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14. ABSTRACT The goal of this study is preclinical testing of Deferiprone (DFP), an iron chelator pre-clinical breast cancer models. DFP increased M1 polarization (even in absence of IL4 or LPS) and in the presence of LPS. DFP enhanced the efficiency of phagocytosis by RAW264.7 cells and was significant in all the culture conditions (p = 0.0004 for Control, p = 0.0007 for IL-4, p = 0.0045 for LPS) but most prominent in the LPS treated group. We measured the tumor cell doubling times including an additional cell line (BT474) and the effect of DFP and found the results similar in all 3 breast cancer cell lines. The IC50 values for the BT474 were similar to the 4T1 and MBA-231 cells. We previously reported the effect of DFP on oxygen metabolism and consumption and extended these results by increasing the n value from n=3 to n=5. Unfortunately, we found no benefit in in vivo studies to DFP in enhancing survival in 3 breast cancer models					
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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose, and scope of the research.*

The goal of this study is preclinical testing of Deferiprone (DFP), an iron chelator in clinical use for nononcologic diseases. We propose to demonstrate the sensitivity of TNBC to DFP as a single agent, and in combination with immune modulation therapy (checkpoint inhibitors) and chemotherapy (paclitaxel and cisplatin). Studies were planned to be performed sequentially by first studying in vitro effects of DFP, followed by in vivo effects, and the effects of adding paclitaxel (taxol), cisplatin, and checkpoint inhibitors. We also measure the immune effects of DFP on cells (in vitro) and organs (ex vivo) including tumors.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Breast cancer, iron imaging, MRI, metabolism, cytokines, immune effects, deferiprone, CD4, CD8

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1 Determine how inhibition of iron metabolism and OXPHOS impedes tumor cell metabolism, TAM function, and inhibits proliferation of macrophages and TNBC cells.

Aim 2: Develop and validate non-invasive MRI methods to quantitatively and spatially, monitor tumor and tissue iron and TAM infiltration, to detect changes induced by macrophage focused therapy.

Aim 3: Determine if inhibition of iron metabolism, TCA cycle, and OXPHOS by DFP: i) inhibits in vivo tumor growth, and ii) enhances responses to chemotherapy and immune checkpoint inhibitors in orthotopic TNBC

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1 We completed 1) studies on the changes in MDA-MB-231 and 4T1 metabolism (**Koutcher Lab**) by DFP 2) but the methodology surprisingly did not work for macrophages. We developed an alternate technique that could be used to study macrophage metabolism in future endeavors to complement tumor cell studies. We measured changes in oxygen consumption induced by DFP in both tumor cells and macrophages (**Merghoub and Koutcher Labs**), 3) the effect of DFP on macrophage and their immune function and phagocytosis (**Merghoub and Koutcher lab**). We present the non-invasive MRI measurement of iron and TAM infiltration (Aim 2), and macrophage imaging by MRI (Aim 2)(not presented previously). The main focus of Year 4,5 were in vivo studies of DFP efficacy (not reported previously) which were disappointing; with the assent of Dr. Shuman-Moss we studied the doubling times and IC50 for the BT474 including in vivo studies (Aim 3) in addition to MDA-231 and 4T1 cells. These results were disappointing (see Section 2 – DFP showed no effect in vivo) but complete the proposed aims.

The report is configured as 8 separate sub-reports, each written as an independent section. The references and figures are similarly numbered separately by section. The first two sections (Aims 2, 3) have not been presented in previous submissions and represent new material for the reviewer. The other material (sections 3-8) were presented in previous years and are noted as such. Since some of this work has been done jointly in conjunction with the Partnering PI (originally Dr. Blasberg, subsequently Dr. Merghoub), we cite in which reports and years the data were presented previously

Section 1: Aim 2: Develop and validate non-invasive MRI (magnetic resonance imaging) methods to quantitatively and spatially monitor tumor and tissue iron and TAM infiltration, to detect changes induced by macrophage focused therapy. (Koutcher Laboratory). Not reported previously. While completed earlier in the grant interval, we delayed presenting it to see if further (not proposed) studies could be done but time did not allow.

Three papers resulted from these studies (1-3) – unfortunately one paper failed to cite the correct DOD grant (1). All three papers are appended and provide further details of the work performed and results. These results are presented in detail in appendices 1-3

Results:

Iron-deposition is a metabolic biomarker of macrophages in both normal and pathological situations, but the presence of iron in tumor and metastasis-associated macrophages (TAMs) is not known. We mapped and quantified hemosiderin-laden macrophage (HLM) deposits in murine models of metastatic breast cancer using iron and macrophage histology, and in vivo MRI. Iron MRI detected high-iron pixel clusters in mammary tumors (See Leftin, et al, Figure 2, in Appendix 1), lung metastasis (See Leftin, et al, Figure 3, in Appendix 1), and brain metastasis (See Leftin, et al, Figure 4, in Appendix 1) as well as liver and spleen tissue (See Leftin, et al, Figure 1, in Appendix 1) known to contain the HLMs. Iron histology showed these regions contained clustered macrophages identified by their common iron status and tissue-intrinsic association with other phenotypic macrophage markers. The in vivo MRI and ex vivo histological images were further processed to determine the frequencies and sizes of the iron deposits, and measure the number of HLMs in each deposit to estimate the in vivo MRI sensitivity for these cells.

Hemosiderin accumulation is a macrophage biomarker and intrinsic contrast source for cellular MRI associated with the innate function of macrophages in iron metabolism systemically, and in metastatic cancer (See Leftin, et al, Figure 2, in Appendix 1).

To further delineate the capability of MRI to quantitate tumor macrophage deposits, we extended our studies by using computer vision image analysis to enhance our ability to define polarized tumor associated macrophages. Magnetic resonance imaging applications utilizing nanoparticle agents for polarized macrophage detection are conventionally analyzed according to iron-dependent parameters averaged over large regions of interest (ROI). However, contributions from macrophage iron deposits are usually obscured in these analyses due to their lower spatial frequency and smaller population size compared with the bulk of the tumor tissue. We hypothesized that, by addressing MRI and histological pixel contrast heterogeneity using computer vision image analysis approaches rather than statistical ROI distribution averages, we could enhance our ability to characterize deposits of polarized tumor-associated macrophages (TAMs). We tested this approach using in vivo iron MRI (FeMRI) and histological detection of macrophage iron in control and ultrasmall superparamagnetic iron oxide (USPIO) enhanced mouse models of breast cancer. Automated spatial profiling of the number and size of iron-containing macrophage deposits according to localized high-iron FeMRI or Prussian blue pixel clustering performed better than using distribution averages to evaluate the effects of contrast agent injections (See Leftin, et al, Figure 1, in Appendix 2). This analysis was extended to characterize subpixel contributions to the localized FeMRI measurements with histology that confirmed the association of endogenous and nanoparticle-enhanced iron deposits with macrophages in vascular regions and further allowed us to demonstrate the polarization status of the macrophage iron deposits detected by MRI. These imaging studies demonstrate that characterization of TAMs in breast cancer models can be improved by focusing on spatial distributions of iron

deposits rather than ROI averages and indicate that nanoparticle uptake is dependent on the polarization status of the macrophage populations (See Leftin, et al, Figure 1 and 2, in Appendix 2).

Iron deposits are a phenotypic trait of tumor-associated macrophages (TAMs). Histological iron imaging and contrast-agent free magnetic resonance imaging (MRI) can detect these deposits, but their presence in human cancer, and correlation with immunotherapeutic response is largely untested. Using these iron imaging approaches, we evaluated the spatial distribution of polarized macrophage populations containing high endogenous levels of iron in preclinical murine and human tumor models, and used them as metabolic biomarkers to correlate TAM infiltration with response to immunotherapy in preclinical trials. Macrophage-targeted inhibition of the colony stimulating factor 1 receptor (CSF1R) by immunotherapy was confirmed to inhibit macrophage accumulation and slow mammary tumor growth in mouse models while also reducing hemosiderin iron-laden TAM accumulation as measured by both iron histology (See Leftin, et al, Figure 3 and 4, in Appendix 3) and in vivo iron MRI (FeMRI) (See Leftin, et al, Figure 1, 2 and 5, in Appendix 3). Spatial profiling of TAM iron deposit infiltration defined regions of maximal accumulation and response to the CSF1R inhibitor, and revealed differences between microenvironments of human cancer according to levels of polarized macrophage iron accumulation in stromal margins (See Leftin, et al, Figure 6, in Appendix 3). We therefore demonstrate that iron deposition serves as an endogenous metabolic imaging biomarker of TAM infiltration in breast cancer that has high translational potential for evaluation of immunotherapeutic response (See Leftin, et al, Figure 5g and Figure 6a-d, in Appendix 3).

References

1. Leftin A, Ben-Chetrit N, Klemm F, Joyce JA, Koutcher JA Iron imaging reveals tumor and metastasis macrophage hemosiderin deposits in breast cancer. .PLoS One. 2017 Sep 12;12(9):e0184765. doi: 10.1371/journal.pone.0184765
2. Leftin A, Koutcher JA. Quantification of Nanoparticle Enhancement in Polarized Breast Tumor Macrophage Deposits by Spatial Analysis of MRI and Histological Iron Contrast Using Computer Vision. Contrast Media Mol Imaging. 2018 Oct 30;2018:3526438. doi: 10.1155/2018/3526438
3. Leftin A, Ben-Chetrit N, Joyce JA, Koutcher JA. Imaging endogenous macrophage iron deposits reveals a metabolic biomarker of polarized tumor macrophage infiltration and response to CSF1R breast cancer immunotherapy. Sci Rep. 2019 Jan 29;9(1):857. doi: 10.1038/s41598-018-37408-7.

Section 2: Aim 3: Effect of DFP on Tumor Growth/Volume (Koutcher Lab) (not reported previously)

Methods

Aim 3Ai: We studied orthotopic MDA-231 human triple negative breast cancer (TNBC) tumor models (implanted in nude mice) and 4T1 TNBC murine tumors (implanted in Balb/C mice). Orthotopic breast tumors were generated by injection of 10^6 4T1 or MDA-MB-231 tumor cells into the mammary fat pad of nude or BALB/c mice, as we previously described (RARC Protocol 08-07-011). Tumors were allowed to grow to ~50- 100 mm³ (measured by calipers) and at that point mice began daily DFP (150mg/kg by gavage, or saline control) until sacrifice (~ 500mm³). Primary breast tumor volumes were measured sequentially by calipers over time. At the initial (50-100 mm³) and final (500mm³) imaging sessions, 3 mice were sacrificed for correlative studies which were presented previously by Dr. Merghoub in his report.

Aim 3Aii: Effect of DFP (in combination with chemotherapy drugs); neoadjuvant treatment model: The same animal tumor models (described above – Aim 3Ai) were studied. The neoadjuvant treatment paradigm included paclitaxel (25mg/kg; 2x/week) and cisplatin (4mg/kg ip weekly), drugs active in TNBC. We compared tumor growth (control vs chemotherapy alone vs DFP alone vs. chemotherapy + DFP);

Aim 3Aiii: Effect of DFP (in combination with immune checkpoint inhibitors); neoadjuvant treatment model:. Our pilot study of a PD-1 inhibitor showed that the combination of Bio-X-cell RMP1-14 and DFP was significantly better ($p < 0.01$) than either drug alone in MycCaP prostate tumors. Only the syngeneic murine 4T1 tumor model in immune competent Balb/C mice was proposed for study in this application. We did not propose to study human xenografts in nude mice, since T cell functionality is critical for checkpoint blockade. Mice bearing orthotopic 4T1 TNBC tumors received daily doses of DFP starting at 50-100 mm³ and 48 h later started treatment with a murine anti PD-1 antibody (RMP1-14) and continuing for the duration of the study. Bio-X-cell RMP1-14 is a PD-1 antibody (2x/week– 250ug/mouse ip in 200ul phosphate buffered saline), which blocks binding of mouse PD-L1 and PD-L2 to PD-1. We compared tumor growth rates (control vs RMP1-14 alone vs DFP alone vs. combination RMP1-14 + DFP

Unfortunately, as noted below, these experiments were not very successful. We did ask for permission to study a HER2⁺ breast cancer model. The rationale for this was that in our preliminary experiments the response in a transgenic HER2⁺ breast cancer model was better than in the triple negative breast cancer model. Hypothesizing that the biology might be different, we repeated the study outlined in Aim 3Ai in the BT474

Statistical analysis: The main tool used in this analysis is the linear mixed model, which takes into account potential clustering effects of tumor and/or cohort. That is, multiple observations from each tumor or each cohort may be dependent. Using the R package lme4, we examine the correlation between tumor volume (log base 2) and various factors such as treatment, growth time, and model, which are incorporated in the model as fixed effects, while tumor and cohort are entered into the model as random effects.

Results

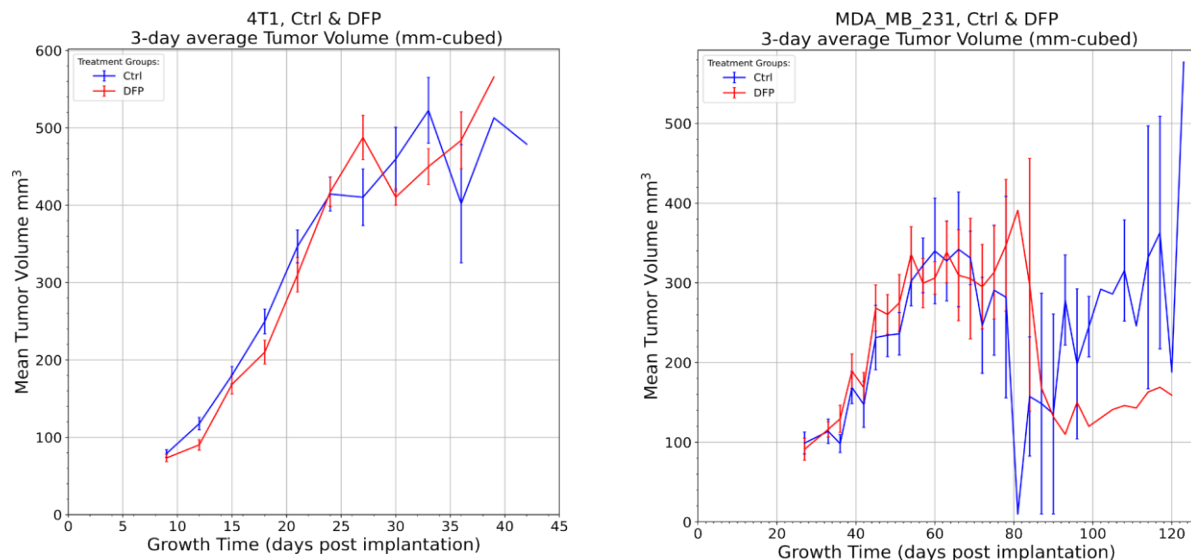


Figure 1. Left: Graph of tumor growth post injection (y axis) vs number of days post injection (x axis) in 4T1 mice tumors. Treatment started at tumor volumes of between 50-75 mm³. Tumor growth was not different between control and DFP treated tumors ($p=0.23$). Right: Graph of tumor volume (MDA-MB-231) vs number of days post injection. By day ~ 80 mice were being sacrificed due to some having large tumors, leading to a drop and subsequent in tumor volume. The MDA-MB-231 is a xenograft and grows more slowly than the 4T1.

4T1 Studies

Control Studies: For the 4T1 model – it is noted in Figure 1 (left) (control and DFP), Fig. 2 (left) (CDDP (cisplatin)), Figure 3 (left) (paclitaxel), Figure 4 (RMP1-14), that the growth rates are very similar for these various single agent treatments ($p\text{-value} = 0.2478$). The 4T1 does show some modest sensitivity to paclitaxel but the overall p value for the effect of any drugs on the growth of 4T1 was not significant. In general (except for DFP since this was the main thrust of the study), if the “overall test” was insignificant, then the statistician found no reason for subset pairwise tests since family-wise type 1 error rate can be greatly inflated (meaning that we may identify false positives, aka, signals that might be due to random noise). Also, if further tests would be done, we would need to adjust for multiple testing, which would require an even more stringent p value. We therefore deferred to Dr. Zhang (the statistician). These data suggest that the 4T1 is a very inherently resistant tumor model to both active chemotherapy drugs and also immune therapy, and from hindsight was probably a poor choice of a tumor model.

Effect of DFP Alone: Figure 1 (left) shows the results for the 4T1 study where we compared the effect of DFP with saline control injections. The results were disappointing showing no added effect by treatment with DFP. The growth curves are virtually overlapping ($p<0.23$).

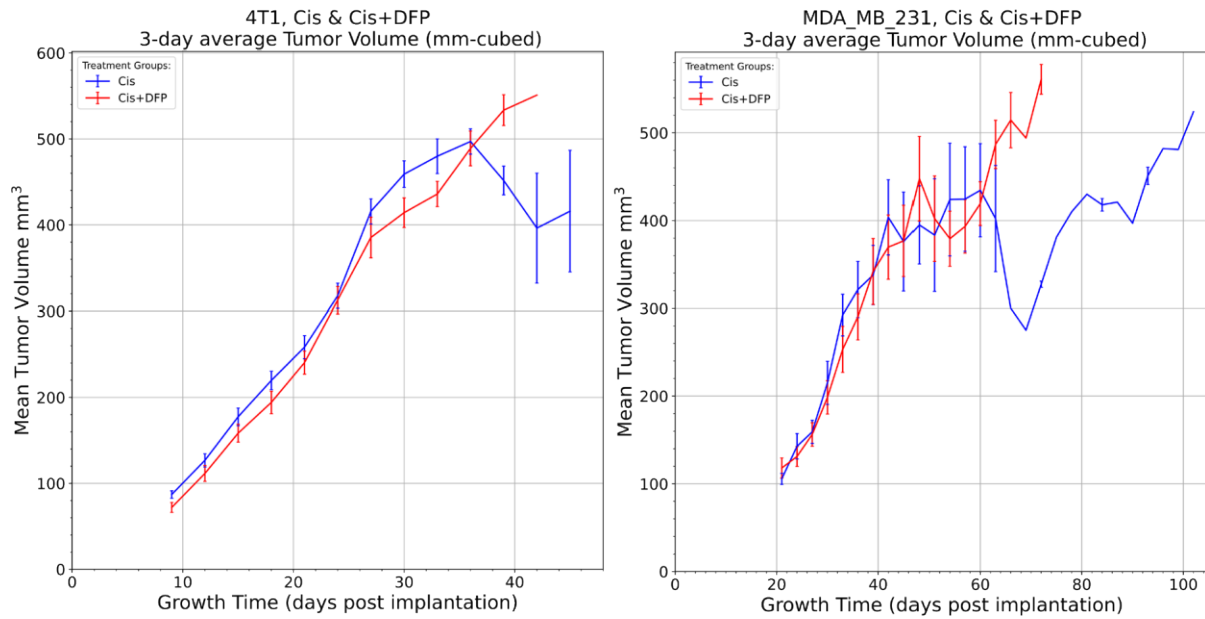


Figure 2. Tumor bearing mice were treated with CDDP +/- DFP. Left: Graph of growth post tumor injection (y axis) vs number of days post injection (x axis) in 4T1 mice tumors. Treatment started at tumor volumes of between 50-75 mm³. Tumor growth was not different between CDDP (control) vs CDDP + DFP treated tumors. Right: Graph of tumor volume (MDA-MB-231) vs number of days post injection. By day ~ 60 mice were being sacrificed due to having large tumors, leading to a drop and subsequent in tumor volume. The MDA-MB-231 is a xenograft and grows more slowly than the 4T1. P=0.99

Effect of Adding DFP to CDDP or Paclitaxel: Similar to the studies on the 4T1 tumor using different single agents, we similarly found no effect of DFP added to CDDP vs CCP alone (Fig 2 left; p=0.10). The growth curves of tumor volume vs time essentially was identical and showed overlapping (Fig. 2, left) error bars.

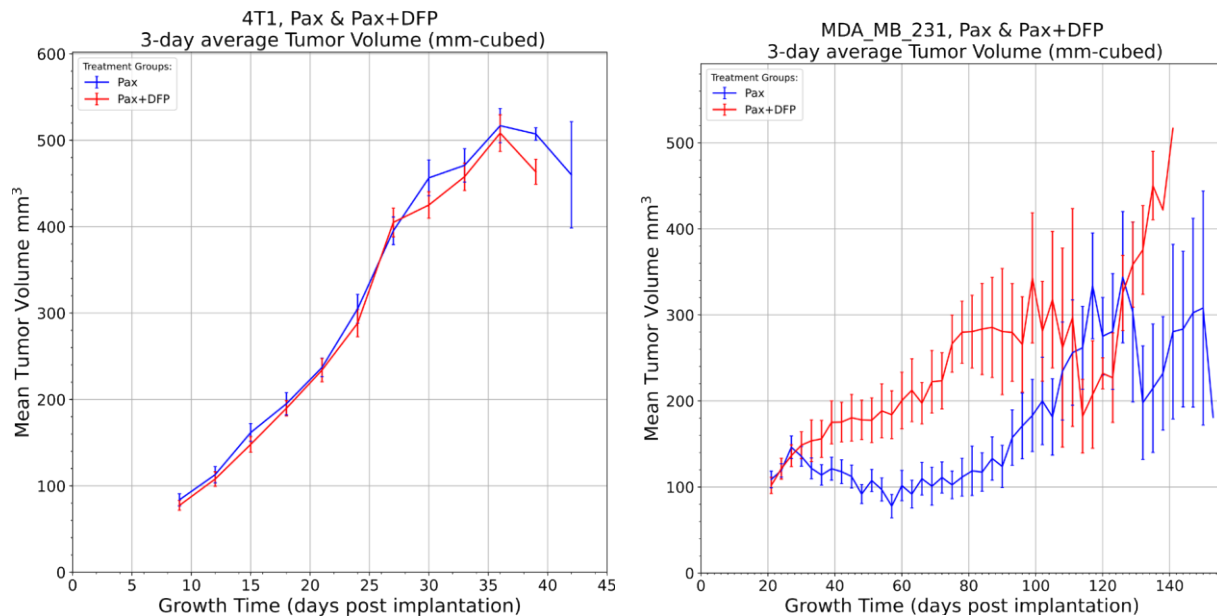


Fig. 3. Tumor bearing mice were treated with Paclitaxel (Pax) +/- DFP. Left: Graph of growth post tumor injection (y axis) vs number of days post injection (x axis) in 4T1 mice tumors. Treatment started at tumor volumes of between 50-75 mm³. Tumor growth was not different between paclitaxel (control) vs paclitaxel + DFP treated tumors. Right: Graph of tumor volume (MDA-MB-231) vs number of days post injection. The MDA-MB-231 is a xenograft and grows more slowly than the 4T1.

Similar to the studies on the 4T1 tumor using single agents, we similarly found no effect of DFP added to paclitaxel ($p=0.39$) (Fig. 3 left). The growth curves of tumor volume vs time are essentially overlapping

Thus, in the 4T1 TNBC, DFP as a single agent showed no benefit in tumor growth delay, nor did it add to the effect of CDDP or paclitaxel, drugs that are used clinically in TNBC. As noted above, the 4T1 is inherently a resistant tumor and showed no response to CDDP or paclitaxel, drugs that are used in treating this tumor

MDA-MB-231 Studies

Control Studies - In contrast to the 4T1 control studies with single drugs, a similar analysis for the human MDA-MB-231 tumor comparing growth rates for control, CDDP, and paclitaxel, showed a difference in growth rate ($p<0.0001$). Since there was an effect, Dr. Zhang performed additional statistical analyses. It was noted that both CDDP and paclitaxel delayed tumor growth ($p=0.0049$ and $p=0.0028$ respectively) in the MDA-MB-231 model. We also compared paclitaxel vs CDDP ($p<0.0001$) with paclitaxel being the more effective agent. This is readily seen in Figs 2 (right) and 3 (right) where the time to grow from $\sim 100 \text{ mm}^3$ to $\sim 300 \text{ mm}^3$ was about 100 days for paclitaxel, vs approximately 15 days (CDDP).

Effect of DFP Alone (MDA-MB-231)

In the study of the human tumor xenograft, MDA-MB-231, we found no effect of DFP ($p=0.42$) (Fig. 1 right). This was similar to the lack of effect noted in the 4T1

Effect of DFP added to CDDP (MDA-MB-231)

Figure 2 (right) shows the results for the MDA-MB-231 study comparing the effects of DFP + CDDP vs CDDP. The curves are clearly overlapping and the statistical analysis shows that $p=0.99$, indicating no difference in effect, by adding DFP to CDDP

Effect of DFP added to Paclitaxel

As noted above, paclitaxel was very effective in treating MDA-MB-231. Despite this, DFP actually inhibited the effect of paclitaxel and accelerated tumor growth ($p<0.015$) (Fig. 3 right). This was disappointing although mechanistically, since the taxanes are microtubule inhibitors, their mechanism of action may be unrelated to metabolism and hypoxia. Nevertheless, this was a surprising and disappointing effect

We had shown and described in the final report from Dr. Merghoub, that exhausted or immunosuppressed T cells often show an increase in their surface expression of T cell inhibitory receptors such as Lag-3, Tim-3 and PD-1. In addition, T cells that co-express 2 or more of these exhaustion markers are thought to be the most exhausted and dysfunctional. We found (see Merghoub report submitted earlier this year) that cisplatin alone or paclitaxel alone induces CD8 T cells in the tumor to increase co-expression of Tim-3 and PD-1. Importantly, this effect was partially reversed when DFP was added to the chemotherapy regimens. This data indicated that while chemotherapies such as cisplatin or paclitaxel can lead to T cell exhaustion as a mechanism of resistance, DFP can partially abrogate this effect and restore T cell function. The cytokine data with RAW264.7 macrophages in Fig. 2 (Section 3) supports this hypothesis. Unfortunately, this did not lead to a statistically significant enhancement in vivo tumor response to chemotherapy.

We were surprised by the disappointing findings since in Dr. Merghoub's final report submitted earlier this year, we had noted that in the spleen, treatment with DFP (+/- cisplatin) decreased spleen size, increased the percent of both CD4 effector T cells (CD4+ Foxp3-) and CD8 T cells, and decreased CD4 T cells that do not express Foxp3 but express high levels of the T cell inhibitory receptor PD-1. Cytokine stimulation showed that DFP increased the number of CD4 and CD8 T cells in the spleen that make either IFN γ and/or TNF α alone or in combination, with cisplatin. Thus we expected an enhanced effect of CDDP + DFP.

In the report we noted that Paclitaxel alone increased expression of these cytokines but did not have any additional benefit in combination with DFP. With DFP, there was an increase in the ability of CD4 and CD8 T cells to make both IFN γ or TNF α , a marker of polyfunctionality in T cells. Similar findings were observed in the

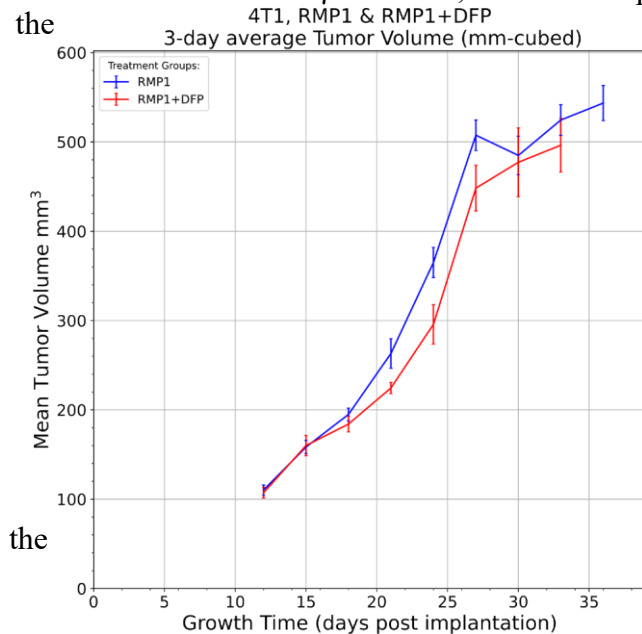


Fig. 4. Effect of RMP1-14 in 4T1 tumor bearing mice. The curves are approximately parallel with the RMP1-14 treated cohort always slightly greater than the RMP1-14 +DFP treated cohort, but does not attain statistical significance. (P=0.09)

tumors especially within the CD4 T cells. These polyfunctional T cells correlated inversely with both spleen and tumor weight indicating a positive role in these cells in controlling tumor growth. We showed that cisplatin or paclitaxel can lead to tumor T cell exhaustion as a mechanism of resistance, but DFP can partially abrogate this effect and restore T cell function, thus our disappointment with

tumor response data.

AIM 3Aiii: Effect of DFP on immunotherapy (RMP1-14) PD1 inhibitor

We compared the effect of treating 4T1 tumor bearing mice with RMP-1-14, a PD-1 inhibitor vs RMP1-14 + DFP. (Fig. 4) A small tumor growth delay was noted in the cohort treated with DFP + RMP1-14 which approached but did not attain statistical significance (p=0.09). These results suggest that any future studies with DFP should focus on immune therapy and not chemotherapy drugs

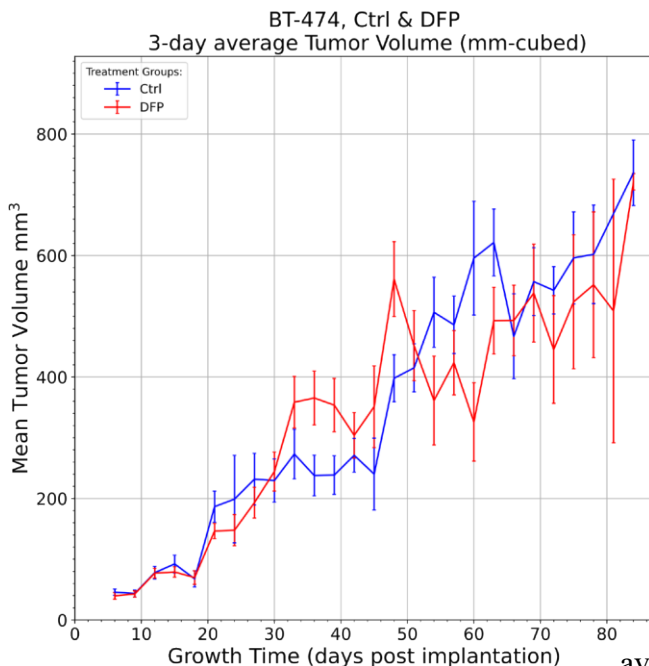


Fig. 5. Effect of DFP in BT-474 tumor bearing mice. The curves are approximately parallel and no effect is noted. (p=0.65)

Additional Studies – unplanned but with permission given:

Unfortunately, as noted, these experiments were unsuccessful. We did ask and receive permission to study a HER2⁺ breast cancer model (Fig. 5). The rationale for this was that in our preliminary experiments the response in a transgenic HER2⁺ breast cancer model was better than in the triple negative breast cancer model. Hypothesizing that the biology might be different, we repeated the study outlined in Aim 3Ai in the BT474. Unfortunately, the results were not any better. DFP had no effect on tumor growth (p=0.60) and with both time and money not available, no further studies were performed.

Conclusions:

The 4T1 was found to be highly resistant to chemotherapy, with or without DFP. Had time and money allowed, the study of a different model would have been considered since the MDA-MB-231 was highly sensitive to paclitaxel

1. DFP had no activity in the two TNBC models (add in the BT474 model) studied as a single agent or in combination with chemotherapy.
2. DFP had a mild effect ($p=0.09$) in the 4T1 when combined with anti-PD1 antibody. If further studies with DFP in breast cancer are done, they would do well to focus on immune therapy and not chemotherapy
3. Studies on the HER-2 positive BT 474 with DFP as a single agent were also disappointed with a p value of 0.65 in comparing control and DFP treated cohorts

Section 3: Aim 1A: Evaluate *in vitro* the effects of deferiprone (DFP) on tumor cell oxygen consumption rates (OCR), macrophage polarization, proliferation, and function. (Koutcher/Merghoub/Blasberg Laboratory)

Data on macrophage proliferation were previously presented (2019) as part of the partnering PI (Dr. Blasberg) report, but was done jointly. Data on macrophage OCR were presented in 2020,2021 (see Section 8 of this report). Data on doubling time and EC50 is in Section 4

Effect of DFP on macrophage polarization, proliferation and function

In this section, we have examined the effect of DFP on macrophage polarization from M0 to M1 and M2 cells (exposure to lipopolysaccharide (LPS) and IL4 respectively) in vitro and function.

To examine the effects of DFP on macrophage polarization and effector function, we cultured RAW 264.7 cells in the presence of LPS (100 ng/ml) and murine IL-4 (10 ng/ml) for 48 hours to polarize these cells to M1 and M2 macrophages, respectively. We confirmed the polarization of RAW 264.7 cells by flow cytometry by examining CD206 and MHC class II expression. The macrophage cell line RAW264.7 (RAW) maintains an M0 phenotype (Control) when in culture, while the presence of IL-4 polarized these cells to a M2 phenotype (CD206+MHC II-) and LPS polarized them to a M1 phenotype (Fig. 1A). When DFP (100 μ M) was added to the cultures, there was an increase in M1 polarization in the control conditions without IL-4 or LPS (control, from 3% to 7%, $p = 0.0016$) and also in the presence of LPS (from 26% to 37%, $p = 0.0016$) (Fig. 1B). However, we did not observe any effects on M2 polarization (Fig. 1B, left). Data are reported in this section as mean $\pm$ SD.

Studies on Macrophage Functional Response to DFP

To assess whether any of the phenotypic changes observed translated into functional changes in the RAW 264.7 cells, we tested their ability to 1) secrete cytokines, 2) phagocytose IgG-coated latex beads and 3) synthesize reactive oxygen species (ROS). We tested cell culture supernatants from RAW 264.7 cells that have been treated with IL-4, LPS (+/- DFP) for three cytokines (IL-10, IL-12, and TNF α) that are known to be associated with M1 or M2 macrophage polarization using the Luminex multiplex cytokine detection system (Millipore). While we were able to detect very small amounts of IL-12 in the supernatants from the cultures, their numbers were inconsistent between replicate experiments (data not shown). We found that RAW 264.7 cells polarized to M2 macrophages with IL-4 secrete more anti-inflammatory cytokines, such as IL-10 (Fig. 1C left), and less pro-inflammatory cytokines such as TNF α (Fig. 1C, right). The addition of DFP to these cells appear to decrease both IL-10 and TNF α but this effect is subtle and was not observed in a second replicate experiment (data not shown). When RAW 264.7 cells were polarized to M1 macrophages with LPS, they secrete more pro-inflammatory cytokines such as TNF α (Fig. 1D). Unexpectedly, LPS also stimulated RAW 264.7 cells to secrete IL-10. This has been reported in the literature as a late phase response to LPS that may be a feedback mechanism to curtail the pro-inflammatory effects of LPS (1,2). While the addition of DFP did not enhance the secretion of TNF α , it did decrease the amount of IL-10 made in response to LPS by ~45% ($p = 0.0007$). Thus, DFP may negate the feedback mechanisms that are induced by LPS to promote a stronger pro-inflammatory response.

We tested the ability of RAW 264.7 cells, polarized with IL-4 and LPS, to phagocytose IgG-coated FITC-conjugated latex beads (via Fc-receptors). Unpolarized RAW cells had some baseline phagocytosis where ~18% of the cells phagocytosed latex beads (Fig. 2 A, B *Control*). Phagocytosis was not affected when cells were polarized to an M2 phenotype with IL4 (Fig. 2 A, B *IL-4*), however, phagocytosis was significantly enhanced (18% vs. 45% FITC+ cells, $p < 0.0001$) when cells are polarized to an M1 phenotype (Fig. 2 A,B *LPS*). The addition of DFP to these cultures has a subtle (with mixed statistical significance) increase in phagocytosis overall. While the effects of adding DFP were subtle with respect to the percentage of RAW 264.7 cells that have phagocytosed latex beads, DFP significantly enhanced the amount of latex beads that have been phagocytosed by each RAW 264.7 cell (Fig. 2 A,B *FITC MFI*). Therefore, DFP enhanced the efficiency of phagocytosis by RAW 264.7 cells. This effect was significant in all the culture conditions ($p = 0.0004$ for Control, $p = 0.0007$ for IL-4, $p = 0.0045$ for LPS) but most prominent in the LPS-treated group. This data is consistent with the finding that DFP decreased the production of IL-10 induced by LPS. This decrease in IL-10 may play a contributing role to the increased efficacy of phagocytosis of LPS-polarized RAW 264.7 cells.

Once macrophages phagocytose their targets, they can kill by several mechanisms. The most characterized mechanism is the release of reactive oxygen species (ROS) which includes both hydrogen peroxide (H_2O_2) and nitric oxide (NO). We tested the ability of RAW 264.7 cells, polarized with IL-4 and LPS, to make ROS. H_2O_2 and NO can be detected fluorometrically using fluorescent substrates (Amplex Red for H_2O_2 and DAF2-DA for NO). RAW 264.7 cells, even when polarized to M1 macrophages do not make detectable amounts of H_2O_2 (Fig. 2C). Unpolarized or IL-4 polarized RAW 264.7 cells make detectable amounts of NO, however, LPS polarized macrophages make significantly more NO ($p = 0.0003$) (Fig. 2D). Thus, RAW 264.7 cells can make NO at a resting state, but this is significantly increased when they are polarized to M1 macrophages. Unlike what we observed in the phagocytosis and cytokine experiments, the addition of DFP does not seem to enhance their ability to make NO.

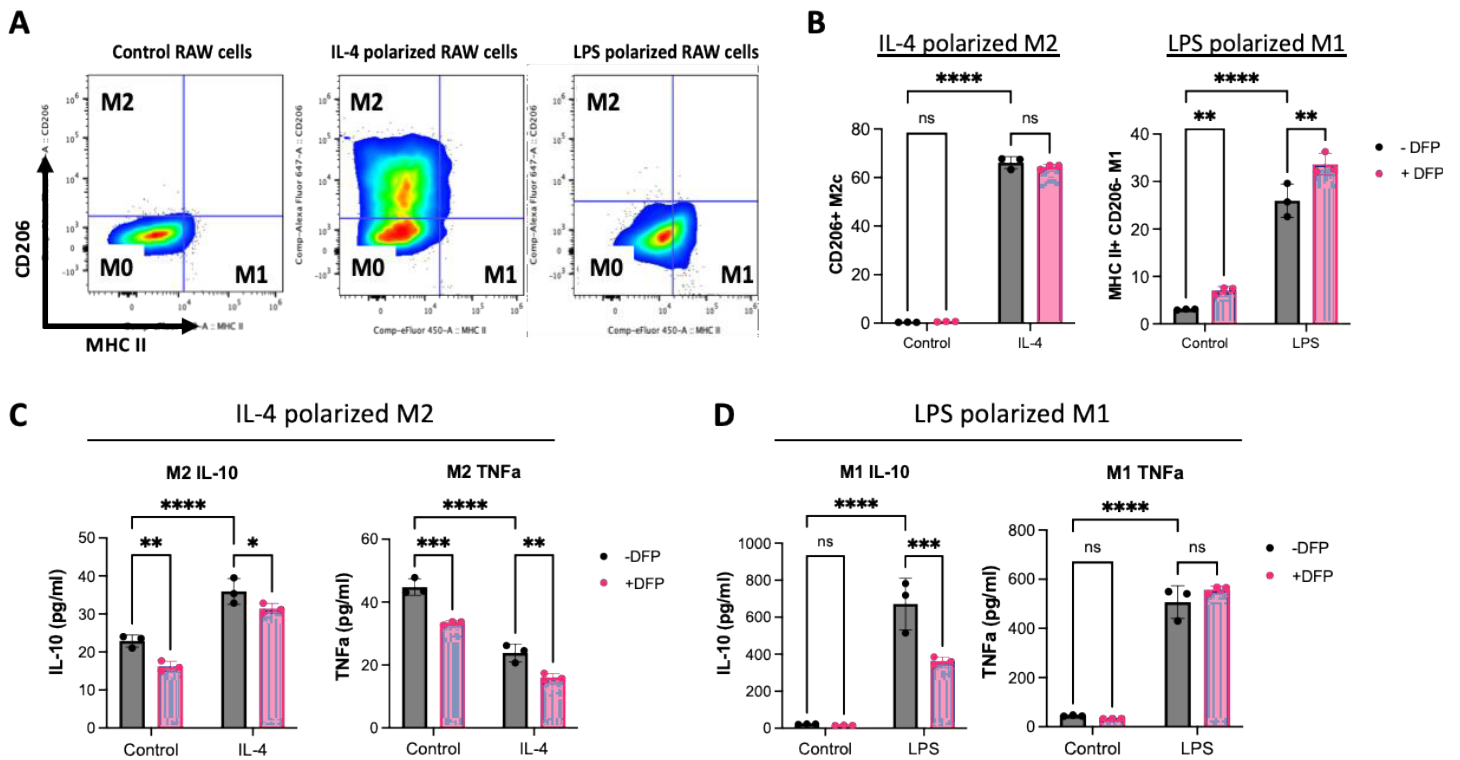


Figure 1: The effects of DFP on macrophage polarization and cytokine production. RAW 264.7 cells were seeded in 24-well plates, treated with IL-4, LPS and/or DFP and incubated for 48 hrs. **A, B.** For phenotypic studies, cells were detached and stained for fluorophore conjugated CD206 and MHC II antibodies for flow cytometry. Shown are representative bivariate plots of MHC II versus CD206 expression (**A**) and quantification of M2 and M1 macrophage polarization (**B**) ($n = 1$). **C, D.** Cell culture supernatants were collected and analyzed for cytokines using the Luminex multiplex assay. Shown are the amounts of IL-10 and TNF α produced by RAW 264.7 cells polarized to M2 (**C**) or M1 phenotypes (**D**) from triplicates samples from one representative experiment ($n = 2$ experiments performed). Statistics were calculated using 2-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

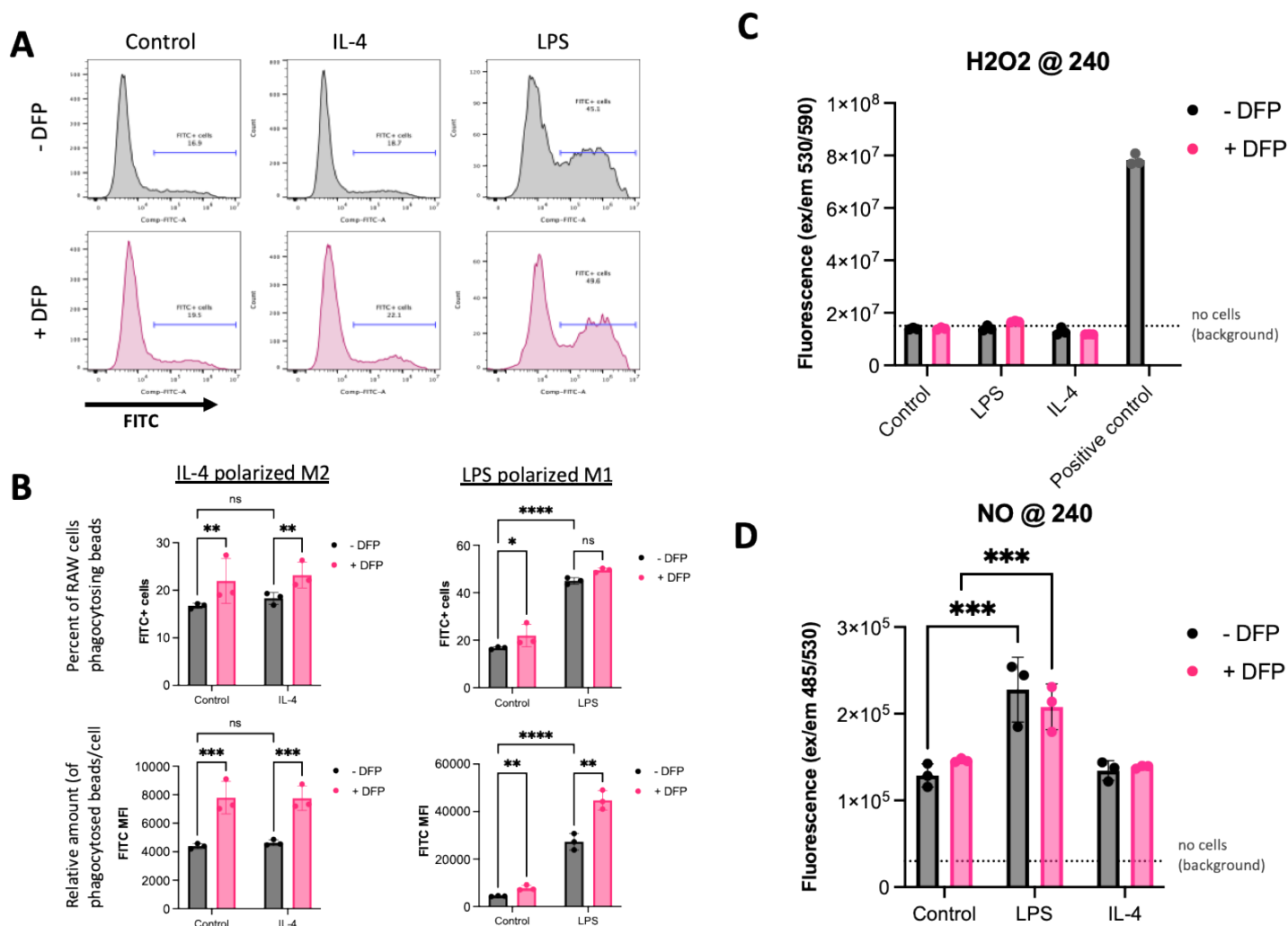


Figure 2: The effects of DFP on macrophage phagocytosis and ROS production. RAW 264.7 cells were seeded in 24-well plates, treated with IL-4, LPS and DFP and incubated for 48 hrs. **A, B.** For phagocytosis: PBS-containing FITC-beads were added to each well and incubated at 37 °C for 2 hrs. Cells were detached and resuspended in 50 μ l Trypan Blue to quench non-phagocytosed beads, stained with a viability dye and then analyzed by flow cytometry. **A** Representative histogram plots of RAW264.7 cells that have taken up FITC beads. **B** Quantification of phagocytosing cells (FITC+ cells, top) and the mean fluorescence (MFI, bottom) of the beads that have been phagocytosed ($n = 1$). **C, D** For ROS production: 50,000 polarized RAW 264.7 cells were plated in 96-well flat bottom plates in the presence of Amplex Red substrate solution (H_2O_2) or DAF2-DA substrate solution (NO). Fluorescence was determined at an excitation/emission of 530/590 for H_2O_2 and 485/530 for NO every 30-60 mins for up to 240 mins. For the positive control condition, diluted H_2O_2 was added into a control well at 240 mins. Shown are relative fluorescence ($n = 1$). Statistics were calculated using 2-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

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Section 4: Tumor Cell Doubling Time in Response to DFP (Koutcher Laboratory) (Aim 1B) – presented previously in 2021

In addition to the proposed cell lines under study, we also investigated the effect of DFP on BT-474 cells, after discussion with Dr. Shuman-Moss. We did this after we advised her that our preliminary in vivo studies with Deferiprone in 4T1 and MDA-MB-231 tumors were disappointing. She agreed that it was reasonable to try the study on an additional different tumor model. We chose the BT-474 HER2⁺ breast cancer model purposely because it is not a triple negative breast cancer (TNBC) model (both, MDA-MB-231 and 4T1 are TNBC). It is a model for clinical HER2-positive breast cancer, a very common and difficult to treat tumor. Our initial in vitro studies are presented below. These include measurements of tumor cell doubling time, EC50 (50% inhibitory or effective concentration), and EC90 (concentration of DFP affecting 50% and 90% of cells respectively). We include these data with our studies of the 4T1 and MDA-MB-231 doubling time measurements as proposed. We report 1) doubling times for the 3 tumor cell lines and 2) EC50, EC90 of the BT474 to deferiprone. The EC50 and EC90 data for 4T1 and MDA-MB-231 were presented previously.

