=7) . Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

c) HR+ BC Patient-derived xenograft (PDX) outgrowth in six/eight-week-old female NSG mice in the different treatment arms (Control n=4; TMX n=4; Dexa n=4; and TMX+Dexa n=5). Data are shown as mean ± SEM and analysed by linear mixed-effects model (P-value of last day is shown).

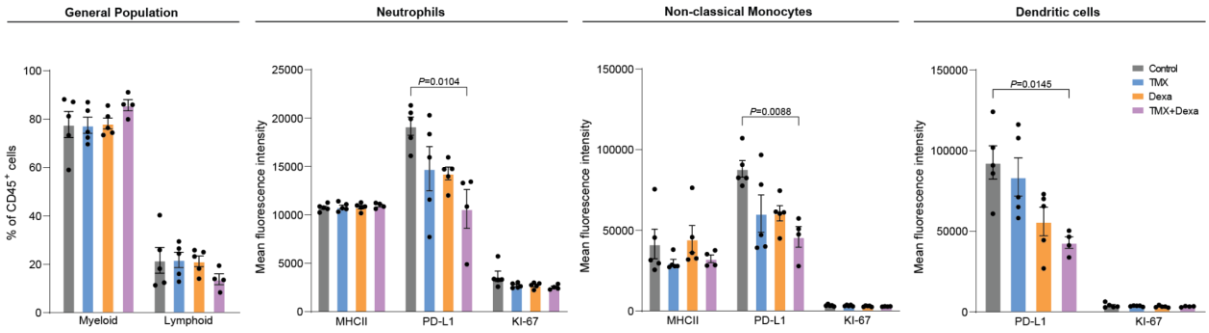
d) TSAE1 syngeneic tumour growth in immunocompetent mice. Data are shown as mean ± SEM and analysed by linear mixed-effects model.

e) Survival curves of mice engrafted with TSAE1 cells and treated with either TMX or TMX+Dexa.

To better understand the impact of glucocorticoid signalling in the anticancer immune response, we performed immuno-profile of xenografted mice treated with the several treatment arms. Overall, no significant alterations in immune cell populations were observed following 7 days of treatment with 4 mg/kg dexamethasone, as assessed by Ki-67 expression, suggesting that dexamethasone does not substantially impact immune cell proliferation under these conditions. Furthermore, the antigen-presenting capacity of neutrophils and monocytes, as indicated by MHC-II expression, was unaffected by treatment supporting the notion that dexamethasone does not compromise their potential anti-tumour function. Importantly, we observe a significant reduction of PD-L1 expression in neutrophils, non-classical monocytes and dendritic cells upon combination of dexamethasone with TMX (**Extended Data Fig. 7f** and **Figure for Reviewer 25**). Reduced PD-L1 levels represent a critical marker of immune

cell activation and anticancer properties in the immune cells thus (10.1016/j.immuni.2007.05.016). Based on these results, we conclude that corticoids combined with endocrine therapy not only decreases BC tumour growth, but may also primes the adaptive immune system for tumour cell killing.

Fig. 25



Barplots show the mean fluorescence intensity for selected markers for systemic myeloid cells and lymphoid cells ($CD45^+$ $CD11b^+$ and $CD45^+$ $CD11b^-$ respectively), neutrophils ($CD45^+$ $CD11b^+$ $Ly6G^+$), non-classical monocytes ($CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^-$) and dendritic cells ($CD45^+$ $F4/80^-$ $CD11c^{high}$ $MHCII^{high}$) from TSAE1--bearing mice treated with three rounds of control treatment,TMXn, Dexae, or TMX+Dexa. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test.

3) Statistics: were appropriate and well described.

We thank the reviewer for critically assessing the scientific rigor of our statistical analyses. We are happy the reviewer finds these appropriate and well described.

4) Conclusions—robustness limited to the limitations described in the data and methodology section. While the findings that GR activation may contribute importantly to fasting's ability to enhance response to endocrine therapy for breast cancer, the authors need to be cautious regarding the strength]f their conclusions. Improvements: more rigorous in vivo data are needed to strengthen the findings

We thank the reviewer for the highly constructive feedback and pinpointing that the extent of *in vivo* datasets of our primary submission needed to be substantially expanded to further solidify our conclusions. These critical suggestions by the reviewer stimulated us to extensively expand our in vivo experimentations, which resulted in the incorporation on 8 additional figure panels, using four independent *in vivo* models to strengthen our original observations, including syngeneic TSAE1 tumour cells in immunocompetent animals (**Fig. 4g; Extended Data Fig. 7d-f, Figure for Reviewers 24**, above), xenograft studies with a second well-established ERα+ human cell line (T47D; **Extended Data Fig. 6f-g, Figure for Reviewers 24**, above), and patient-

derived xenografts (**Fig. 4e; Extended Data Fig. 6h-i** and **Figure for Reviewers 24**, above); all of which were orthotopically implanted. We strongly believe these valuable recommendations from the reviewer greatly added to the quality of our work, and we are sincerely grateful to the reviewer for steering us into this direction.