Methods

For EC50 experiments, 1×10^5 BT-474 cells (ATCC) were seeded per well in 3 separate 12 well plates for each experiment (Falcon® 12-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate, with Lid, Individually Wrapped, Sterile, 50/Case, Product Number 353043) and allowed to attach for 24 h. For the cell doubling time experiments, 3.2×10^4 and 1.3×10^5 for untreated and DFP-treated 4T1 cells (Fred R. Miller, Karmanos Cancer Institute, Detroit, Michigan) respectively, 5×10^4 MDA-MB-231 cells (ATCC), 6×10^4 BT-474 cells (ATCC), and 9×10^4 RAW 264.7 cells (ATCC) were seeded per well in 24-well plates (Corning 3524 or BD Falcon 353047) and allowed to attach for 24 h. Cell culture media was prepared by the Media Preparation Facility (Dulbecco's Modified Essential Medium containing 25 mM Glucose (Thermo Fisher Scientific, Cat # 12100), supplemented with 2 mM glutamine to a final concentration of 6 mM Glutamine and supplemented with 10% Fetal bovine serum, 100 U/ml Penicillin and 100 µg/ml Streptomycin). Deferiprone was purchased from Toronto Research Chemicals (Cat#D4740000) and stored as a solid at -20 °C and, for the duration of the experiment, in solution at 4 °C.

For EC50 experiments after 24 h, the cell culture medium was exchanged for culture medium with 0 µM, 16 µM, 30 µM, 66 µM, 100 µM, 160 µM, 300 µM, 660 µM, 1000 µM for every 3 wells, respectively. Then cells were grown for an additional 72 h with daily medium change. At ~73 h, cells were harvested by trypsinization and counted with a Luna cell counter using the Trypan Blue exclusion assay. For each experiment, the total number of live cells for each sample were averaged over the triplicate samples and the corresponding effective concentration to achieve 50% and 90% effect respectively calculated as described below:

$$100 \cdot \frac{T - T_0}{C - T_0}, \text{ with}$$

T₀: Number of viable cells at time 0 (~24 h post seeding and pre-treatment)

C: Number of viable cells in untreated control cells at time t

T: Number of viable cells at time t

Fitting equation for EC50 (50% effect between top and bottom plateau, with top plateau 100 % (untreated control)), using a 4-parameter fit, i.e. fitting of Top, Bottom, HillSlope and LogEC50:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogEC50} - \text{LogDrugDose}) \cdot \text{HillSlope}}}$$

Calculation of EC_{anything} (e.g. used to calculate EC90):

$$\text{logEC50} = \text{logECF} - (1/\text{HillSlope}) \cdot \text{log}(F/(100-F)), \text{ with}$$

F: Constant between 0 and 100, referring to the percentage between top and bottom plateau (e.g. 50 for ED50, 10 for EC90, as the Y response decreases with increasing drug dose).

For cell doubling time experiments after 24 h, 3 triplicated wells were counted for each cell line (and for 4T1 for both seeding densities) to obtain a time zero count. Then, the cell culture medium was exchanged for culture medium with 0 µM or 100 µM DFP for 24 wells (1 plate for each cell type & condition), respectively. Every 24 h, the medium was exchanged for fresh culture medium with or without DFP to avoid nutrient depletion and

medium acidification. At the same time, cells in 3 wells for each cell type & condition were harvested every 24 h until at least ~ 2 cell doubling times had past and counted with the Trypan Blue exclusion assay using a Luna cell counter (Logos Biosystems, Annandale, VA). The natural logarithm of the cell number at each time point was plotted over the growth time and fitted by linear regression to obtain the cell doubling time. The resulting cell doubling times were averaged for each cell line and condition and the standard error calculated. As the growth rate is inhibited by DFP, we performed a one-tailed T test to compare the doubling times of DFP-treated with untreated cells. Where each independent experiment that had both, treated and untreated cells (here for BT-474), the T Test was paired, while otherwise an unpaired T test was performed (all other cell lines and conditions).

Results

Fig. 1A shows a typical EC₅₀ experiment curve. In this model, deferiprone is largely cytostatic but does also include some cytotoxicity effects. The EC₅₀ was measured as $43.5 \pm 6.2 \mu\text{M}$ which is comparable to that found previously for the other breast and prostate cancer cell lines and provided in the 2018 report.

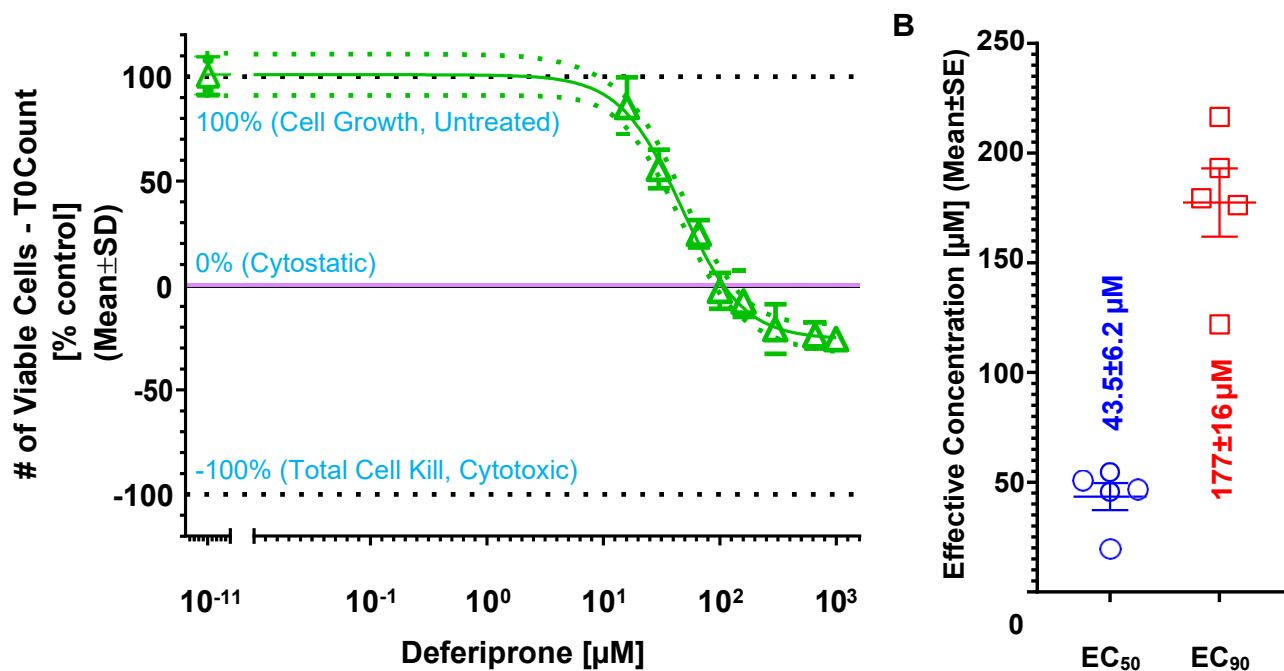


Figure 1: Effect of Deferiprone on BT-474 cells. (A) A representative curve displaying the effect of a 73 hour exposure of BT-474 cells to increasing DFP concentrations on the cell growth and viability. T0 count is the number of viable BT-474 cells at the start of the exposure. (B) Average EC₅₀ and EC₉₀ values for BT-474 cells from 5 independent experiments with triplicate samples within each experiment.

Figure 2A show the natural logarithm of the cell numbers at various time points and the corresponding linear fits with 95% confidence intervals of the fits used to calculate the doubling times for each independent experiment. For each separate independent experiment, each time point is the average of 3 replicates. Figure 2B summarizes the cell doubling times for 4 cell lines, untreated or exposed to 100 μM DFP. Deferiprone significantly reduces the growth rate, i.e. prolongs the cell doubling times, in 4T1 and BT-474 cells. The same effect is observed for MDA-MB-231 cells, though there are only 2 experiments for untreated MDA-MB-231 cells. We also complete the doubling time experiments for RAW 264.7 cells (Fig. 2B) as we stated that we would at the time that this part of the report was originally submitted. Table 1 shows the IC₅₀ values for macrophage exposure (M0, M1, M2) to DFP

Table 1. IC ₅₀ values (μM) for M0, M1, M2 macrophages for DFP (N=3)		
M0	M1	M2
79.04 ± 5.3	92.95 ± 3.2	113.67 ± 5.2

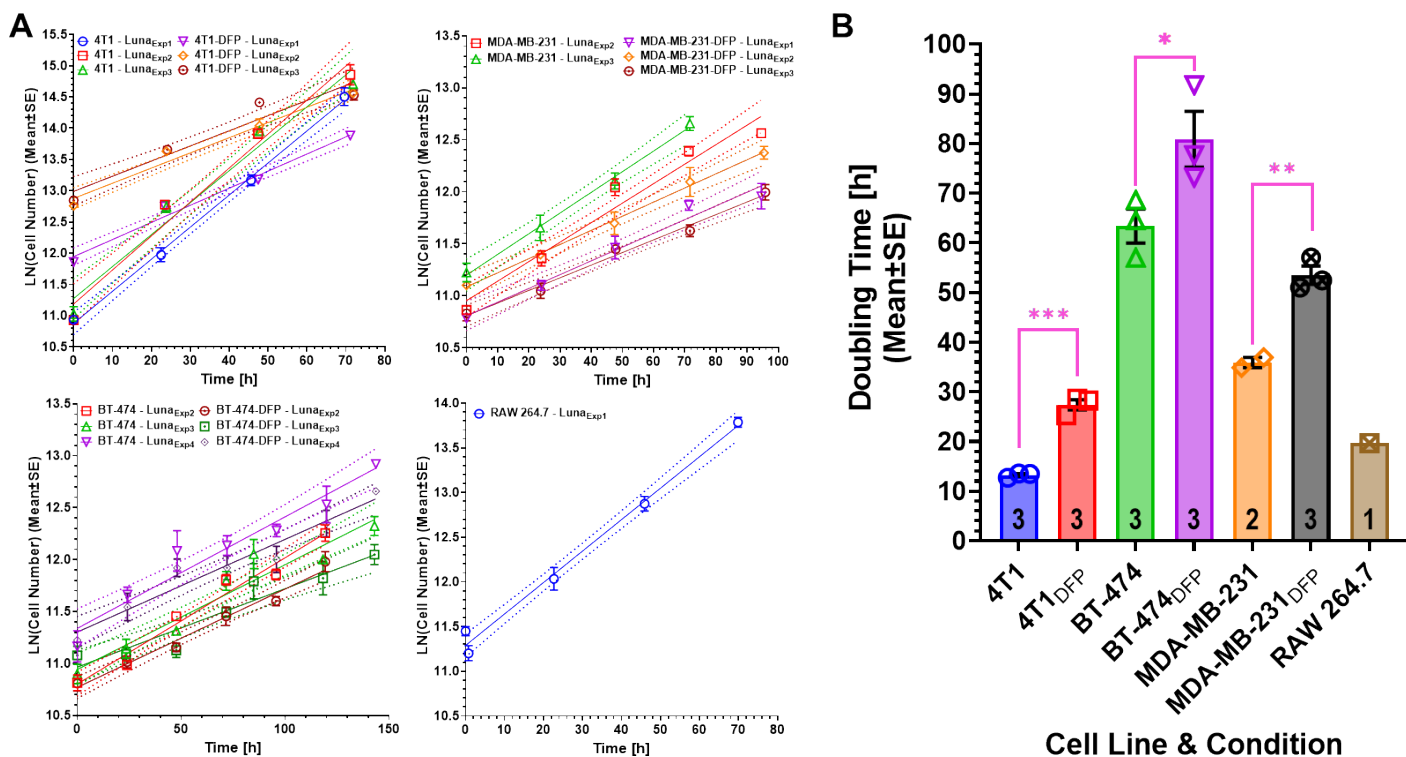


Figure 2. Effect of DFP on in vitro cell growth. **(A)** Natural logarithm of the cell numbers obtained from triplicate samples at each time point and the corresponding linear fits with 95% confidence intervals of the fits for each independent experiment for 4T1 (top left), MDA-MB-231 (top right), BT-474 (bottom left), and untreated RAW 264.7 cells. **(B)** Average cell doubling times for untreated and DFP-treated cells. Deferiprone significantly reduces cell growth in BT-474 ($p = 0.0139$, 1-tailed, paired T test), 4T1 cells ($p = 0.0009$, 1-tailed, unpaired T-test assuming both populations have the same standard deviation) and MDA-MB-231 cells ($p = 0.0029$, 1-tailed, unpaired T-test assuming both populations have the same standard deviation). * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$.

Section 5: Effect of DFP on Tumor Cells using Live Perfused cells (Koutcher Laboratory) (Aim 1)

Section 5A; Studies of 4T1 cells (presented in 2019)

Section 5B: Studies of MDA-MB-231 cells (presented in 2020)

In the 2019 report we presented the 4T1 studies (both ^{31}P and ^{13}C) of changes in tumor metabolism induced by exposure to deferiprone and appropriate control studies, and analyzed the data in terms of changes of metabolite ratios. The data were presented at the recent Molecular Imaging Society meeting (1). **Appendix 4 summarizes the data for the 4T1 cells (1), appendix 5 summarizes the data for the MBA-MB-231 cells (presented in 2022 (2)), and appendix 6 is a comparison of the studies os being written up for a single manuscript.** They provide the details how the perfused cells appear when growing on microcarrier beads, the setup in the magnet to ensure viability (bioreactor), the growth rate during the experiment (demonstrated that as in cell culture, DFP slows growth of tumor cells), and ^{31}P and ^{13}C MR spectra, and time course effect of DFP on metabolism.

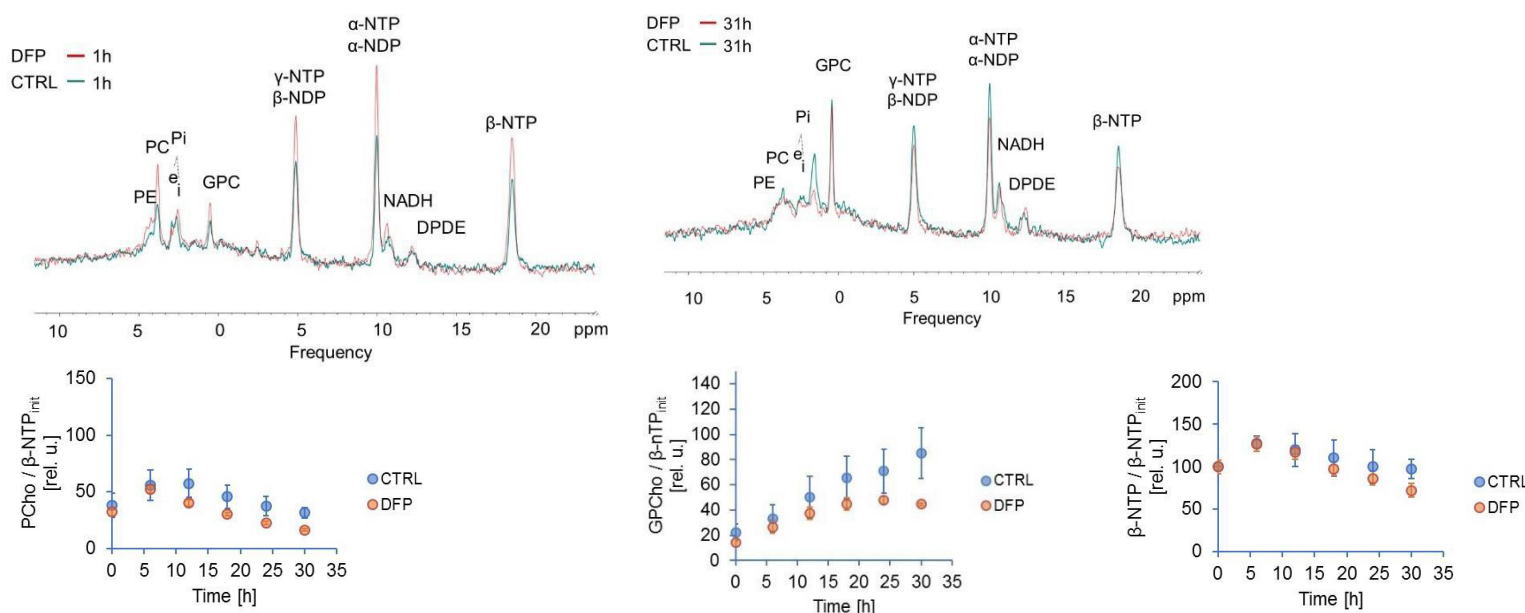


Fig. 1 Top left. ^{31}P NMR spectra obtained in the first hour of cell perfusion with and without DFP as labelled (baseline). Top Right. ^{31}P NMR spectra obtained after 31 hours of cell perfusion with and without DFP. Peak areas ratios were normalized to $\beta\text{-NTP}$ which has been shown to be a measure of cell number. Bottom: Changes in glycerophosphocholine (center), phosphocholine (left), and $\beta\text{-NTP}$ (right) over time. Changes were significant after 18 hours. PE = phosphoethanolamine, PC=phosphocholine, Pi = inorganic phosphate (2 peaks - intra and extracellular), GPC = glycerophosphocholine, NDP= nucleoside diphosphate, NTP = nucleoside triphosphate, DPDE = diphosphodiester, NADH = Nicotine adenine dinucleotide (reduced and oxidized)

The ^{31}P MR spectra for 4T1 cells are shown in Fig. 1. We compared DFP-treated and control cells, acquired at the start (left) and end (right) of the experiment. Time course curves for phosphocholine (Pcho), glycerophosphocholine (GPCho), and $\beta\text{-NTP}$ (nucleoside triphosphate) showed statistically significant changes ($p < 0.05$, unpaired t-test) between DFP-treated and control cells (bottom panels). Three independent experiments were done for untreated and DFP treated cells. To analyze biochemical changes that occurred in response to DFP in greater depth, we perfused the cells with $1\text{-}^{13}\text{C}$ -glucose and monitored changes in metabolism (Fig. 2). Quantitative evaluation of ^{13}C NMR spectral metabolites of DFP-treated cells revealed lower residual glucose (higher glucose consumption), decreased glutamine- $^{13}\text{C}_4$ (decreased tricarboxylic acid cycle activity), and fatty acid chain synthesis, decreased glycerol-3-phosphate, and less alanine- $^{13}\text{C}_3$ labeling, among other changes, compared to control (CTRL) cells (Fig. 3). We are comparing these findings with our previous results in prostate cancer (4) and have accumulated corresponding data for the MDA-231 (see below Section 5B and reference 2).

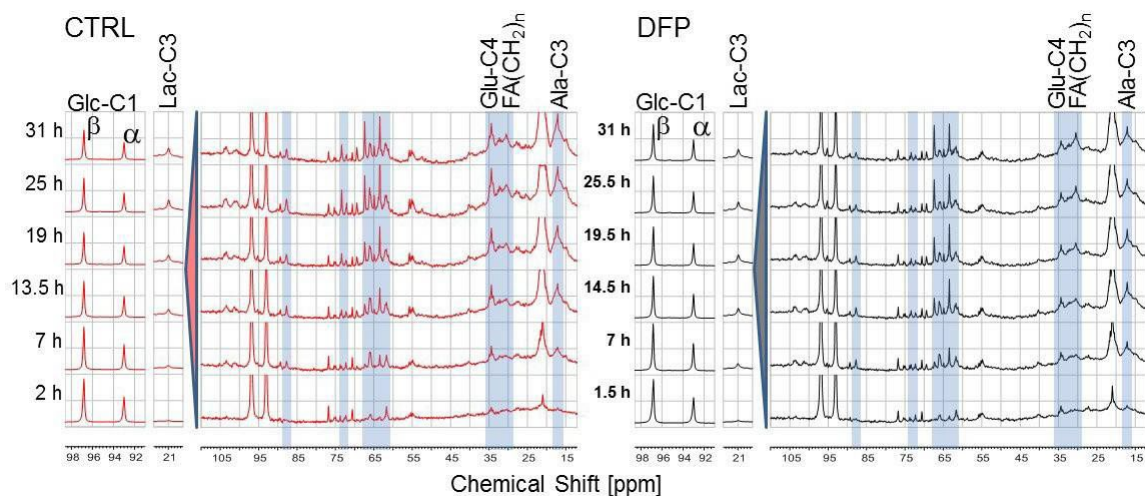


Fig. 5. Serial ^{13}C NMR spectra of 4T1 cells perfused with 1- ^{13}C glucose as a label and media or 100 μM DFP. Not all peaks are labelled because of their density but over 200 peaks were assigned and analyzed. We have performed these measurements on 3 independent studies of control and DFP treated 4T1 cells

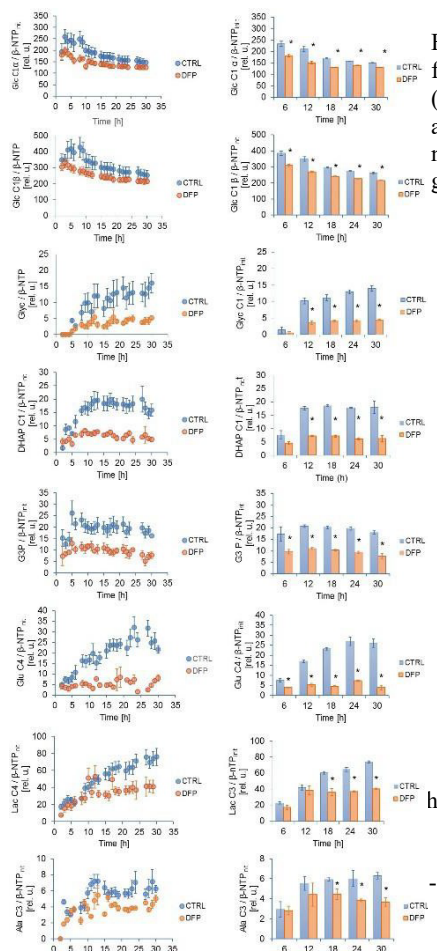


Fig. 3. Changes in metabolites over time for 4T1 cells untreated or treated with DFP (100 μM). DFP induces changes in lipid and TCA metabolism based on changes noted in glycerol-3-P04 and ^{13}C -glutamate.

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2. Porcari, P., Ackerstaff, E., LeKaye, H.C., Koutcher, J.A. Metabolic Effects of Deferiprone on Triple Negative Breast Cancer. Digital Poster #1843, 29th Ann Mtg & Exhibit of the International Soc for Magn Reson in Med (ISMRM), ISMRM & SMRT, Virtual Conference & Exhibition, May 15 – 20, 2021 (Appendix 5)
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4. Simoes, R.V., et al., *Inhibition of prostate cancer proliferation by Deferiprone*. NMR Biomed, 2017. **30**(6).

Section 5B: The effect of iron chelation on the human TNBC MDA-MB-231 cell line – Metabolic Studies (Koutcher Laboratory). Aim 1 - Presented in 2020 report

1. Methods

1.1.1. Cells: The human, epithelial TNBC cell line MDA-MB-231 was grown in complete Dulbecco's Modified Essential (DME_{compl}) medium, containing 25 mM glucose (Glc), 6 mM glutamine (Gln), 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in humidified 5% CO₂/ 95% air at 37 °C, split every 6 to 7 days and used up to passage 10.

1.1.2. Bioreactor Studies: For the magnetic resonance (MR)-cell bioreactor studies, 6×10⁶ MDA-MB-231 cells were seeded on microcarrier beads (0.5 ml; 125-212 µm diameter; PP3772, Corning, New York, NY) and cultured for five days, with daily medium change, in a Nunc™ Deep Form Bacteriology Petri dish (n = 7; Thermo Fisher Scientific, Pittsburgh PA, USA) until reaching ~ 70% confluence. Cell number and viability were estimated from a representative sample at the start (#cells_{init}) and end (#cells_{end}) of each MR experiment by Trypan Blue exclusion assay.

1.2. Monitoring Metabolism Changes: Multinuclear magnetic resonance spectroscopy (MRS)

Continually perfused, live cells were studied in an MR-compatible cell bioreactor (1,2) on a vertical-bore 500 MHz AVANCE III Bruker scanner equipped with a 10-mm broad-band probe (Bruker BioSpin, Billerica, MA) for ¹H and ³¹P/¹³C scans. To monitor metabolic changes, cells were continuously perfused for 31 hours with DME_{compl} media. After

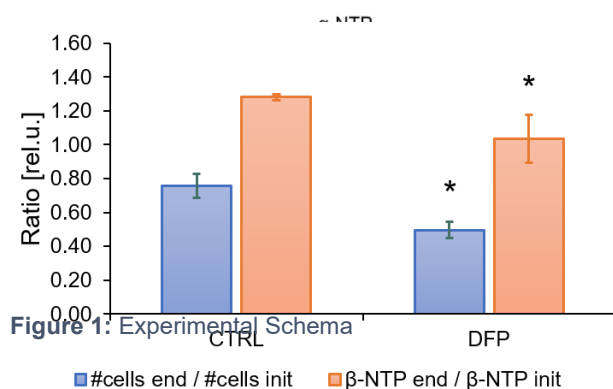


Figure 1: Cell mass and cellular energy ratio. Comparison between the ratio of the cell numbers at the end and start of the experiment (#cells_{end} / #cells_{init}, blue bars) and the corresponding cellular energy ratio (β-NTP_{end} / β-NTP_{init}; orange bars), calculated for both, control (CTRL) and Deferiprone (DFP)-treated MDA-MB-231 Triple Negative Breast Cancer cells. Data are mean ± standard deviation (SD), n = 3 each. * Statistically significantly different from the corresponding values in the control (CTRL) MDA-MB-231 cells (P < 0.05).

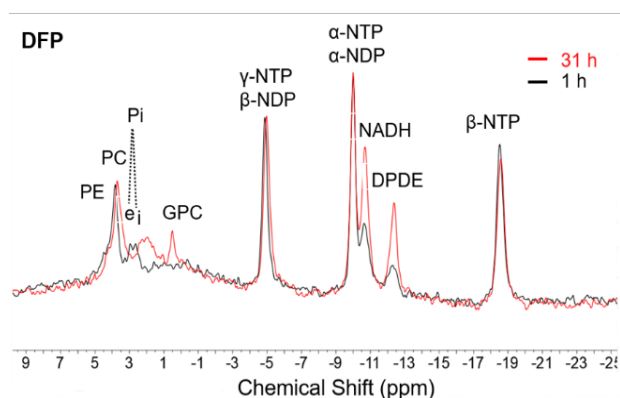


Figure 2: Representative ³¹P MR spectra of DFP- treated and untreated MDA-MB-231 cells, depicting energy level metabolite changes over time. Top panel: Comparison between representative ³¹P MR spectra of untreated (CTRL) MDA-MB-231 cells acquired at the start (t = 0-1 h, black spectrum) and end (t = 31 h; teal spectrum) of the experiment. Bottom panel: Comparison between representative ³¹P MR spectra of DFP-treated MDA-MB-231 cells, acquired at the start (t = 0-1 h, black spectrum) and the end (t = 31 h; red spectrum) of the experiment. PE = phosphoethanolamine, PC = phosphocholine; Pi = inorganic phosphate, (e = extracellular, i = intracellular); GPC = glycerophosphocholine, NTP = nucleotide triphosphate; NDP = nucleotide diphosphate, NADH = total nicotinic adenine dinucleotides and nicotinic adenine dinucleotide phosphates (NAD⁺, NADH, NADP⁺, NADPH); DPDE = diphosphodiester.

the setup and acquisition of the first ³¹P MR spectrum, the unlabelled perfusion medium was replaced with DME_{compl} medium containing 25 mM 1-¹³C-labeled glucose for the remaining 30 h of the experiment. During each experiment, ¹H-decoupled, Nuclear

Overhauser Effect-enhanced ¹³C MR spectra (1200 s relaxation time (TR), 45° flip angle, 30 kHz sweep width (SW), 8 k spectral points (np), 1000 averages, 20 min acquisition time) were acquired repetitively inter-spaced every six hours with two consecutive ³¹P spectra (TR(repetition interval) = 1200 s, 45° flip angle, 20 kHz SW(spectral width), 2 k (np), 1800 averages, 30 min acquisition time). Three independent experiments each, were performed in regular medium conditions (CTRL) and three in medium containing 100 µM DFP (DFP-treated). For ¹³C and ³¹P MR spectra an exponential line broadening of 7 Hz and 10 Hz were applied respectively before fast Fourier transformation, followed by 0 and 1st order phase correction. Quantification of MR spectral peaks was performed by time domain fitting of the metabolite signal area using AMARES (advanced method for accurate, robust and efficient spectral fitting)(jMRUI v5.2, <http://www.jmrui.eu/>), as described previously (2). All metabolite signal areas were normalized to the β-NTP signal area of the first ³¹P MR spectrum (β-NTP_{init}). The intra- and extra-cellular pH were calculated from the chemical shift of the respective intra- and extra-cellular inorganic phosphate (Pi) signal, as described in detail previously (2,3).

1.3. Statistical Analysis: Data were analysed using GraphPad Prism version 8.0 (GraphPad software, Inc., CA) and presented as mean ± standard error (SE), unless stated otherwise. A 2-tailed,

unpaired Student's t test was used to compared DFP-treated and untreated (CTRL) MDA-MB-231 cell data. A P value < 0.05 was considered significant.

2. Results

2.1.1. Cells

2.1.2. Cell count ratio and cellular energy ratio

Figure 1 shows the comparison between the cell count ratios ($r_c = \#cells_{end}/\#cells_{initial}$) of each experiment and the corresponding cellular energy ratios ($r_E = [\beta\text{-NTP}_{end}/\beta\text{-NTP}_{init}]$) (NTP = nucleoside triphosphate) determined for both DFP-treated and untreated cells. In DFP-treated MDA-MB-231 cells, the cell mass and the cellular energy ratios were significantly lower than in untreated MDA-MB-231 cells. This agrees with our EC_{50} studies (last year's report) showing decreased proliferation and some cytotoxicity in MDA-MB-231 ($EC_{50} = 58.5 \pm 5.6 \mu\text{M}$, $EC_{90} = 163 \pm 32 \mu\text{M}$ with ~50% of cells at time 0 lost after 48 h exposure to 100 μM DFP) caused by DFP. The cell loss seen in untreated MDA-MB-231 cells is in line with generally occurring cell loss in nearly confluent cells observed for ≤ 1 cell doubling time (here, 31 h versus a ~40 h MDA-MB-231 doubling time). While the cell count ratio r_c decreases by $37.7 \pm 4.7\%$ between untreated and DFP-treated MDA-MB-231 cells, the cellular energy ratio r_E decreases by $19.3 \pm 9.1\%$, resulting in a ~18% decrease difference ($P = 0.057$, 2-tailed, unpaired t test).

2.2. Assessment of Metabolism Changes by Multinuclear MRS

2.2.1. ^{31}P MRS Results & Discussion: The comparison of representative ^{31}P spectra of DFP-treated and untreated (CTRL) MDA-MB-231 cells are respectively reported in the bottom and top panel of **Figure 2**. In each panel, spectra acquired at the start ($t = 0$, black spectrum in both panels) and end ($t = 31$ h, red spectrum for DFP-treated in the bottom panel, teal spectrum for CTRL in the top panel) of an experiment for each group of cells are reported. Spectra were analysed to measure peak ratios representing metabolite concentration ratios.

Figure 3A shows representative ^{31}P MR spectra of DFP-treated and untreated MDA-MB-231 cells acquired at 31 h. The time course of $\beta\text{-NTP}$ levels is similar between DFP-treated and control MDA-MB-231 cells, except for the last two time points where $\beta\text{-NTP}$ levels plateau and are stable in the DFP-treated cells while it keeps increasing in the untreated cells (**Figure 3B**). This observation, together with the ~18% decrease difference of r_E and r_c in response to DFP treatment (**Figure 1**), indicate not only an increase of cellular $\beta\text{-NTP}$ in both, DFP-treated and untreated MDA-MB-231 cells, but the increase of cellular $\beta\text{-NTP}$ is diminished in response to DFP. This is in line with our observation of impaired bioenergetics after 23 h of DFP exposure in TRAMP- C2 prostate cancer cells (1). The phospholipid precursor PC (**Figure 3C**) exhibits higher values in DFP-treated than untreated MDA-MB-231 cells, with a significant difference at the earliest time point (6 h), while phosphoethanolamine (PE) is lower in DFP-treated MDA-MB-231 cells with a statistically significant difference at 18 h (**Figure 3D**). Post 6 h, the phospholipid precursor GPC increases similarly over

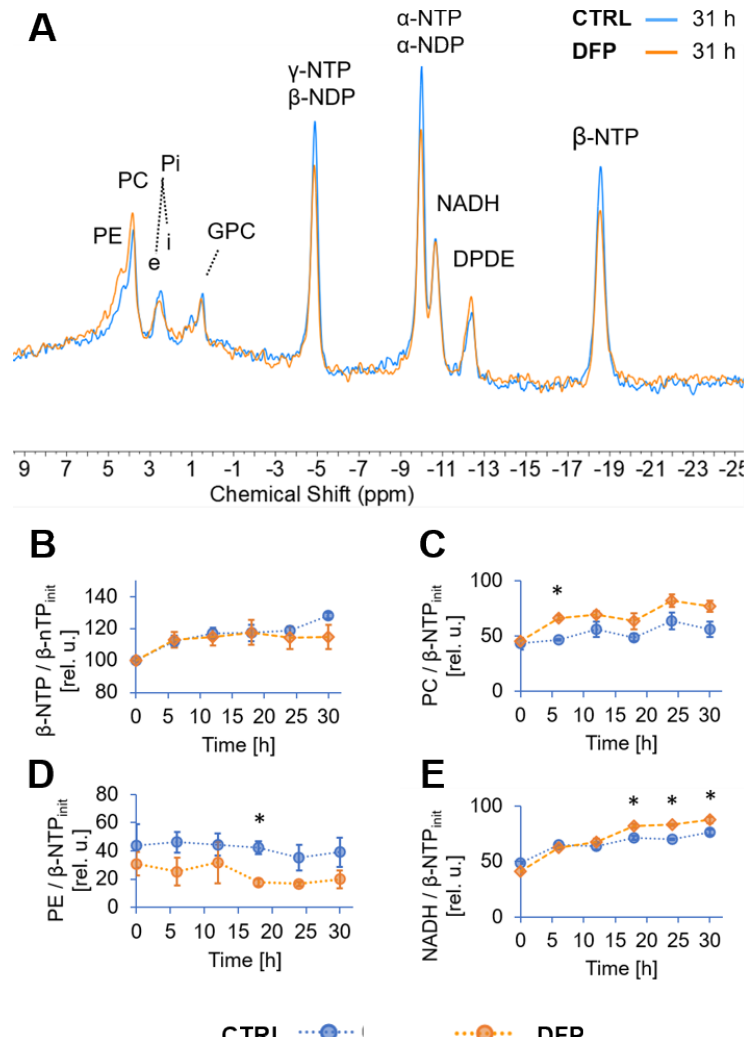


Figure 3: ^{31}P MRS results on DFP-treated and untreated MDA-MB-231 cells. A: Representative ^{31}P spectra of DFP-treated (red spectrum) and untreated MDA-MB-231 cells (CTRL; blue spectrum), acquired at 31 h. B, C, D, E: Time course of selected metabolites: $\beta\text{-NTP}$ (B), PC (C), PE (D) and NADH, i.e. the combined signals of NAD^+ , NADP^+ , NADH, NADPH (E). *Statistically significantly different from the corresponding values in control (CTRL) MDA-MB-231 cells ($P < 0.05$).

the time frame of 31 h in DFP-treated and untreated cells (data not shown). Contrary to other treatments (3,4) and TRAMP-C2 (1) cells, the GPC/PC ratio is increasing similarly for DFP-treated and untreated MDA-MB-231 cells at 12 h – 31 h. The difference between TRAMP-C2 and MDA-MB-231 may be in part due to the longer cell doubling of MDA-MB-231 leading to less phospholipid membrane turnover. The three phospholipid precursors PC, PE, and GPC are metabolic markers of phospholipid membrane turnover (4-6), with the higher cellular PC in DFP-treated cells possibly related to decreased phosphatidylcholine synthesis as a result of cell growth inhibition by DFP (1). The combined signal of nicotinamide adenine dinucleotides NAD(H), and nicotinamide adenine dinucleotide phosphates NADP(H) is significantly higher in DFP-treated than untreated MDA-MB-231 cells beyond 12 h (**Figure 3E**). The intracellular pH was stable throughout and unaffected by DFP (data not shown). The extracellular pH dropped by ~ 0.19 pH units in untreated MDA-MB-231 and overlapped with DFP-treated MDA-MB-231 cells up-to 18 h, after which the intra- and extra-cellular Pi overlapped and were indistinguishable in DFP-treated cells, precluding the calculation of extracellular pH for the late time points (data not shown).

2.2.2. ^{13}C MRS Results & Discussion: Figure 4 shows representative ^{13}C MR spectra of DFP-treated (bottom panel) and untreated (CTRL) MDA-MB-231 cells (top panel), acquired at the start ($t = 1$ h; black spectrum in both panels) and

the end (31 h; red spectrum for DFP treated in the bottom panel and teal spectrum for CTRL in the top panel) of a representative experiment. A direct comparison between ^{13}C MR spectra of DFP-treated (orange spectrum) and untreated MDA-MB-231 cells (CTRL, blue spectrum) at 31 h is shown in Figure 5.

The quantitative evaluation of ^{13}C spectra metabolites of DFP-treated and control MDA-MB-231 cells (**Figure 6**) shows various average metabolite levels that significantly differ between DFP-treated and untreated MDA-MB-231 cells. The dynamic ^{13}C MRS monitoring of MDA-MB-231 cells while perfused with $1\text{-}^{13}\text{C}$ -glucose (Glc) allows the evaluation of metabolic changes induced by DFP treatment (**Figure 6B**). A significant decrease in uptake and metabolism of Glc 1C α and Glc 1C β respectively were

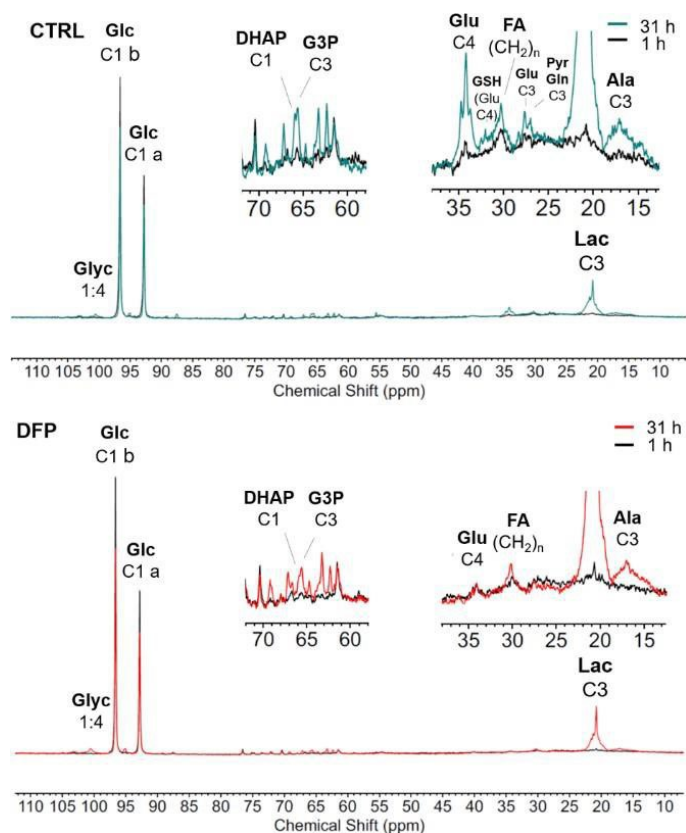


Figure 4: Representative ^{13}C MR spectra of DFP-treated and untreated MDA-MB-231 cells. Top Panel: Comparison between representative ^{13}C MR spectra of untreated (CTRL) MDA-MB-231 cells acquired at the start ($t = 0$ h, black spectrum) and end ($t = 31$ h; teal spectrum) of the experiment. **Bottom panel:** Comparison between representative ^{13}C MR spectra of DFP-treated MDA-MB-231 cells, acquired at the start ($t = 0$ h, black line) and the end ($t = 31$ h; red line) of an experiment. Glc = glucose; DHAP = dihydroxyacetone phosphate; G3P = glycerol-3- phosphate; Glu = glutamate; FA = fatty acids; GSH = glutathione; Pyr = pyruvate, Ala = alanine.

observed in the presence of DFP at 12 h and 18 h of perfusion for Glc 1C α and at 12 h – 31 h of perfusion for

Glc 1C β . At the same time, the incorporation of Glc into glycogen (Glyc) is significantly higher in DFP-treated than

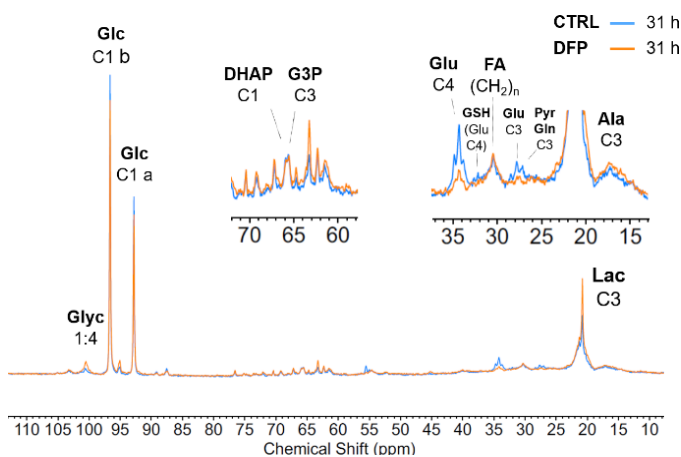


Figure 5: Representative ^{13}C MR spectra of DFP-treated and untreated MDA-MB-231 cells acquired at 31 h. Comparison between ^{13}C MR spectra acquired at 31 h on DFP-treated (orange spectrum) and untreated (CTRL) MDA-MB-231 cells (blue spectrum). Peak assignments are the same as in Figure 4.

untreated MDA-MB-231 cells while de-novo glutamate (Glu) synthesis significantly decreases in DFP-treated compared

to untreated MDA-MB-231 cells (**Figure 6B**). Along the whole experiment (31 h), levels of dihydroxyacetone phosphate (DHAP), glycerol-3-phosphate (G3P) and alanine (Ala) do not show significant differences between DFP-treated and untreated MDA-MB-231 cells while the lactate labelling rate is significantly lower in DFP-treated than untreated MDA-MB-231 cells at each time point (**Figure 6**). These data suggest a decrease both in glycolysis (decreased lactate), Krebs cycle activity (decreased C4 glutamate), decreased overall glucose utilization, and decreased phosphatidylcholine synthesis as a result of cell growth inhibition by DFP. The combined signal of nicotinamide adenine dinucleotides NAD(H), and nicotinamide adenine dinucleotide phosphates NADP(H) is significantly higher in DFP-treated than untreated MDA-MB-231 cells beyond 12 h (**Figure 3E**), which would require further studies of the PPP to probe these changes.

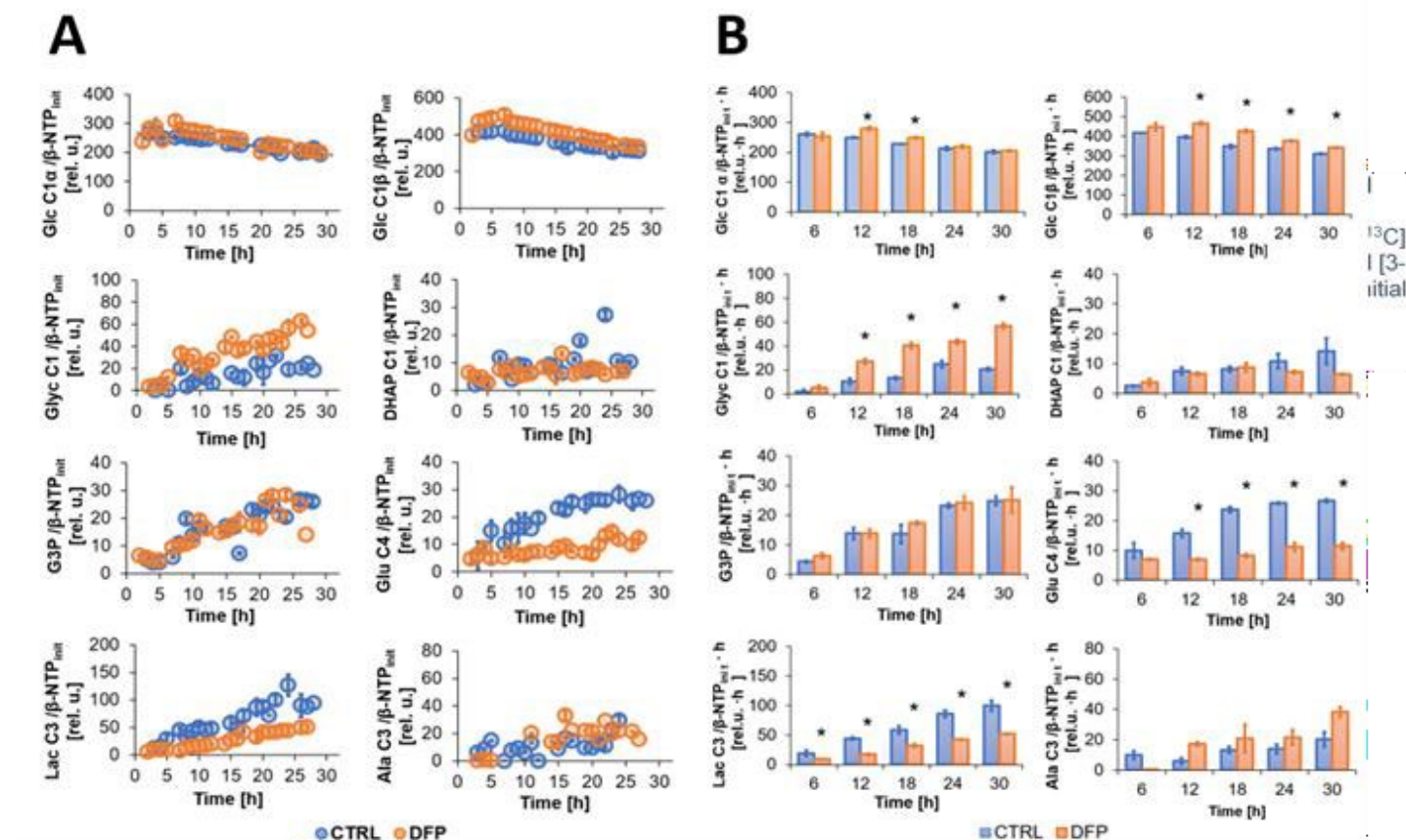


Figure 6: Time course effect of DFP on live MDA-MB-231 cell metabolism during perfusion experiments, as detected by ^{13}C MRS. A: Average time course effect of $1\text{-}^{13}\text{C}$ -labeled glucose (Glc) consumption and synthesis of $[1\text{-}^{13}\text{C}]$ glycogen (Glyc), $[1\text{-}^{13}\text{C}]$ dihydroxyacetone phosphate (DHAP); $[3\text{-}^{13}\text{C}]$ glycerol-3-phosphate (G3P), $[4\text{-}^{13}\text{C}]$ glutamate (Glu), $[3\text{-}^{13}\text{C}]$ lactate (Lac) and $[3\text{-}^{13}\text{C}]$ alanine (Ala), are shown for both DFP-treated and untreated MDA-MB-231 cells. Metabolites were normalised to the initial value of β -nucleoside triphosphate (β -NTP_{init}). B: Average metabolite levels (mean $\pm$ SE) at different time intervals.

* Significantly different from untreated MDA-MB-231 cells (p < 0.05, unpaired Student t test).

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Section 6: Cell Migration Studies (Koutcher Laboratory) (Aim 1; presented 2019, 2021)

Data below are for MDA-MB-231 presented in 2021. Studies on cell migration in the 4T1 were presented in 2019 and are presented at end of this section.

TNBC Cell Migration - Methods

The effect of deferiprone (DFP) exposure on the migration of 4T1 and MDA-MB-231 cells was assessed in up to 6 independent experiments per time point per cell line using the wound healing assay.¹ For each experiment, a 24-well plate (Falcon® Plates, Corning Life Sciences, Teterboro, NJ, USA) was seeded with 3.1×10^4 4T1 cells or 25×10^4 MDA-MB-231 cells per well in 0.4 ml culture medium. Cells were cultured until reaching a confluent monolayer in each well, which was scraped with a plus sign motion (Fig. 1A) 48 hours and 3-3.5 days after the seeding of 4T1 and MDA-MB-231 respectively using a standard 200 µl pipette tip.¹⁻³ Wells were subsequently washed once with phosphate-buffered saline (PBS) to remove the cellular debris, and each of them refilled with 0.4 ml of medium containing either no DFP (control = 0 µM DFP), 50 µM DFP, or 100 µM DFP corresponding to three different treatment conditions. The culture medium was changed every 24 h post seeding to avoid nutrient depletion and medium acidification. Following a pilot experiment to assess the timing of the wound closures for each cell line, the total imaging time to monitor cell migration into the cell-free area was set to 48 hours for 4T1 and 60-72 hours for MDA-MB-231 cells, with 12 hour time resolution for both of them. For each cell line, one image per plate was acquired approximately 1 hour after DFP exposure ($t = 0$) and then every 12 hours through brightfield microscopy (ZEN microscope, Carl Zeiss, Germany) using a $5\times$ objective lens and 45 frames per well. Using the ZEN software, a single image of each well was obtained at each time point by stitching the frames together and exporting to Tiff format for further processing, which includes the image conversion to 8-bit, the background subtraction with the subsequent application of filters such as de-speckling and unsharp filters followed by the variance and Gaussian blur filters. To automate the image processing and determine the open wound area per well at each time point, a custom-written macro in Fiji (ImageJ; <https://imagej.nih.gov/ij/>)⁴ was used. For each cell line, a mean relative open wound area was calculated for each treatment condition and time point per experiment by dividing the absolute open wound area in μm^2 for each well by the average of the absolute open wound area in μm^2 in all wells at $t = 0$, as described before.^{2,3} This allowed the investigators to account for the inter- and intra-experimental variations at the start of each experiment. For each independent experiments and cell line, the mean and standard deviation (SD) of the relative open wound area were calculated for each condition and time point. Then for each cell line, the mean average relative open wound area at each time point and for each condition was averaged for the up-to 6 independent experiments and the corresponding SD calculated by error propagation (maximum error, assuming Gaussian distribution). For the latest time points, when all wells per experiment and treatment condition had a fully closed wound area (relative open wound area = 0%, see e.g. Fig. 1A at ~48 h for 50 µM and 100 µM DFP), the measurement error was defined to be zero.

Results

Figure 1A shows the effects of the “scratch” on tumor growth using 0 µM (control), 50 µM and 100 µM DFP. Figure 1B shows that MDA-MB-231 cells treated with DFP had a modestly greater cell migration at 24 hours, which is different than what was noted with the 4T1 cells. As presented in the 2019 report, exposure of 4T1 cells to 100 µM DFP (~EC90 for 4T1 cells) delays full wound closure (i.e. inhibits cell migration required in the metastatic process) to 48 h, resulting in a significantly higher relative open wound area at 24 h ($P < 0.0001$) and 36 h ($P = 0.0003$) compared to untreated 4T1 cells and 4T1 cells treated with 50 µM DFP. In both cell lines, wound closure is dominated by migration within the first doubling time and after that influenced by both cell migration and growth. Thus, wound closure differences in response to DFP between 4T1 and MDA-MB-231 may be due to their different growth rates depicted in Section 4, Figure 2 (cell doubling time).

A

B

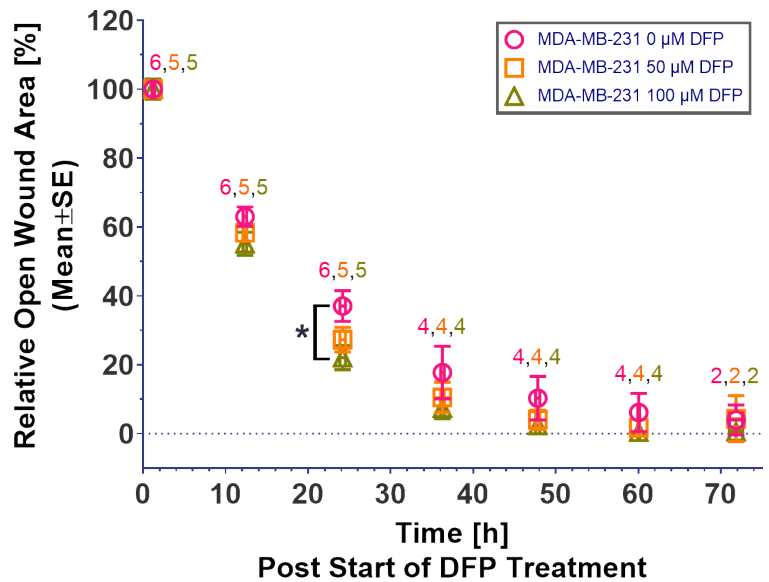


Figure 1: Wound healing assay for MDA-MB-231 cells exposed to DFP for up-to 72 h. **(A)** Single sample well images overlaid with outlines of open wound area (cross in center outlined in pink) at different time points; cyan indicates thresholded cell counts outside of scratch, while darker pink (at 36 and 48 hours) indicates threshold for cell counts when the wound has been closed with cells and no open wound area can be detected. No open wound area is left in the 100 μ M DFP $\sim$ 36 h and $\sim$ 48 h and in the 50 μ M DFP samples at $\sim$ 48 h. Due to differences in contrast between dense cells outside of the original wound area and the single layer, flatter cells migrated into the wound area, a single threshold is not a reliable way to assess cell counts and leads to the cell count void visible in the images when the wound area is closed. (Hence, cell counts have not been used, due to described difficulty of reliable cell counts in dense samples across the wells using these images). **(B)** Wound closures in untreated and treated MDA-MB-231 cells averaged over 2-6 independent experiments (indicated for each condition color-coded above each average), with 3-6 repeats within each experiment. As can be seen from the somewhat faster wound closure, cell migration increased in cells exposed to increasing doses of deferiprone, reaching significance between control and 100 μ M DFP only at 24.17 ± 0.34 h ($p < 0.05$). At later time points, control cells catch up with the wound closure which might in part be explained by their faster cell doubling time DT than the DT for cells exposed to DFP (see Fig. 2 in section 4 (above – cell doubling times)).

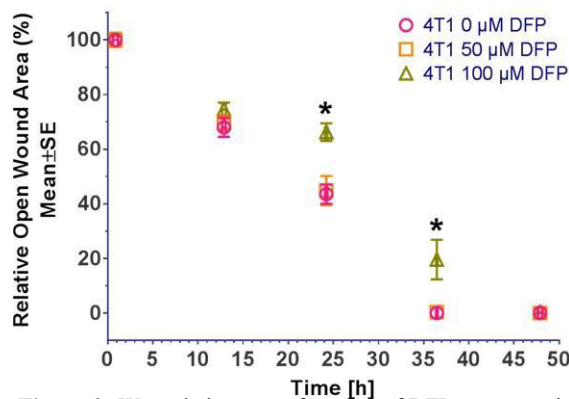


Figure 2: Wound closure in response to DFP exposure time for cells treated with 0 μ M, 50 μ M, and 100 μ M DFP, respectively. Average of 3 independent experiments, with number of wells / experiment and DFP dose of $n=5, 5, 5$ for 0 μ M DFP, $n=5, 6, 6$ for 50 μ M, and $n=6, 6, 6$ for 100 μ M DFP respectively

4T1 Data (Presented in 2019 report)

Overall relative wound closure over time in response to DFP exposure is displayed for 4T1 as mean $\pm$ SE ($n = 3$) in **Fig. 2**. Statistical differences between treatment groups were assessed by 2-way ANOVA with Tukey multiple comparison (swapped direction of comparisons). As shown in **Fig. 2**, untreated 4T1 cells closed the scratches completely by 36 h, undistinguishable from 4T1 cells treated with 50 μ M DFP ($\sim$ IC₅₀ for 4T1 cells). Exposure of 4T1 cells to 100 μ M DFP ($\sim$ IC₉₀ for 4T1 cells) delays full woundclosure (i.e. inhibits cell migration required in the metastatic process) to 48 h, resulting in a significantly higher relative open wound area at 24 h ($P < 0.0001$) and 36 h

($P=0.0003$) compared to untreated 4T1 cells and 4T1 cells treated with 50 μ M DFP (**Fig. 2**). Parallel experiments are being completed on the MDA-231 breast cancer cell line.

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Section 7: Testing Corning Dissolvable Microcarriers for feasibility of culturing RAW 264.7 Cells for metabolic studies (Aim 1) (Koutcher Laboratory). Presented in 2021

In a previous report, we noted successful completion of metabolic measurements of both MDA-MB-231 and 4T1 cells (2019, 2020). We also noted that we had begun parallel measurements on RAW264.7 macrophage cells but had hit a serious impediment in that the cells adhered poorly to the beads which were necessary to keep them alive and stable in the perfusion apparatus. THIS WORK WAS NOT PART OF THE ORIGINAL APPLICATION BUT WAS NECESSITATED BY THE UNEXPECTED FINDING THAT THE RAW264.7 CELLS DID NOT GROW ON THE USUAL MICROCARRIER BEADS WE STUDIED. We reported testing different types of microcarrier beads unsuccessfully. We report here that further attempts on some novel commercial beads that were in the “pre-commercial” state were successful. Dissolvable microcarriers (Product Number 7285, Corning®, New York, NY, USA) were tested to culture the RAW 264.7 cells with the aim to monitor cellular and energy metabolism through ^{31}P and ^{13}C magnetic resonance spectroscopy (MRS) while live cells growing on microcarriers in our compatible MR perfusion system.

Dissolvable microcarriers represent a new product from Corning. For this reason, it required a long time for procuring and testing them. They are made of polygalacturonic acid (PGA) polymer chains, cross-linked via calcium ions. When fully hydrated, they have an average size of 200 to 300 μm , suitable for our perfusion system, and a density of 1.01 to 1.03 g / cm^3 . We tested them along with the Collagen-coated Microcarriers (Corning, Product Number 3786) after a meeting with the Corning Scientific support team, which allowed us to have different options following the unsatisfactory results obtained by culturing the RAW 264.7 cells on the Enhanced Attachment Microcarrier (Product Number 3779, Corning®, New York, NY, USA), those previously used to culture TNBC cell lines 4T1 and MDA-MB-231.

Hydration of dry dissolvable microcarriers and cell seeding

1 g of dry dissolvable microcarriers were hydrated in 150 ml of sterile water within a siliconized glass bottle of 250 ml (Corning, Cat. No. 1395-250). After gently swirling the suspension, the microcarriers were hydrated for 10 min. To confirm complete hydration, a sample of 2 ml of microcarriers was inspected under the microscope and imaged to compare their hydration state with that recommended in the instructions, as shown in **Fig. 1**.

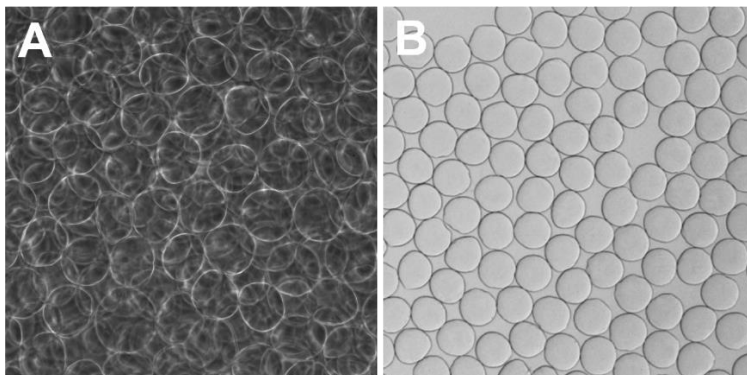


Figure 1. Comparison between fully hydrated dissolvable microcarriers imaged under the microscope (10x magnification) after 10 min hydration time (**A**) and those shown in figure (**B**), provided in the product instructions to confirm the complete hydration by direct comparison.

Subsequently, the water used for microcarrier hydration was exchanged with high glucose DME culture medium added with 2 mM glutamine, the same culture medium used for all other studies in this report, and the microcarriers allowed to settle for 30 minutes. For the seeding procedure, 3×10^6 RAW 264.7 cells were seeded on 0.5 ml of dissolvable microcarriers in:

- i. a deep-form bacteriological Petri dish of 25 mm height ($n = 3$; 100 mm diameter; Cat # 4031, Thermo Scientific Nunc™, Waltham, MA, USA);
- ii. an ultra-low attachment bacteriological Petri dish of 20 mm height ($n = 1$; 100 mm diameter; 20 mm height; Cat # 3262; Corning®, New York, NY, USA);

- iii. an ultra-low attachment bacteriological Petri dish of 15 mm height (n = 2; 100 mm diameter; 15 mm height; Cat # 351029; Corning®, New York, NY, USA),

where 15 ml of complete high glucose DME culture medium were added afterwards. The different plates (i), (ii) and (iii) were used to evaluate the best option to adapt for preculturing and growing the cells on microcarriers before loading them into the bioreactor for the cell perfusion experiments. Immediately after seeding the cells, each plate was inspected under the microscope and imaged at 10x and 20x magnification as shown in **Fig. 2** for a representative plate.

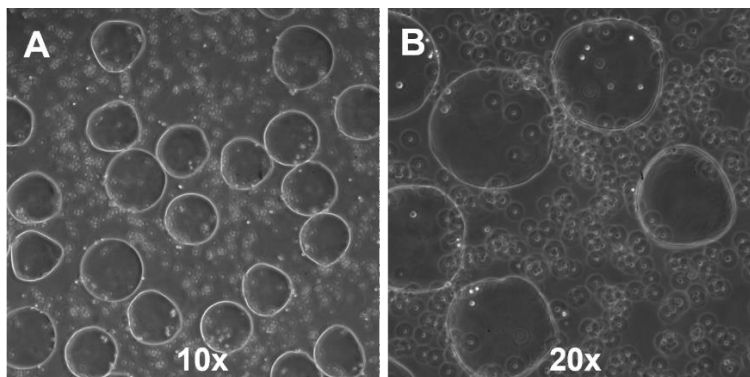


Figure 2. Representative images of dissolvable microcarriers inspected under the microscope and imaged at 10x (**A**) and 20x (**B**) magnification immediately after seeding the RAW 264.7 cells.

Monitoring RAW 264.7 cell growth on dissolvable microcarriers

The RAW 264.7 cells were observed growing on the dissolvable microcarriers for up to four days after the seeding in both, ultra-low-attachment bacteriological Petri dishes of 15 mm height (iii) and one of the deep-form bacteriological Petri dishes of 25 mm height (i); for up to 5 days for the remaining two deep-form bacteriological Petri dishes of 25 mm height (i), indicated as NUNC in some figures, and the ultra-low attachment bacteriological Petri dish of 20 mm height (ii).

Twenty-four hours after cell seeding on the dissolvable microcarriers, the medium was changed in all plates except in one of the two ultra-low attachment bacteriological Petri dishes of 15 mm height (type iii; Plate 1). This exception was made to evaluate whether changing, or not changing the culture medium 24 hours post seeding (on day 1), were both viable options. There was concern that since the dissolvable microcarriers were barely visible, there was a risk of losing them during the medium changing procedure on the first day, hence a possible advantage to not changing the media 24 hours after plating. To evaluate this, images from both Petri dishes, (iii, Plate 1 (**top panel (media not changed)**) and Plate 2 (**bottom panel, media changed** at 24 hours), acquired at day 1 before changing the culture medium in plate 2 (**Fig. 3**), and at day 2 (48 hours following the seeding) after changing the medium (**Fig. 4**) in both plates, were compared at 10x (**A, D**), 20x (**B, E**) and 40x (**D, F**) as shown in both **Fig. 3** and **Fig. 4**. The comparison between the images acquired on both Petri dishes at day 1, before changing the culture medium in Plate 2 (**Fig. 3**), and at day 2, immediately after changing the medium in both plates (**Fig. 4**), do not show substantial differences between the cells cultured on microcarriers in both plates. In addition, no acidification process was revealed as indicated by the color of the culture medium in Plate 1 at day 2. These observations along with the high probability of losing a significant number of microcarriers when changing the medium on day 1 because of their low visibility, suggest that not changing the culture medium 24 hour after the cell seeding might be the best procedure to adopt.

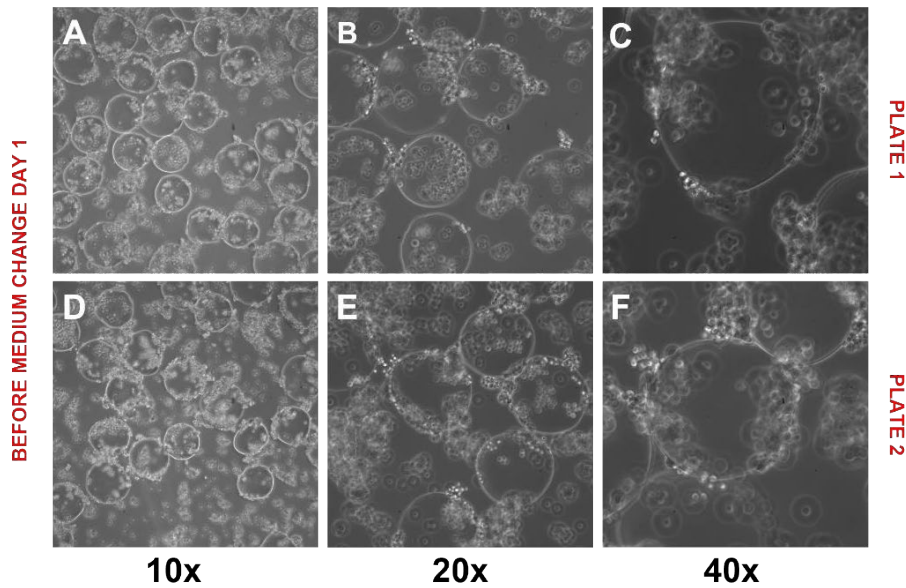


Figure 3. Comparison between images acquired **before** changing the culture medium at day 1 (24 hours following the seeding) from the growing cells on dissolvable microcarriers in the two ultra-low attachment bacteriological Petri dishes of 15 mm height (iii), Plate 1 (**top panel**; A, B, C) and Plate 2 (**bottom panel**; D, E, F). Images A and D, B and E, and C and F, were respectively acquired at 10x, 20x and 40x magnification.

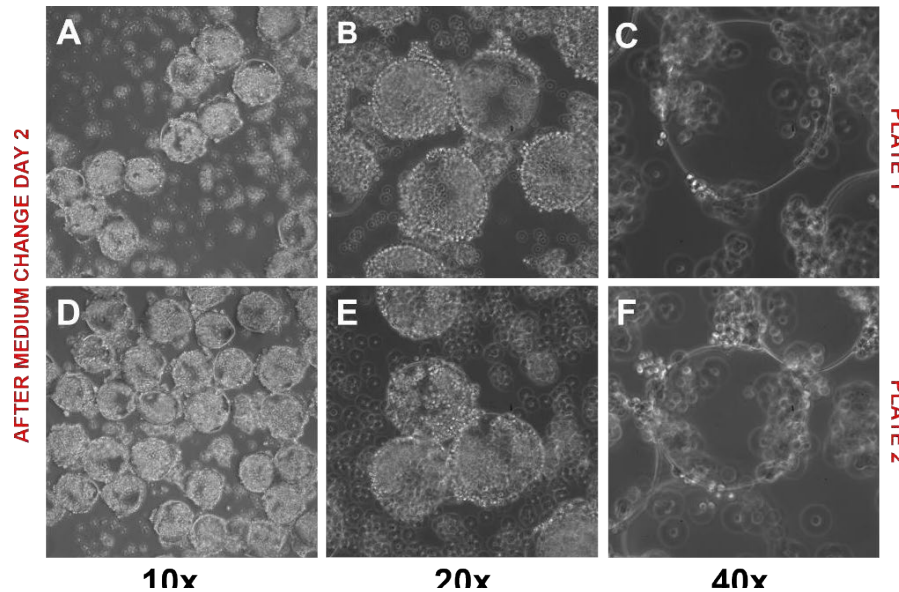


Figure 4. Comparison between images acquired **after** changing the culture medium at day 2 (48 hours following the seeding) from the growing cells on dissolvable microcarriers in two ultra-low attachment bacteriological Petri dishes of 15 mm height (iii), Plate 1 (**top panel**; A, B, C) and Plate 2 (**bottom panel**; D, E, F). Images A and D, B and E, and C and F, were respectively acquired at 10x, 20x and 40x magnification. Qualitatively no differences were noted whether the media was changed at 24 hours or not

After day 1, the culture medium was changed daily in each plate and the growing cells on the dissolvable microcarriers were inspected under the microscope and imaged at 10x, 20x and 40x magnification before and after medium change. Representative images of growing cells on dissolvable microcarriers in different type of plates (i, ii and iii) are shown below for each day following the seeding procedure. **Fig. 5, 7, 9** and **12** show the comparison between images of growing cells on dissolvable microcarriers acquired on the same deep-form bacteriological Petri dish of 25 mm height (i) immediately before (**top panel**) and after changing the culture medium (**bottom panel**) at day 1 (24 hour following cell seeding), day 2 (48h), day 3 (72h) and day 4 (96h), respectively. **Fig. 6, 8, 10** and **13** show the same type of comparison for the ultra-low attachment

bacteriological Petri dish of 20 mm height (ii) at day 1, day 2, day 3 and day 4, respectively. Both ultra-low attachment bacteriological Petri dishes of 15 mm height (iii), which were used to evaluate whether changing medium 24 hours following the seeding (**Fig. 3** and **4**), were inspected each day and harvested on day 4 (96 hour following cell seeding). Representative images of one of the plates (iii) at day 3 (72h following cell seeding) are shown in **Fig. 11**.

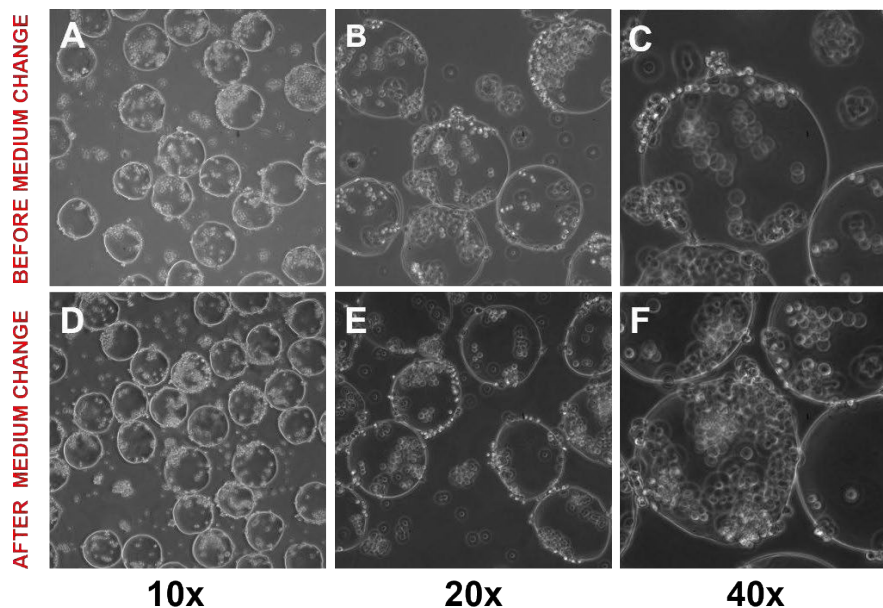


Figure 5. Representative images of growing cells on dissolvable microcarriers in a deep-form bacteriological Petri dish of 25 mm height (i) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 1 (24 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

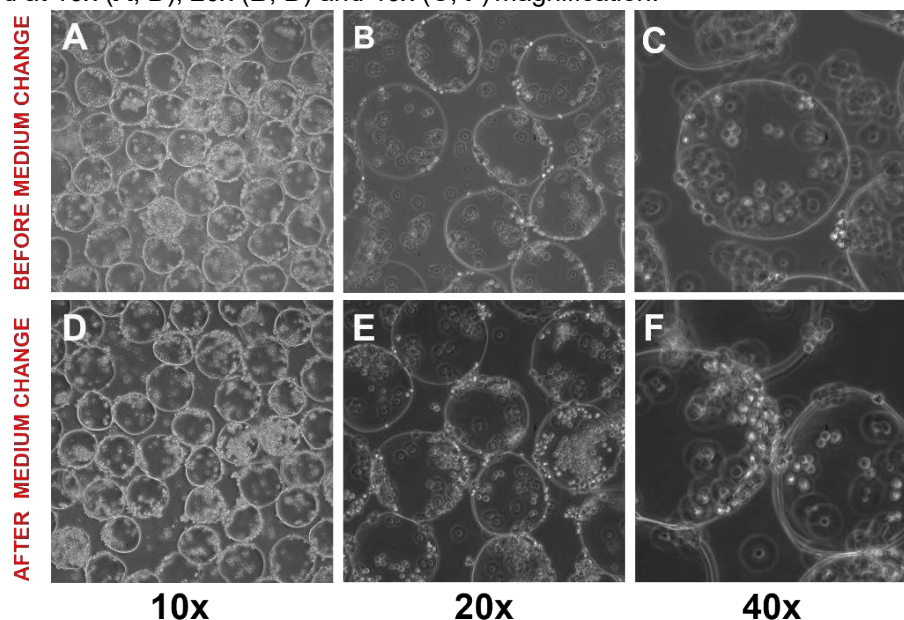


Figure 6. Representative images of growing cells on dissolvable microcarriers in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 1 (24 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

The comparisons between **Fig. 5** and **6**, **Fig. 7** and **8**, **Fig. 9** and **10**, and **Fig. 12** and **13** respectively acquired at day 1, 2, 3 and 4 on microcarriers growing on a representative deep-form bacteriological Petri dishes (i) (**Fig. 5, 7, 9, 12**) and on those growing on the ultra-low attachment plate (ii) (**Fig. 6, 8, 10, 13**) do not show any difference depending on the type of plate used. The same consideration was revealed by comparing **Fig 9, 10**

and **11** acquired at day 3 respectively on cells growing on microcarriers in a representative deep-form bacteriological Petri dishes (i) (**Fig. 9**) and on those growing on the ultra-low attachment plate (ii) (**Fig. 10**) and on a representative plate (iii) (**Fig. 11**). The absence of substantial differences between cells growing on microcarriers in the different type of plates (i, ii, iii) suggest the use of the deep-form bacteriological Petri dishes as more appropriate because their height (25 mm) is easier for handling.

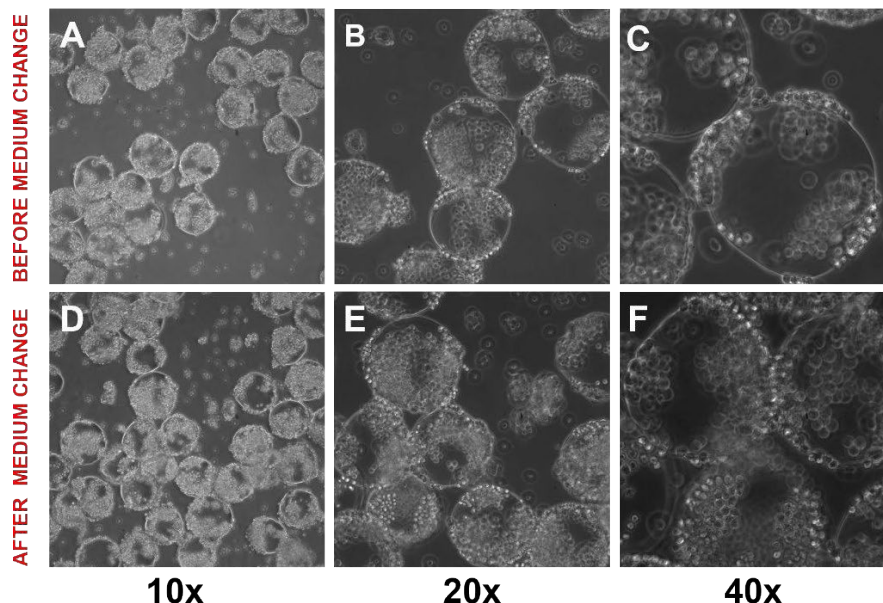


Figure 7. Representative images of growing cells on dissolvable microcarriers in a deep-form bacteriological Petri dish of 25 mm height (i) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 2 (48 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

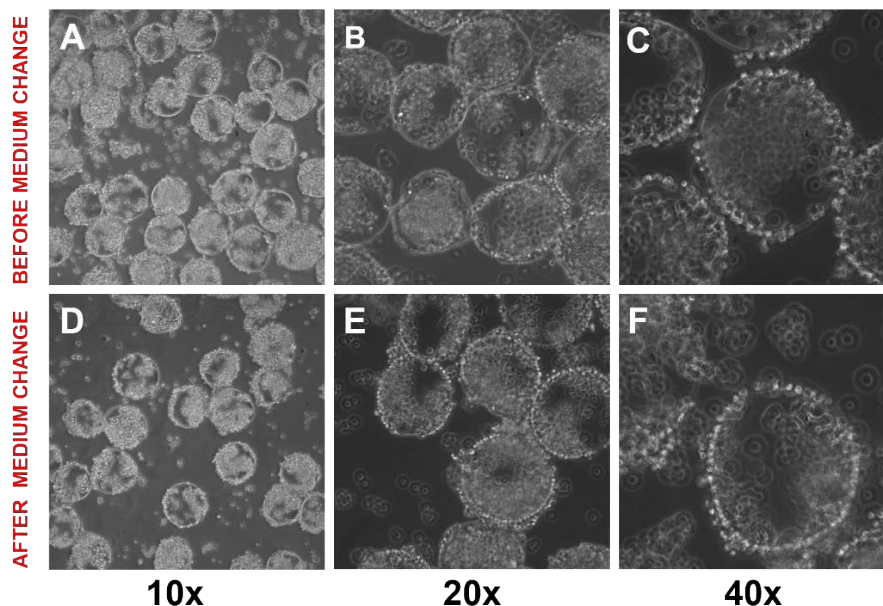


Figure 8. Representative images of growing cells on dissolvable microcarriers in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 2 (48 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

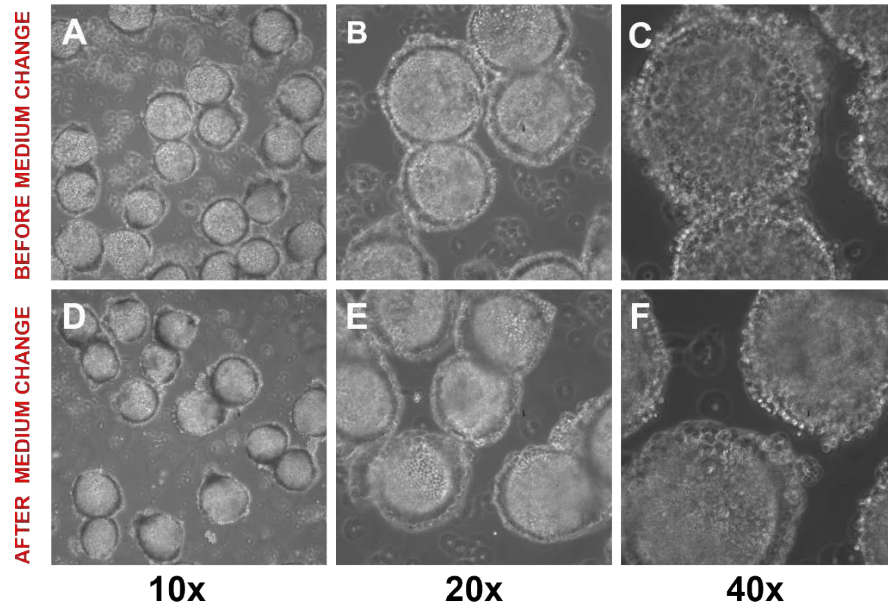


Figure 9. Representative images of growing cells on dissolvable microcarriers in a deep-form bacteriological Petri dish of 25 mm height (i) inspected under the microscope before (top panel; **A, B, C**) and after changing the culture medium (bottom panel; **D, E, F**) at day 3 (72 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

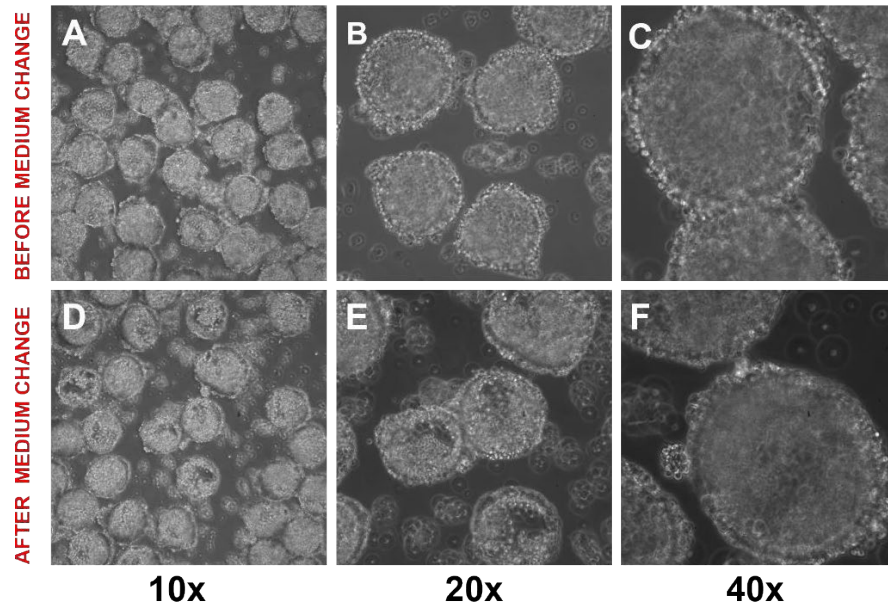


Figure 10. Representative images of growing cells on dissolvable microcarriers in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) inspected under the microscope before (**top panel; A, B, C**) and after changing the culture medium (**bottom panel; D, E, F**) at day 3 (72 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

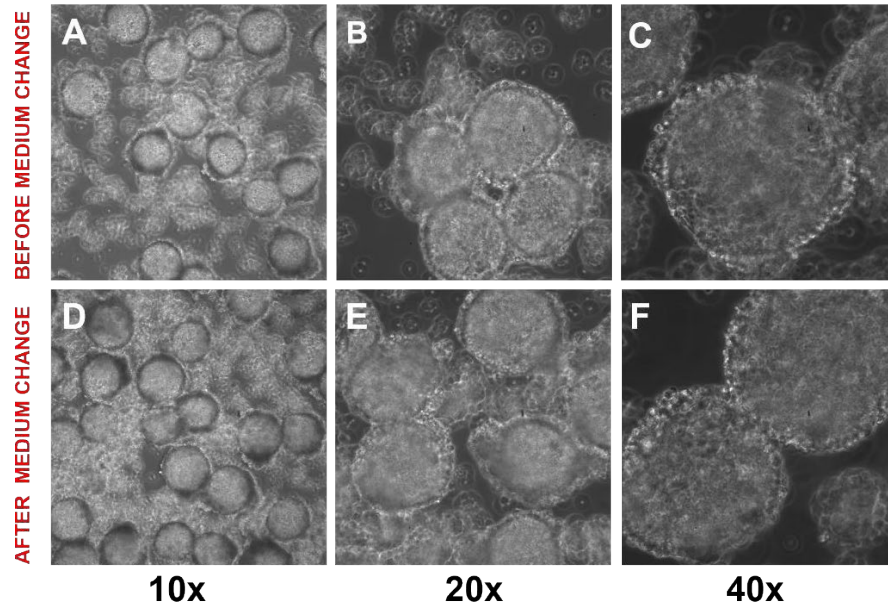


Figure 11. Representative images of growing cells on dissolvable microcarriers in an ultra-low attachment bacteriological Petri dish of 15 mm height (iii) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 3 (72 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

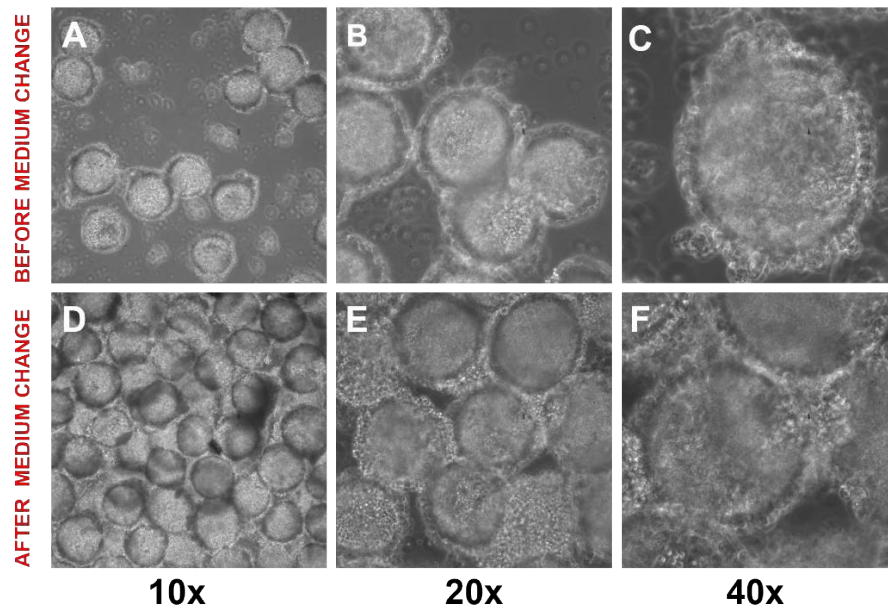


Figure 12. Representative images of growing cells on dissolvable microcarriers in a deep-form bacteriological Petri dish of 25 mm height (i) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 4 (96 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

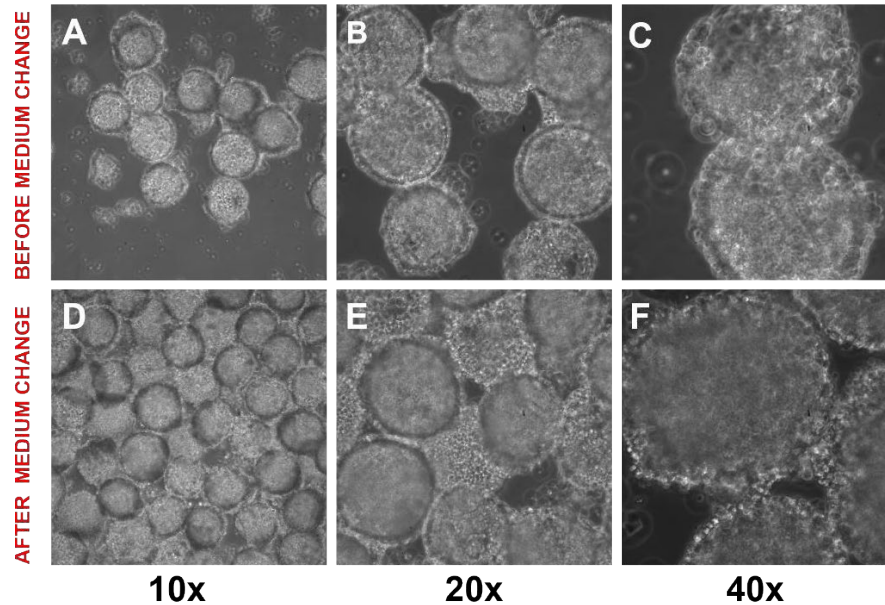


Figure 13. Representative images of growing cells on dissolvable microcarriers in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) inspected under the microscope before (**top panel**; **A, B, C**) and after changing the culture medium (**bottom panel**; **D, E, F**) at day 4 (96 hours following cell seeding). Images were acquired at 10x (**A, D**), 20x (**B, E**) and 40x (**C, F**) magnification.

Cell Harvest of RAW 264.7 cells grown on dissolvable microcarriers

RAW 264.7 cells in both ultra-low attachment bacteriological Petri dishes of 15 mm height (iii) along with those in one plate of a deep-form bacteriological Petri dish of 25 mm height (type i; Plate 2, indicated as NUNC) were harvested on day 4 (96 hours following cell seeding). The cells in the remaining two deep-form bacteriological Petri dishes of 25 mm height (type i; Plate 1 and 3) along with those in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) were harvested on day 5 (120 hours following cell seeding). Cells were harvested following the microcarrier dissolution achieved by adding a solution containing EDTA, which chelates calcium ions and destabilises the polymers crosslinking, and pectinase, responsible of PGA polymer degradation. The harvested solution was prepared by adding 1.32 ml of pectinase (Sigma Cat. No. P2611; 3800 U/ml) and 1 ml of 0.5 M EDTA (USB Corp. Cleveland No.15694) in 57.44 ml of 0.25% Trypsin / 0.25% EDTA in HBSS w/o Calcium & Magnesium.

To dissolve the microcarriers and harvest the cells, the same procedure was used for all plates. It consisted of removing the medium, washing two times the cells with 1x phosphate buffered saline (PBS), add the pre- warmed harvesting solution and stir suspension gently for 10 to 20 min. The only difference between the harvested cells on day 4 and day 5 consisted of the way used to allow the microcarriers to settle before removing the medium or PBS. On day 4, the microcarriers were settled by 1 min centrifugation at 200 x g, while on day 5 they were settled by waiting for ~5 to 10 min before removing the medium or PBS.