5) Clarity and context: The introduction was generally well written, although there were a few issues regarding literature support for the premise. The WHI paper by Chlewbowski, et al was cited as one of the main clinical studies supporting the protective effects of fasting. The WHI intervention arm was a low fat, high fruit and vegetable regimen that induced minimal weight loss, so it seemed misleading to claim that study supported fasting as an anticancer approach.

We thank the reviewer for pointing out this incorrect representation of the WHI study. Our intention was to highlight that dietary interventions can impact the outcome of breast cancer treatment, but we realize now that our original positioning of this study in our introduction, may have misinformed the reader. Therefore, we now reformulated this section of the introduction accordingly.

6) Another point made in the introduction that seemed confusing was the argument that insulin, IGF-1 and leptin have only a partial impact on ER+ tumour cell proliferation, and therefore other pathways must be more important. However, the paper cited focused on OSI-906, an IGF_1R inhibitor shown to have relatively modest effects on tumour cell proliferation, at least compared with other inhibitor that inhibit other receptor tyrosine kinases along this pathway besides IGF-1R. Most/all studies to date have not simultaneously manipulated multiple pathways, such as IGF-1R and leptin receptor, so the claim that fasting effects must be due to pathways other than insulin/IGF-1/leptin signaling seemed an overinterpretation of the evidence.

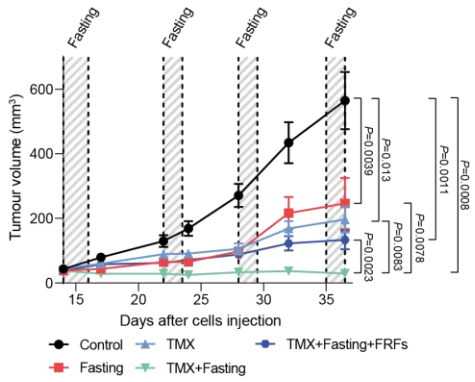
We thank the reviewer for raising an important point in our introduction. The reviewer is absolutely correct to state that the referenced paper did not study the combinatorial effect of the three growth hormones (IGF1/leptin/insulin; Fasting-reducing factors, FRFs), and that therefore no conclusive data have been provided thus far, to state that FRFs as combination would not be sufficient to drive the phenotype. We have updated the introduction accordingly.

To address further this point, we expanded the results sections describing the roles of these FRFs in relation to GR activation, where we feel this is better positioned and allows for deeper description of the literature in relation to our novel findings.

We reperformed the animal studies as we described in the original manuscript (**Fig. 1a and b**), but now with the addition of a 5th treatment arm where mice were treated with a combined Fasting+TMX regimen, along with a combination of FRFs) (**Extended data Fig. 3b and Figure 26 for reviewers**). As the reviewer can appreciate, the addition of FRFs sufficed to partly rescue the growth that was impaired by the Fasting+TMX combination. However, a difference is still appreciable to the TMX alone

treatment. These results imply that addition of these growth factors does not fully impair the beneficial effects of fasting.

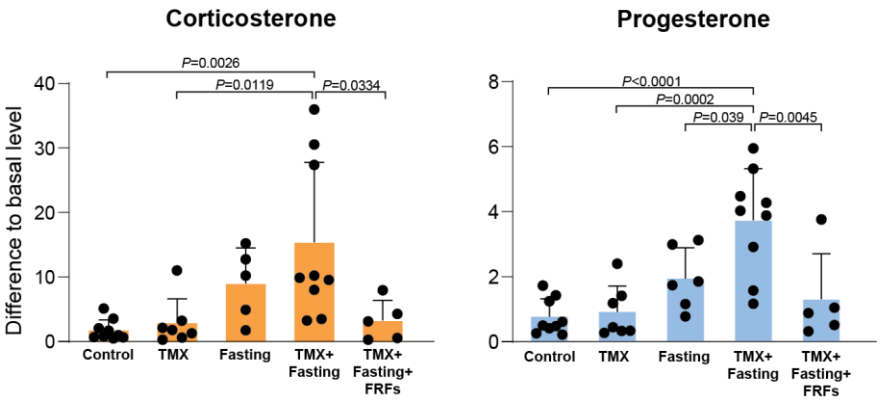
Fig. 26



Xenograft tumour growth in six/eight-week-old female athymic nude mice in the different treatment arms. Data are shown as mean ± SEM.

Moreover, while circulating corticosterone and progesterone levels in the blood of these mice were increased upon TMX+Fasting exposure, this effect was prevented by FRFs co-treatment in mice (**Extended Data Fig. 3a** and **Figure for Reviewers 27** below). Jointly, these results support a conclusion that FRFs treatment impairs GR activity in these tumours due to a systemic reduction of corticosterone levels. We have updated the manuscript accordingly - *Lines 116-120: "Fasting and FMDs were previously found to reduce blood IGF-1, insulin and leptin levels, here referred to as fasting-reducing factors (FRFs), in mice and humans with BC[3,6,21,22]. The re-introduction of these FRFs prevented the beneficial effects of fasting on tumour growth3 (Extended Data Fig. 3b) and the elevation of circulating corticosterone and progesterone levels in mice treated with TMX+Fasting (Extended Data Fig. 3a)."*

Fig. 27



Corticosterone and progesterone levels, in relation to basal levels, on mice blood after 4 cycles of all treatment conditions in relation to basal levels (pre-treatment). Data are shown as mean ± SD and analysed by one-way ANOVA followed by Tukey multiple comparison test.