Fig. 14 shows the comparison between the images acquired on day 4 before proceeding to dissolve the microcarriers and cell harvest on both, ultra-low attachment bacteriological Petri dishes of 15 mm height (iii) (**top panel, A – C**, Plate 1; **middle panel, D – F**, Plate 2) and one deep-form bacteriological Petri dish of 25 mm height (i) (**bottom panel, G – I**, Plate 2 indicates as NUNC), and the corresponding images (**left column, J – L**) acquired 20 minutes after adding the harvesting solution. Before starting with the dissolution procedure to harvest the cells, each plate was inspected under the microscope and imaged at 10x (**A, D, G**), 20x (**B, E, H**), and 40x (**C, F, I**) magnification. When the microcarrier dissolution procedure was completed, each plate was inspected again under the microscope and imaged at 40x magnification (**left column, J – L**). For each plate, harvested cells were counted using the Trypan Blue exclusion assay. For Plate 1 and 2 of the ultra-low attachment bacteriological Petri dishes of 15 mm height (iii), the numbers of cells were respectively $28.86 \cdot 10^6$ and $27.60 \cdot 10^6$ while for the deep-form bacteriological Petri dish of 25 mm height (i) indicated as NUNC Plate 2 in **Fig. 14** resulted as $14.76 \cdot 10^6$. This lower number was due to an error in aspirating the culture medium on day 1 when microcarriers were barely visible, as previously addressed.

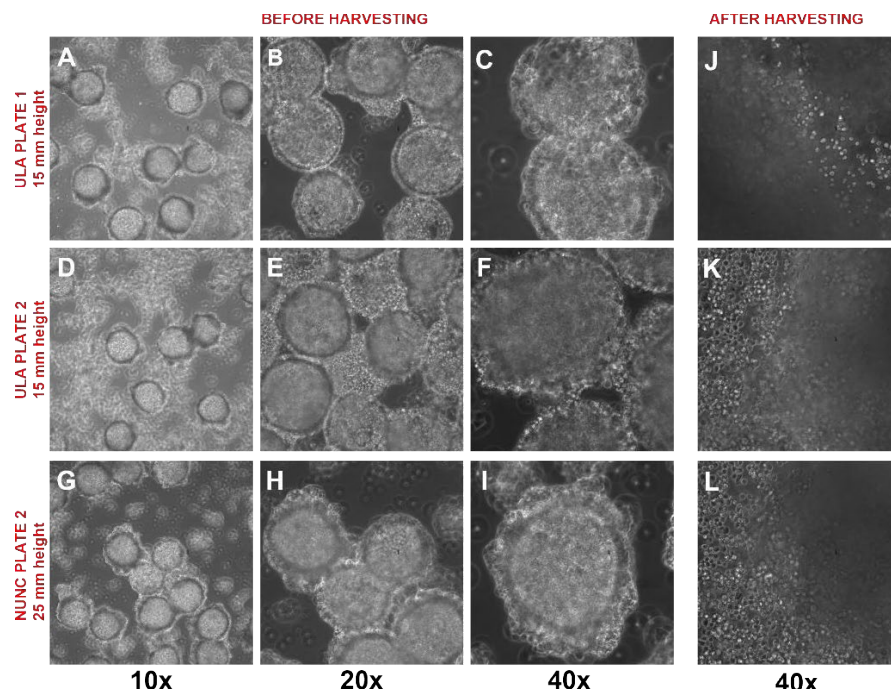


Figure 14. Right panel: Representative images acquired before harvesting the growing cells on dissolvable microcarriers in both ultra-low attachment bacteriological Petri dishes of 15 mm height (iii) (**top panel, A – C, Plate 1; middle panel, D – F, Plate 2**) and in one deep-form bacteriological Petri dish of 25 mm height (i), indicated as NUNC Plate 2 (**bottom panel, G – I**). **Left panel:** Images acquired 20 min after adding the harvested solution showing the microcarriers dissolution for each plate (**J – L**). During the dissolution procedure microcarriers were settled using the centrifuge for 1 min.

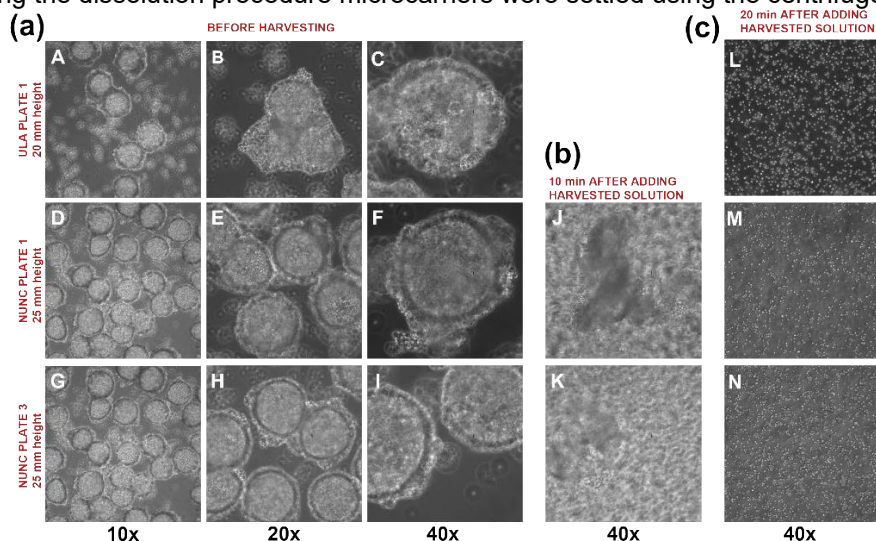


Figure 15. (a): Representative images acquired before dissolving the microcarriers in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) (**A – C**) and in the deep-form bacteriological Petri dishes of 25 mm height (i), (**middle panel; D – F, NUNC Plate 1; bottom panel, J – I, NUNC Plate 3**). **(b):** Images acquired 10 min after adding the harvested solution showing the dissolution state of microcarriers for NUNC Plate 1 (**J**) and NUNC Plate 3 (**K**). **(c):** Images acquired after adding the harvesting solution showing the microcarriers dissolution for each plate (**J – L**). During the dissolution procedure the microcarriers were settled by waiting for 5 up 10 min.

Fig. 15 (a) shows growing cells on dissolvable microcarriers at day 5 before dissolving the microcarriers. Images were acquired in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii), indicated as ULA Plate 1 (**top panel, A – C**) and in the deep-form bacteriological Petri dishes of 25 mm height (i) (**middle panel, D – F, NUNC Plate 1; bottom panel, G – I, NUNC Plate 3**) with 10x (**A, D, G**), 20x (**B, E, H**), and 40x (**C, F, I**) magnification. In **Fig. 15 (b)**, images show the dissolution state of microcarriers in NUNC plate 1 (**J**) and NUNC plate 3 (**K**) 10 minutes after adding the harvested solution. **Fig. 15 (c)**, show the complete dissolution of microcarriers in ULA Plate 1 (**L**), NUNC Plate 1 (**M**) and NUNC Plate 3 (**N**) 20 min after adding

the harvested solution. Images in Fig. 15 (b) and (c) were acquired with 40x magnification. The number of live cells harvested in the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) was $19.14 \cdot 10^6$. Cells harvested in both remaining deep-form bacteriological Petri dishes of 25 mm height (i), indicated as NUNC, were $24.46 \cdot 10^6$ and $22.47 \cdot 10^6$ for Plate 1 and 3, respectively.

Cell attachment on dissolvable microcarriers (response to pipetting)

To verify the solid attachment of RAW 264.7 cells on the dissolvable microcarriers, a sample of 2 ml growing cells on microcarriers was obtained from the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) on day 5. Microcarriers were manually stimulated by pipetting with increasing strength from gently to very strong. After applying increasing strength, the sample was inspected under the microscope and imaged with 20x and 40x magnification, as shown in **Fig. 16**. Following an increased strength in the manual stimulation (pipetting), RAW 264.7 cells continued to be attached to the dissolvable microcarriers, even after applying a very strong stimulation (**Fig.16**). This indicates that RAW 264.7 cells are expected to remain attached to the dissolvable microcarriers during the loading into the perfusion system and subsequently continuously growing on them to allow the MRS monitoring of their metabolism, with and without treatment with DFP or other drugs.

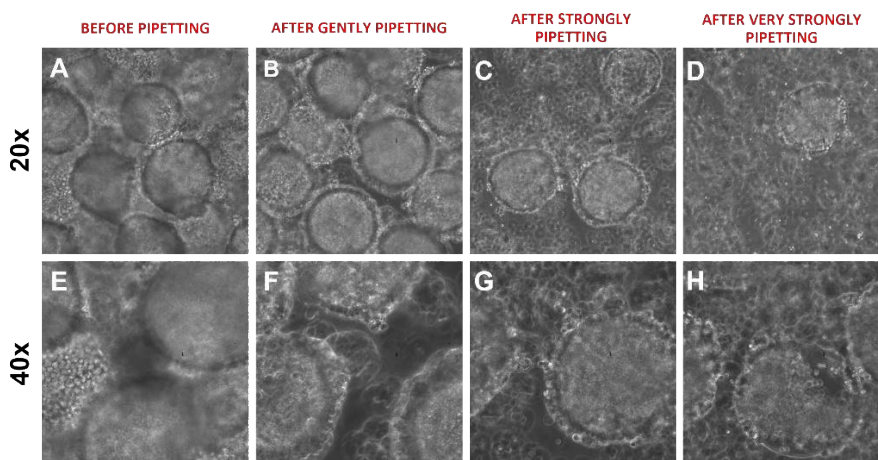


Figure 16. Representative images acquired on a sample of dissolvable microcarriers (2 ml) obtained from the ultra-low attachment bacteriological Petri dish of 20 mm height (ii) 5 days after cell seeding. Images were acquired before manually stimulating the microcarriers (**A, E**) and immediately after gently pipetting (**B, F**), strongly pipetting (**C, G**) and very strongly pipetting (**D, H**). For each different strength, images were acquired with 20x (top panel, **A - D**) and 40x (bottom panel, **E - H**) magnification.

Final considerations

As shown from the previous images, dissolvable microcarriers are appropriate for growing the RAW 264.7 cells and monitoring them with ^{31}P and ^{13}C MR spectroscopy using our MR-compatible bioreactor. As shown from **Fig. 16**, RAW 264.7 cells remain attached to the dissolvable microcarriers also after a very strong manual stimulation, indicating that they can very likely remain attached to the dissolvable microcarriers when loaded into the perfusion system and grow on them during the subsequent monitoring of their metabolism by MRS.

Fig. 3 and **Fig. 4** further show that it might not be necessary to change the medium 24 hours after the seeding. In addition, images acquired on the ultra-low attachment plates (ii; iii) (**Fig. 6, 8, 10, 11, 13**) compared with those acquired on the deep-form bacteriological Petri dishes (i) (**Fig. 5, 7, 9, 12**) do not show substantial differences. Therefore, the use of the deep-form bacteriological Petri dishes of 25 mm height (i) appear more suitable, as their height eases their handling. Finally, **Fig. 12** and **Fig. 13** show that day 4 (96 hours following cell seeding) might be the best day for loading the cells on the perfusion system.

Section 8: Aim 1: Effect of DFP on Oxygen Consumption of Breast Tumor Cells and Macrophages (Koutcher and Merghoub/Blasberg laboratories). Presented in 2021 report

The focus of oxygen consumption rate (OCR) measurements has been to measure the effect of DFP on triple negative breast tumor (TNBC) cells, specifically the effect of DFP on basal and maximal respiration as a measure of oxygen utilization and its possible relation to metabolism at the cellular level. As macrophages are an important part of the tumor stroma that play a significant role in tumor progression and regression, OCR measurements of a macrophage cell line were included in these experiments. In an earlier report, we presented data on these studies but only had an n=3 and had focused on optimization and normalization experiments. We now (2021) present data with n=5 independent studies for MDA-231 (human TNBC), 4T1 (murine TNBC) as well as unpolarized and polarized RAW 264.7 cells (murine macrophages). Initially, we summarize our methodology for the convenience of the reviewers before summarizing the results.

Methods

We evaluated the mitochondrial response to Deferiprone (DFP), an iron chelator, in 2 triple negative breast cancer cell lines, the human MDA-MB-231 and murine 4T1, as well as a macrophage cell line RAW 264.7, polarized (M1, M2) and unpolarized (M0). We measured the oxygen consumption rate (OCR) using the Seahorse XF Cell Mito Stress Test Kit (Agilent, Santa Clara, CA) on a Seahorse XFe96 flux analyzer (Agilent), following the manufacturer's instructions. This assay assesses mitochondrial function by measuring in live cells the effects of the sequential exposure to Oligomycin (Oligo; inhibition of ATP synthase), Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; protonophore, uncoupler of mitochondrial oxidative phosphorylation) and Rotenone & Antimycin A (Rot/AA; inhibition of mitochondrial complexes III & I respectively) on cellular OCR (Fig. 1A). From the OCR measurements, 8 quantitative parameters are derived (Fig. 1A): Non-mitochondrial oxygen consumption (Non-Mito OC), Basal Respiration (Basal Resp), Proton Leak, ATP Production (ATP Prod), Maximal Respiration (Max Resp), Spare Respiratory Capacity (SRC), % Spare Respiratory Capacity (% SRC), and % Coupling Efficiency (% CE).

All cell lines were cultured at 37 °C in 5% CO₂ in a humidified incubator using high-glucose Dulbecco's modified essential medium (DMEM HG), supplemented with 10% fetal calf serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM glutamine, bringing the final glutamine concentration to 6 mM glutamine. Based on previous data (1), a 48 h exposure time to 100 µM DFP (~EC₉₀ for most cell lines) was chosen. Cells were seeded 1 day prior to DFP exposure on a Seahorse XF96 V3 PS Cell Culture Microplate (Agilent), specific plates facilitating the xFe96 analyzer measurements.

In the 2020 report, we summarized preliminary pilot experiments to optimize cell seeding densities and assay acquisition parameters. Final cell seeding numbers / well were 40k, 6k, 1.7k, and 7k for MDA-MB-231, RAW 264.7, untreated 4T1, and DFP-treated 4T1 respectively. An additional group of untreated, unpolarized RAW 264.7 at 4k seeding density was included to confirm that the observation of the lack of cell seeding density effect in RAW 264.7 cells, due to cell density saturation at time of the flux analyzer measurements for seeding densities above 6k cells, was not further seen. The RAW 264.7 cells were polarized to M2 by exposure to 20 ng/ml IL-4 for 48 h and to M1 by exposure to 100 ng/ml LPS for 48 h (>12 h exposure time for IL-4 or LPS are needed for significant polarization (data not shown)). The optimized acquisition parameters were 3 repeat measurements (cycles) / assay injection stage (Oligo, FCCP, and Rot/AA respectively), a 3 min mixing time and a 2 min measurement/cycle.

After the XFe96 flux analyzer measurements, the total cell number / well and the total cell number in the sensor area of the Seahorse flux analyzer measurements (centered ~55% of total well area) as well as total protein / well were measured to facilitate normalization of the OCR measurements in each single well. Well images depicting the cell layer (Fig. 1B) were acquired by using the direct cell counting protocol on a Celigo Imaging Cytometer (Nexcelom Bioscience, Lawrence, MA) with a customized Seahorse XFe96 plate layout. Total cell numbers for each entire well and the sensor area (area between the posts in the well) were obtained by adjusting the analysis parameters for each cell line separately, due to their differences in contrast and cell shape (Fig. 1B). Of note is that 4T1 cells have a tendency, when confluent, to come off and fold over at well edges, potentially confounding the cell count. Thus, counting cells in the sensor area only versus the entire well area alleviates the impact of an inhomogeneous cell distribution on the OCR curve normalization. Here, this was overall less of an issue, as cells were – by design – all at high confluence (>80%) at the time of the Seahorse analyzer experiment. Total protein, historically considered the gold standard for normalization of

OCR curves, was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) as described previously (1,2). Total protein is assumed to be directly proportional to cell number. However, this might not be true for all cell lines and might differ between cells lines, as well as be affected by treatments. Hence, we also evaluated the normalization by cell number. As the sensor of the sensor plate covers only ~55% of the well area, normalizing the OCR to the cell number in that area would reflect most closely the true OCR value / cell, while normalization to total cell number / well allows the comparison to the total protein measurements and how well total protein and total cell number are related. Data are presented using the sensor region for normalization although data were analyzed by all three methods

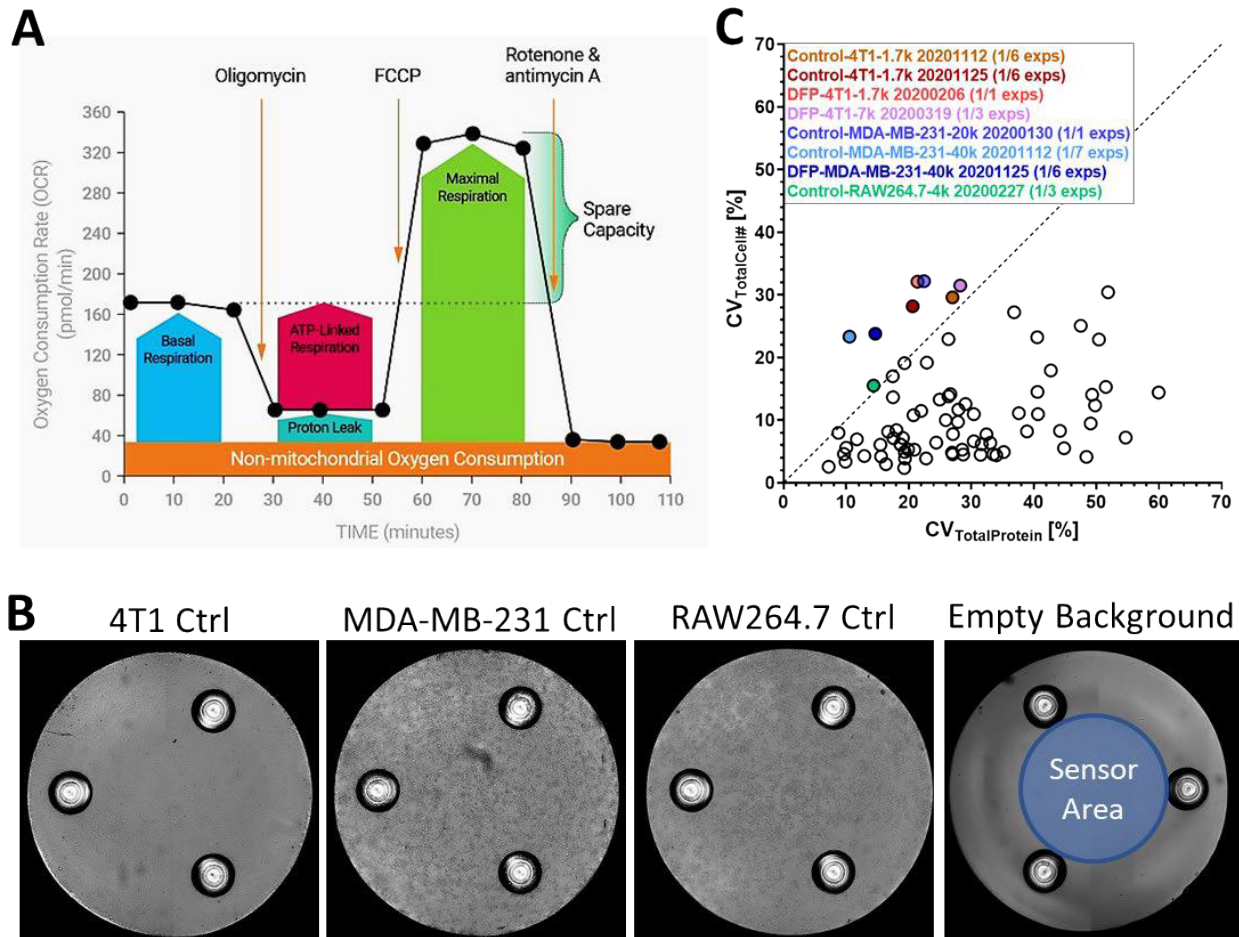


Figure 1: (A) Scheme of Cell Mito Stress Test and associated quantitative parameters calculated from each stage. The minimum OCR after Rot/AA injection delivers Non-Mito OC; the last rate measurement before Oligomycin injection is used to calculate the Basal Resp; the minimum OCR measurement after Oligo injection and maximum OCR measurement after FCCP injection are respectively used to calculate the remaining quantitative parameters. (B) Representative brightfield images of single wells with untreated cells of each cell type and an empty well. The 4 empty corner wells on the Seahorse plate serve as background controls for the Seahorse assay. The sensor area is a circular area within the three posts in each well. The differing contrast, cell density and growth homogeneity for each cell line can be discerned. (C) Total Protein measurements are overall more variable than Total Cell Counts. Each circle represents one experimental condition at one independent experiment with data derived from a total of 27 experimental conditions over 7 independent (separate) experiments, leading to a total of 78 different cohorts. Only 8 of the 78 cohorts (marked in color) have a smaller CV for Total Protein than for Total Cell Number. Only 3 of 78 cohorts had a similar (within < 1.5%) CV for Total Protein and Total Cell Number. (Data from 2 of 3 independent optimization experiments are included in this analysis. One optimization experiment had no cell count data, due to the Celigo imager being under repair.)

The Seahorse data were analyzed using the Wave 2 software (Agilent). For all wells, the OCR curves were visually inspected for experimental issues, such as defective loading of Oligo, FCCP, or Rot/AA and wells with loading issues at any of the 3 injection stages were excluded from further analysis. Some experiments had empty background wells, leading to unreliable background correction and distortion of Seahorse analyzer curves. We found that while the background correction had a large impact on the curves with improperly treated, i.e. empty, background wells, the impact on the curves, and thus, the derived quantitative parameters

was marginal in experiments with properly treated (non-empty) background wells. Hence, we analyzed the Seahorse analyzer curves for all experiments without the inbuilt background correction.

The OCR curves normalized by the three different normalizations and resulting 8 quantitative parameters were exported for further graphing and statistical analysis in GraphPad Prism 9 (GraphPad Software, LLC, San Diego, CA).

Statistical Analyses:

The statistical analysis was done separately for each normalization, i.e. data based on protein, total well cell counts and sensor area cell counts but we only present data based on sensor area cell number normalization.

To obtain for each quantitative parameter and each group the error of the mean averaged over all experiments, we propagated the SD of the mean from single experiments using the Gaussian error propagation, i.e. the square root of the sum of the squared single experiment SDs.

How TNBC cell type, macrophage polarization and/or exposure to DFP lead to significant differences in mitochondrial quantitative parameters between various groups was addressed by statistical comparisons. While a non-parametric analysis using the Friedman Test with Dunn's multiple comparison is the most rigorous statistical analysis, it significantly reduces the power to detect differences for comparably few (here 5) independent experimental repeats. As in each single experiment, there were anywhere from 4-10 (mostly 8) replicate wells for each of the 11 groups with very few outliers, and we repeated the experiment 5 times, we are confident of the intra- and interexperimental repeatability. Thus, we used a two-tailed, 1-way ANOVA with Geisser Greenhouse correction (intra-experimental group means paired by experiment date) followed by Bonferroni post hoc analysis to assess statistically significant differences of each mitochondrial quantitative parameter between groups.

Results and Discussion

As shown in last year's report (2020) and updated with the additional experiment for a total of $n = 5$ independent experiments (2021 report), we compared 3 different normalizations of OCR curves and derived quantitative parameters: OCR curves normalized to (i) Total Protein, (ii) Total Cell Number, and (iii) Total Cell Number in Sensor Area. In agreement with last year's data, we found that total protein does not relate very well to total cell number at high density cultures (data not shown). Additionally, the total protein measurements had a higher coefficient of variation than the total cell number measurements (Fig. 1C). While the results across the different normalizations are comparable (data not shown), we will present the detailed results based on the normalization to the total cell number in the sensor area for the purpose of this report, as it most closely reflects the true OCR parameter for the cells

As each experiment contained 11 different groups, there are many potential comparisons. While including data for all quantitative parameters, we concentrate on the results for changes in basal and maximal respiration which were the focus of the initial application. We include tables with P values for each comparison grouping covering all quantitative parameters.

Figure 2 shows a typical result of normalized Seahorse analyzer curves from a single experiment for the 11 groups. For 4T1 and MDA-MB-231 cells, the normalized OCRs in the Rot/AA stage are similar between the respective untreated and DFP-exposed cells. That means that any differences visible in the BL (1st) stage of the curve can be attributed to differences in basal respiration, while the lowest values in the Oligo stage reflect H⁺ Leak and ATP Prod changes, and the highest values in the FCCP stage reflect directly changes in maximal respiration and corresponding SRC. In RAW 264.7 cells, DFP leads to a lowering of the non-Mito OC in M1-polarized cells, while polarization per se and DFP exposure of M0 and M2 RAW 264.7 cells lead to only modest variations of the non-Mito OC as reflected in the Rot/AA stage of the curves.

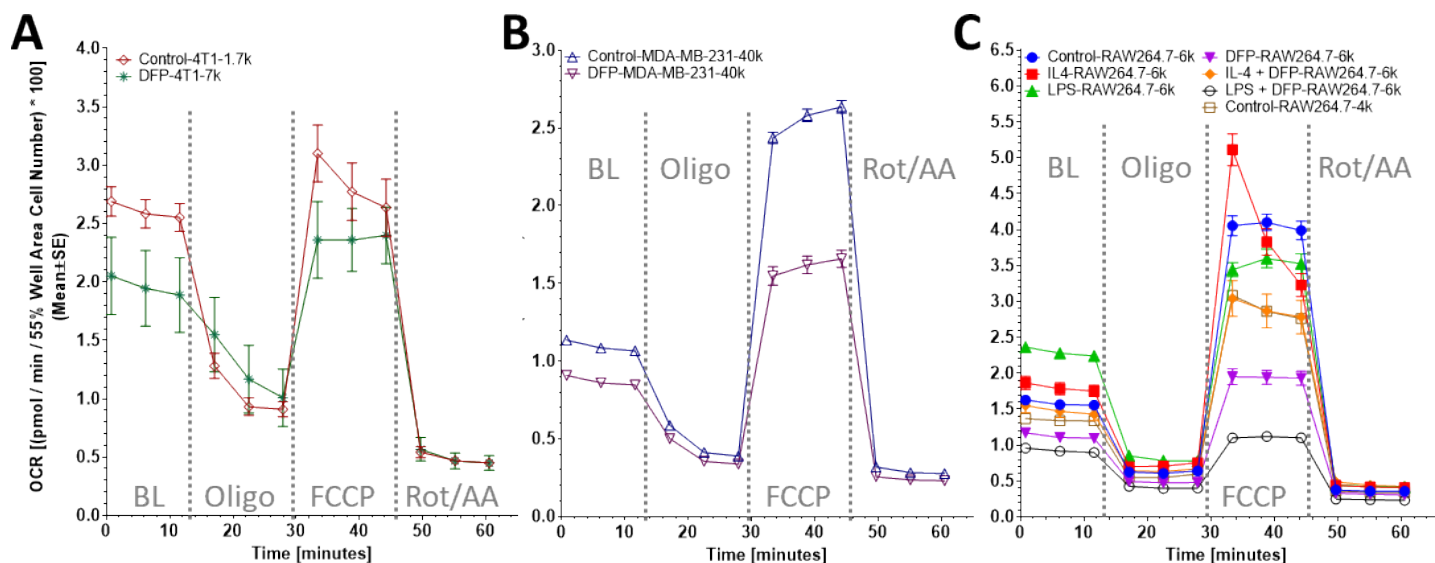


Figure 2: Oxygen Consumption Rates (OCRs) normalized to cell number in the sensor area of each well for either untreated or DFP-treated (A) murine TNBC cells 4T1 (n=10 Control; n=5 DFP), (B) human TNBC cells MDA-MB-231 (n=8 Control; n=8 DFP), and (C) unpolarized and polarized RAW 264.7 cells (n=8 control, except n=7 for LPS-RAW264.7; n=8 DFP). (C) also includes a RAW 264.7 cell number control. Representative data from 1 of 5 independent experiments.

The repeatability of the quantitative parameters within and across independent experiments, i.e. the intra- and inter-experimental variability, is demonstrated representatively for all 11 groups by basal respiration in Figure 3. For sensor area cell number normalization, group membership accounted for ~26.5% to ~58% of the Total Variance while experiment date which accounted between ~1% to ~7.4% of Total Variance across all 8 quantitative mitochondrial parameters (2-way ANOVA with Tukey multiple comparison test). Also, an interaction between groups and experiment date contributed significantly to the % Total Variance. Similarly, group membership, experiment date and their interaction were significant sources of variation for each quantitative parameter obtained from either total protein or total cell number normalization. Thus, all statistical analyses of quantitative parameters (and curves) were performed paired to experiment date. Excepting SRC and % SRC, intra-experimental variability increased for all other mitochondrial quantitative parameters with decreasing cell doubling time. That is MDA-MB231 cells with the longest cell doubling time had the lowest intraexperimental variability followed by RAW 264.7 cells and 4T1 cells as the cell doubling time decreases (Figure 3).

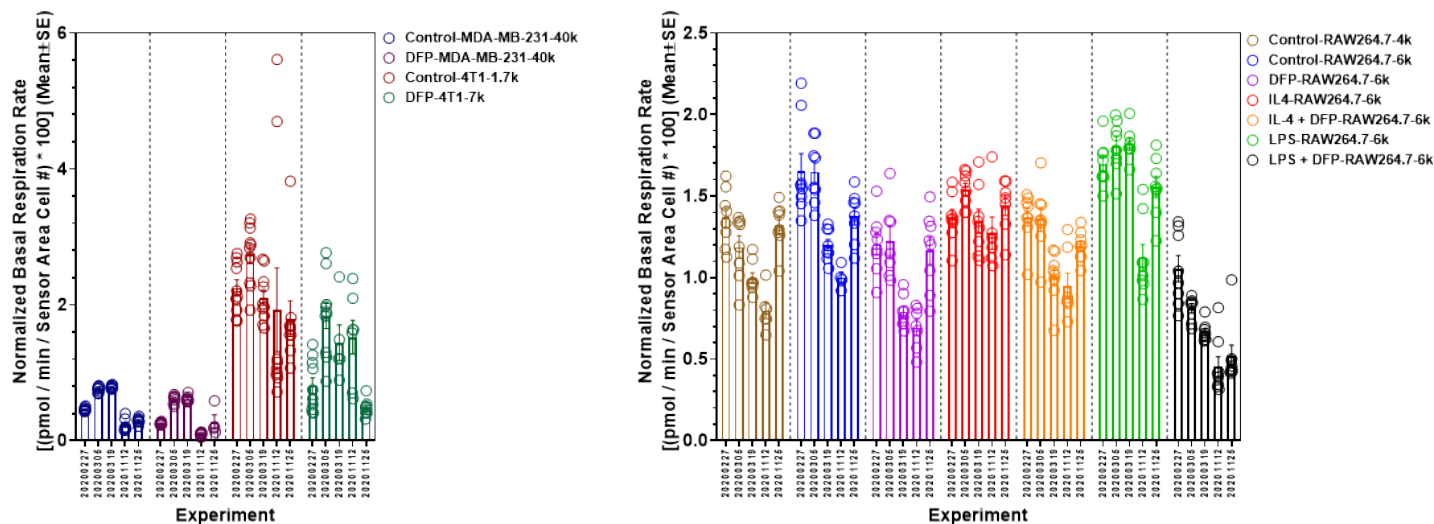


Figure 3: Inter- and intra-experimental variability of basal respiration of the 2 TNBC lines, untreated or DFP-treated (left panel) as well as unpolarized (M0), M1 (by LPS)- and M2 (by IL-4)-polarized RAW 264.7 cells. The bars display the mean±SE of separate experiments, while the open circles represent single wells within each experiment.

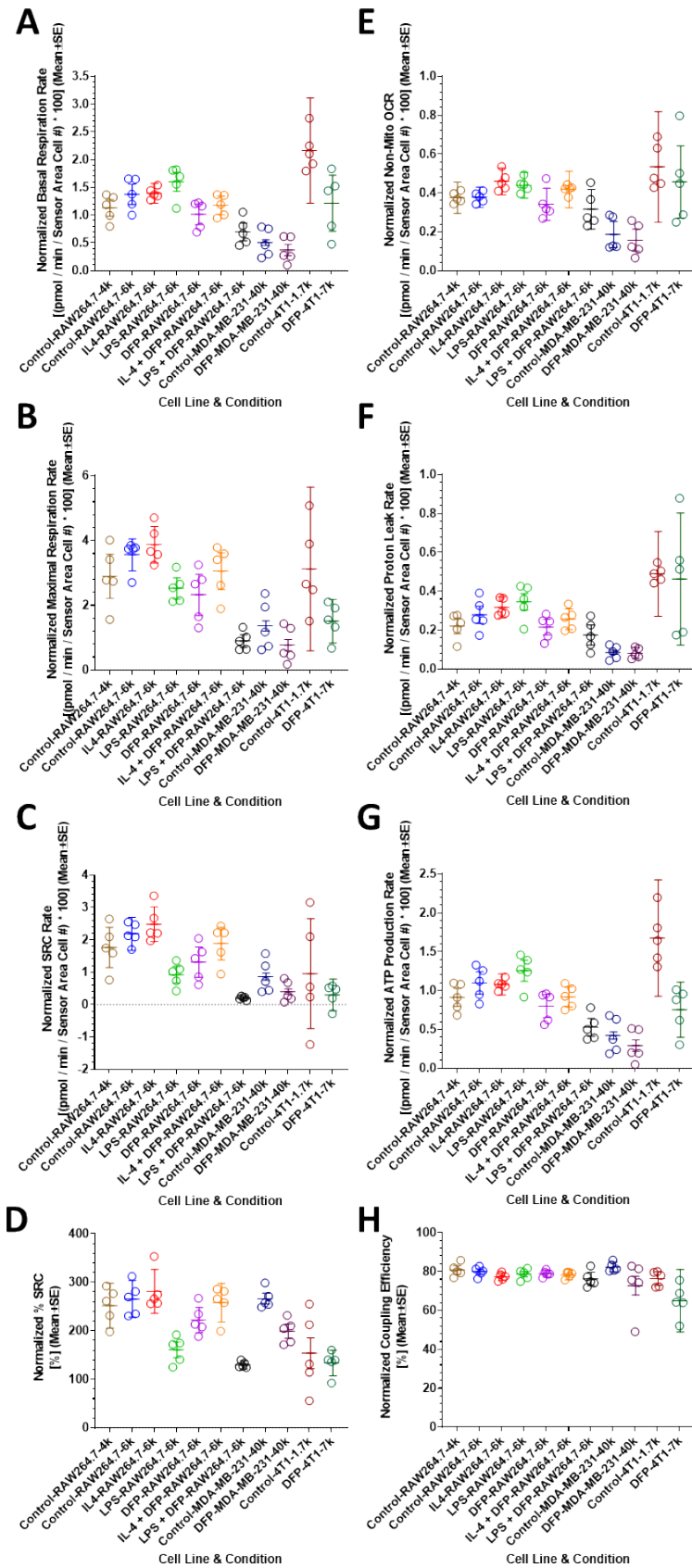


Figure 4: Quantitative mitochondrial parameters for the 11 experimental groups. **A-H** display single experiment means (open circles, $n = 5$) overlaid with the overall mean $\pm$ SE for Basal Resp, Max Resp, SRC, % SRC, Non-Mito OC, H^+ Leak, ATP Prod, and % CE respectively. Overall SE was calculated by propagating the SD error of the means from each single experiment.

The summary graphs for the 8 quantitative mitochondrial parameters based on sensor area cell number normalization are shown for all experimental groups in Figure 4.

Table 1-5 list the P Values for the group comparisons based on various scientific inquiries, i.e. A) The comparison of 2 different TNBC cell types in the presence and absence of DFP, B) the effect of DFP on cancer cells or macrophages, C) the effect of polarization of macrophages in the presence and absence of DFP and D) the differences between unpolarized macrophages and TNBC cell lines in the absence and presence of DFP.

We found that human versus murine TNBC cells have very different mitochondrial parameters: Basal respiration (Fig. 4A), NonMito OC (Fig. 4E), H^+ Leak (Fig. 4F), and ATP Production (Fig. 4G) are significantly higher, with % Coupling Efficiency (Fig. 4H) significantly lower in untreated 4T1 than untreated MDA-MB-231 cells (Table 1, 2).

In DFP-exposed TNBC cells, the differences between 4T1 and MDA-MB-231 are reduced for basal respiration (Fig. 4A), H^+ Leak (Fig. 4F) and ATP Production (Fig. 4G), while % SRC (Fig. 4D) and NonMito OC (Fig. 4E) are respectively significantly lower and higher in DFP-treated 4T1 than DFP-treated MDA-MB-231 cells (Table 1, 2). This is consistent with the observed prolongation of the cell doubling time, i.e. reduced cell growth, in response to DFP.

DFP exposure of 4T1 cells reduces significantly their basal respiration and ATP production (Fig. 4 A, G and Table 1, 2). In MDA-MB-231 cells, DFP reduces significantly basal respiration, maximal respiration, ATP Production, SRC and %SRC (Figure 4, Table 1, 2).

These differences in mitochondrial parameter between the 2 TNBC cell lines and their response to DFP exposure are consistent with their differences in growth rates as well as their respective growth rate reductions in response to DFP. We hypothesize that a higher aerobic glycolysis in 4T1 cells than MDA-MB-231 cells contributes to these differences as well.

The observed TNBC DFP response agrees with our previous observation in 3 prostate

cancer lines, where a 24 h exposure to DFP significantly diminished Basal Resp, Max Resp, H⁺ Leak (in 2 of 3 cell lines), ATP Prod (in 2 of 3 cell lines), and SRC (in 2 of 3 cell lines), while not affecting NonMitoOC [9].

Table 1: Statistical analysis to assess the differences of NonMito OC, Basal Resp, and Max Resp between the human and murine TNBC line and the effects of DFP exposure on either. Uncorrected P values (Fisher's LSD test) color coded for Bonferroni correction, based on multiple comparison group size n to achieve 95% significance: n = 1, **P ≤ 0.05**; n = 2, **P ≤ 0.025**; n = 3, **P ≤ 0.0167**; n = 4, **P ≤ 0.0125**; n = 5, **P ≤ 0.01**.

TNBC Cell Line Differences in the Presence and Absence of DFP			
	NonMito OC	Basal Resp	Max Resp
Control-4T1-1.7k vs. Control-MDA-MB-231-40k	0.0055	0.0001	0.1245
DFP-4T1-7k vs. DFP-MDA-MB-231-40k	0.0199	0.0401	0.0722
Effect of 48 h DFP Exposure on a Human and a Murine TNBC Cell Line			
	NonMito OC	Basal Resp	Max Resp
DFP-4T1-7k vs. Control-4T1-1.7k	0.3436	0.0083	0.0652
DFP-MDA-MB-231-40k vs. Control-MDA-MB-231-40k	0.0531	0.0125	0.0057

Table 2: Statistical analysis to assess the differences of H⁺ Leak, ATP Prod, SRC, % SRC and % CE between the human and murine TNBC line and the effects of DFP exposure on either. Uncorrected P values (Fisher's LSD test) color coded for Bonferroni correction, based on multiple comparison group size n to achieve 95% significance: n = 1, **P ≤ 0.05**; n = 2, **P ≤ 0.025**; n = 3, **P ≤ 0.0167**; n = 4, **P ≤ 0.0125**; n = 5, **P ≤ 0.01**.

TNBC Cell Line Differences in the Presence and Absence of DFP					
	H ⁺ Leak	ATP Prod	SRC	% SRC	% CE
Control-4T1-1.7k vs. Control-MDA-MB-231-40k	<0.0001	0.0004	0.9213	0.0373	0.0215
DFP-4T1-7k vs. DFP-MDA-MB-231-40k	0.0359	0.0294	0.6998	0.0199	0.4104
Effect of 48 h DFP Exposure on a Human and a Murine TNBC Cell Line					
	H ⁺ Leak	ATP Prod	SRC	% SRC	% CE
DFP-4T1-7k vs. Control-4T1-1.7k	0.8347	0.0038	0.3798	0.557	0.0619
DFP-MDA-MB-231-40k vs. Control-MDA-MB-231-40k	0.6243	0.0065	0.0063	0.0015	0.1641

The mitochondrial parameter values for unpolarized and polarized RAW 264.7 cells tend to fall in between the corresponding parameter values observed for the TNBC cell lines (Fig. 4).

In RAW 264.7 cells, DFP exposure significantly reduces basal respiration and ATP production, independent of polarization, while maximal respiration, proton leak, and SRC were significantly affected in M0 and M1- polarized RAW 264.7 cells (Figure 4, Table 3, 4). M1 (by LPS)-polarized RAW 264.7 cells were the most sensitive to DFP and displayed significantly reduced NonMito OC in response to DFP. This sensitivity to DFP parallels the observation in Section 1 of this report where M1-polarized RAW 264.7 cells release the highest phagocytosis and release the highest amount of NO in response to DFP (Section 1, Figure 2). Of note is also that not all RAW 264.7 cells are polarized in cultures exposed to 48 h of IL-4 and LPS respectively. As described in Section 1, Figure 1 of this report, approximately 60-70% of untreated and DFP-treated RAW 264.7 cells are M2 polarized, while ~26% in untreated and ~37% in DFP-treated RAW 264.7 are M1 polarized as a result of exposure to IL-4 and LPS respectively.

Exposure of the 2 TNBC lines as well as unpolarized and M2-polarized RAW 264.7 cells to 100 μM DFP, did not significantly affect NonMito OC (Fig. 4B, Table 1, 3), consistent with the iron chelation effect on mitochondrial, iron-dependent enzymes and corresponding effect on cell proliferation (see also Section 2, Figure 2. (2).

The M1 (LPS) polarization of RAW 264.7 cells reduced maximal respiration, SRC, and % SRC in the presence and absence of DFP, with basal respiration and ATP production lower in the presence and not absence of DFP, compared to unpolarized and M2-polarized RAW 264.7 cells (Fig. 4, Table 3, 4). In the presence of DFP,

M2 (by IL-4) polarization of RAW 264.7 cells decreases the basal respiration, maximal respiration, proton leak, ATP Production, and SRC compared to unpolarized RAW 264.7 cells (Fig. 4, Table 3, 4). These observations are consistent with the findings by others that M1 macrophages typically feature increased pentose phosphate pathway and glycolytic fluxes, supplemented by a dysfunctional Krebs (TCA) cycle (3,4). Due to the impaired reactions catalyzed by isocitrate dehydrogenase and succinate dehydrogenase, citrate and succinate accumulate in M1 macrophages (3,4). The higher ATP production of M1 RAW 264.7 cells in the absence of DFP compared to M0- and M2-polarized RAW 264.7 cells is reflective of ATP preferentially derived from glucose. The higher reliance of M0 and M2 macrophages on the Krebs cycle agrees with their higher Max Resp, SRC, and % SRC compared to M1 macrophages. The lower % SRC (Max Resp / Basal Resp) in M1 than in M2 macrophages might be the result of their reliance on the faster glycolysis and some modest increase in OXPHOS, similar to what has been observed in other studies(5) [13]. Taken together, this would also explain why M1 macrophages are more strongly impacted by DFP which inhibits aconitase in the TCA cycle and results in further accumulation of citrate and inhibition of TCA cycle, similar to our observation in prostate cancer (1).

Table 3: Statistical analysis to assess the effects of DFP on NonMito OC, Basal Resp, and Max Resp of unpolarized and polarized macrophages as well as the effects of macrophage polarization in the presence and absence of DFP. Uncorrected p values (Fisher's LSD test) color coded for Bonferroni correction, based on multiple comparison group size to achieve 95% significance: n =1, **P ≤ 0.05**; n = 2, **P ≤ 0.025**; n = 3, **P ≤ 0.0167**; n = 4, **P ≤ 0.0125**; n = 5, **P ≤ 0.01**.

Effect of 48 h DFP Exposure on Unpolarized and Polarized Macrophages			
	NonMito OC	Basal Resp	Max Resp
DFP-RAW264.7-6k vs. Control-RAW264.7-6k	0.3349	0.0012	0.0091
LPS + DFP-RAW264.7-6k vs. LPS-RAW264.7-6k (M1)	0.0144	0.001	0.0031
IL-4 + DFP-RAW264.7-6k vs. IL4-RAW264.7-6k (M2)	0.1215	0.0242	0.1043
Effect of Macrophage Polarization in the Absence of DFP			
	NonMito OC	Basal Resp	Max Resp
IL4-RAW264.7-6k vs. Control-RAW264.7-6k	0.0565	0.8622	0.2257
LPS-RAW264.7-6k vs. Control-RAW264.7-6k	0.0529	0.0919	0.0075
LPS-RAW264.7-6k vs. IL4-RAW264.7-6k	0.3633	0.1255	0.0004
Effect of Macrophage Polarization in the Presence of DFP			
	NonMito OC	Basal Resp	Max Resp
IL-4 + DFP-RAW264.7-6k vs. DFP-RAW264.7-6k	0.0693	0.0148	0.0067
LPS + DFP-RAW264.7-6k vs. DFP-RAW264.7-6k	0.1973	0.0321	0.0092
LPS + DFP-RAW264.7-6k vs. IL-4 + DFP-RAW264.7-6k	0.048	0.0021	0.0018

Table 4: Statistical analysis to assess the effects of DFP on H⁺ Leak, ATP Prod, SRC, and % SRC of unpolarized and polarized macrophages as well as the effects of macrophage polarization in the presence and absence of DFP. Uncorrected p values (Fisher's LSD test) color coded for Bonferroni correction, based on multiple comparison group size to achieve 95% significance: n =1, **P ≤ 0.05**; n = 2, **P ≤ 0.025**; n = 3, **P ≤ 0.0167**; n = 4, **P ≤ 0.0125**; n = 5, **P ≤ 0.01**.

Effect of 48 h DFP Exposure on Unpolarized and Polarized Macrophages					
	H ⁺ Leak	ATP Prod	SRC	% SRC	% CE
DFP-RAW264.7-6k vs. Control-RAW264.7-6k	0.0221	0.0005	0.0306	0.146	0.1204
LPS + DFP-RAW264.7-6k vs. LPS-RAW264.7-6k	0.0003	0.0013	0.0142	0.0397	0.1201
IL-4 + DFP-RAW264.7-6k vs. IL4-RAW264.7-6k	0.0352	0.0243	0.1552	0.368	0.1646
Effect of Macrophage Polarization in the Absence of DFP					

	H ⁺ Leak	ATP Prod	SRC	% SRC	% CE
IL4-RAW264.7-6k vs. Control-RAW264.7-6k	0.3185	0.7923	0.1782	0.3388	0.1187
LPS-RAW264.7-6k vs. Control-RAW264.7-6k	0.0173	0.1686	0.0015	0.0005	0.3019
LPS-RAW264.7-6k vs. IL4-RAW264.7-6k	0.5288	0.0793	0.002	0.0043	0.5009
Effect of Macrophage Polarization in the Presence of DFP					
	H ⁺ Leak	ATP Prod	SRC	% SRC	% CE
IL-4 + DFP-RAW264.7-6k vs. DFP-RAW264.7-6k	0.0073	0.023	0.0173	0.0528	0.3006
LPS + DFP-RAW264.7-6k vs. DFP-RAW264.7-6k	0.1954	0.0206	0.0119	0.0043	0.1021
LPS + DFP-RAW264.7-6k vs. IL-4 + DFP-RAW264.7-6k	0.0332	0.0008	0.0033	0.0022	0.2824

Unpolarized, untreated RAW 264.7 cells have a significantly lower basal respiration, proton leak, and ATP production than untreated 4T1 cells and a significantly higher basal respiration, proton leak, and ATP production than untreated MDA-MB-231 cells (Table 5). These differences are maintained in DFP exposed cells for MDA-MB-231 but not 4T1 cells (Fig. 4, Table 5). Maximal respiration and SRC (and to a lesser extent % SRC) are lower in untreated MDA-MB-231 cells than RAW 264.7 cells; a similar pattern is observed for DFP-treated cells (Fig. 4, Table 5). In the presence of DFP, unpolarized RAW 264.7 cells have a higher NonMito OC than MDA-MB-231 cells. In the presence of DFP, 4T1 cells have a lower % SRC and % CE than unpolarized RAW 264.7 cells Fig. 4, Table 5).

Table 5: Statistical analysis to assess the differences between unpolarized macrophages and TNBC cells in the presence and absence of DFP on NonMito OC, Basal Resp, Max Resp, H⁺ Leak, ATP Prod, SRC, % SRC, and % CE. Uncorrected p values (Fisher's LSD test) color coded for Bonferroni correction, based on multiple comparison group size to achieve 95% significance: n = 1, **P ≤ 0.05**; n = 2, **P ≤ 0.025**; n = 3, **P ≤ 0.0167**; n = 4, **P ≤ 0.0125**; n = 5, **P ≤ 0.01**.

Effect of Macrophage and TNBC Cell Line Differences in the Absence and Presence of DFP					
	NonMito OC	Basal Resp	Max Resp	H ⁺ Leak	
Control-4T1-1.7k vs. Control-RAW264.7-6k	0.0607	0.003	0.6101	0.0047	
Control-MDA-MB-231-40k vs. Control-RAW264.7-6k	0.0118	0.0031	0.0018	0.0019	
DFP-4T1-7k vs. DFP-RAW264.7-6k	0.2128	0.5492	0.2348	0.1359	
DFP-MDA-MB-231-40k vs. DFP-RAW264.7-6k	0.0027	0.0095	0.0253	0.0065	
Effect of Macrophage and TNBC Cell Line Differences in the Absence and Presence of DFP					
	ATP Prod	SRC	% SRC	% CE	
Control-4T1-1.7k vs. Control-RAW264.7-6k	0.0097	0.209	0.0243	0.2545	
Control-MDA-MB-231-40k vs. Control-RAW264.7-6k	0.0049	0.0025	0.9593	0.291	
DFP-4T1-7k vs. DFP-RAW264.7-6k	0.8237	0.0397	0.0024	0.0127	
DFP-MDA-MB-231-40k vs. DFP-RAW264.7-6k	0.012	0.0433	0.2482	0.4002	

In summary, cell type impacted the quantity of the 8 mitochondrial parameters with human TNBC cell line having typically the lowest values. DFP reduced basal respiration and maximal respiration (less so for 4T1) in the TNBC lines, consistent with our previous observations in prostate cancer cell lines. The results for the unpolarized and polarized RAW 264.7 cells reflect the induction of metabolic shifts between TCA cycle, glycolysis, and pentose phosphate pathway in response to intracellular iron chelation and/or macrophage polarization.

References

1. Simoes, R.V., et al., *Inhibition of prostate cancer proliferation by Deferiprone*. NMR Biomed, 2017. **30**(6).

2. Simoes, R.V., et al., *Metabolic plasticity of metastatic breast cancer cells: adaptation to changes in the microenvironment*. Neoplasia, 2015. **17**(8): p. 671-84.
3. Thapa, B. and K. Lee, *Metabolic influence on macrophage polarization and pathogenesis*. BMB Rep, 2019. **52**(6): p. 360-372.
4. Viola, A., et al., *The Metabolic Signature of Macrophage Responses*. Front Immunol, 2019. **10**: p. 1462.
5. Zuo, H. and Y. Wan, *Metabolic Reprogramming in Mitochondria of Myeloid Cells*. Cells, 2019. **9**(1).

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This was not a training grant so there is nothing to report. Nevertheless, in Year 1 this supported Dr. Leftin who went on to write his own peer reviewed funding based on experiments begun under the auspices of this proposal and subsequently joined the faculty at SUNY Stony Brook. He used his training on iron studies and metabolism to advance to a full-time tenure track position. Dr. Porcari has been working on this project and is a postdoctoral fellow and has gone to three conferences to present findings from this project and has been promoted to a more senior position in another laboratory. She has now been promoted although works in a different laboratory

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

1. Leftin A, Ben-Chetrit N, Klemm F, Joyce JA, Koutcher JA Iron imaging reveals tumor and metastasis macrophage hemosiderin deposits in breast cancer. .PLoS One. 2017 Sep 12;12(9):e0184765. doi: 10.1371/journal.pone.0184765
2. Leftin A, Koutcher JA. Quantification of Nanoparticle Enhancement in Polarized Breast Tumor Macrophage Deposits by Spatial Analysis of MRI and Histological Iron Contrast Using Computer Vision. Contrast Media Mol Imaging. 2018 Oct 30;2018:3526438. doi: 10.1155/2018/3526438
3. Leftin A, Ben-Chetrit N, Joyce JA, Koutcher JA. Imaging endogenous macrophage iron deposits reveals a metabolic biomarker of polarized tumor macrophage infiltration and response to CSF1R breast cancer immunotherapy. Sci Rep. 2019 Jan 29;9(1):857. doi: 10.1038/s41598-018-37408-7.
4. Porcari, P., **Ackerstaff, E.**, LeKaye, H.C., Koutcher, J.A. *Metabolic Effects of Deferiprone on Triple Negative Breast Cancer*. Digital Poster #1843, 29th Ann Mtg & Exhibit of the International Soc for Magn Reson in Med (ISMRM), ISMRM & SMRT, Virtual Conference & Exhibition, May 15 – 20, 2021.
5. Porcari P, Ackerstaff E, Winkleman DP, Veeraperumal S, Kruchevsky N, Lekaye HC, Koutcher JA. **The Role of Iron Chelation in the Tumour Microenvironment of Triple-Negative Breast Cancer**. 28th ISMRM Annual Meeting, Virtual Conference 2020, August 8-14. Oral Presentation, 0144. (selected for presentation at the **Cancer Imaging Highlights Sessions** of the ISMRM 2020).
6. **"Iron Chelation Affects Cell Growth and Metabolism in the Triple-negative Breast Cancer Cell Line 4T1"** Porcari P, LeKaye HC, Koutcher JA, Ackerstaff E. World Molecular Imaging Congress 2019, September 4-7, Montreal, Canada. Poster presentation, P300

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing further to report

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

In view of the fact that DFP is in clinical use, our most significant finding was that it enhanced immune response in the 4T1 murine TNBC, a tumor that is known to be immunologically "cold" (relatively inactive). In the spleen 1) DFP increased the percent of CD4 and CD8 effector T cells, 2) enhanced these cell numbers when used in combination with cisplatin, 3) decreased cells that expressed high level of the inhibitory PD-1 receptor, indicating a shift to a pro-inflammatory environment, and a decrease in immune suppressive cells. In both spleen and tumor, DFP induced an increase in the ability of CD4 and CD8 T cells to make both IFN γ or TNF α , a marker of polyfunctionality in T cells. DFP induced an increase in macrophage cells that made either IFN γ or TNF α . DFP enhanced the efficiency of phagocytosis by RAW 264.7 cells. This effect was significant in all the culture conditions (p = 0.0004 for Control, p = 0.0007 for IL-4, p = 0.0045 for LPS) but most prominent in the LPS-treated group. In DFP-exposed TNBC cells, the differences between 4T1 and MDA-MB-231 are reduced for basal respiration, H⁺ Leak, and ATP Production). DFP exposure of 4T1 cells reduces significantly their basal respiration and ATP production. In MDA- MB-231 cells, DFP reduces significantly basal respiration, maximal respiration, ATP Production, SRC and %SRC. These differences in mitochondrial parameter between the 2 TNBC cell lines and their response to DFP exposure are consistent with their differences in growth rates as well as their respective growth rate reductions in response to DFP. These data suggest possible implications for developing treatment with drugs that inhibit energy production and indicate that sensitivity may depend on growth rate. However, DFP was not active in vivo in the tumor models studied as opposed to some of our previous studies, although it approached significance in PD-1 inhibitor study

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

In year 2, we have shown that MRI can be used to measure and quantitate iron in vivo. This MAY go to clinical use (**Koutcher Lab**). Otherwise, nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We present a lengthy study to develop methods of keeping cells alive in a perfusion apparatus which maybe relevant to other investigations (study 4 above). We have also investigated an additional tumor model since our preliminary in vivo results were disappointing which in this report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

COVID-19 had a major impact. The laboratories were shut down for two months and a key person was stuck overseas. Fortunately, she had a computer and data to analyze but being away 6 months was a major loss to this project. Thus, the analysis of the metabolism studies on MDA-MB-231 which where the data were acquired in 12/19-3/20 were just completed and not truly digested in terms of biochemical significance. These data are being written up and a manuscript is almost complete. The major problem was that the tumor lines selected were not sensitive to DFP although DFP showed immunologic effects

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Loss of technician in year 2 for about 8 months allowed us to carry funds over to a no cost extension year. Gap in postdoctoral fellows due to recruiting an experienced person from overseas has also allowed carry over of funds which was necessary to complete the studies due to these delays.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No human subjects

Significant changes in use or care of vertebrate animals

No change in vertebrate animals

Significant changes in use of biohazards and/or select agents

None

6 PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
Report only the major publication(s) resulting from the work under this award.

1. Leftin A, Ben-Chetrit N, Klemm F, Joyce JA, Koutcher JA Iron imaging reveals tumor and metastasis macrophage hemosiderin deposits in breast cancer. PLoS One. 2017 Sep 12;12(9):e0184765. doi: 10.1371/journal.pone.0184765
2. Leftin A, Koutcher JA. Quantification of Nanoparticle Enhancement in Polarized Breast Tumor Macrophage Deposits by Spatial Analysis of MRI and Histological Iron Contrast Using Computer Vision. Contrast Media Mol Imaging. 2018 Oct 30;2018:3526438. doi: 10.1155/2018/3526438
3. Leftin A, Ben-Chetrit N, Joyce JA, Koutcher JA. Imaging endogenous macrophage iron deposits reveals a metabolic biomarker of polarized tumor macrophage infiltration and response to CSF1R breast cancer immunotherapy. Sci Rep. 2019 Jan 29;9(1):857. doi: 10.1038/s41598-018-37408-7.
4. Porcari, P., **Ackerstaff, E.**, LeKaye, H.C., Koutcher, J.A. Metabolic Effects of Deferiprone on Triple Negative Breast Cancer. Digital Poster #1843, 29th Ann Mtg & Exhibit of the International Soc for Magn Reson in Med (ISMRM), ISMRM & SMRT, Virtual Conference & Exhibition, May 15 – 20, 2021.
5. Porcari P, Ackerstaff E, Winkleman DP, Veeraperumal S, Kruchevsky N, Lekaye HC, Koutcher JA. **The Role of Iron Chelation in the Tumour Microenvironment of Triple-Negative Breast Cancer**. 28th ISMRM Annual Meeting, Virtual Conference 2020, August 8-14. Oral Presentation, 0144. (selected for presentation at the **Cancer Imaging Highlights Sessions** of the ISMRM 2020).
6. **3. “Iron Chelation Affects Cell Growth and Metabolism in the Triple-negative Breast Cancer Cell Line 4T1”** Porcari P, LeKaye HC, Koutcher JA, Ackerstaff E. World Molecular Imaging Congress 2019, September 4-7, Montreal, Canada. Poster presentation, P300

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Leftin A, Ben-Chetrit N, Klemm F, Joyce JA, Koutcher JA Iron imaging reveals tumor and metastasis macrophage hemosiderin deposits in breast cancer. PLoS One. 2017 Sep 12;12(9):e0184765. doi: 10.1371/journal.pone.0184765 Wrong acknowledgement
2. Leftin A, Koutcher JA. Quantification of Nanoparticle Enhancement in Polarized Breast Tumor Macrophage Deposits by Spatial Analysis of MRI and Histological Iron Contrast Using Computer Vision. Contrast Media Mol Imaging. 2018 Oct 30;2018:3526438. doi: 10.1155/2018/3526438. Support acknowledged
3. Leftin A, Ben-Chetrit N, Joyce JA, Koutcher JA. Imaging endogenous macrophage iron deposits reveals a metabolic biomarker of polarized tumor macrophage infiltration and response to CSF1R breast cancer immunotherapy. Sci Rep. 2019 Jan 29;9(1):857. doi: 10.1038/s41598-018-37408-7. Support acknowledged

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Porcari, P., **Ackerstaff, E.**, LeKaye, H.C., Koutcher, J.A. *Metabolic Effects of Deferiprone on Triple Negative Breast Cancer*. Digital Poster #1843, 29th Ann Mtg & Exhibit of the International Soc for Magn Reson in Med (ISMRM), ISMRM & SMRT, Virtual Conference & Exhibition, May 15 – 20, 2021.
2. Porcari P, Ackerstaff E, Winkleman DP, Veeraperumal S, Kruchevsky N, Lekaye HC, Koutcher JA. **The Role of Iron Chelation in the Tumour Microenvironment of Triple-Negative Breast Cancer**. 28th ISMRM Annual Meeting, Virtual Conference 2020, August 8-14. Oral Presentation, 0144. (selected for presentation at the **Cancer Imaging Highlights Sessions** of the ISMRM 2020).
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• **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

None.

• **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

None in Year 4.

• **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None in Year 4.

• **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*

- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Participants:

Koutcher Laboratory: W81XWH-17-1-0525 (Initiating PI)

Jason Koutcher; Corresponding PI, 10% (1.2 CAL months); Dr. Koutcher directs the overall project.

Ellen Ackerstaff ~20% (2.4 CAL months)- (Co-Investigator) Dr. Ackerstaff supervises all the metabolic studies performed both in vivo and in vitro.

Soe Min – 50% (6 CAL months) - she takes the place of Ms. Kruchevsky and provides technical support (cell studies and in vivo studies)

Paola Porcari = 50% (6 CAL months)- postdoctoral fellow; performs all the MR studies and some cell studies

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a

change in active other support significantly impacts the effort on the project that is the subject of the project report.

See below.

KOUTCHER, JASON

New Grant Since Last Submission

1S10 OD032202-01 (PI: Koutcher)

09/01/22-08/31/23

Bruker AV-NEO Console for 7T MRI

Purchase of a new console to replace a 20 year old console on the 7.0T magnet; NIH \$600,000

Role: Principal Investigator

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

None.

& SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Partnering PI award (W81XWH-17-1-0526) ended September 14, 2021 and thus an independent final report will be submitted in January 2022. No annual technical report will be provided.

QUAD CHARTS: *Not applicable.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

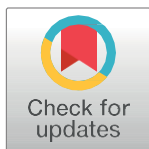
RESEARCH ARTICLE

Iron imaging reveals tumor and metastasis macrophage hemosiderin deposits in breast cancer

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Abstract

Iron-deposition is a metabolic biomarker of macrophages in both normal and pathological situations, but the presence of iron in tumor and metastasis-associated macrophages is not known. Here we mapped and quantified hemosiderin-laden macrophage (HLM) deposits in murine models of metastatic breast cancer using iron and macrophage histology, and in vivo MRI. Iron MRI detected high-iron pixel clusters in mammary tumors, lung metastasis, and brain metastasis as well as liver and spleen tissue known to contain the HLMs. Iron histology showed these regions to contain clustered macrophages identified by their common iron status and tissue-intrinsic association with other phenotypic macrophage markers. The in vivo MRI and ex vivo histological images were further processed to determine the frequencies and sizes of the iron deposits, and measure the number of HLMs in each deposit to estimate the in vivo MRI sensitivity for these cells. Hemosiderin accumulation is a macrophage biomarker and intrinsic contrast source for cellular MRI associated with the innate function of macrophages in iron metabolism systemically, and in metastatic cancer.

Introduction

Macrophage accumulation is an emerging hallmark of the breast tumor and metastasis micro-environment [1–4]. At early stages of neoplasia, macrophages mount inflammatory response to initial tumor proliferation, and at later stages these macrophages adopt pro-tumor phenotypes associated with angiogenesis, metastatic dissemination, and immune-suppression [5, 6]. Histological evaluation of cells stained for macrophage surface receptor markers such as CD68 can provide a score of immune cell accumulation, or infiltration, that has been correlated with disease stage [7–12], and can also provide predictive information on expected outcomes of therapeutic intervention [13]. These assessments of tumor-associated macrophages (TAMs)

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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are largely limited to biopsy of tumor tissue in situ, or sampling of lesions following bulk surgical resection. Collection of these specimens is routine, but invasive in primary breast tumors, and it becomes increasingly difficult to assess metastasis-associated macrophages (MAMs) in metastatic sites such as the lung and brain. Therefore, alternative non-invasive translational techniques are required that enable imaging and characterization of TAMs in vivo.

Macrophages function to recycle iron from senescent erythrocytes and have evolved to sequester the salvaged iron collected while mounting their innate immune response to tissue insult and pathogens [14–16]. To package their iron cargo, macrophages accumulate it in crystalline ferritin granule aggregates called hemosiderin.

So-called hemosiderin-laden macrophages (HLMs) also confer high MRI contrast and have long been used to detect macrophages preclinically and clinically [17–20]. Additionally, clinical MRI protocols have been developed that are sensitive to these cellular iron deposits [21, 22], and are similar to those utilized currently as standard-of-care breast tumor detection methods [23] suggesting that simultaneous tumor and HLM imaging can be performed.

As iron metabolism is a major area of research in breast and other cancers [24–28], but the association of hemosiderin deposits in tumor and metastasis has been relatively unexplored, we sought to determine the extent to which iron deposition occurs in the breast tumor and metastasis microenvironment, and evaluate our ability to map these deposits histologically and in vivo by MRI. We found that hemosiderin iron is indeed a metabolic biomarker of a population of macrophage deposits, and that the association of these iron(III)⁺ CD68⁺ macrophages with pro-inflammatory (allograft inflammatory factor-1, AIF1) and anti-inflammatory (mannose receptor, CD206) subsets varies according to tissue type. The association between hemosiderin and macrophages also provided a way to detect deposits of these cells in vivo by iron MRI with cellular level sensitivity. Using image feature detection algorithms we measured the frequency and size of the deposits in the iron MRI and histology. These findings show that iron deposition defines a metabolically distinct subset of immune infiltrates in tumors and metastasis, and provides an in vivo means to map and quantify the size of the deposits according to their innate iron status.

Materials and methods

Animal procedures

All animal work was approved by the Animal Care and Use Committee of MSKCC and performed according to their guidelines.

Use of humane endpoints

The specific criteria used to determine when animals should be euthanized follow the humane endpoints for the cancer models used including: (a) primary tumor reached 1 cm³, (b) first sign of respiratory distress due to lung metastasis, (c) first sign of neurological impairment, such as head tilting, changes in ambulation or disequilibrium due to brain metastasis. Humane endpoints were used in all studies, and once animals reached the humane endpoint they were euthanized immediately.

Study design

A total of 8 mice bearing primary mammary tumors, 17 mice harboring lung metastases, and 19 mice displaying brain metastases were imaged upon presenting with signs associated with their humane endpoint to (1) confirm tumor burden as part of parallel investigations, and (2) to simultaneously map cellular iron deposits for the current studies. Tissues for ex vivo

histology were available from 8 of the primary tumor models, 8 of the lung metastasis models, and 5 of the brain metastasis models due to sampling requirements for downstream analysis in parallel investigations. None of the animals died prior to, or after the current studies, and all animals were euthanized immediately after meeting humane endpoint criteria. Mice were inspected daily to minimize suffering and distress by veterinary staff and principal scientific investigators trained to identify clinical, physiological, and behavioral signs anticipated in the cancer models.

Tumor models

In the primary tumor models, female 6 week-old FVB/n mice underwent orthotopic injection of 1×10^6 syngeneic TS1 MMTV-PyMT tumor cells [29] suspended in 100 μ L of 50% Matrigel (BD Bioscience) into the lower mammary fat pad. MRI and histological measurements are made in tumors approximately 1cm³ in volume. In the lung metastasis models, female 6 week-old FVB/n mice underwent intravenous injection of 2.5×10^4 TS1 MMTV PyMT tumor cells [29], and underwent MRI and histological analysis once presenting visible changes in respiration. In the brain metastasis models, female 6 week-old C57BL6 mice underwent intracardiac injection of 1×10^4 brain metastasis cells obtained by propagation of the primary MMTV-PyMT mammary tumor line in 3D Matrigel culture followed by in vivo passage as brain metastasis [30], and were imaged by MRI and histology at first sign of neurological impairment.

Bio-iron collection

Blood was withdrawn by vein puncture and collected in heparinized tubes. Crude hemosiderin was obtained from fresh mouse spleen by magnetic separation using MACS columns and running buffer (Miltenyi).

Iron MRI

All MRI images were acquired with a 7T/30 cm horizontal bore MRI system (Biospec, Bruker Corp., MA) with a custom-built 30 mm inner-diameter transmit-receive quadrature radio-frequency coil. 2D multi-gradient echo (MGE) T_2^* relaxometry was used to acquire 16 multi-echo images with an inter-echo image acquisition time of 3.0ms, and an inter-scan time of 3s. Axial images were acquired with a slice thickness of 500 μ m and an in-plane pixel resolution of 0.01 mm² (0.1 mm $\times$ 0.1 mm). Pixel-by-pixel determination of T_2^* relaxation times was performed by fitting the magnitude of each pixel in the MGE image echo series with a standard mono-exponential fit function in Matlab (Natick, MA) and Fiji [31] [32]. T_2^* relaxation times were measured over the working range of iron(III) concentrations at 7T (0.0–0.3 mg iron(III) g⁻¹ from iron(III) nitrate nonahydrate, Fisher Scientific). The linear relation between the relaxation rate $R_2^* = 1/T_2^*$ and iron(III) concentration was found, and was subsequently used to generate the parametric iron(III) MRI maps. The iron(III) MRI maps were then stratified by concentration (total range, 0.0–0.3 mg g⁻¹; high, 0.15–0.3 mg g⁻¹). Spatial characteristics of the high-iron(III) pixels were characterized by performing cluster analysis over the stratified iron MRI images using the Fiji Analyze Cluster tool. During the MRI scans mice were anesthetized with 1–3% isoflurane in O₂ gas, and image acquisition was gated with the monitored respiration of the animal.

Histology

Sections of PBS-perfused tissue were collected and fixed in 4% PFA for 24 hours at 4°C, and then washed with H₂O, and re-suspended in 70% ethanol (Fisher Scientific). Tissues were

paraffin embedded and 5µm sections cut onto glass slides for histology. The Prussian blue histochemical iron(III) assay was performed by placing de-paraffinized slides in a working solution of equal parts 5% potassium ferricyanide (Fisher Scientific) and 5% hydrochloric acid (Fisher Scientific) prepared in distilled water and stained for 30 minutes. Slides were then rinsed in distilled water, counter-stained with nuclear-fast red, and cover-slipped with Permount (Fisher Scientific). The immunohistochemical detection of CD68, and immunofluorescent detection of CD3, CD31, and CD68, AIF1, and CD206 was performed with a Discovery XT processor (Ventana Medical Systems). The tissue sections were de-paraffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems), and sections were blocked for 30 minutes with Background Buster solution (Innovex), followed by Avidin/biotin blocking for 8 minutes. For immunohistochemistry, anti-CD68 (Boster, cat# PA1518, 5µg/ml) was applied and sections were incubated for 5 hours, followed by 60 minutes incubation with biotinylated goat anti-rabbit antibodies (Vector Labs, cat#PK6101) at 1:200 dilution. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer's instructions. Slides were counter-stained with hematoxylin and cover-slipped with Permount (Fisher Scientific). For immunofluorescence, anti-CD3 antibody (DAKO, cat# A0452, 1.2µg/ml), anti-CD31 (Dianova, cat# DIA-310 1µg/ml), anti-CD68 (Boster, cat# PA1518, 5µg/ml), anti-CD206 (Abcam, cat#ab64693, 1µg/ml), anti-AIF1 (Wako, cat#019-19741, 0.5µg/ml) were applied and sections were incubated for 5 hours, followed by 60 minutes incubation with biotinylated goat anti-rabbit antibodies (Vector Labs, cat#PK6101) for CD31, CD3, CD68, AIF1 and CD206 at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide Alexa Fluor A488 (Invitrogen, cat# T20922), TSA-Alexa 546 (Invitrogen, cat# T20933), TSA-Alexa 568 (Invitrogen, cat#T20914) TSA-Alexa 594 (Invitrogen, cat# T20935), or TSA-Alexa 647 (Invitrogen, cat# T20936) prepared according to manufacturer's instructions with predetermined dilutions. After staining, slides were counterstained with DAPI (Sigma Aldrich, cat# D9542, 5 µg/ml) for 10 min, and coverslipped with Mowiol.

Slides were digitized with a Mirax Scan system and read with Panoramic Viewer (3DHIS-TECH, Budapest Hungary). Images were exported to Fiji, and pixels associated with macrophages were identified by their Prussian blue iron(III), DAB positive staining, or immunofluorescent positive labeling, and their size ($>20\mu\text{m}^2$), and threshold masks of these pixels were generated using the Fiji Analyze Cluster tool. MRI resolution-matched iron(III) cluster maps were generated by resizing the threshold masks (1:100) by pixel averaging and bilinear interpolation with Fiji. The spatial characteristics of resolution-matched clusters were measured using the Fiji Analyze Cluster tool. The number of iron(III)⁺ macrophages per cluster was determined by using the cluster maps to define regions for cell counts in the full-resolution histological images. Metastasis surveys were conducted in a similar manner, with the exception that the lesions were first identified automatically by thresholding the neutral red counter-stained Prussian Blue tissue sections to identify individual lesions, and within these lesions, we performed the macrophage counts and resolution-matched cluster quantification using the Fiji Analyze Cluster tool.

Statistics

Results are presented as mean $\pm$ s.e.m. unless otherwise indicated. Comparison between groups and distributions was performed using two-tailed unpaired student's t-tests, or Mann-Whitney tests as appropriate. All statistical calculations were performed in GraphPad Prism 7 Software.

Results

Multi-gradient echo (MGE) T_2^* -weighted MRI exhibits reduced pixel intensity with increasing concentration of iron(III) (Fig 1a). As a quantitative measure of this sensitivity to iron, we calculated the rate of MGE image pixel decay ($R_2^* = 1/T_2^*$) in solutions of increasing iron concentrations and found a positive linear relation between these experimental variables (Fig 1b). Pixel-wise parametric maps of iron concentration were generated using this relation for the iron standards over the full range of the iron(III) contrast at 7T (Fig 1c, 0–0.3 mg iron g^{-1}).

When applied to other bio-iron sources this iron mapping method confirmed that iron levels of blood, and iron(III)-transferrin ($\sim 20\mu g$ transferrin iron g^{-1}) were at the lower limit of the detectable range of the iron MRI maps (Fig 1c). [33–35]. By contrast, superparamagnetic hemosiderin from spleen [36] [37, 38] and iron nanoparticles mimicking the bio-iron deposits ($200\mu g$ iron g^{-1}) were found in the high-iron range.

These tests demonstrated the ability to map iron in vivo, and further suggested the ability to differentiate between different bio-iron sources using in vivo MRI. We then imaged orthotopic MMTV-PyMT mammary tumors, livers, and spleens in vivo (Fig 1d). The iron MRI examinations revealed “hot-spot” high-iron(III) pixel clusters that were quantified by automated image cluster analysis of high-iron stratified MRI maps (0.15–0.3 mg iron(III) g^{-1}) to measure the size (Fig 1e) and frequency of each of the clusters in the various tissues (Fig 1f). The total number and size of the high-iron(III) clusters varied significantly between tissue types suggesting different quantities, and different spatial distributions of HLMs. High-iron clusters were found

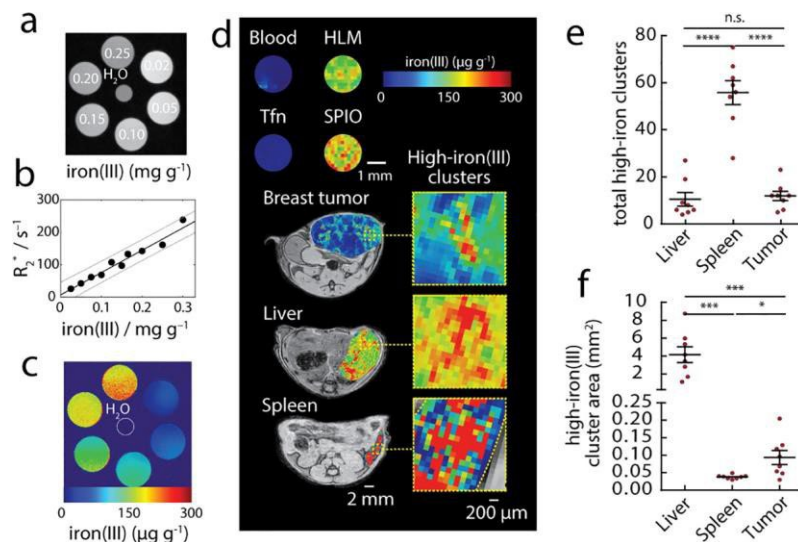


Fig 1. Quantitative contrast agent-free MRI of macrophage iron deposits. (a) Multi-gradient echo (MGE) MRI image (first-echo) showing variation of contrast with increasing concentration of iron(III) (0–0.25 mg g^{-1}). (b) Quantitative MRI measurements of signal relaxation rates R_2^* ($= 1/T_2^*$) for the MGE image series as a function of iron(III) concentration (solid-line linear fit, dashed lines 95% confidence interval). (c) Parametric iron(III) maps generated for the standards, and H_2O (dashed circle). (d) Iron MRI maps of whole blood, transferrin (Tfn, $\sim 20\mu g$ iron/mL), macrophage hemosiderin from mouse spleen, superparamagnetic iron oxide (SPIO) nanoparticles in aqueous solution ($200\mu g/mL$), and in vivo in mammary tumors, livers (no tumor), and spleens (no tumor) of the MMTV-PyMT breast cancer models. Upper scale bar, 1mm, lower scale bar 2mm. Expansions show high-iron pixel cluster regions indicative of hemosiderin deposition. Scale bar $200\mu m$. High-iron(III) MRI clusters quantified automatically by (e) counting the total number of high iron pixel clusters in the iron MRI images (0.15–0.3 mg iron(III) g^{-1} range), and (f) measuring the average cross-sectional areas of the clusters (mm^2). Points are individual animals (mean $\pm$ s.e.m.; $n = 8$ animals; n.s. not significant $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ two-tailed unpaired t-test).

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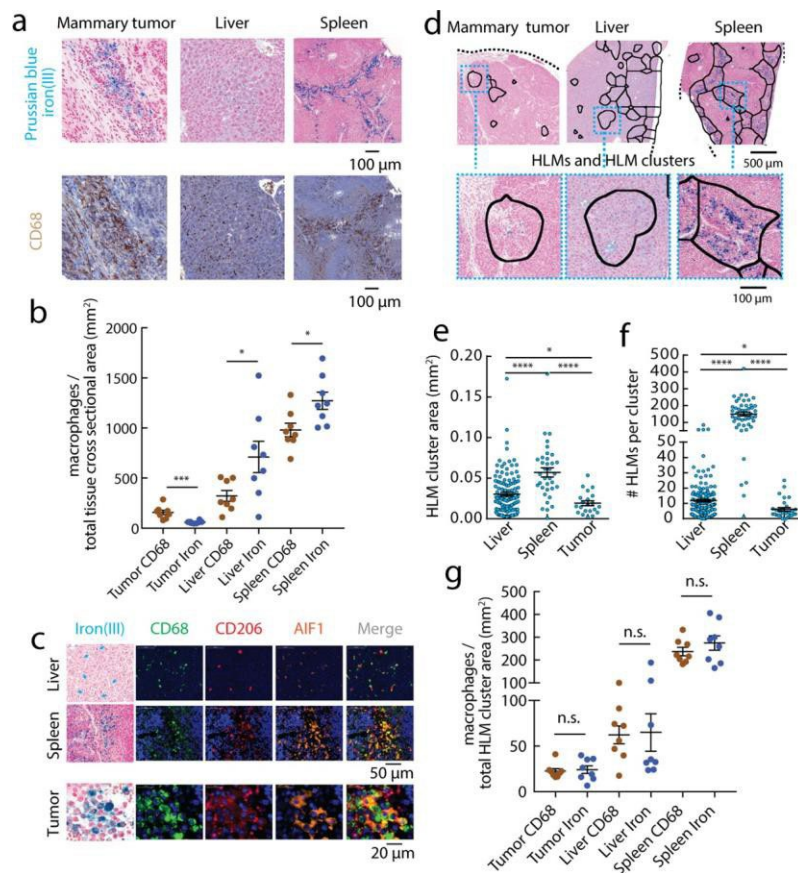


Fig 2. Hemosiderin iron is a biomarker of macrophage deposits in systemic and tumor microenvironments. (a) Prussian blue iron(III) and CD68 macrophage histology from orthotopic MMTV-PyMT mammary tumor, liver, and spleen. Scale bar 200µm. (b) The total number of Prussian blue iron(III)⁺ and CD68⁺ macrophages per mm² of the whole tissue cross-sectional area measured by automated cell counting. Points are individual animals (mean±s.e.m.; n = 8 animals; n.s., not significant *p<0.05, ***p<0.001 two-tailed unpaired t-test). (c) Iron(III) histochemistry beside single color channel and merged CD68, CD206, and AIF1 immunofluorescent markers. Liver (arrows denote iron(III)⁺ features) and spleen scale bar 50µm, mammary tumor scale bar 20µm. (d) The Prussian blue iron(III) images of MMTV-PyMT mammary tumors, livers, and spleens processed by down-sampling the resolution of the digitized histology to the MRI pixel scale (1:100, 0.01mm²/pixel) to generate resolution-matched clusters centered around iron(III)⁺ HLM deposits (solid lines). Scale bar 100µm. Automated size distribution analysis of (e) cross-sectional area of the clusters, and (f) frequency of the resolution-matched HLM clusters. Each point is a cluster detected in whole cross-sections of orthotopic MMTV-PyMT mammary tumors, livers, and spleens (mean±s.e.m.; n = 8 animals; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Mann-Whitney test). (g) The number of Prussian blue iron(III)⁺ and CD68⁺ macrophages per mm² of the total cluster area. Points are individual animals (mean±s.e.m.; n = 8 animals; n.s., not significant *p<0.05 two-tailed unpaired t-test).

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sparsely in discrete deposits around peritumoral regions. In the liver, large clusters suggested the known distribution iron-laden Kupffer cells throughout the liver. In the spleen where HLMs are densely deposited in the red-pulp, focal high-iron(III) clusters were significantly more frequent than both tumors (p<0.0001) and liver (p<0.0001).

To further confirm the presence of these HLMs at cellular resolution, whole tissue cross-sections of orthotopic murine MMTV-PyMT mammary tumors, livers, and spleens were stained by Prussian blue iron(III) and the general marker CD68 (Fig 2a), revealing iron(III)⁺ cells characterized as HLMs co-localized with CD68⁺ macrophages. Counting the total number of iron(III)⁺ macrophages and CD68⁺ macrophages per mm² of the whole tissue cross-

section area (Fig 2b) showed both total HLMs and CD68 macrophages increased in frequency from tumor < liver < spleen. The relative numbers of iron(III)⁺ HLMs to CD68⁺ macrophages was slightly greater for Kupffer cells ($p < 0.05$) and red-pulp macrophages ($p < 0.05$) of the reticuloendothelial organs; iron-laden populations were less frequent than CD68 infiltrates in the tumors ($p < 0.001$). Additional immunofluorescence staining was performed to assess the association of the iron(III)⁺ cells with other macrophage subsets at cellular resolution, confirming the immunohistochemical determination of the co-localization with CD68, and further revealing that these macrophages also express both pro-inflammatory (AIF1⁺, allograft inflammatory factor 1) and anti-inflammatory (CD206⁺, mannose receptor) markers in the mammary tumors, livers, and spleens (Fig 2c). These measurements indicate that at the whole tumor level of analysis, macrophages determined by their association with iron comprised a unique population present systemically, and a unique sub-population of the macrophage subsets found in the tumors.

To estimate the contributions of the HLM deposits to the high-iron(III) MRI clusters, all HLMs in the Prussian Blue stained cross-sections were automatically detected, and thresholded images of these cellular deposits were downsampled to the MRI pixel dimensions (1:100 pixel resolution scale factor) to generate “resolution-matched” HLM clusters in the histology (Fig 2d). These regions were quantified by the same image cluster analysis as the iron MRI yielding the cluster areas (Fig 2e), and high-resolution counts of HLMs within these regions (Fig 2f). HLM clusters ranged from approximately 0.01–0.1 mm² (on the order of 1–10 MRI pixel²), and the number of macrophages per deposit ranged from approximately 10 HLMs/cluster in the mammary tumors to >100 HLMs/cluster in the spleen. The total iron (III)⁺ and CD68⁺ macrophages in the deposits were found to be the same in all the tissues ($P > 0.05$) (Fig 2g), and increased from breast < liver < spleen as in the whole cross-section histological scores. This proves the association of macrophages with hemosiderin iron in the deposit regions, and further corroborates the iron MRI measurements by confirming the location, size, and cellularity of the HLM deposits in the tissues.

As HLMs were detected in primary tumors, we hypothesized that we could also detect them in advanced stages of cancer such as lung metastases. We initiated an intravenous injection model of MMTV-PyMT mammary-to-lung metastasis, and imaged the HLM deposits by histology and in vivo by iron MRI. Immunofluorescence detected CD68⁺ macrophages at the same lesion margins as iron(III)⁺ HLMs stained by Prussian blue. These cells were found in the proximity of CD31⁺ vasculature, where CD3⁺ T-cells were also localized indicating a functional tumor immune microenvironment rather than necrotic regions (Fig 3a). The number of iron(III)⁺ HLMs was significantly smaller than CD68⁺ macrophage counts per mm² lung metastases showing that in this metastatic setting, they are a sub-population of the total infiltrating macrophages ($p < 0.0001$) (Fig 3b). At cellular resolution, these HLMs were found to express high levels of CD68 and CD206 with either no or low expression of AIF1 indicating that they are primarily acting in an anti-inflammatory capacity in the lung metastases (Fig 3c). The histological analysis also revealed that the iron(III)⁺ macrophage deposits occurred in only 24% of the lesions detected (42 iron(III)⁺ metastases / 169 total metastases), indicating that HLMs are a subpopulation of macrophages in lung metastasis.

In vivo iron(III) MRI examinations were also performed in the lung metastasis models. Iron(III) maps revealed numerous high-iron(III) clusters (Fig 3d), and image segmentation and automatic cluster analysis of the high-iron(III) regions revealed a highly-significant association of clusters with metastases ($p < 0.0001$, Fig 3e). However, statistical analysis of the high-iron cluster distribution revealed large areas that we could not rationalize to be discrete deposits of macrophages based on our findings in livers, spleens, and mammary tumors. This indicated to us that the known difficulties using MRI to image air-tissue interfaces in the lung

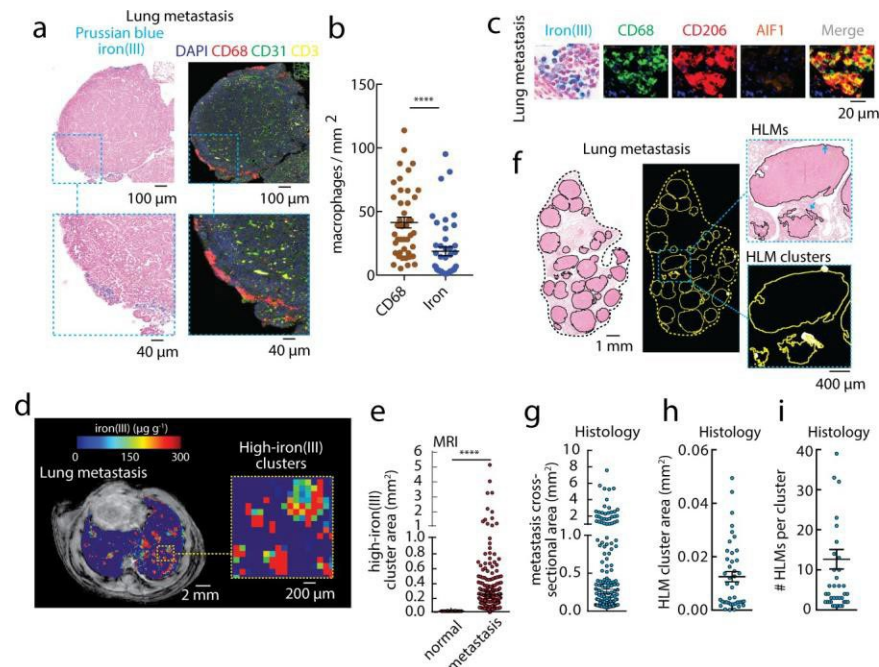


Fig 3. Imaging HLM deposits in lung metastasis. (a, left) Prussian blue iron(III) histology, and (right) immunofluorescence staining (DAPI, CD68, CD31, CD3) of lung metastasis from MMTV-PyMT mammary-lung model (intravenous injection). Scale bar 100µm. Expansion shows HLM deposits. Scale bar 40µm. (b) The total number of iron(III)⁺ HLMs and CD68⁺ macrophages per mm² lung metastasis measured by automated cell counting. Points are individual metastases. (mean±s.e.m.; n = 42 metastases, n = 8 lung sections; ****p<0.0001 Mann-Whitney test). (c) Iron(III) histochemistry beside single color channel and merged CD68, CD206, and AIF1 immunofluorescent markers. Scale bar 20µm. (d) In vivo iron(III) MRI maps measured in MMTV-PyMT mammary-lung metastasis models. Scale bar 2mm. Expansion shows high-iron (III) cluster regions and individual metastases. Scale bar 200µm. (e) High-iron(III) MRI clusters automatically analyzed to determine the average cross-sectional area of the clusters (mm²). Points are individual high-iron (III) clusters measured in metastasis and normal lung regions (mean±s.e.m.; n = 166 metastases, n = 47 normal lung regions, n = 17 mice; ****p<0.0001 Mann-Whitney test). (f) Whole lung cross sections of the metastasis models stained for Prussian blue iron(III), and processed images showing individual metastasis (outlines), HLM deposits (arrows), and resolution-matched HLM clusters in the individual metastasis (filled regions). Scale bar 1mm. Expansion scale bar 400µm. Prussian blue iron(III) histology automatically assessed for (g) metastasis cross-sectional area (mean±s.e.m. n = 169 metastases, n = 8 lung sections), (h) MRI-resolution matched HLM cluster area, and (i) number of iron(III)⁺ HLMs per cluster (mean±s.e.m.; n = 42 iron(III)⁺ metastases out of 169 counted, n = 8 lung sections).

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[39] instead led to high-iron clusters delocalized to the size to each metastasis rather than detecting small HLM deposits within the metastatic lesions. Measurement of lung metastasis areas from histology confirmed that the cluster sizes measured by iron(III) MRI in lung were indeed on the order of the metastatic lesions themselves (Fig 3f and 3g). Nonetheless, we performed the resolution-matched HLM cluster assessment to demonstrate the potential of the method if more tractable lung MRI conditions were available, and indeed the HLM cluster areas (Fig 3h), and the counts of HLMs in these discrete regions were on the order of those measured in primary tumors (Fig 3i). These measurements indicate that while hemosiderin deposition and clustering is a feature of CD68⁺ CD206⁺ macrophages in lung metastasis, it is not detectable in a straightforward application of the iron MRI measurements because of the constraint that magnetic susceptibility mismatch imposes on MRI measurements of the lung.