Referees' comments:

Referee #1 (Remarks to the Author)

I have no additional concerns. Congrats on a great study.

We would like to sincerely thank the Reviewer for positively evaluating our revised manuscript and for supporting our study for publication.

Referee #2 (Remarks to the Author):

The authors have addressed Reviewers comments with further data and explanations.

We want to thank the Reviewer for taking the time of reviewing our revised manuscript, and are grateful for the positive feedback.

Few minor things to consider:

Fig 19, please show tornado plots. H3K27ac meta profiles are not very strong.

We thank the reviewer for raising this interesting minor point for consideration. Below, we present the tornado plots that are associated to the average profiles that were included in the prior submission. Indeed, the signal is not very strong for these regions, yet detectable and selectively elevated for the fasting-associated regions. A possible explanation for the relatively modest increase of H3K27ac signal in this regions, is the discrepancy between long-term (1 month*) in vivo* intervention versus short-term (7 days) *in vitro* exposure to dexamethasone.

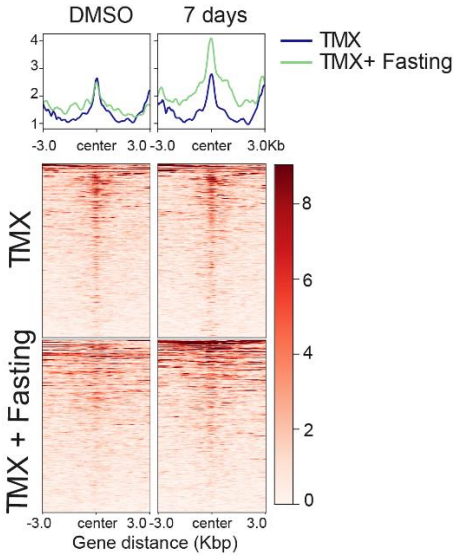


Fig 20a. More information would be helpful on PDX and its genomic profile? Where is the pdx from?

We thank the reviewer raising this point. The PDX used (IDC186) was generated by the team of Jos Jonkers (NKI; an expert in mouse modelling of breast cancer) and was generated from a premenopausal breast cancer patient, with a grade 2 ERα+, PR+ and HER2- tumor. After surgery, the tumour was collected and xenografted in mice to generate a PDX model. We updated our material and methods section, incorporating all relevant clinicopathological data for the PDX model used.

"The IDC186 MIND-PDX model was previously established from a pre-menopausal Caucasian grade 2 BC patient, confirmed positive for ERα (95%) and PR (95%) but negative for HER2 (Extended Data Fig. 6h)."

Fig 20b. The effect of the combination in T47D while significant is biologically modest as tamoxifen has a good effect.

We thank the reviewer for mentioning this point, and we fully agree. Indeed, the T47D clone we have appears particularly sensitive to tamoxifen *in vivo*, which was also the case in our prior study in which responses to fasting were tested (Caffa et al. 2020 Nature). Irrespective of above-mentioned strong response to tamoxifen, we still observe a significantly lower tumour volume when Dexa is combined with TMX, as compared to TMX alone.

Referee #3 (Remarks to the Author):

The authors were highly responsive to the previous review, did a huge amount of additional work to strengthen their story, and their data is now quite convincing and exciting. They used 4 hormone receptor-positive (HR+) mouse models of breast cancer to more clearly establish that fasting, in combination with endocrine therapy, induces extensive epigenetic reprogramming. Importantly, the authors have linked the anticancer effects of fasting in this model to activation of glucocorticoid receptor (GR) and progesterone receptor (PR) signaling, and the have repeated many of their key experiments with additional controls to add more rigor and clarity, establishing: a) GR-knockout resulted in attenuation of the beneficial effects of fasting in combination with tamoxifen; b) exogenous administration of GR-ligands mimicked fasting-enhanced anti-estrogen action and tumour regression. They also show in fasted patients that systemic levels of progesterone and cortisol were elevated. Moreover, transcriptomic analysis of GR activation signatures in tumours from fasted (v unfasted) patients showed inverse correlations with proliferation and immune markers. Their findings established that GR activation underlies fasting's ability to enhance endocrine drug activity in breast cancer. The findings also strongly support further interrogation of therapeutic strategies that mimic the beneficial effects of fasting on responses to endocrine therapy for HR+ breast cancer.

We want to thank the reviewer for the thoughtful, highly supportive and positive evaluation of our work. We appreciate the recognition of the additional efforts we undertook to strengthen our study, and delighted to hear that the revised data and conclusions were well received.