To investigate HLM deposition in another metastatic setting, we used an in vivo MMTV-PyMT brain metastasis model. Tumor cells were introduced by intracardiac injection, and we

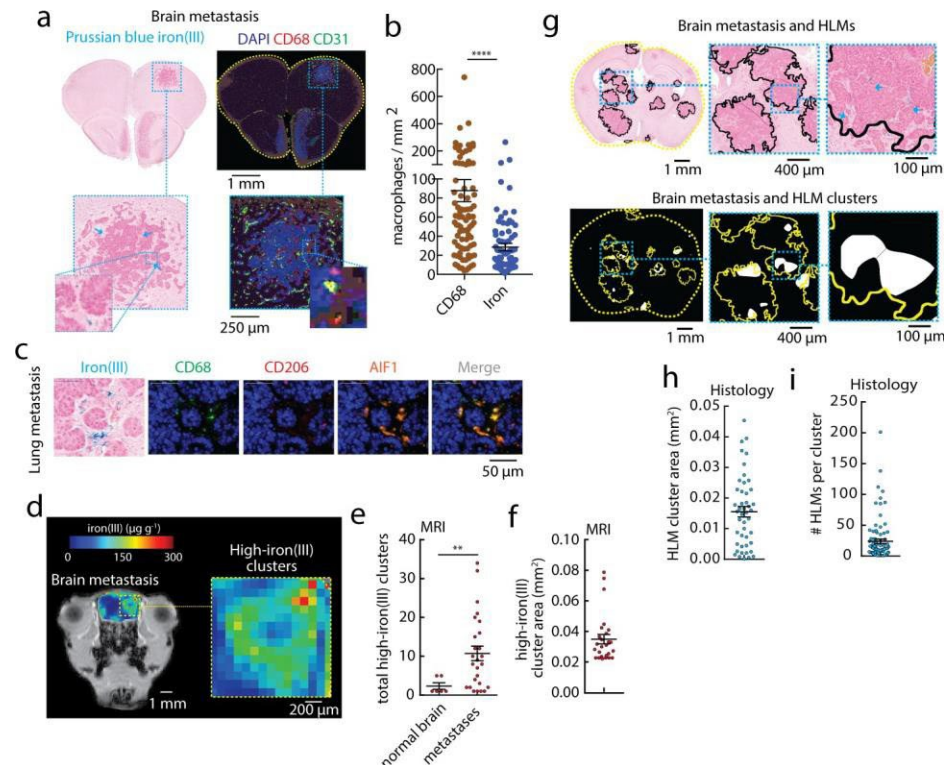


Fig 4. Imaging HLM deposits in brain metastasis. (a, left) Prussian blue iron(III) histology, and (right) immunofluorescence staining (DAPI, CD68, CD31) of brain metastasis from MMTV-PyMT mammary-brain model (intracardiac injection). Scale bar 1 mm. Expansion shows individual metastasis, and individual iron (III)⁺ HLM deposit (5x). Scale bar 250µm. (b) The total number of iron(III)⁺ HLMs and CD68⁺ macrophages per mm² brain metastasis measured by automated cell counting. Points are individual metastases. (mean±s.e.m.; n = 86 iron(III)⁺ and CD68⁺ metastasis; n = 16 brain sections, n = 5 animals; ****p<0.0001 Mann-Whitney test). (c) Iron(III) histochemistry beside single color channel and merged CD68, CD206, and AIF1 immunofluorescent markers. Scale bar 50µm. (d) In vivo iron(III) MRI maps measured in MMTV-PyMT mammary-brain metastasis models. Scale bar 2mm. Expansion shows high-iron(III) cluster regions and individual metastases. (e) High-iron(III) MRI clusters automatically counted in normal brain regions and the metastases, and (f) the average cross-sectional areas of the high-iron(III) clusters (mean±s.e.m.; n = 97 metastasis, n = 26 high-iron(III) clusters; n = 66 normal regions, n = 6 high-iron(III) clusters, n = 19 animals; **p<0.001 Mann-Whitney test). (g) Whole brain cross sections of the metastasis models stained for Prussian blue iron(III). The processed images show individual metastasis (outlines), HLM deposits (arrows), and MRI resolution-matched HLM clusters in the individual metastasis (filled regions). Scale bar 1mm. Expansion scale bar 400µm. Prussian blue iron(III) histology automatically assessed for (h) the average HLM cluster cross-sectional area measured per brain metastasis, and (i) number of iron(III)⁺ HLMs per cluster (mean±s.e.m.; n = 46 brain metastasis; n = 212 HLM clusters, n = 16 brain sections, n = 5 animals).

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mapped HLMs in the brain metastases by histology and iron MRI. Prussian blue histology confirmed iron(III)⁺ HLM deposits at the edges of the invasive metastatic lesions (Fig 4a), and immunofluorescence revealed these macrophages to be a sub-population of the CD68⁺ macrophages as in the other breast cancer tissues (p<0.0001) (Fig 4b). In the brain metastasis, these iron⁺ CD68⁺ cells also expressed high levels of inflammatory AIF1, and no or very low levels of CD206 (Fig 4c) suggesting that they are activated microglia. These iron(III)⁺ cells were also present in the majority ~81% of the lesions (86 iron(III)⁺ metastases / 105 total metastases).

Iron MRI examinations revealed focal small high-iron(III) MRI pixel clusters associated with HLM deposits, and elevations in low level contrast that distinguished brain metastases from tissue background (Fig 4d). Counts of high-iron(III) clusters in these metastases compared to normal brain regions revealed a significant association of these clusters with the lesions

($p < 0.01$, Fig 4e). Areas of the high-iron clusters (Fig 4f) were consistent with the small cluster sizes of mammary tumors and spleens, and HLM clusters generated automatically within each metastasis from histology (Fig 4g) revealed that HLM cluster areas (Fig 4h) were also on the order of the *in vivo* measurements, and measuring the number of HLMs per cluster by these parallel histological assessments (Fig 4i) support the use of iron MRI in mapping HLM deposition in brain metastasis with cellular sensitivity.

Discussion and conclusions

The association of iron deposits with macrophages underscores their metabolic role in recycling iron for heme biosynthesis. The function of macrophages in storing iron is highlighted in the livers and spleens, organs known to harbor HLMs and are central to iron homeostasis.

Here, iron(III)⁺ and CD68⁺ macrophages occurred with similar frequency, and were also associated with AIF1 and CD206 populations indicating that most of the macrophages were engaged in iron-handling roles. In mammary tumors, however, iron(III)⁺ macrophages were relatively fewer than in the liver and spleen, and only a fraction of the HLMs were associated with the total CD68⁺, AIF1⁺, and CD206⁺ macrophages also found in the mammary tumors. Similarly, in lung and brain metastases, HLM populations were even more rare, presumably because of the restrictive cellular barriers (blood-brain/lung) that limit deposition of immune cells and erythrocytes, and thus limits accumulation of iron in macrophages in these organs [40–42]. These observations indicate that while the iron deposition in tumor and metastasis associated macrophages is not as prominent as in organs such as the spleen and liver, iron storage in hemosiderin is an important conserved function for macrophages in both those iron-handling tissues and in the tumor and metastatic microenvironments.

In the innate immune response, resident macrophages and circulating myeloid cells from bone marrow respond to and transmit cytokine cues signaling inflammatory and anti-inflammatory programs, leading to adaptive signaling and vascular remodeling of the tumor microenvironment [43–45]. In these situations macrophages can change their surface receptor expression giving rise to so-called polarization in a given tissue setting [5, 46]. The surface receptor CD68 is a pan-macrophage marker and is used commonly for both pre-clinical and clinical assessment of macrophage infiltration, and thus, the majority of our studies relied on this marker to evaluate the iron staining in relation to this general macrophage population.

We also assessed the association of the HLMs with other specialized functional markers including AIF1 (pro-inflammatory, M1) and CD206 (anti-inflammatory, M2). We found that in mammary tumors, livers, and spleens, iron(III)⁺ CD68⁺ HLMs expressed both AIF1 and CD206 to a similar degree indicating that in these tissues the HLM subpopulations were not polarized to either phenotypic extreme. In metastasis, however, HLMs were strongly polarized. The iron⁺ CD68⁺ HLMs in lung metastasis exhibited high levels of CD206 not AIF1, and in brain iron⁺ CD68⁺ HLMs were predominantly associated with high levels of AIF1, not CD206. Interestingly, this demonstrates that while our imaging investigations identify specific populations of macrophages according to their hemosiderin deposits in all the tissues studied, the association of the iron-laden macrophage deposits with the phenotypic polarization markers appears to be related more to the tissue where they are found, rather than the association with iron deposition alone.

Mapping iron deposit size and frequency provides a new application of iron MRI techniques that enables the localization and quantification of macrophage deposits systemically, and in cancer microenvironments. As the basic iron MRI experiments are commonly performed clinically, and the new spatial image analysis approaches rely on simple cluster map processing procedures, their combination has high translational potential. Applications for the

iron MRI and cluster mapping methods include the quantification of cellular distributions following iron-oxide contrast agent injections [47, 48], monitoring macrophage depletion using injections of clodronate liposomes [49], or tracking cellular response to administration of macrophage targeted cancer therapies [48, 50, 51].

The studies presented here link a hallmark phenotype of macrophages, hemosiderin iron deposition, with the presence of infiltrating macrophages in metastatic breast cancer models, and we describe an in vivo approach for the quantitative mapping of these HLM deposits and measurement of their frequency and size. Taken together, imaging macrophages according to their endogenous iron levels reveals a cellular biomarker associated with their innate role in iron metabolism, and bridges a gap in our ability to quantitatively assess macrophage accumulation in vivo.

Author Contributions

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Research Article

Quantification of Nanoparticle Enhancement in Polarized Breast Tumor Macrophage Deposits by Spatial Analysis of MRI and Histological Iron Contrast Using Computer Vision

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Magnetic resonance imaging applications utilizing nanoparticle agents for polarized macrophage detection are conventionally analyzed according to iron-dependent parameters averaged over large regions of interest (ROI). However, contributions from macrophage iron deposits are usually obscured in these analyses due to their lower spatial frequency and smaller population size compared with the bulk of the tumor tissue. We hypothesized that, by addressing MRI and histological pixel contrast heterogeneity using computer vision image analysis approaches rather than statistical ROI distribution averages, we could enhance our ability to characterize deposits of polarized tumor-associated macrophages (TAMs). We tested this approach using *in vivo* iron MRI (FeMRI) and histological detection of macrophage iron in control and ultrasmall superparamagnetic iron oxide (USPIO) enhanced mouse models of breast cancer. Automated spatial profiling of the number and size of iron-containing macrophage deposits according to localized high-iron FeMRI or Prussian blue pixel clustering performed better than using distribution averages to evaluate the effects of contrast agent injections. This analysis was extended to characterize subpixel contributions to the localized FeMRI measurements with histology that confirmed the association of endogenous and nanoparticle-enhanced iron deposits with macrophages in vascular regions and further allowed us to define the polarization status of the macrophage iron deposits detected by MRI. These imaging studies demonstrate that characterization of TAMs in breast cancer models can be improved by focusing on spatial distributions of iron deposits rather than ROI averages and indicate that nanoparticle uptake is dependent on the polarization status of the macrophage populations. These findings have broad implications for nanoparticle-enhanced biomedical imaging especially in cancer.

1. Introduction

Widespread efforts have succeeded in integrating nanoparticles in virtually all areas of medical imaging. The appeal of these formulations derives from their ability to be tailored to specific applications ranging from neuroscience to oncology by chemical manipulation of nanoparticle composition rendering them visible to multiple imaging modalities such as MRI, PET, and optical imaging systems [1]. Moreover, the ability to functionalize these particles using delivery systems such as polymers or lipids and bioaffinity tags such as antibodies further enhances our ability to probe,

monitor, and control ubiquitous biological processes spanning drug delivery and cell tracking [2].

Nanoparticles formulated from iron are ubiquitous contrast agents for MRI. One common agent used consists of ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles solubilized with dextran polymer to facilitate suspension and delivery in aqueous solution [3, 4]. Cancer research is a premier application of these USPIO nanoparticles, and they have been used to investigate virtually every cancer type. They provide information about both vascular and immune cell properties of the tumor that are key determinants of the pharmacodynamic behavior of

drugs and the cellular immune response to therapies [5–7].

Quantification of tissue uptake of iron nanoparticles and deposition in macrophages is conventionally performed using region of interest (ROI) analysis of MRI images [8]. Pixel contrast levels, relaxation times, rates, or the susceptibility phase [9–12] are measured over tissue cross-sectional areas and analyzed according to pixel distribution statistics. Changes in iron concentration following USPIO injection can be further quantified from these pixel distributions according to parametric relations between these observables and known iron standards, iron-labeled cells, or biopsy iron measurement to provide quantitative estimates on nanoparticle delivery, cellular uptake, and concentration [13–16]. So-called iron MRI (FeMRI) approaches can estimate iron nanoparticle uptake by the measurement of parametric distribution statistics. However, it has long been an accepted caveat of the quantitative interpretation of most cellular MRI applications that ROI-based distribution analysis is biased by contributions from the abundant low-iron areas of the tissue, i.e., those not containing the iron deposit or contrast agent, which limits the specificity of the MRI pixel distribution analysis for iron accumulation in rare cellular targets such as macrophages in tumors.

Macrophages are important imaging targets in cancer because they can function in both inflammatory and wound-healing roles that influence tumor growth and therapeutic response [17–19]. Nanoparticle injection and uptake is dependent upon and can influence this polarization status of the targeted cellular populations as these cells exhibit plasticity in these functional roles that in turn is coupled to their innate role in iron metabolism [20, 21]. While MRI studies do not directly report on polarization, immunofluorescence imaging can be used to evaluate changes in TAM iron deposit polarization and provide subpixel information about the dependence of the USPIO contrast agent uptake on TAM polarization [22–24]. However, nanoparticle delivery to the tumor rarely exceeds a few percent of the injected dose [25], and therefore, only a small fraction of macrophages present in the tumor will be engaged by the nanoparticles and give rise to localized detectable iron contrast. Therefore, similar to cellular iron MRI, whole ROI analysis of histological polarization state measurements is more representative of the nanoparticle-free macrophage populations rather than subpopulations containing iron. Therefore, scoring macrophage polarization with immunofluorescence using whole ROI distribution analysis also will generally not be representative of the relatively rare iron-handling populations targeted by the nanoparticle injections. Thus, alternative unbiased analysis approaches that better quantify the local distributions of these iron-containing cells and their phenotypes are required to evaluate the polarization status of the macrophage targets and to provide cellular level corroboration of FeMRI applications.

In the current study, we advance a computer vision approach to localize polarized macrophages according to iron status in order to improve their quantification in USPIO-enhanced cellular imaging by MRI and histology. We have previously shown that, by addressing the spatial

heterogeneity of iron-dependent image contrast, we could enhance the quantification of these macrophage deposits without contrast agents using MRI and histology because the type of iron stored in the macrophages generates stronger MRI contrast enhancement compared with venous or hemorrhagic blood [26–30] and Prussian blue only labels macrophage with solid iron deposits corresponding to those of the highest iron concentration. These endogenous stores conferred sufficient cellular sensitivity and specificity to detect macrophage iron deposits in multiple cancer models including prostate cancer and breast cancer, in both primary and distant disseminated metastatic locations of the lung and brain, as well as systemically in organs which naturally or pathologically accumulate macrophage iron such as the liver and spleen [29, 30]. In the current study, we continue our translational development of this approach by combining it with the USPIO contrast agent injection to evaluate iron deposition in polarized breast tumor macrophages.

2. Results

To demonstrate the difference between the ROI average distribution measurements and the localized measurements of cellular iron deposition with and without dextran-USPIO injection, we initiated orthotopic MMTV-PyMT murine mammary tumors and injected USPIO (0.5 mmol/kg) or saline intravenously once tumor volumes reached approximately 1 cm³. Mapping iron-dependent contrast 24 hrs after injection at 7T showed that both the nanoparticle-free (Figure 1(a), –USPIO) and contrast-enhanced tumors (Figure 1(b), +USPIO) evidenced heterogeneous distributions of pixel iron levels, with both cohorts exhibiting numerous high-iron pixel regions, i.e., clusters indicative of iron deposits, at the periphery of the tumor, and iron oxide-injections increased the numbers of these clusters.

Analysis of the iron MRI maps was then performed to quantify these pixel distributions. Parametric pixel distributions expressed as number of pixel clusters (Figure 1(c)) and cluster size measured in mm² (Figure 1(d)) were reconstructed as a function of iron concentration with high and low ranges defined with respect to the median iron level of the distribution range. Frequency (number of pixel clusters) and size distribution maxima fell well within the low-iron contrast range in both control and USPIO-injected tumors and appeared to shift nominally towards higher concentrations with USPIO injection. In the high iron range of the frequency distribution, clear increases of pixel clusters occurred with the USPIO uptake, while the size of these high-iron clusters appeared to remain constant in the control and nanoparticle-treated groups suggesting that iron injection changed only the number of the deposits but not their size. Statistical analysis of the ROI-based parametric iron distributions was then performed to quantify the observations. First, median values were calculated from ROI analysis over whole tumor cross sections as is conventionally performed. Median iron levels of the parametric pixel distributions from the ROI showed small but significant increases in frequency (Figure 1(e); $p < 0.05$) and size (Figure 1(f); $p < 0.05$) with USPIO enhancement. In

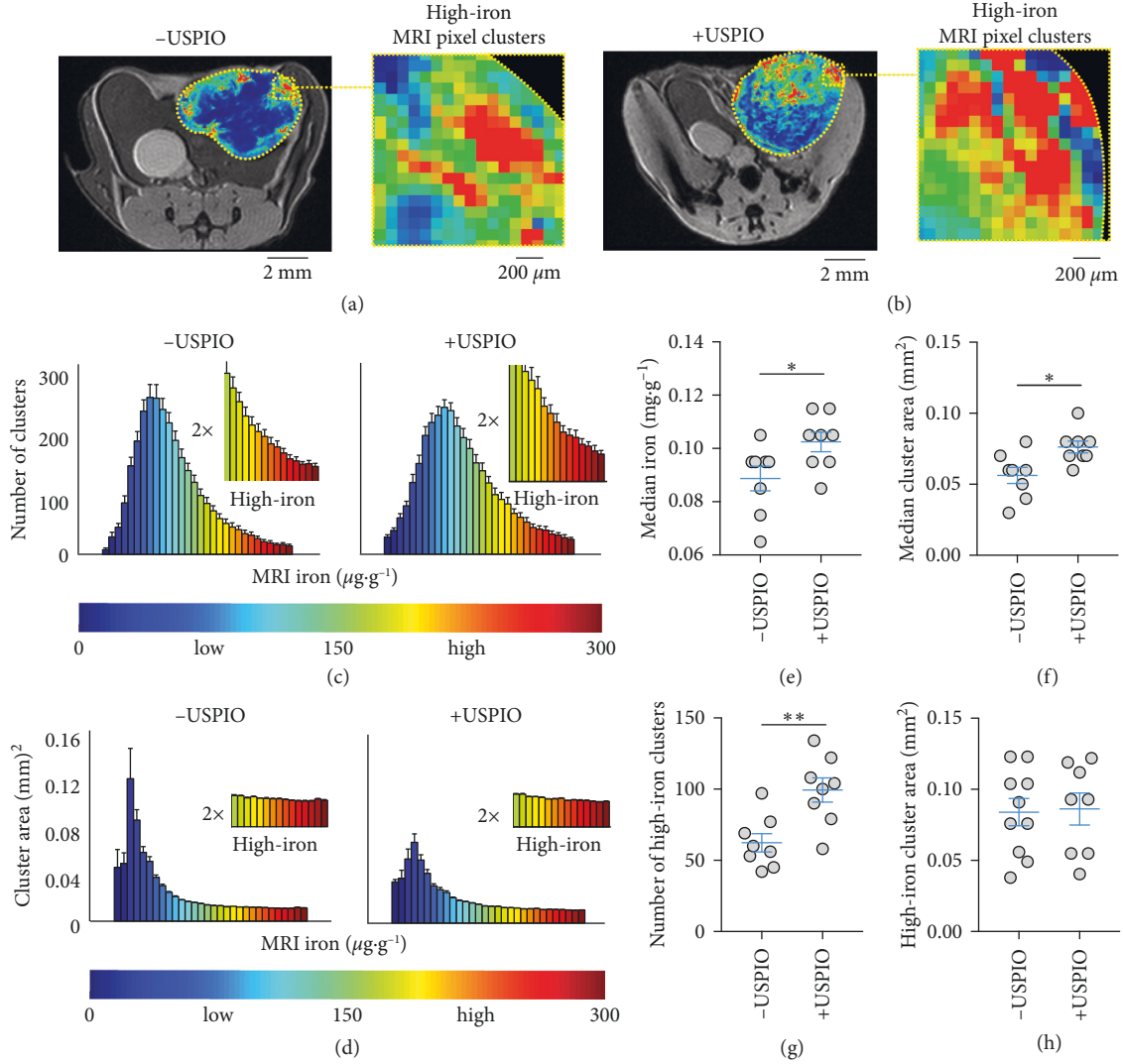


FIGURE 1: Spatial quantification of endogenous and nanoparticle-enhanced iron deposits with MRI *in vivo*. T₂-weighted MRI and iron concentration overlay images of (a) control (-USPIO) and (b) iron nanoparticle-injected (+USPIO) tumors. Expansion shows high-iron pixel contrast in clustered areas. (c) Number (#) of clusters and (d) area of the pixel clusters in control (-USPIO) and nanoparticle-injected (+USPIO) tumors as a function of iron concentration. Distributions are from whole cross-sectional regions of interest (ROI) areas of tumors measuring approximately 1 cm³ (mean $\pm$ SEM shown, $n = 8$ tumors/group). MRI iron concentration range at bottom corresponds to values in iron images above. Control (-USPIO) and nanoparticle-injected (+USPIO) (e) median iron concentrations and (f) pixel cluster sizes. (g) Number (#) of high-iron pixel clusters and (h) size of the high-iron clusters from localized computer vision analysis (mean $\pm$ SEM shown, $n = 8$ tumors/group, n.s. $p > 0.05$, * $p < 0.05$, *** $p < 0.001$, two-tailed unpaired Student's t -test). Scale bars are shown for all images.

order to more specifically quantify local accumulation of iron deposits indicated by the high-iron range stratification, the frequency of high-iron clusters in the maps was counted, and the areas were measured. This revealed significant increases of the high-iron pixel clusters with iron nanoparticle injection (Figure 1(g); $p < 0.001$) and provided an additional measurement of the size of these regions of iron accumulation with and without contrast agents (Figure 1(h); $p > 0.05$). This demonstrates an improvement over the ROI quantification in terms of the significant increases in iron deposit accumulation detected and confirms the observation that the number of high-iron clusters increase but the size remains relatively unchanged with USPIO injection.

To confirm the cellular identity of the iron⁺ species and characterize the microenvironment of the iron deposits, Prussian blue iron histochemistry (Figure 2(a)) and immunofluorescence for F4/80⁺ (Figure 2(b)) macrophages and CD31⁺ vasculature (Figure 2(c)) was performed. Iron staining of the MMTV-PyMT tumor sections evidenced iron⁺ cellular species in stromal regions almost exclusively at peripheral tumor margins. These localized cellular iron sources clustered as multicellular deposits in both the control and USPIO-treated tumors. Macrophage immunofluorescence of these tissue cross sections further confirmed that the iron⁺ cells identified in the Prussian blue histology were colocalized with F4/80⁺ macrophages. These iron⁺ F4/80⁺ macrophages were

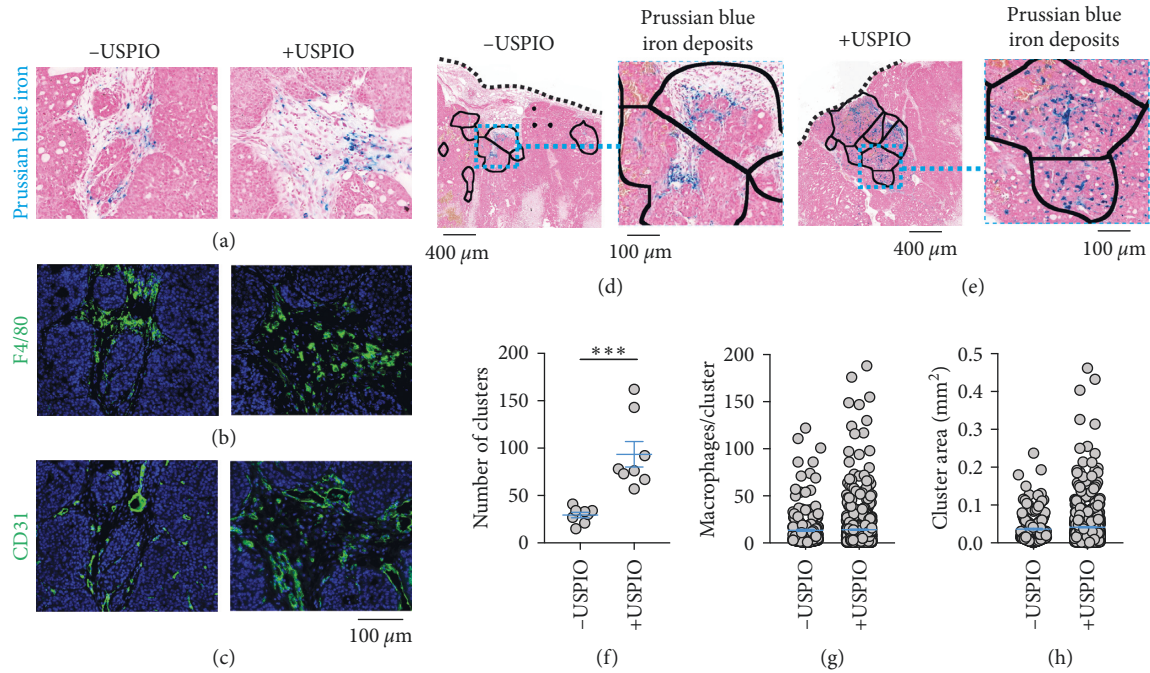


FIGURE 2: Spatial quantification of endogenous and contrast-enhanced macrophage deposits by iron histology. Paraffin-embedded sections from control (-USPIO) and iron nanoparticle-injected (+USPIO) tumors. (a) Prussian blue iron staining, (b) F4/80 macrophage immunofluorescence, and (c) CD31 vascular immunofluorescence were performed in the same sections to demonstrate the colocalization of iron deposits with infiltrating macrophages in vascular areas of the tumor microenvironment. Histological fields from (d) control and (e) iron nanoparticle-injected mice showing deposits of Prussian blue iron⁺ macrophages localized in infiltrative border regions in discrete clusters. Black borders are regions of interest drawn automatically around deposits of cells according to iron status. Expansion shows iron⁺ macrophages in the deposit regions. (f) Number of iron deposits (clusters) per tumor cross section in control (-USPIO) and nanoparticle-injected (+USPIO) mice (mean $\pm$ SEM shown, $n = 8$ tumors/group, *** $p < 0.001$, two-tailed unpaired Student's t -test). (g) Number of iron⁺ macrophages per deposit, and (h) size of each of the deposits measured over the whole Paraffin-embedded tumor cross sections stained by Prussian blue (mean $\pm$ SEM shown, -USPIO $n = 235$ total clusters/group, +USPIO $n = 748$ total clusters/group, n.s. $p > 0.05$). Scale bar is shown for all images.

invariably found in close proximity to CD31⁺ vasculature in both control and USPIO-injected cohorts suggesting that the accumulation of metabolic or nanoparticle-derived iron is dependent upon their spatial distribution beside tumor vasculature.

The spatial characteristics of the histologically detected macrophage iron deposits were then analyzed in a manner similar to the FeMRI pixel cluster analysis. The endogenous (Figure 2(d)) and USPIO-enhanced deposits (Figure 2(e)) found in the localized regions were automatically identified and the number of the clusters (Figure 2(f)), the number of iron⁺ macrophages per deposit (Figure 2(g)), and the areas of the clusters (Figure 2(h)) were measured exhaustively from whole tumor axial cross sections. The number of deposits containing iron⁺ macrophages increased significantly with USPIO injection ($p < 0.001$), and deposit areas in the control and injected groups were found to be equivalent, approximately a few MRI square-pixels ($p > 0.05$). These regions also equivalently contained an average of approximately 14 iron⁺ macrophages per control or USPIO-enhanced deposit ($p > 0.05$) supporting the cellular sensitivity of the FeMRI measurement and specificity of the pixel cluster analysis for these cellular species.

To determine the polarization status of general macrophage populations in the tumors and their changes with injection of USPIO, immunofluorescence staining of the tumor cross sections was conducted for pan-macrophage (CD68), inflammatory (M1-like AIF1 (allograft inflammatory factor 1)), and wound-healing surface marker phenotypes (M2-like CD206 (mannose receptor)), besides Prussian blue iron histology as the primary observable (Figures 3(a)–3(e)). Absolute counts of CD68⁺ macrophages conducted over whole tissue cross-sectional areas were performed to score CD68 infiltrates (Figure 3(f); $p > 0.05$). AIF1⁺ and CD206⁺ polarization markers were similarly quantified to evaluate phenotypic shifts. Exhaustive counts performed over the control tumor cross sections and calculation of frequency of these populations with respect to total numbers of these macrophages (M1 or M2/(M1+M2)) (Figure 3(g)) showed that USPIO injection did not lead to significant changes in absolute counts of macrophages ($p > 0.05$). Phenotypic populations in the control mice were significantly biased towards M2-like polarization with 42% M1-like AIF1⁺ and 58% of macrophages CD206⁺ ($p < 0.05$). This analysis of TAM

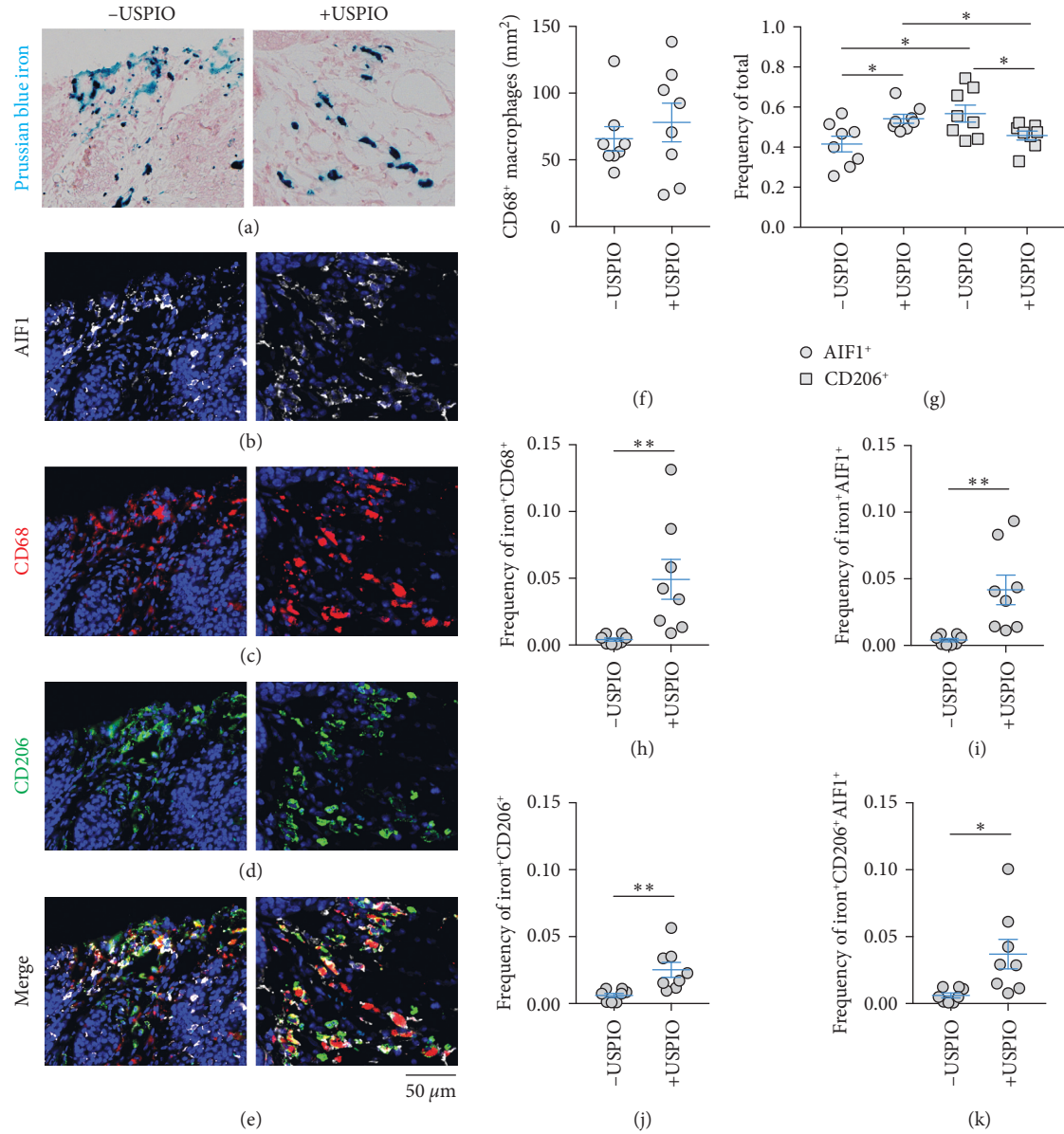


FIGURE 3: Characterization of endogenous and contrast-enhanced macrophage iron deposit polarization. Fields of paraffin-embedded tumor cross sections from control (left, -USPIO) and iron nanoparticle-injected (right, +USPIO) mice showing deposits of (a) Prussian blue iron⁺ macrophages and colocalized (b) AIF1, (c) CD68, (d) CD206, and (e) merged immunofluorescent markers. (f) Absolute count of CD68⁺ macrophages per mm² tumor cross section and (g) frequency of total M1-like (AIF1) and M2-like (CD206) macrophages determined by whole tumor cross section ROI analysis of immunofluorescent images in control (-USPIO) and nanoparticle-injected (+USPIO) cohorts (mean $\pm$ SEM shown, $n = 8$ tumors/group, * $p < 0.05$, two-tailed Student's t -test). Frequency of total iron⁺ macrophage calculated as (h) iron⁺CD68⁺/CD68⁺, (i) iron⁺AIF1⁺/AIF1⁺, (j) iron⁺CD206⁺/CD206⁺, and (k) iron⁺CD206⁺AIF1⁺/CD206⁺AIF1⁺ cells (mean $\pm$ SEM shown, $n = 8$ tumors/group, * $p < 0.05$, ** $p < 0.01$, two-tailed Student's t -test). Scale bar is shown for all images.

polarization was also performed in the USPIO-injected animals and showed that M1 and M2 status was significantly different with 54% AIF1⁺ and 46% CD206⁺ TAMs present ($p < 0.05$) indicating that USPIO injection caused a significant increase in M1-like cells and reduction of M2-like cells ($p < 0.05$). This demonstrates that TAM polarization and not number of macrophages in the tissue changed as a function of iron nanoparticle injection in these measurements.

We then measured the frequency of specifically iron⁺-polarized TAMs with respect to their respective general population (iron⁺M1⁺ or iron⁺M2⁺/M1⁺ or M2⁺), performed counts of iron⁺CD68⁺ cells as a function of total CD68, and also counted the frequency of the iron⁺AIF1⁺CD206⁺ population expressing both general macrophage polarization markers selected in this study (Figures 3(h)–3(k)). The numbers of iron⁺ cells were less frequent than the general populations assessed above. In control tumors, 0.41% were

iron⁺, 0.40% of AIF1⁺ were iron⁺, 0.61% of CD206⁺ TAMs were iron⁺, and 0.60% were iron⁺AIF1⁺CD206⁺ cells. Injection of USPIO led to significant increases in all these macrophage subsets with 4.9% iron⁺CD68⁺ cells ($p < 0.01$), 4.1% iron⁺AIF1⁺ ($p < 0.01$), 2.5% iron⁺CD206⁺ ($p < 0.01$), and 3.7% iron⁺AIF1⁺CD206⁺ cells observed after USPIO injection ($p < 0.05$). This indicates that SPIO injection increases iron in all polarized macrophage subsets but also indicates that inflammatory subsets take up relatively, but not significantly ($p > 0.05$), more iron than M2-like populations.

3. Discussion

The main purpose of the current work was to evaluate a computer vision method for detection and quantification of USPIO-enhanced macrophages by MRI and extend this analysis to histological images in order to characterize sub-MRI pixel phenotypes of the cells. By targeting the spatial heterogeneity in iron-based pixel contrast arising from endogenous or iron contrast agent-enhanced cellular iron deposits, this approach improved statistical quantification of macrophages over conventional ROI-average distribution analysis, and provided measured constraints on the size and frequency of the polarized macrophage deposits.

The MRI analysis approach presented here improves on current conventional ROI-based approaches by parsing the spatial distributions of iron image contrast. This was accomplished by constructing parametric iron MRI maps and then quantifying the number and size of pixel cluster areas as a function of stratified iron concentration range. This indicated that most of the area of tumor pixels in both control and nanoparticle-injected groups were predominantly of low iron contrast, reflecting a cellular distribution characteristic of the location of low-iron cancer cells and stroma in the tumor. The ROI distribution analysis only revealed minor shifts towards high iron concentrations with USPIO injection, while counting of the localized iron clusters increased the statistical differences between the control and USPIO-injected groups. A similar approach was used to evaluate the size of macrophage iron deposits in the histological analysis with and without contrast agents. This revealed areas of iron-laden macrophage colonies that were on the order of the size and frequency of the high-iron MRI clusters. This side-by-side spatial analysis confirmed the cellular sensitivity and specificity of the MRI and histological methods for detecting localized macrophage deposits according to the iron status, and provided per unit area cellularity estimates of the iron-laden macrophages detected *in vivo* by MRI.

To complement the iron histology analysis, we also performed immunofluorescent imaging focusing on the microenvironment and polarization characteristics of both general macrophage populations and specifically the iron⁺ macrophage subpopulations. The polarization of macrophages is a multifactorial process that depends on the tissue and microenvironment in which they are found as well as complex signaling between tissue resident and infiltrating immune cells with the macrophages [31, 32].

As such, myriad intracellular and surface protein markers have been developed that allow one to specify their position along the continuum of accessible polarization states [33]. Iron itself is a central metabolic factor in macrophage function and is associated with many polarization states [21]. To determine the association of iron deposits with the tumor microenvironment and polarization, we adopted a panel of general tissue and macrophage biomarkers which showed the iron⁺ macrophages were predominantly found in vascularized CD31⁺ regions of the stroma where they likely serve iron-handling functions in heme homeostasis [34–36]. Overall, these iron⁺ macrophages were found as a subpopulation of the total macrophages detected in the whole cross sections of stained tissues. Characterization of these general populations indicated that frequency of CD68⁺ macrophage infiltrates remained approximately unchanged with USPIO injection and further confirmed previous studies indicating that phenotypic inflammatory M1-like macrophage markers are increased and protumor M2-like phenotypes decreased with USPIO injection.

In our spatial analysis, we further characterized effects of USPIO on polarized endogenous and nanoparticle-enhanced iron⁺ macrophage deposits. We observed that USPIO injections increase iron in pan-macrophage CD68⁺ populations as expected and also found relative differences in frequency of iron-laden populations following USPIO injection in polarized subsets. Here, although inflammatory (AIF1⁺), wound-healing (CD206⁺), and double-positive macrophage populations (AIF1⁺CD206⁺) all increased frequency with USPIO injection, inflammatory macrophage populations experienced the largest relative increases presumably due to their predominant role in iron scavenging during the acute inflammatory response caused by the iron nanoparticles [22–24]. Therefore, spatial histological segmentation approaches based on iron status combined with macrophage polarization measurements in these regions allow for the further characterization of subpixel phenotypes of the iron-laden macrophage giving rise to the contrast measured by MRI. This provides further insight into the biological function of the macrophages detected and reveals differences in their iron handling roles in the tumor microenvironment.

4. Conclusions

While the spatial image analysis approach described here is based on identification of the macrophage according to the iron status, similar machine-based analyses are envisioned to be conducted utilizing other parametric MRI contrasts and other multimodality imaging contrasts. Further, in the current work, the approach is specific for the macrophage due to their high innate capacity for iron uptake; however, integrating other endogenous and contrast-agent enhanced parameters with these analysis tools can also potentially provide spatial information about different cellular populations in diverse tissues settings. We anticipate that the current findings will motivate the use of computer-assisted image analysis routines and accelerate the translation of these methods towards the clinic to aid in our imaging investigations of complex cellular microenvironments and physiological processes in diseases such as cancer.

5. Methods

5.1. Animal Procedures. All animal work was approved and performed according to the guidelines of the Animal Care and Use Committee of MSKCC. Mice were anesthetized with 1–3% isoflurane in O₂ gas, and respiration was monitored during all imaging sessions. Female 6-week-old FVB/N mice underwent orthotopic injection into the lower mammary fat pad of 1×10^6 syngeneic TS1 MMTV-PyMT tumor cells grown under standard tissue culture conditions and suspended in 100 μ L 50% Matrigel (BD Bioscience). Mice bearing orthotopic TS1 MMTV-PyMT tumors (approximately 1 cm³) were injected with either 0.5 mmol/kg dextran coated superparamagnetic 5 nm iron oxide nanoparticle contrast agent (Ocean NanoTech) or saline and were imaged 24 hr after injection.

5.2. MRI. ¹H MRI was conducted on a 7T/30 cm horizontal bore Biospec MRI system (Bruker BioSpin Corporation) with a custom-built 30 mm inner-diameter transmit-receive quadrature coil. A 2D multigradient echo (MGE) relaxation pulse sequence with fat suppression was used with the following parameters: 16 evenly spaced 3 ms TEs, TR 1.2 s, matrix 256 $\times$ 256 in the plane data matrix with 25–49 slices, an in-plane spatial resolution of 0.1 mm $\times$ 0.1 mm with a slice thickness of 0.5 mm, and RF flip angle of 90°. Each phase encode acquisition was gated on the animal's respiratory cycle. The first image of the gradient-echo series was used as reference images shown in the figures.

Aqueous solutions of Fe³⁺(NO₃)₃ (Fisher Scientific) were used as reference iron concentrations over the 0.0–0.3 mg iron(III) g⁻¹ range at 7T [29]. The T_2^* values for these solutions were determined by pixel-wise mono-exponential fitting of the MGE images using Matlab (Mathworks) and/or Fiji [37]. A linear relation between the relaxation rate $R_2^* = 1/T_2^*$ and known iron concentration was found and was subsequently used to generate parametric iron MRI maps. Iron MRI maps were stratified by high concentration (total range, 0.0–0.3 mg·g⁻¹; high, 0.15–0.3 mg·g⁻¹). Spatial characteristics of the high-iron pixels were then quantified by performing cluster analysis over whole tissue MRI cross sections with the Fiji Analyze Cluster tool.

5.3. Histology. Whole tissue cross sections were sliced from the axial midpoint regions of PBS-perfused tumors following MRI studies, fixed in 4% PFA for 24 hours at 4°C, and then washed with H₂O and resuspended in 70% ethanol (Fisher Scientific). Tissues were paraffin embedded, cut into 5 μ m sections, and placed on glass slides for immunohistochemistry.

The Prussian blue histochemical detection of iron(III) was performed by manually deparaffinizing in xylene and rehydration in series of alcohol dilutions (100%, 95%, and 70%) and tap water. Slides were then placed in a working solution of equal parts of 10% potassium ferricyanide (Fisher Scientific) and 10% hydrochloric acid (Fisher Scientific) prepared in distilled water and stained for 30 minutes. Slides were rinsed in distilled water, counterstained with nuclear-fast red, and cover slipped with Permount (Fisher Scientific).

The immunofluorescent detection of F4/80, CD31, CD68, AIF1, and CD206 was performed using a Discovery XT processor (Ventana Medical Systems). The tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with the CC1 buffer (Ventana Medical Systems), and sections were blocked for 30 minutes with Background Buster solution (Innovex) followed by avidin/biotin blocking for 8 minutes. F4/80 (Abcam, cat# ab6640, 5 μ g/ml), CD31 (Dianova, cat# DIA-310 1 μ g/ml), CD68 (Boster, cat# PA1518, 5 μ g/ml), AIF1 (Wako, cat# 019–19741, 0.5 μ g/ml), and CD206 (Abcam, cat# ab64693, 1 μ g/ml) were applied, and sections were incubated for 5 hours, followed by 60 minutes incubation with biotinylated goat anti-rabbit antibodies (Vector Labs, cat# PK6101) at 1:200 dilution. The detection was performed with Streptavidin-HRP D (part of the DABMap kit, Ventana Medical Systems), followed by incubation with Tyramide Alexa Fluor A488 (Invitrogen, cat# T20922), Tyramide Alexa Fluor 568 (Invitrogen, cat# T20948), or Tyramide Alexa 647 (Invitrogen, cat# B40958), respectively, prepared according to manufacturer's instructions with predetermined dilutions. After staining, slides were counterstained with DAPI (Sigma-Aldrich, cat# D9542, 5 μ g/ml) for 10 min and coverslipped with Mowiol. Histological sections were digitized with a Mirax Scan system and read with Panoramic Viewer (3DHISTECH, Budapest, Hungary). Images were first visually inspected, and then the whole images were exported and processed in Fiji. Iron deposits were quantified as described by Leftin et al. [29]. Briefly, iron deposit maps were generated by resizing the histological images by using pixel averaging and bilinear interpolation to down-sample the image size (1:100) to the resolution of the MRI experiment. The resulting masks of regions containing iron⁺ macrophages were discretized by watershed gradient processing, and spatial characteristics of the clusters were determined using the Fiji Analyze Cluster tool. The number of iron⁺ macrophages per cluster was then determined by using the cluster maps to define regions of interest for cell counts in the full-resolution histological images. Polarization state was determined by either exhaustive whole cross section counting of AIF1 or CD206 immunostained macrophages or localized analysis that was restricted to colocalized iron⁺ macrophages contained in the iron deposit regions.

5.4. Statistics. Two-tailed Student's *t*-tests were performed with significance determined as $p < 0.05$. All statistical calculations indicated in the text were performed with the GraphPad Prism 7 Software.

Data Availability

All supporting data are found in the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

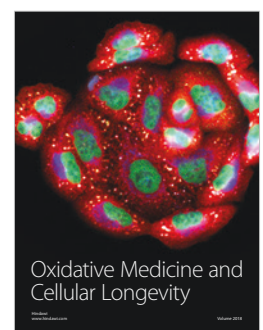
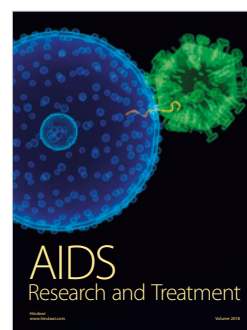
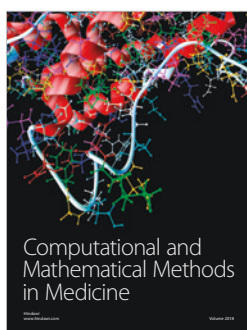
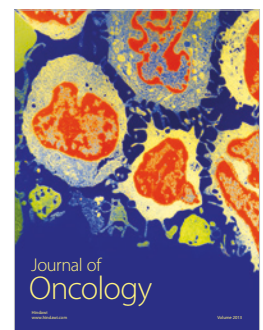
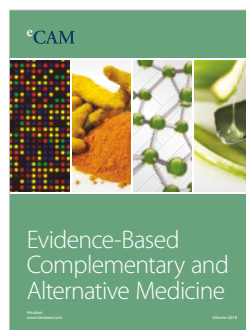
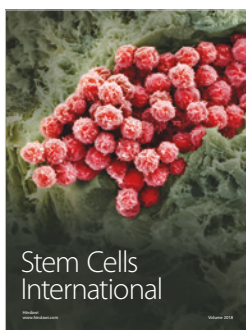
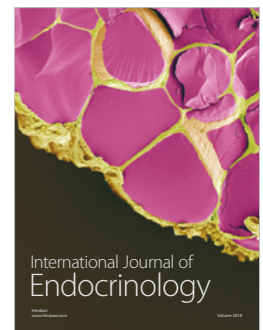
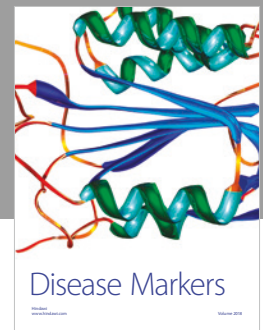
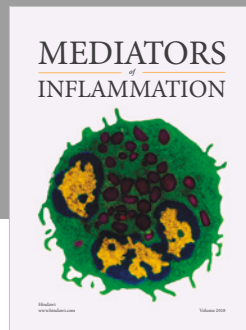
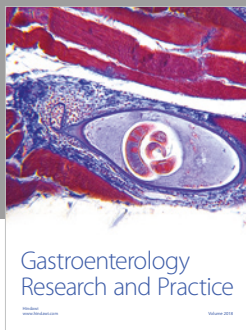
Acknowledgments

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Imaging endogenous macrophage iron deposits reveals a metabolic biomarker of polarized tumor macrophage infiltration and response to CSF1R breast cancer immunotherapy

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Iron deposits are a phenotypic trait of tumor-associated macrophages (TAMs). Histological iron imaging and contrast-agent free magnetic resonance imaging (MRI) can detect these deposits, but their presence in human cancer, and correlation with immunotherapeutic response is largely untested. Here, primarily using these iron imaging approaches, we evaluated the spatial distribution of polarized macrophage populations containing high endogenous levels of iron in preclinical murine models and human breast cancer, and used them as metabolic biomarkers to correlate TAM infiltration with response to immunotherapy in preclinical trials. Macrophage-targeted inhibition of the colony stimulating factor 1 receptor (CSF1R) by immunotherapy was confirmed to inhibit macrophage accumulation and slow mammary tumor growth in mouse models while also reducing hemosiderin iron-laden TAM accumulation as measured by both iron histology and *in vivo* iron MRI (FeMRI). Spatial profiling of TAM iron deposit infiltration defined regions of maximal accumulation and response to the CSF1R inhibitor, and revealed differences between microenvironments of human cancer according to levels of polarized macrophage iron accumulation in stromal margins. We therefore demonstrate that iron deposition serves as an endogenous metabolic imaging biomarker of TAM infiltration in breast cancer that has high translational potential for evaluation of immunotherapeutic response.

In most cancers, macrophage infiltration is linked to negative clinical outcomes such as poor survival, metastatic dissemination, and evasion of anti-tumor immune mechanisms^{1–4}. Major efforts are underway to understand the function of macrophage infiltrates in the tumor microenvironment in order to develop new treatments such as immunotherapies that target macrophages and inhibit these deleterious outcomes. To support these efforts, there is an increasing need for macrophage biomarkers and imaging approaches that allow for the localization of the targeted macrophage populations according to metabolic phenotype or function and measurement of their response to therapy. Histological methods are useful for quantification of macrophage behavior, but *in vivo* characterization is not possible, and definition of specific phenotypic properties such as polarization status or metabolism can be difficult to generalize from selective biopsy due to tissue intrinsic aspects of macrophage function and the heterogeneous nature of the tumor microenvironment^{5,6}. *In vivo* approaches such as positron emission tomography (PET) can provide information about tumor macrophage presence, but repeated imaging is limited due to the accumulation of radioactive dose, and resolution of infiltrating macrophages is also limited by current

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technology^{7,8}. As an *in vivo* imaging tool, magnetic resonance imaging (MRI) can be used to map many metabolic pathways associated with cancer including glycolysis^{9,10}, the tricarboxylic acid cycle¹¹, phospholipid and ATP metabolism^{12,13}, dependencies on perfusion and hypoxia¹⁴, pH¹⁵, and oxidation/reduction balance¹⁶. Despite this arsenal of anatomical and functional molecular protocols, these non-invasive approaches are usually not able to resolve and assign spatial differences in metabolism to specific immune cell populations within the tumor. This is because the metabolic properties of these populations are often obscured as they share similar metabolic pathways to the cancer cells, have smaller relative population sizes, and more heterogeneous spatial distributions compared to the bulk of the tumor¹⁷. Given the available resolution of most metabolic MRI techniques this leads to an average representation of the spatial distribution of metabolites, often reflecting just the dominant cellular population, i.e. the cancer cells, in the metabolic images.

In order to enable the imaging of macrophages according to their metabolic status, we sought to identify metabolic pathways that exhibit higher specificity for these populations rather than cancer cells or other cellular species. Iron metabolism, the processes by which uptake, storage, and re-export of iron takes place, is conserved in most mammalian cells¹⁸. However, macrophages in particular are known to play a central role in systemic homeostasis of iron according to their unique genetic program that enables them to handle high metabolic flux of this micronutrient systemically and in the tumor microenvironment^{19–21}. In this iron-regulating role, macrophages can exhibit a unique phenotypic trait, namely the accumulation of aggregates comprised of iron known as hemosiderin²². Recently, we identified endogenous hemosiderin iron deposition as a putative pan-tissue bio-marker of TAMs by using clinical iron-sensitive MRI methods (FeMRI) and Prussian blue iron histology with- out contrast agents to detect accumulated iron in hemosiderin-laden macrophages (HLMs) of murine prostate, breast, and metastatic cancer models^{23,24}. Technically, high-iron concentration FeMRI pixel regions and Prussian blue positive regions indicate the location of macrophage iron deposits that sets them apart from other lower concentration bio-iron sources such as blood due to the physical magnetic and chemical properties of the solid iron stores^{25–33}. Similar high-resolution MRI and histological iron imaging approaches can also be used to identify macrophage targets in cancer but traditionally require intravenous injections of iron nanoparticle contrast agents that rely on macrophage phagocytosis rather than metabolism in a manner similar to many PET probes^{34,35}. However, caveats of the nanoparticle-enhanced MRI and histological techniques include off-target delivery following from the enhanced permeability and retention effect contributed by highly vascularized leaky tumors that reduces specificity for the macrophage deposits³⁶, and the nanoparticles themselves can induce polarization of macrophages that can potentially bias the metabolic function and the therapeutic response of the targeted populations^{37–39}. By recognizing the tendency of macrophages to metabolically accumulate hemosiderin—which generates high-iron contrast akin to that produced using iron nanoparticle injections⁴⁰—microscopic deposits of these cells can be quantified in terms of their abundance and spatial distribution by MRI and histology without contrast agents according to their innate iron metabolism. While these prior studies associated HLMs with TAMs and therefore suggest that they can be used as probes of TAM infiltration to gauge efficacy of immune therapy, here we define the spatial correlations of these metabolically-unique TAM infiltrates with immunotherapy response, and prospectively characterize their distribution in human breast cancer using histological iron imaging in order to support the translation of such combination metabolic iron imaging and therapy approaches to the clinic.

Results

Spatial profiling of tumor macrophage iron deposits with iron imaging. Endogenous FeMRI methods are increasingly favored over the use of invasive biopsy using Prussian blue iron imaging for measurement of non-heme iron concentrations in liver, heart, and brain^{26–28,41–44}. While these methods mitigate the sampling bias introduced by selective biopsy, they conventionally rely on whole organ averages of cellular iron loading, and therefore neglect spatial heterogeneity indicative of TAM infiltration. We rationalized that we could also use FeMRI and correlative Prussian blue histology to detect, resolve, and quantify the spatial distributions of localized TAM iron deposits in breast cancer tumors by addressing spatial heterogeneity of cellular iron deposits through utilization of image analysis algorithms that enable the automatic detection, quantification, and localized mapping of HLM deposits in the iron images⁴⁰. As proof-of-concept, we directly compared histological sections stained for iron using Prussian blue that is specific for HLMs (Fig. 1a), with iron maps generated by *ex vivo* FeMRI-microscopy (Fig. 1b) of co-registered tissue sections obtained from an orthotopic TS1 breast cancer model used commonly in TAM research whose tumors are promoted under control of the murine mammary tumor virus which drives expression of the mammary gland specific polyoma virus middle T-antigen (MMTV-PyMT)^{45–49}. Iron⁺ TAMs were determined to be the dominant species generating distinct high-iron pixel clusters in the MMTV-PyMT tumor cross-sections as comparison of Prussian Blue stained macrophage (Fig. 1c) and red blood cells (Fig. 1d), another candidate for contributing to iron contrast because of their heme cargo, showed they do not stain for Prussian blue iron and thus contribute only to low FeMRI contrast, further corroborating the specificity of the method for HLMs²³. We then interrogated the spatial distribution of the HLMs in the histological and FeMRI iron maps to compare these measurements as a score of TAM infiltration. Analysis of the histological (Fig. 1e), and MRI images (Fig. 1f) for high concentrations of iron yielded maps of the iron containing TAMs. The histological iron deposits and FeMRI pixel clusters were then graphed as a function of position in the tumor (% infiltration, Fig. 1g), and the radial infiltration profiles of the histological deposits and MRI clusters were found to be the same (Fig. 1h, $p > 0.05$). This confirmed the equivalence between the HLM measurements by FeMRI and histology, and further provided a novel means to map the spatial distribution of the HLM deposits according to metabolic status with cellular sensitivity.

Correlation between immunotherapeutic CSF1R inhibitor response and polarized iron deposit accumulation. To then further establish these TAM iron deposits as immunotherapy targets, we initiated preclinical CSF1R (colony-stimulating factor-1 receptor) inhibitor (BLZ945) trials in murine breast cancer

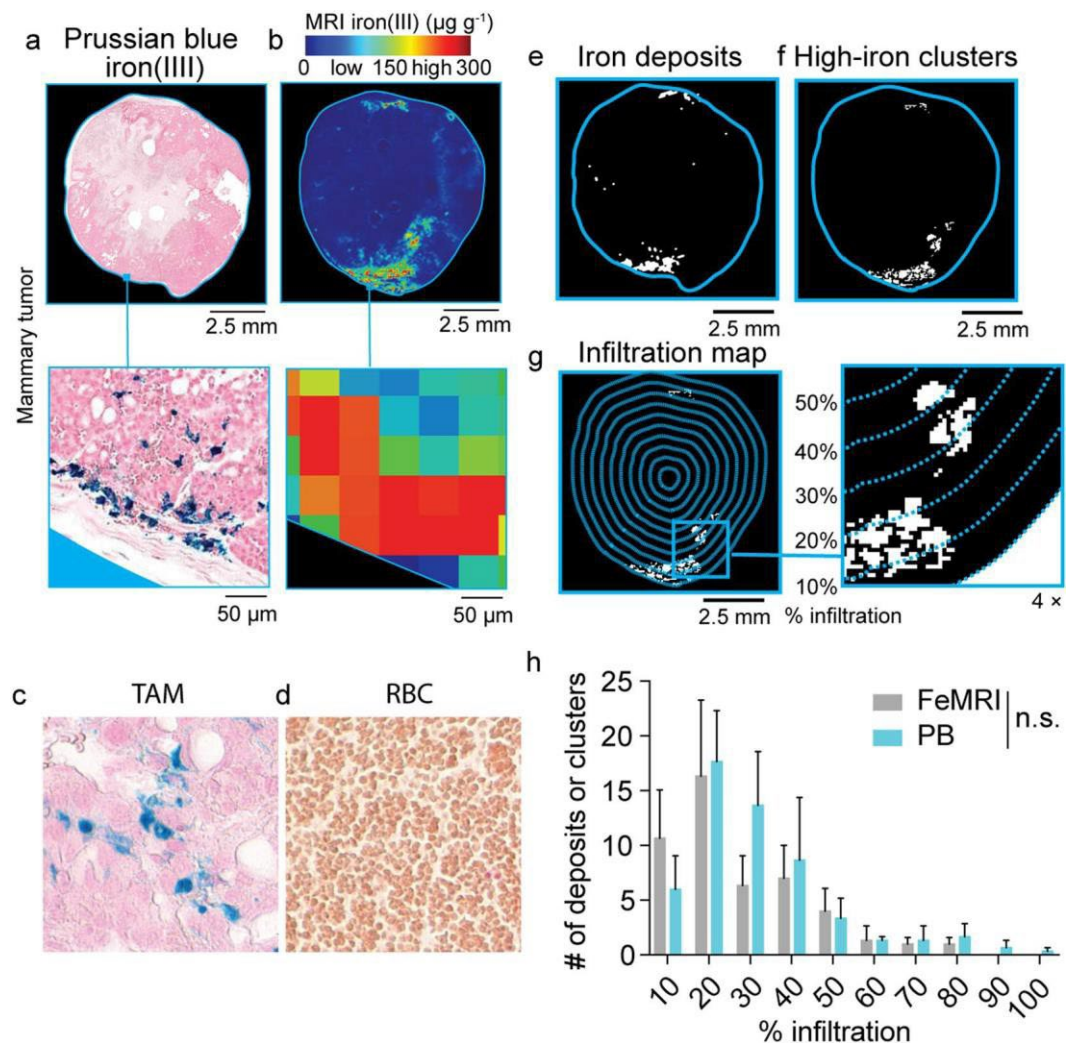


Figure 1. Imaging and spatial profiling of tumor macrophage iron deposits *ex vivo* with iron MRI (FeMRI) and Prussian blue iron histology. **(a)** Representative Prussian blue iron histology of MMTV-PyMT TS1 mammary tumor cross-section. Scale bar 2.5 mm. Expansion shows iron⁺ macrophage deposits. Expansion scale bar 50 μ m. **(b)** Representative *ex vivo* FeMRI of MMTV-PyMT TS1 mammary tumor cross-section. Scale bar 2.5 mm. Expansion shows high-iron pixel clusters. Expansion scale bar 50 μ m. **(c)** Representative Prussian Blue iron histology of tumor associated macrophages (TAM), and **(d)** red blood cells (RBC). Note iron⁺ macrophages and iron⁻ RBCs. **(e)** Iron deposit mask from Prussian blue histology. Scale bar 2.5 mm. **(f)** High-iron FeMRI cluster mask. Scale bar 2.5 mm. **(g)** Infiltration mapping using radial decile region rake sampling overlaid on high-iron MRI cluster mask. Scale bar 2.5 mm. 4x expansion shows high-iron FeMRI clusters and decile boundaries. **(h)** Infiltration profile showing number of histological iron deposits from Prussian blue (PB) and high-iron FeMRI pixel clusters (FeMRI) as a function of percent (%) infiltration into the MMTV-PyMT TS1 mammary tumors. (mean $\pm$ s.e.m. $n = 3$ tissue cross-sections, n.s. $p > 0.05$ Kolmogorov-Smirnov test).

models. This receptor kinase inhibitor blocks the interaction between the cytokine colony stimulating factor 1 (CSF1) that mediates macrophage accumulation in tumors via signaling with its receptor CSF1R^{50,51}. The drug has been shown to have the primary immunological effect of inhibiting the accumulation of TAMs in tumors, making it an excellent candidate for testing the iron imaging approaches, and it has the coincident therapeutic effect of slowing the growth of some breast, cervical, brain, and other cancers^{48,52–55}. Cell-line derived TS1 and 99LN MMTV-PyMT tumors orthotopically implanted in the mammary fat pads of their respective syngeneic FVB/N and C57BL6 hosts were studied. Treatment with the small molecule CSF1R inhibitor BLZ945 was initiated when tumors reached approximately 100 mm³ in the TS1 and 99LN models. Treatment continued until control tumors reached or exceeded 1 cm³ measured by caliper to establish pre-treatment and endpoint imaging time points, and significant tumor growth inhibition was observed in both models with CSF1R inhibition by these endpoints (Fig. 2a, $p < 0.001$ – $p < 0.0001$). Subsequent measurement of the tumor volumes made by MRI in the imaging studies of the control and BLZ945 treated TS1 (Fig. 2b,c) and 99LN models (Fig. 2d,e) recapitulated the reductions in tumor volumes established by the initial pilot trials where TS1 tumor volumes were 51% of

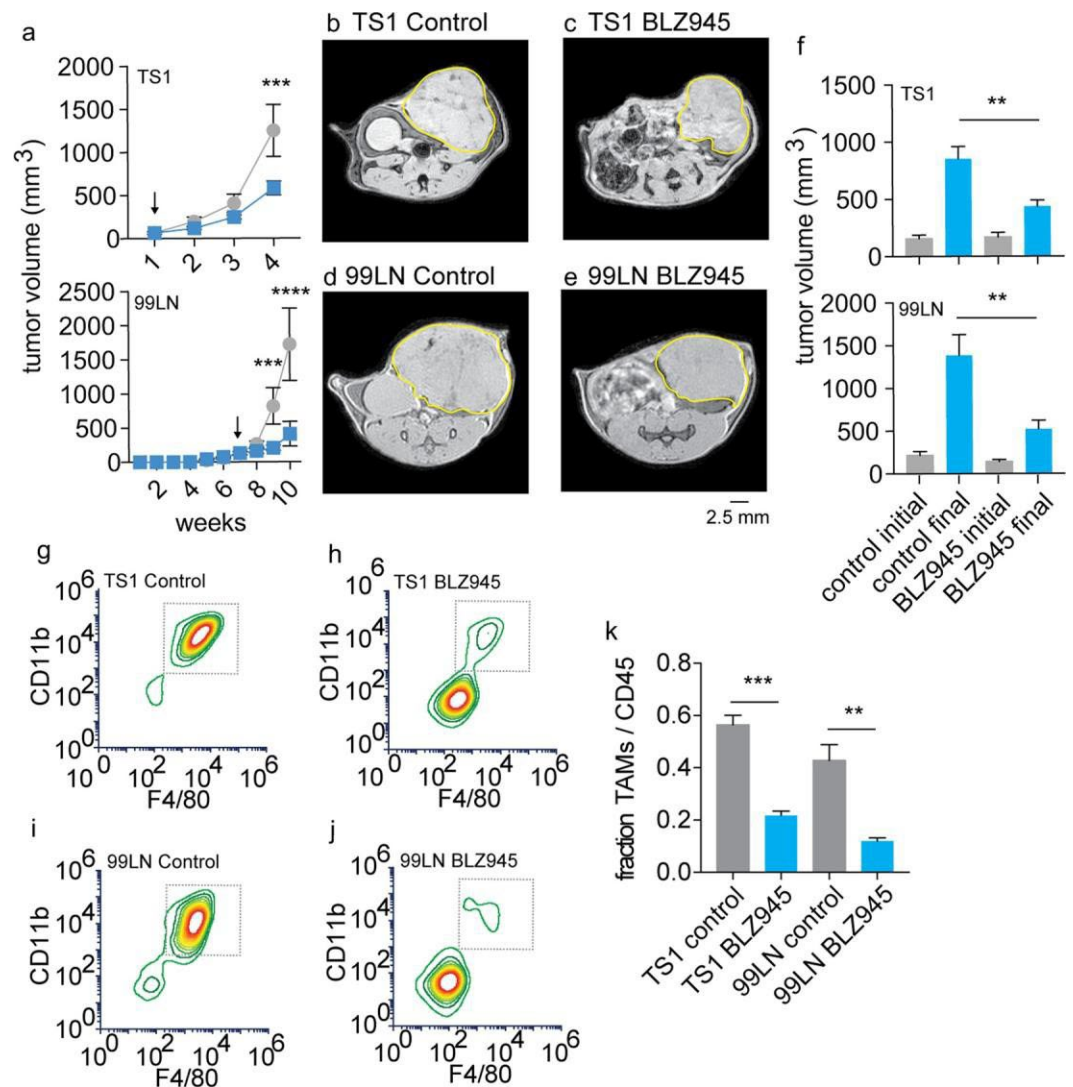


Figure 2. Validation of CSF1R immunotherapy effects on tumor growth and TAM accumulation in PyMT- MMTV breast cancer models. **(a)** Murine breast tumor models were established by orthotopic mammary fat pad injections and measured by caliper in TS1 and 99LN models during administration of the CSF1R inhibitor BLZ945 (200 mg/kg). Arrows indicate start of BLZ945 treatment (mean $\pm$ s.e.m. $n = 5$ mice/group, *** $p < 0.001$, **** $p < 0.0001$, 2-way ANOVA with Tukey's multiple comparison test). Representative first-echo images from MGE MRI experiments made at study endpoints in control and BLZ945 treated **(b,c)** TS1 and **(d,e)** 99LN MMTV-PyMT models. **(f)** Pre-treatment and endpoint tumor volumes measured by MRI in the CSF1R inhibitor trials. (mean $\pm$ s.e.m., $n = 8$ mice/group, ** $p < 0.01$, two-tailed unpaired students t-test). Flow cytometry panels of TAMs (live CD45⁺Ly6c⁻Ly6g⁻ cells gated on CD11b⁺F4/80⁺ cells) obtained from control and BLZ945 treated **(g,h)** TS1, and **(i,j)** 99LN tumors. **(k)** TAM frequency with respect to total CD45⁺ myeloid cells in the TS1 and 99LN CSF1R inhibitor trials (mean $\pm$ s.e.m. $n = 4$ mice/group, ** $p < 0.01$, *** $p < 0.001$, two-tailed unpaired students t-test).

controls, and the drug also limited the growth of the 99LN model tumors to approximately 34% of the untreated groups (Fig. 2f, $p < 0.01$).

To then characterize the primary immunological effect of the CSF1R inhibitor on TAM accumulation as has been done in previous studies in MMTV-PyMT models with the BLZ945 drug and others^{45,48}, whole tumors from the TS1 and 99LN models were collected at imaging endpoints and single-cell suspensions were prepared from the homogenates. Fluorescence staining of the cells for live CD45⁺Ly6c⁻Ly6g⁻CD11b⁺F4/80⁺ TAMs was then performed and the frequency of these cells was quantified by flow cytometry. Treatment with BLZ945 reduced the frequency of TS1 TAMs (Fig. 2g,h), and similarly 99LN models also exhibited reductions in TAMs (Fig. 2i,j) in accord with the previous studies of the inhibitor, thereby providing further preclinical validation for our imaging studies. Overall the CSF1R immune therapy lowered the frequency of TAMs with respect to total CD45⁺ cells in the tumors significantly with levels of BLZ945 treated TAM fraction by approximately 30% of the control levels in both TS1 and 99LN groups (Fig. 2k, $p < 0.001$ – $p < 0.01$).

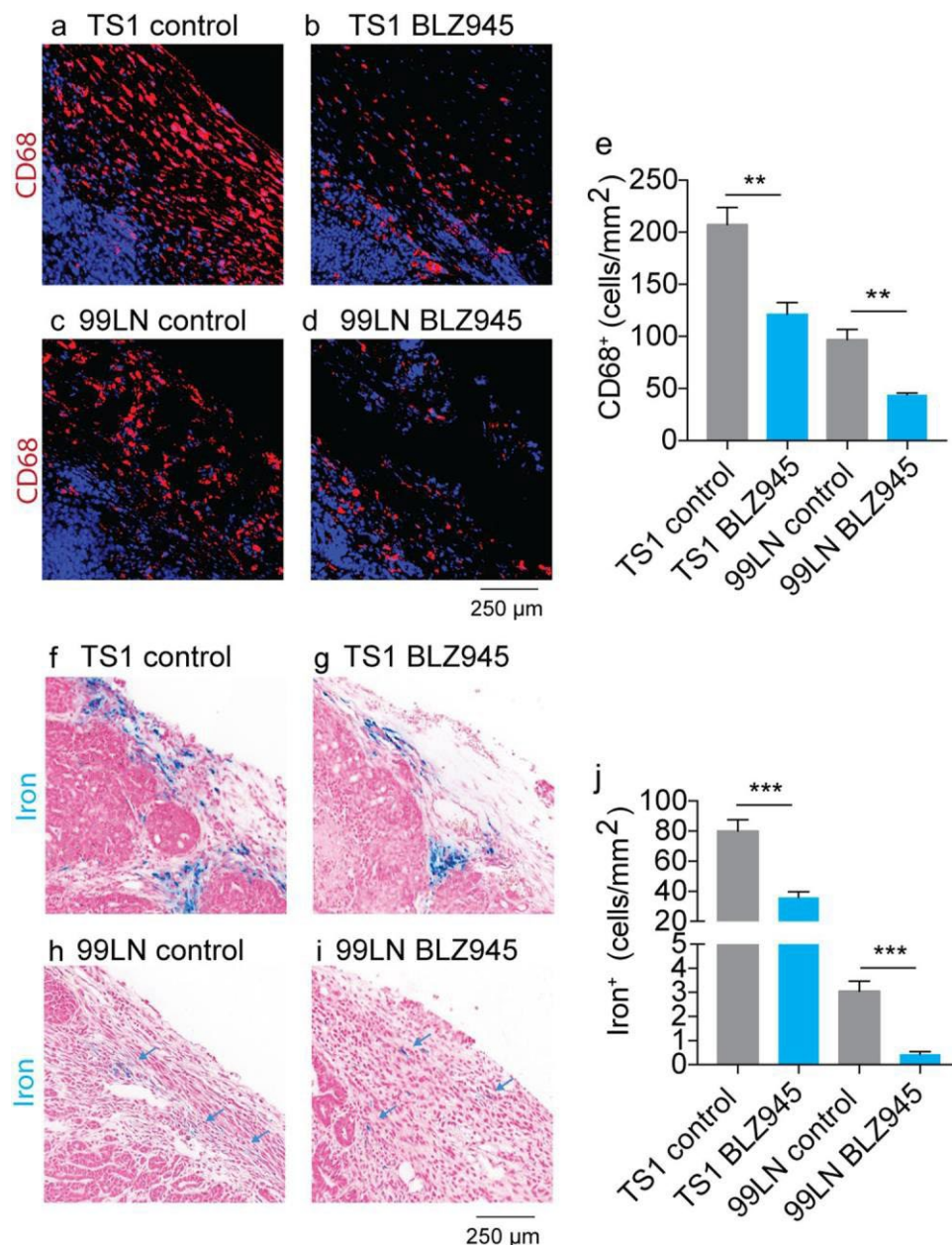


Figure 3. CD68 immunofluorescence and Prussian blue imaging of macrophage iron deposits in MMTV-PyMT murine breast cancer models of CSF1R immunotherapy. Representative CD68 macrophage immunofluorescent imaging in control and BLZ945 treated (a,b) TS1, and (c,d) 99LN tumors. Scale bar 250 μ m (e) Absolute counts of CD68⁺ macrophages per mm² MMTV-PyMT tumor cross-section in BLZ945 CSF1R inhibitor trials (mean $\pm$ s.e.m. n = 4 mice/group, **p < 0.01, two-tailed unpaired students t-test). Representative iron staining using Prussian blue iron histochemistry in control and BLZ945 treated MMTV-PyMT (f,g) TS1, and (h,i) 99LN tumors. Scale bar 250 μ m (j) Absolute counts of iron⁺ macrophages per mm² tumor cross-section in the CSF1R inhibitor trials (mean $\pm$ s.e.m. n = 4 mice/group, ***p < 0.001, two-tailed unpaired students t-test).

In order to quantify the response of iron⁺ TAMs to the BLZ945 inhibitor, we performed Prussian blue histology specific for the TAM iron deposits beside CD68 macrophage histology as a general marker of TAMs. First, digitized images of paraffin-embedded whole axial cross-sections of the tumor collected at CSF1R trial endpoints were analyzed by counting all TAMs according to their CD68 staining to measure general response to the CSF1R inhibitor. Reduced numbers of CD68⁺ infiltrating macrophages were found in both the TS1 (Fig. 3a,b) and 99LN cohorts (Fig. 3c,d) confirming previous studies of this inhibitor's effects on TAM accumulation in MMTV-PyMT models⁴⁵. Overall, CD68⁺ macrophages were lower by approximately 42% in the TS1 model and 55% in the 99LN model following BLZ945 treatment (Fig. 3e, p < 0.01) generally recapitulating the reductions in TAMs measured by flow cytometry in the trials. Histological assessments of HLMs using Prussian blue iron staining were

performed in the same manner as CD68 histology. TS1 tumors in the syngeneic FVB/N background exhibited numerous HLM deposits consisting of colonies of iron⁺ TAMs found largely in stromal margins of the para- fin embedded tumor cross-sections (Fig. 3f), and treatment with BLZ945 reduced the number of these cellular species (Fig. 3g). The iron⁺ TAMs were relatively fewer in the 99LN models, but were still detected in the digital image analysis of the Prussian blue iron-stained 99LN tumors (Fig. 3h), and these iron deposits were also lowered with BLZ945 treatment (Fig. 3i). Administration of the CSF1R inhibitor reduced iron⁺ TAM accumulation by approximately 50% in the TS1 models and 85% in the 99LN models (Fig. 3j, $p < 0.001$), again corroborating the primary effect of the CSF1R inhibitor on TAM accumulation, and further indicating that this drug also effects iron containing TAM populations.

Macrophage accumulation in tumors fulfills both inflammatory and anti-inflammatory roles, but little is known about the polarization and CSF1R status of iron⁺ TAMs. To investigate the polarization status of these macrophage subpopulations, Prussian blue iron-stained histological sections (Fig. 4a,b) were retained using multiplexed immunofluorescence for M1-like (Fig. 4c,d, pro-inflammatory, AIF1; allograft inflammatory factor-1), M2-like (Fig. 4e,f, anti-inflammatory, CD206; mannose receptor), and CSF1R receptor (Fig. 4g,h) markers in the TS1 and 99LN models. Controlling for the primary inhibitory effect on the accumulation of iron⁺ populations, fields containing the iron⁺ TAMs in control tumors and fields still containing iron deposits following BLZ945 treatment were identified in the registered tumor cross-sections in order to quantify the co-positivity of the iron⁺ TAMs as a function of M1-like, M2-like, and CSF1R status in these localized regions. Counts of these macrophage populations in the TS1 and 99LN tumors (Fig. 4k,l) showed that TS1 tumors had relatively higher numbers of macrophages expressing polarization markers and CSF1R compared with the 99LN model in accord with the cell counts made independently of polarization status. The CSF1R inhibitor BLZ945 did not greatly effect these general populations, though a small reduction of CD206⁺ macrophage was observed in the TS1 deposit regions ($p < 0.05$). To specifically assess changes in polarization status of the iron⁺ macrophages with BLZ945 treatment, the fractions of iron⁺ macrophage subpopulations expressing M1-like, M2-like, and mixed M1- and M2-like markers were calculated as a function of the total iron⁺AIF1⁺, iron⁺CD206⁺ and iron⁺AIF1⁺CD206⁺ populations in the TS1 (Fig. 4m) and 99LN models (Fig. 4n). Similarly, the iron⁺AIF1⁺, iron⁺CD206⁺ and iron⁺AIF1⁺CD206⁺ populations were also assessed for CSF1R positivity calculated as a fraction of total iron⁺CSF1R⁺AIF1⁺, iron⁺CSF1R⁺CD206⁺ and iron⁺CSF1R⁺AIF1⁺CD206⁺ macrophages present (Fig. 4o,p). This analysis revealed that while the iron⁺ populations were found co-localized with AIF1 and CD206 polarization markers as well as CSF1R, the fraction of these iron⁺ M1-like and iron⁺ M2-like species and their CSF1R⁺ counterparts in these iron deposit regions were largely unaffected by the CSF1R inhibitor except for small differences in iron⁺AIF1⁺CD206⁺, iron⁺CSF1R⁺AIF1⁺ and iron⁺CSF1R⁺AIF1⁺CD206⁺ populations in the fields assessed ($p < 0.05$). This histological analysis indicates that polarization of the iron⁺ populations, and general populations overall is largely unaffected by the CSF1R inhibitor, and also indicated that the HLMs were not significantly biased towards a given polarization state as they were frequently co-localized with multiple markers.

Iron imaging of macrophage tumor infiltration in CSF1R inhibitor trials. *In vivo* contrast-agent free FeMRI was then used to quantify macrophage iron deposits of the MMTV-PyMT models in the BLZ945 trials, and correlate their detection with the CSF1R inhibitor's primary immunotherapeutic effects on macrophage accumulation and tumor growth. FeMRI images were quantified using image-processing algorithms demonstrated above in the *ex vivo* analysis. Control and BLZ945 treated MMTV-PyMT mammary tumors exhibited high-iron pixel clusters indicative of macrophage iron deposits in both the TS1 (Fig. 5a,b) and 99LN (Fig. 5c,d) models. High-iron pixel clusters found in BLZ945 treated tumors were approximately 37% of control levels in both models (Fig. 5e $p < 0.01$) supporting the flow cytometry and histological measurements as shown in Figs 2 and 3. Tumor growth and accumulation of FeMRI clusters were positively correlated in both TS1 (Fig. 5f) and 99LN models (Fig. 5g), and immunotherapeutic response was indicated by a reduction in tumor growth and inhibition of FeMRI cluster accumulation.

Counts of the high-iron FeMRI pixel clusters and HLM deposits found in histological Prussian blue iron images were also analyzed as a function of position in order to establish spatially-resolved scores of immunotherapeutic response (Fig. 6a–d). The scores of the FeMRI clusters (Fig. 6e,f) and HLM deposits (Fig. 6g,h) both showed higher levels of TAMs at the stromal margins of the tumors with decreasing numbers of iron containing cells found towards the tumor core where less macrophage infiltration generally occurs. Treatment with BLZ945 resulted in overall lower levels of the iron containing regions throughout the tumor cross-sections. The clusters measured by FeMRI and HLM deposits measured by Prussian blue histology were most affected at the outer margins of the tumors by the CSF1R therapy as indicated by the significant reductions observed over these regions ($p < 0.05$ – $p < 0.0001$). This establishes that contrast-agent free *in vivo* FeMRI can detect and map macrophage iron deposits in a similar manner to *ex vivo* iron histology, and that using FeMRI and iron histology during CSF1R immunotherapy provides measurements of TAM infiltration correlated with the regions of maximal immunotherapeutic response. Thus, iron serves as a novel metabolic biomarker indicating response to immunotherapy treatment that can be monitored *in vivo* using non-invasive MRI technology.

Prospective survey of polarized macrophage iron deposit infiltration in human breast cancer.

The eventual clinical translation of iron as a metabolic biomarker for macrophage detection and its combination with CSF1R immunotherapy largely depends on whether iron deposits are detectable in human breast cancer. Therefore, prospective surveys of histological samples containing regions of human carcinoma *in situ* and invasive carcinoma were performed by staining paraffin-embedded sections with the Prussian blue method to identify non-heme iron deposits specific to HLMs. Iron deposits were detected in the stromal margins of carcinoma *in situ* (CIS, Fig. 7a), and were also detected at stromal boundaries of invasive carcinoma (INV, Fig. 7b). In regions densely populated by cancer cells in highly invasive carcinoma where stromal margins were not evident these

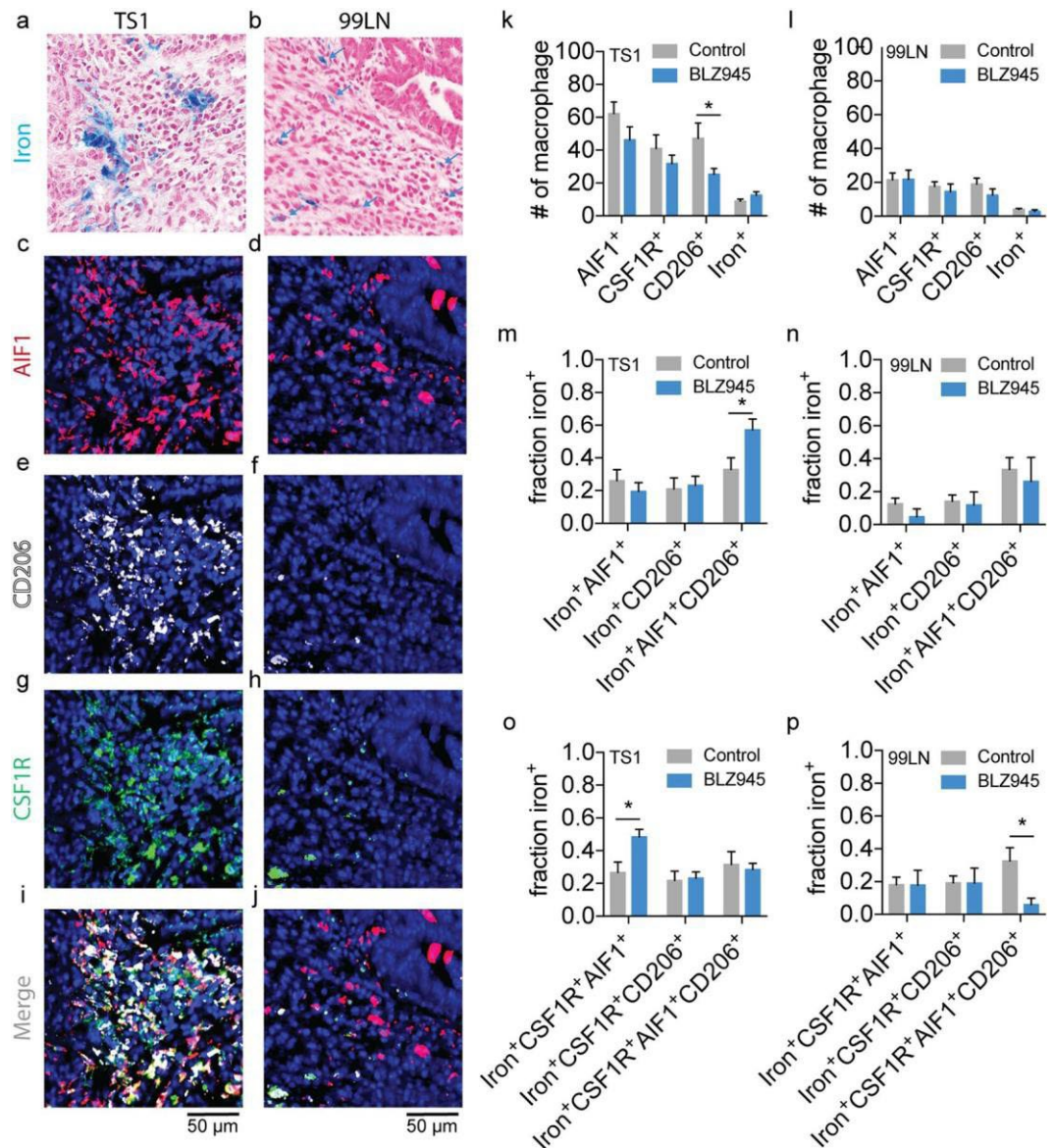


Figure 4. Immunofluorescent and Prussian blue imaging of macrophage iron deposit polarization and CSF1R status in MMTV-PyMT murine breast cancer models of CSF1R immunotherapy. Representative registered fields centered on TAM iron deposits in MMTV-PyMT TS1 and 99LN tumors stained for iron with Prussian blue (**a,b**), allograft inflammatory factor-1 (AIF1, M1-like, **c,d**), mannose receptor (CD206, M2-like, **e,f**), colony stimulating factor 1 receptor (CSF1R, **g,h**) and the combined immunofluorescent channels (**i,j**). Note fields of control tumors are shown, but are representative of both control and BLZ945 treated groups. Each field is 200 $\mu\text{m} \times 200 \mu\text{m}$. Scale bar 50 μm . Blue arrows indicate location of iron⁺ macrophage in 99LN field. Number (#) of AIF1⁺, CSF1R⁺, CD206⁺ and iron⁺ macrophages detected per field for control and BLZ945 treated (**k**) TS1 and (**l**) 99LN MMTV-PyMT mammary tumor models (mean $\pm$ s.e.m. $n = 20$ fields for TS1 control and BLZ945, $n = 20$ fields for 99LN control and $n = 7$ fields for 99LN BLZ945, $*p < 0.05$, Mann-Whitney test). Fraction of total iron⁺AIF1⁺, iron⁺CD206⁺, and iron⁺AIF1⁺CD206⁺ macrophages detected per field in control and BLZ945 treated groups for (**m**) TS1 and (**n**) 99LN mammary tumor models. Fraction of total iron⁺CSF1R⁺AIF1⁺, iron⁺CSF1R⁺CD206⁺ and iron⁺CSF1R⁺AIF1⁺CD206⁺ macrophages detected per field in control and BLZ945 treated groups for (**o**) TS1 and (**p**) 99LN mammary tumor models (mean $\pm$ s.e.m. $n = 20$ fields for TS1 control, $n = 20$ fields TS1 BLZ945, $n = 20$ fields for 99LN control and $n = 7$ fields for 99LN BLZ945, $*p < 0.05$, Mann-Whitney test).

deposits were absent (Fig. 7c). Profiling of HLM deposit infiltration as a function of position across regions indicative of CIS (Fig. 7d) and INV where the HLMS are present (Fig. 7e) further indicated significant spatial differences between the HLMS in these breast cancer microenvironments. Similar to the murine spatial infiltration profiles, human HLMS were more abundant at the outer stromal margins of the tumors, and *in situ* carcinoma was found to exhibit higher numbers of infiltrating HLMS compared with the margins of the invasive carcinoma tumor microenvironments (Fig. 7f, $p < 0.01$ – $p < 0.0001$). This confirmed the association of macrophage iron

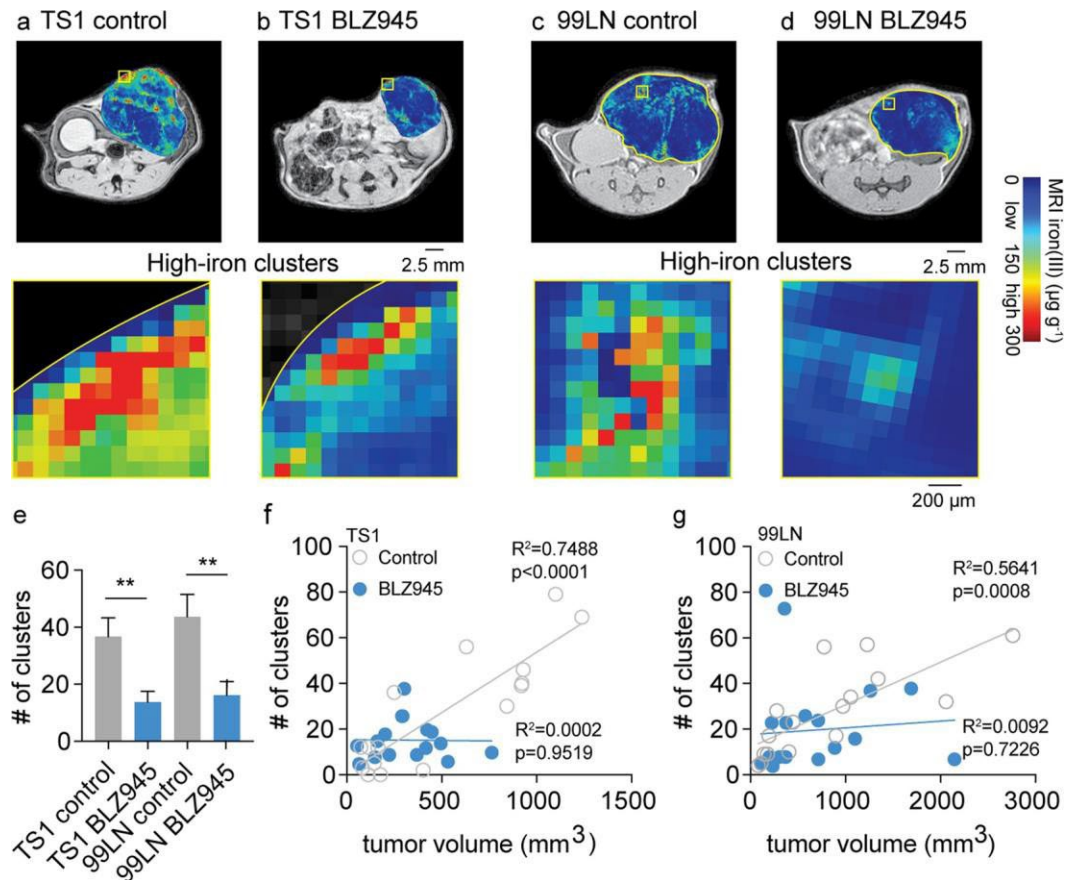


Figure 5. *In vivo* iron MRI (FeMRI) of murine macrophage iron deposits and correlation between immune and therapeutic CSF1R inhibitor response. Representative *in vivo* FeMRI axial cross sections of the mammary tumors are shown in control and BLZ945 treated (a,b) TS1, and (c,d) 99LN models. Scale bar 2.5 mm.

Expansions show high-iron pixel clusters. Scale bar 200 μm . (e) Number (#) of high-iron FeMRI pixel clusters in the TS1 and 99LN tumors in the CSF1R inhibitor trials (mean + s.e.m. $n = 8$ mice/group, ** $p < 0.01$ two-tailed unpaired students t-test). Linear correlations between high-iron FeMRI clusters and tumor volumes in the control (○) and BLZ945-treated (●) (f) TS1 and (g) 99LN MMTV-PyMT tumor models ($n = 8$ mice/group, R^2 and correlation p-value from linear Pearson correlation are shown).

deposits with human breast cancer, and shows that while they are commonly found in the tumor-stroma boundaries of both cancer subtypes, they are more prominently observed in the *in situ* pathologies where the stromal margins are better defined compared to invasive carcinoma where such margins can be less evident. As *in situ* carcinoma is thought to precede the emergence of invasive carcinoma, these findings support the translational value of using iron as an early cancer imaging biomarker of TAMs.

The iron⁺ TAMs in the human cancers were further tested for polarization and CSF1R status using multi-plexed immunofluorescence imaging methods as we performed in the murine CSF1R inhibitor trials⁵⁶. The CIS and INV fields stained for Prussian blue iron⁺ HLMs (Fig. 8a,b) were re-stained for inflammatory M1-like macrophages (Fig. 8c,d; AIF1), anti-inflammatory M2-like macrophages (Fig. 8e,f; CD206), and the CSF1R receptor (Fig. 8g,h) in order to determine the co-positivity of iron with these markers (Fig. 8i,j). The total numbers of macrophages were assessed in fields centered on HLM deposits in the stromal margins of the CIS and INV regions. The AIF1⁺, CSF1R⁺, and CD206⁺ macrophages were significantly different in the CIS and INV regions (Fig. 8k) and corresponded to higher numbers of pro-inflammatory AIF1⁺ cells in INV fields ($p < 0.0001$), while CSF1R⁺ ($p < 0.05$), CD206⁺ ($p < 0.01$), and iron⁺ macrophages ($p < 0.001$) were significantly lower in these same regions. To quantify the association of the iron⁺ macrophages with these markers the fraction of iron⁺AIF1⁺, iron⁺CD206⁺, iron⁺AIF1⁺CD206⁺ macrophage were calculated as a function of the total polarized iron⁺ TAM population (Fig. 8l), and similarly these iron⁺ populations were assessed for co-positivity with the CSF1R marker (Fig. 8m) to determine whether the iron⁺ TAMs in these fields express this receptor to further motivate later immunotherapeutic interventions using CSF1R inhibitors. Overall, significantly higher fractions of iron⁺AIF1⁺ ($p < 0.01$) and iron⁺AIF1⁺CD206⁺ ($p < 0.0001$) macrophages were found in CIS microenvironments, while iron⁺CD206⁺ markers were statistically the same in CIS and INV locations ($p > 0.05$). Additionally, the calculation of the fraction of the iron⁺AIF1⁺ and iron⁺CD206⁺ populations with CSF1R indicated that significantly more iron⁺CSF1R⁺AIF1⁺ ($p < 0.001$) and iron⁺CSF1R⁺CD206⁺ ($p < 0.001$) macrophages were present in CIS fields and iron⁺CSF1R⁺AIF1⁺CD206⁺ populations were largely the same in the CIS and INV.

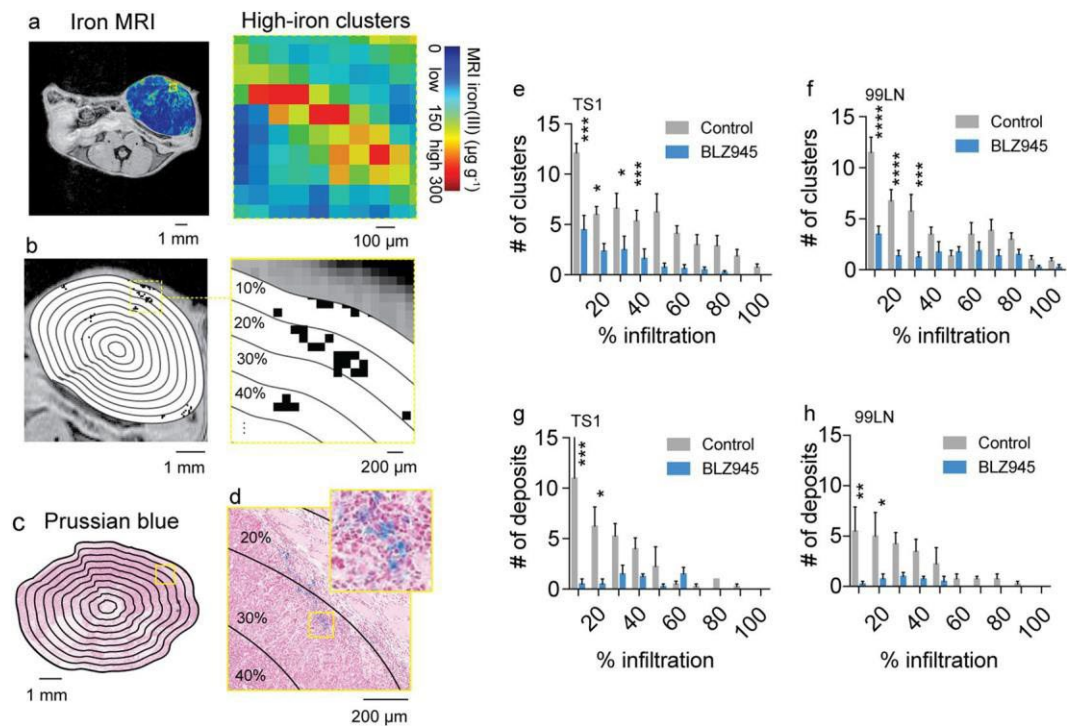


Figure 6. Spatial scores of murine tumor macrophage infiltration in CSF1R immune therapy trials from iron MRI and Prussian blue iron histology. **(a)** *In vivo* iron MRI (FeMRI) of MMTV-PyMT mammary tumor. Scale bar 1 mm. Expansion shows high-iron pixel clusters. Scale bar 100 μ m. **(b)** Binary high-iron pixel cluster mask from FeMRI of MMTV-PyMT tumor cross-section. Scale bar 1 mm. Concentric rake region of interest grid overlay used to profile clusters is shown. Expansion shows detected clusters and concentric counting grid. Scale bar 200 μ m. **(c)** Prussian blue iron stained cross-section of MMTV-PyMT tumor showing the rake grid overlay used to score the iron deposits. Scale bar 1 mm. **(d)** Expansion showing deposit, i.e. colony of iron⁺ macrophages. Scale bar 200 μ m. Infiltration profiles of high-iron clusters from FeMRI in control and BLZ945-treated **(e)** TS1 and **(f)** 99LN MMTV-PyMT models (mean $\pm$ s.e.m. n = 8 mice/group, *p < 0.05, ***p < 0.001, ****p < 0.0001, 2-way ANOVA with Sidak's multiple comparison test). Iron⁺ macrophage deposit infiltration profiles from Prussian blue histology in control and BLZ945-treated **(g)** TS1 and **(h)** 99LN MMTV-PyMT models (mean $\pm$ s.e.m. n = 4 mice/group, *p < 0.05, ***p < 0.001, ****p < 0.0001, 2-way ANOVA with Sidak's multiple comparison test).

microenvironments (p > 0.05). Thus, we demonstrate that TAMs in the human breast cancer microenvironments differ in their phenotype, and that iron accumulation occurs in polarized TAMs of human breast cancer. Interestingly, the iron⁺ macrophages in both cancer types were associated with polarization markers as well as CSF1R, however, the fraction of these polarized iron⁺ subpopulations was biased towards M1-like and CSF1R⁺ status in the CIS regions, but also frequently exhibited mixed phenotypic character in both settings. In the context of our novel iron imaging approaches, this indicates that while macrophage polarization is an important immunological factor in both murine and human cancers, the number of iron⁺ macrophages itself can serve as a TAM imaging biomarker that is sensitive to microenvironment and stage of the cancer with high-potential for *in vivo* detection by MRI.

Discussion

The accumulation of hemosiderin iron in tumor-associated macrophages enables the imaging of these cells in heterogeneous tumor microenvironments according to localized differences in iron metabolism. In earlier studies we reported that iron deposits occur in the stromal margins of murine mammary tumors and prostate cancers using histological imaging and *in vivo* FeMRI^{23,24,40}. Recently, these iron deposits have also been detected in the stromal margins of non-small cell lung cancer tumors³⁷, and we have also shown them to be present in lung and brain metastases²³. In the current study we confirmed that such TAM iron deposits are present in multiple MMTV-PyMT breast cancer models, they are correlated with response to CSF1R breast cancer immunotherapy, and we demonstrated that the spatial infiltration and accumulation of the TAM iron deposits is a factor that is conserved in murine and human breast cancer and differentiates between human breast cancer microenvironments.

From an *in vivo* imaging standpoint our novel measurements also serve to further establish FeMRI as a unique approach for the detection and monitoring of immune cell response to cancer immunotherapy. Alternative MRI approaches label macrophages using injections of iron nanoparticles^{34,35,57}, and injected radiolabelled nanoparticles used in PET imaging can also be used to measure macrophage during immunotherapy trials^{8,58}. Our imaging studies are set apart from these contrast agent-dependent techniques as we do not use contrast agent injections.

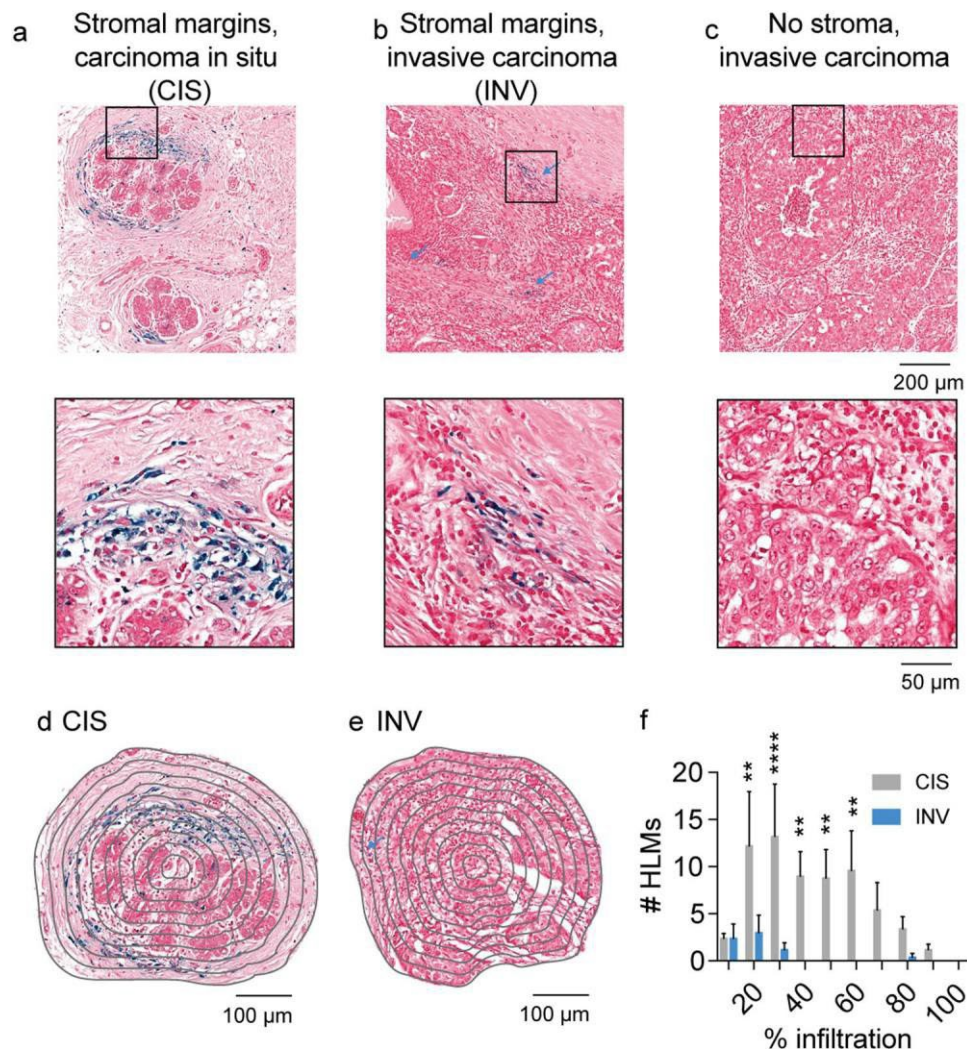
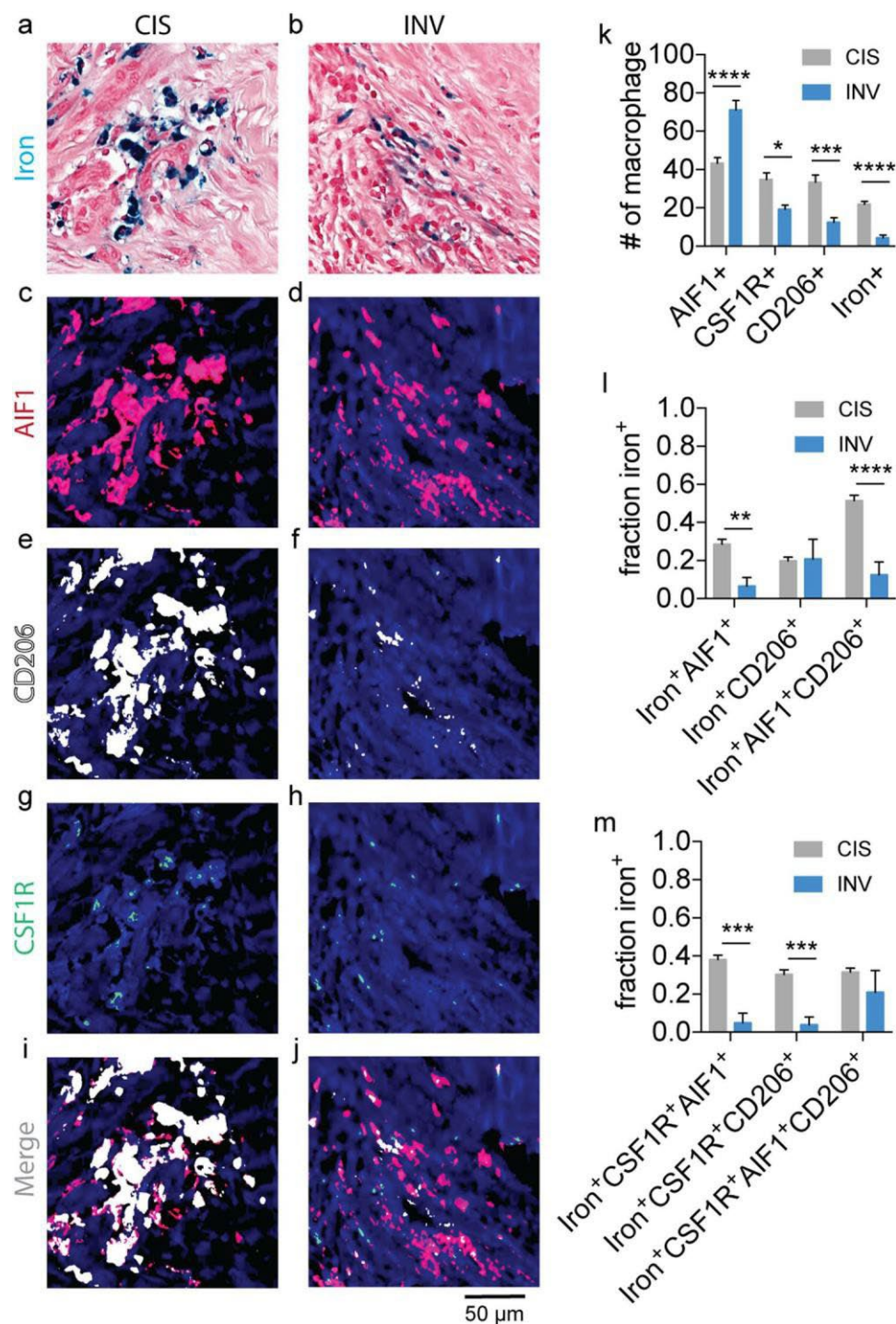


Figure 7. Spatial scores of iron deposits from Prussian blue histology in human breast cancer. Prussian Blue iron histochemistry shows the presence of iron deposits in (a) stromal margins of carcinoma *in situ* (CIS) and (b) invasive carcinoma (INV, blue arrows). No iron deposits were associated with (c) invasive carcinoma exhibiting poorly defined stromal margins. Scale bar 200 μ m. Expansions of boxes in (a–c) shown below. Scale bar 40 μ m. Concentric rake region of interest grid overlay used to profile HLMs in (d) CIS and (e) INV fields. Scale bar 100 μ m. (f) Iron⁺ macrophage (HLM) infiltration profiles from Prussian blue histology in CIS and INV fields. (mean $\pm$ s.e.m. $n = 5$ fields/cancer subtype, $**p < 0.01$, $****p < 0.0001$, 2-way ANOVA with Sidak's multiple comparison test).

We instead capitalize on the high physiological innate iron metabolism of macrophages that results in the phenotypic storage of solid deposits of iron that is readily validated using Prussian blue iron histochemistry of the TAM iron deposits. These magnetic deposits give rise to characteristic high-iron FeMRI pixel contrast that reveals their infiltration in the tumor microenvironment where they fulfill obligate roles in iron storage and recycling²¹. Although our reliance on endogenous iron stores to image TAMs is potentially restricted because we only detect those macrophages engaged in iron handling roles, our previous findings and current studies showing HLMs in the different MMTV-PyMT backgrounds as well as human cancers support the feasibility of using the iron imaging approaches to image them, and supports the combination of this form of endogenous cell-tracking with immunotherapeutic trials targeting macrophage in the clinic.

While we focus on iron as a primary biomarker of tumor macrophages in this contribution, TAMs are also recognized to adopt so-called polarization phenotypes that are closely associated with their role in immune response and communication to other immune cells such as T-cells that is critical for immunotherapeutic efficacy^{48,53}. These multifactorial polarization states reflect the stage of immune response, progression of primary tumor and metastasis growth, as well as the microenvironment and tissue in which they are found⁶. We discovered that macrophage iron deposits are found more prevalently at the stromal margins of tumors in murine models and human breast cancers suggesting that iron is another microenvironmental factor influencing macrophage phenotype and spatial distribution. However, when we investigated the polarization status of the macrophage iron deposits we found them to be associated with various phenotypic polarization markers, and they were often co-localized



with macrophage markers indicating mixed polarization character, rather than adopting a single polarization state. While the current studies do not address the functional significance of such polarization states in the context of their role in signaling to other immune cells, it is evident due to their clear association with iron that they serve a primary functional role in iron metabolism. In this regard it can be speculated that iron⁺ macrophages are primed to fill M1-like inflammatory functions where they sequester iron to shield it from depletion due to hemorrhage or pathogen, as well as function in M2-like wound-healing roles where they serve as stores of iron to sustain cellular proliferation of the microenvironmental milieu during tissue repair¹⁹. This scenario is supported by our observations of the differences in iron⁺ macrophage of *in situ* and invasive human cancers. Here, more iron⁺ macrophages were found in earlier cancer stages suggesting that these macrophages avidly stored iron during initial inflammatory immune response to the cancer. Similarly, in advanced cancers reduced numbers of iron-containing TAMs presumably reflects the depletion of stromal macrophage iron stores in order to fuel the cancer's malignant outgrowth as it has been proposed that cancer cells have a pronounced dependence on iron metabolism that serves to co-opt this critical micronutrient from other cellular species in the tumor microenvironment^{60,61}. In this context, the iron⁺ deposits can themselves be used as specific histological and *in vivo* bio-markers of TAM infiltration which varies with immune status and cancer stage without the need for assessment of tissue-dependent polarization.

Immune therapies such as the small molecule inhibitors of macrophage colony stimulating factor 1 receptor (CSF-1R) including BLZ945 and PLX3397, as well as antibodies directed against this receptor have been shown to reduce the accumulation of TAMs in preclinical models and achieve therapeutic gains as monotherapy and in combination with other therapies^{48,52}. We confirmed the primary immunotherapeutic effects of the CSF1R inhibitor in the MMTV-PyMT mammary tumor models by demonstrating that the drug achieves reduction in macrophage accumulation together with tumor growth inhibition. We also confirmed that the iron⁺ macrophages express CSF1R in murine and human breast cancers supporting their role as targets of these immunotherapies. Indeed, inhibition of macrophage accumulation by CSF1R therapy resulted in significant reductions of iron-laden macrophage deposits in the orthotopic MMTV-PyMT models. Therefore, these studies validated the immunotherapeutic effect of the drug in the breast tumor models, further identified the iron-laden macrophage populations as responsive to the CSF1R immunotherapy, and confirm their CSF1R status for future therapeutic targeting in human cancer.

Cancer therapies targeting iron metabolism under current investigation have sought to either achieve therapeutic gains by iron loading using nanoparticle theranostic injections, or by causing iron depletion with iron chelators^{24,37,39,62}. In the current studies we identify another means of targeting cellular iron for therapy. By capitalizing on the observation that iron deposits are restricted to TAMs, we demonstrate that CSF1R inhibition that directly targets TAMs can also be used to indirectly prevent accumulation of TAM iron deposits. As our murine investigations and surveys of human breast cancers confirm the presence of these cells and characterize their spatial dependencies, targeting the iron deposits using CSF1R inhibitors and spatially monitoring them using iron imaging has notable potential value as a translational therapeutic cancer imaging strategy. Further applications of this approach include diseases such as iron overload disorder, neurodegeneration, inflammation, and hemorrhage where iron deposits may be present and their detection, mapping, and subsequent therapeutic reduction can be desirable^{41,42,59,63}.

Conclusions

In sum we identified iron deposition as a metabolic biomarker of macrophage infiltration in murine and human breast cancer that identifies responsive polarized TAM populations to CSF1R immunotherapy using histological and *in vivo* iron imaging together with preclinical cancer research approaches. While the current studies support our ability to image polarized TAMs according to iron status in breast cancer, and suggest that iron deposits are associated with specific types of cancer, given the diverse types of human breast cancers encountered in the clinic, further histopathology will be required to more completely characterize the association of the deposits with the myriad immunological markers involved in immunotherapeutic studies, as well as correlate their accumulation with cancer stage, and clinical outcome. Also, though these findings support the translation of the FeMRI approaches to human breast cancer, and our detection of iron deposits in human cancer strongly supports the feasibility of this approach, further imaging validations will ultimately determine whether the approaches presented here in preclinical models will find their niche in clinical cancer imaging and immunotherapy trials in the future. Therefore, these demonstrations support such future studies that seek to image immune cells and harness their innate functional and metabolic dependencies for therapy.

Methods

Magnetic resonance imaging. MRI was performed on a 7T/30 cm horizontal bore Bruker Biospec MRI system (Bruker Biospin Corp.) with a custom-built 30 mm inner-diameter transmit-receive quadrature radio-frequency coil.

Iron MRI. 2D multi-gradient echo (MGE) relaxometry pulse sequence was used with the following parameters: TR/TE 1.2 s/3 ms × 16 echos, *in vivo* spatial resolution 0.1 mm × 0.1 mm × 0.05 mm, *ex vivo* 0.05 mm × 0.05 mm × 0.5 mm, RF flip angle 60°, and each spatial encoding phase encode acquisition was gated on the animal's respiratory cycle. The first image of the gradient-echo series was used as reference images shown in the figures and overlays, and all images shown and analyzed correspond to axial mid-section slices of the tumors. Aqueous solutions of iron(III) nitrate (Fisher Scientific) were used as standards for iron concentration measured at 7T as described in refs^{23,24,40}. The T₂* values for these solutions was determined by pixel-wise monoexponential fitting of the MGE images using Fiji⁶⁴, and the linear relation between the relaxation rate R₂* = 1/T₂* and known iron concentrations was found and subsequently used to generate parametric maps of iron concentration.

Quantification of high-iron FeMRI clusters was conducted by binary stratification with respect to the median of the concentration range of the iron maps followed by counting the frequency of the high-iron pixel clusters with the Fiji Analyze Cluster tool. Infiltration profiles of the high-iron clusters were generated by measuring them as a function of position according to a concentric ring pattern generated with the Fiji ROI Manager tool macros.

Animal procedures. All animal studies were approved by the MSKCC IACUC committee and performed in accordance with their guidance and regulations.

In vivo MRI. Mice were anesthetized with 1–3% isoflurane in O₂ gas, and respiration was monitored during all imaging sessions.

Primary tumor models. Female 6 week-old FVB/N or C57BL6 mice underwent orthotopic mammary fat pad injection of 1×10^6 syngeneic TS1 MMTV-PyMT or 99LN tumor cell lines grown and collected using standard tissue culture conditions and suspended in a 50% solution of Matrigel and saline (BD Bioscience). Endpoint was defined as when the control cohorts average tumor size reached approximately 1 cm³ determined by caliper, MRI, or veterinary staff inspection notice.

CSF1R inhibitor administration. The CSF1R inhibitor BLZ945 (Novartis) was administered by oral gavage (200 mg kg⁻¹ in captisol vehicle, 5×/week). Dosing commenced once tumors exceeded approximately 100 mm³ and continued until control cohorts average tumor size reached approximately 1 cm³ determined by caliper, MRI, or veterinary staff inspection notice.

Flow cytometry. Tumor-bearing mice at endpoint were perfused with 20 mL of PBS prior to mammary tumor retrieval at endpoint. Tumors were washed once in PBS and enzymatically digested for 45 minutes into a single cell suspension using a tumor dissociation kit (Miltenyi Biotec). Cell suspensions were filtered twice through 70 µm cell strainers, and Fc-blocked for 30 minutes on ice (1:50; clone 2.4G2, BD Bioscience). Cells were then immunostained using antibodies (BioLegend) for CD45 (1:400; clone: 30-F11), Ly-6C (1:400; clone HK1.4), Ly-6G (1:400; clone 1A8), CD11b (1:200; clone M1/70), and F4/80 (1:250; clone BM8) for 45 minutes on ice. Cells were then washed twice in PBS and suspended in FACS-buffer (2% FBS in PBS) containing DAPI (2.5 µg/mL; Invitrogen) for exclusion of dead cells. TAMs were identified as DAPI⁺CD45⁺Ly6C⁻Ly6G⁻CD11b⁺F4/80⁺ cells, and frequency of TAMs was determined from counts in the F4/80⁺CD11b⁺ flow cytometry gates with respect to total live CD45⁺ cells using FCS Express (De Novo Software).

Histology. Cross-sections from the PBS-perfused MMTV-PyMT tumors were sliced at the axial tumor mid-point and fixed in 4% PFA for 24 hours at 4 °C, and then washed with H₂O and re-suspended in 70% ethanol (Fisher Scientific). Human tissue sections donated to the Molecular Cytology Core Facility by the Pathology Department were acquired under MSKCC Institutional Review Board informed consent and provided for the study without any unique patient identifier except diagnosis. They were fixed in 10% neutral buffered formalin. All tissues were paraffin embedded and 5 µm sections cut onto glass slides and coverslipped prior to histological quantification.

The Prussian blue histochemical detection of iron(III) was performed by standard methods using 30 minute staining of de-paraffinized slides in equal parts 10% potassium ferricyanide (Fisher Scientific) and 10% hydrochloric acid (Fisher Scientific) followed by nuclear-fast red counter-staining for 30 minutes.

The immunofluorescent detection of CD68 (Boster, cat# PA1518, 5 µg/ml, 5 hours) was performed using standard Discovery XT processor protocols and reagents (Ventana Medical Systems) with biotinylated goat anti-rabbit antibodies (Vector Labs, cat#PK6101, 60 minutes) at 1:200 dilution, and visualized with Tyramide Alexa Fluor A546 (Invitrogen, cat# T20933) and DAPI (Sigma Aldrich, cat# D9542, 5 µg/ml) prepared according to manufacturer's instructions with predetermined dilutions.

Multiplex immunofluorescent stainings were performed as previously described⁵⁶. Prussian blue stained slides were differentiated, and sections were then sequentially incubated with anti-CSF1R (Santa Cruz, cat#sc-692,

0.5 µg/ml, 5 hours) and biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101, 60 minutes) at 1:200 dilution, anti-CD206 (Abcam, cat#ab64693, 1 µg/ml, 5 hours) and biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101, 60 minutes) at 1:200 dilution, and finally anti-AIF1 (Wako, cat#019-19741, 0.5 µg/ml, 5 hours) and biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101, 60 minutes) at 1:200 dilution. The detection was performed successively for each of the antibodies using streptavidin-HRP D (part of DABMap kit, Ventana Medical Systems) followed by incubation with Tyramide Alexa 488 (Invitrogen, cat# B40953) for CSF1R, Tyramide Alexa 647 (Invitrogen, cat# B40958) for CD206, and Tyramide Alexa 568 (Invitrogen, cat# T20948) for AIF1, and DAPI (Sigma Aldrich, cat# D9542, 5µg/ml) prepared according to manufacturer instruction with predetermined dilutions.

Histological sections were digitized with a Mirax scan system and read with Panoramic Viewer (3DHISTECH). Images were first visually inspected for quality and then processed to remove background fluorescence and provide maximal signal for binary spot counting performed over whole cross-sections or selected fields from exported images using Fiji according to staining or immunofluorescent labeling. Deposits of iron containing macrophages and their infiltration profiles were quantified from the Prussian Blue histology using the Fiji Analyze Cluster tool and ROI Manager macro tools as described in refs.^{23,24,40} and also applied to the FeMRI data. Evaluation of iron⁺ macrophage polarization phenotype in murine and human tissue was conducted by co-registering Prussian blue and triple-stained immunofluorescent images, identification of 200 µm × 200 µm fields centered on iron deposits, and performing exhaustive binary counts made of the iron⁺ cells as a function of AIF, CD206 and CSF1R positivity in Fiji. Fractional populations were calculated by dividing the total counts of a given set of co-localized macrophage markers by the sum of the markers being compared, for example M2-like polarization of iron containing macrophage was calculated by dividing the iron⁺CD206⁺ population by the sum of iron⁺AIF⁺, iron⁺CD206⁺, and iron⁺AIF⁺CD206⁺ populations.

Statistics. Statistical tests are indicated in the figure legends when performed and significance is determined as $p < 0.05$ in all analyses. All statistical analyses were performed with GraphPad Prism 7 (GraphPad Software).

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Author Contributions

A.L. designed the study. A.L. collected MRI and histological data, and performed MRI and histological analysis.

A.L. and N.B.C. initiated and maintained mouse models. A.L. and N.B.C. collected and analyzed FACS data. A.L. wrote the paper, and A.L., N.B.C., J.A.J. and J.A.K. edited and approved the paper.

Additional Information

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Appendix 4

The Role of Iron Chelation in the Tumour Microenvironment of Triple-Negative Breast Cancer

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Synopsis

Intracellular iron, essential for cancer cell proliferation and metabolism, is modified in cancer cells. Triple-negative breast cancers are metastatic cancers associated with a high recurrence rate, poor prognosis and lack of effective targeted therapies. We are investigating the potential of Deferiprone, a clinically approved intracellular iron chelator for non-cancer related diseases, to improve chemotherapeutic treatment response in triple-negative breast cancer by altering iron-dependent proliferation and metabolism. The effectiveness of Deferiprone to impair triple-negative breast cancer cell growth and affect cellular metabolism was evaluated by monitoring live cells, exposed to Deferiprone, in an MR-compatible cell bioreactor using multi-nuclear MRS.

Introduction

Triple-negative breast cancers (TNBC) lack estrogen (ER⁻), progesterone (PR⁻) and HER2⁻ receptors and are metastatic cancers associated with a high recurrence rate, poor prognosis and lack of effective targeted therapies. Infiltration of iron-rich tumour-associated macrophages promotes tumour progression.^{1,2} Cellular iron is essential for cell proliferation³ and metabolism and increases cancer cell susceptibility to modest iron deficiency.⁵⁻⁷ Deferiprone (DFP), a clinically approved iron chelator for non-cancer related diseases,⁸ enters cancer cells and alters iron-dependent enzyme activities by decreasing intracellular iron.⁹⁻¹¹ Based on preliminary data showing a DFP-induced cytostatic effect in multiple breast cancer cell lines (Table 1), we investigate the mechanism of DFP to induce cell cytostasis in TNBC and its potential to improve chemotherapeutic treatment response in combination therapy. Specifically, the effectiveness of DFP to impair TNBC cell growth and affect cellular metabolism was evaluated in live cells in an MR-compatible cell bioreactor¹² by measuring the metabolization of ¹³C-1-Glucose using ¹³C-MRS and cell energy state by ³¹P-MRS.

Methods

Murine metastatic TNBC 4T1 cells were grown in Dulbecco's Modified Essential (DME) medium, containing 25 mM glucose, 6 mM glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C in humidified 5% CO₂ / 95% air. For the MR-cell bioreactor studies, 4T1 cells were cultured on microcarriers (125-212 µm diameter; PP3772, Corning, New York, NY; 3×10⁶ cells/0.5 ml beads×6) to ~70% confluence. Cell number and viability were estimated from a representative sample at the start (#cells_{initial}) and end (#cells_{end}) of each MR experiment by Trypan Blue exclusion. Continually perfused, live cells were studied in an MR-compatible cell bioreactor¹² on a 500 MHz MR system (Bruker BioSpin, Billerica, MA) equipped with a 10-mm broad-band probe for ¹H and ³¹P/¹³C scans. To monitor metabolism changes, cells were continuously perfused for 31 hours with DME media. After 1 h setup and the first ³¹P MR spectrum, the un-labeled perfusion medium was replaced with medium containing 25 mM 1-¹³C-labeled glucose for the remaining 30 h of experiment. Three independent experiments were performed in regular medium conditions (CTRL) and three in medium containing 100 µM DFP (DFP-treated). During each experiment, ¹H-decoupled, Nuclear Overhauser Effect-enhanced ¹³C MR spectra (TR=1200s, 45 degree flip angle, 30 kHz sweep width, 8k points, 1000 averages, 20 min acquisition time) were acquired repeatedly interspaced with two consecutive ³¹P spectra (TR=1200s, 45 degree flip angle, 20 kHz sweep width, 2k points, 1800 averages, 30 min acquisition time) every six hours. Quantification of MR spectral peaks was performed in time domain using AMARES (jMRUI v5.2). Metabolites were normalized to the β-NTP signal of the first ³¹P MR spectrum (βNTP_{init}). A two-tail unpaired Student's t test was used to compare DFP-treated and untreated (CTRL) 4T1 cell data. The effect of DFP on the migration of confluent 4T1 cells was assessed by wound healing assay.³

Results

Figure 2A shows the comparison between the cell count ratios $r_c = [\#cells_{end}/\#cells_{initial}]$ of each experiment and the corresponding cellular energy ratios ($r_e = [\beta-NTP_{end}/\beta-NTP_{init}]$) determined for both DFP-treated and untreated cells. In DFP-treated 4T1 cells the cell mass was significantly lower than in untreated 4T1 cells, whereas r_e decreases significantly slower than the cell loss determined by cell counting. This agrees with our IC₅₀ studies showing decreased proliferation caused by DFP. Wound healing is significantly reduced in DFP-treated 4T1 cells at 24 and 36 h exposure, delaying wound closure to about 48 h compared to the 36 h in untreated and 50 µM DFP-treated 4T1 cells (Fig. 2B). Figure 3A shows representative ³¹P spectra of DFP-treated and untreated 4T1 cells acquired at 31 h. The time course of β-NTP levels is similar between DFP-treated and control 4T1 cells (Fig. 3B). The phospholipid precursors PCho (Fig. 3C) and GPCho (data not shown) exhibit significant differences between DFP-treated and control 4T1 cells at the latest time points. The intracellular pH was stable throughout and unaffected by DFP (data not shown). Representative ¹³C spectra acquired over time in control and DFP-treated cells are shown in Figure 4. The quantitative evaluation of ¹³C spectra metabolites of DFP-treated and control 4T1 cells (Fig. 5), show various average metabolite levels that differ significantly between DFP-treated and untreated 4T1 cells. Specifically, less glucose-C1 signal loss, lower lactate-C3, glutamine-C4, fatty acid chains (CH₂)_n, and Alanine-C3 ¹³C-labelling were found in DFP-treated than in control 4T1 cells.

Discussion

Our findings that DFP exposure of TNBC 4T1 cells can impair their migration and cell growth (Fig. 2) agrees with our previous findings in prostate cancer.³ Changes in PCho (Fig. 3C), GPCho and GPCho/PCho indicated subtle phospholipid membrane turnover differences between DFP-treated and control cells. The time course effects of DFP on 4T1 cell metabolism, assayed by ¹³C MRS (Fig. 5), suggest that DFP exposure affects the Krebs cycle, cell energy and fatty acid metabolism in accordance with our previous study using live TRAMP-C2 cells.³

Conclusion

In conclusion, this study shows the cytostatic effect of DFP on the TNBC 4T1 cells and can be considered as a first step for a potential clinical translation in combination therapy into humans. Clinical translation is straightforward considering that Deferiprone is already approved clinically for non-cancer related diseases.

Acknowledgements

This work was supported by grants W81ZWH-17-1-0525 (JAK) and P30CA008748 (Cancer Center Support Grant). We thank Dr. RV Simões for helpful discussions and the Molecular Cytology Core Facility for their assistance with the wound healing assay.

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Figures

Table 1 IC ₅₀ values for DFP	
Cell Lines	IC ₅₀ (μM)
4T1-WT	40.2 ± 2.6
4T1-NC	39.9 ± 4.1
4T1-KD9	41.6 ± 2.5
E0771	60.6 ± 1.8
MDA-MB-231	58.5 ± 5.6
MDA-MB-231-LM2	64.5 ± 4.3

Each cell line was exposed to Deferiprone (DFP) for 48 h in each of three independent experiments. Data are mean ± SD

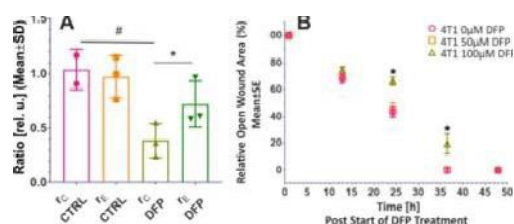


Figure 2. A. Cell growth. Comparison between the ratio, rC , of counted cells at the end and start of each experiment ($rC = \#cells_{end} / \#cells_{int}$), and the corresponding cellular energy ratio ($rE = \beta\text{-NTP}_{end} / \beta\text{-NTP}_{init}$) in DFP-treated and untreated 4T1 cells. **B.** Cell migration. Wound closure as function of DFP exposure for cells treated with 0, 50 and 100 μM DFP, respectively. * $p < 0.05$ 2-way ANOVA, Tukey's multiple comparison (swapped directions).

Data in **A** and **B** are averaged over three independent experiments, respectively.

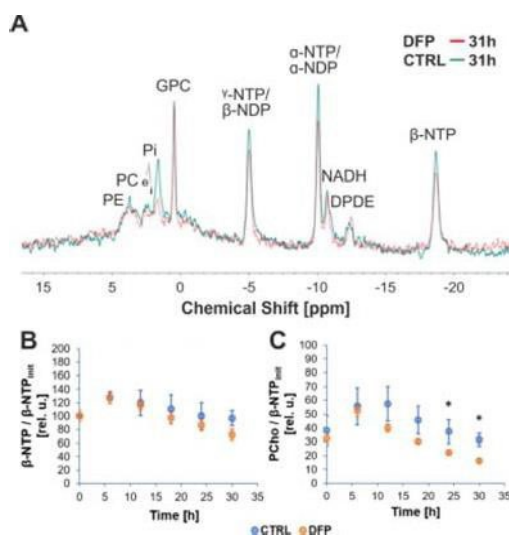


Figure 3: A Representative ^{31}P spectra of DFP-treated and untreated 4T1 cells, acquired at 31 h. **B,C:** Time course of $\beta\text{-NTP}$ (**B**) and PCho (**C**). PCho levels differ significantly between untreated and DFP-treated (100 μM DFP) 4T1 cells (* $p < 0.05$, unpaired t test) at the latest time points. PCho changes indicates phospholipid membrane turnover differences between DFP-treated and control cells. Data are mean $\pm$ standard error, $n = 3$.

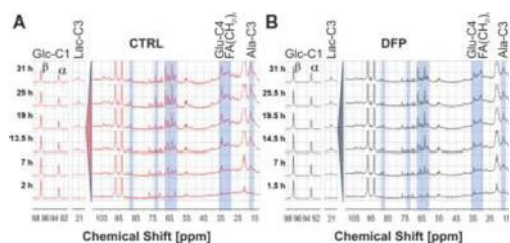


Figure 4 Representative ^{13}C spectra of DFP-treated (100 μM) and untreated 4T1 cells over time.

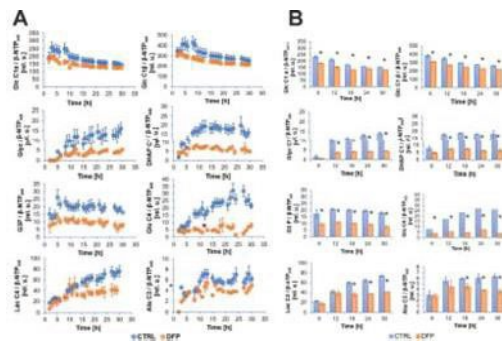


Figure 5: A Time course effect of DFP on live 4T1 cell metabolism, as detected by ^{13}C -MRS. B Average metabolite levels (mean $\pm$ SE) at different time intervals. * Significantly different from untreated cells ($p < 0.05$, unpaired Student t test).

Metabolic Effects of Deferiprone on Triple Negative Breast Cancer

Synopsis

Triple-negative breast cancer (TNBC) metastasizes and is currently associated with poor prognosis and lack of effective targeted therapies. We are studying the potential of Deferiprone, an intracellular iron chelator clinically used for non-cancer-related diseases, to improve the chemotherapeutic treatment response in TNBC by altering cellular iron-dependent proliferation and metabolism. Here, cellular and energy metabolism changes in response to Deferiprone were assayed in live TNBC cells by multi-nuclear MRS in our MR-compatible bioreactor. Metabolic changes in a human TNBC cell line are compared to previous findings in murine TNBC cells.

Introduction

Methods

Results & Discussion

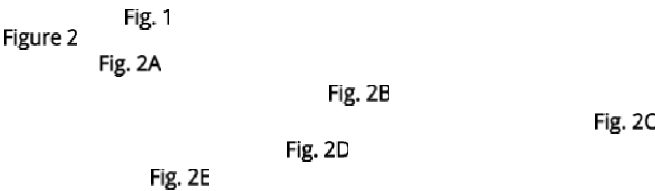


Figure 3

Fig. 4

Figure 5

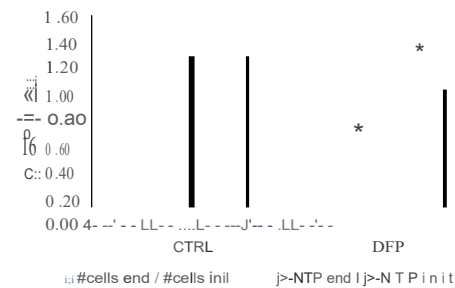
Fig. 4E

Conclusion

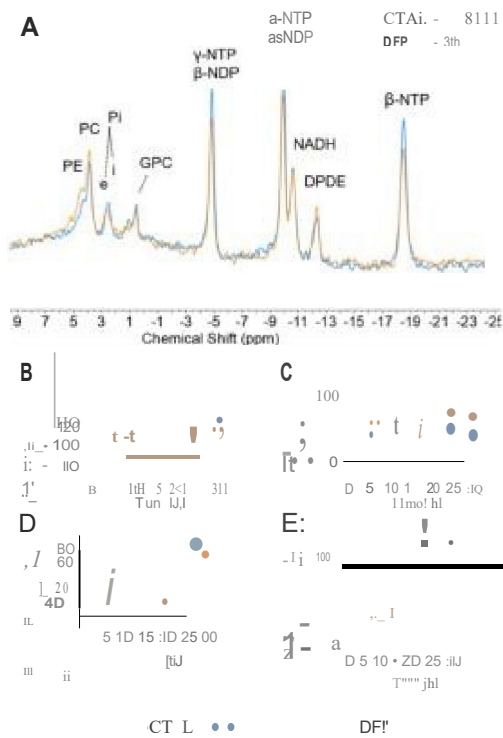
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References

Figures



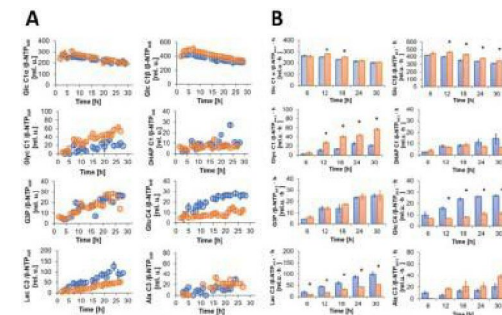
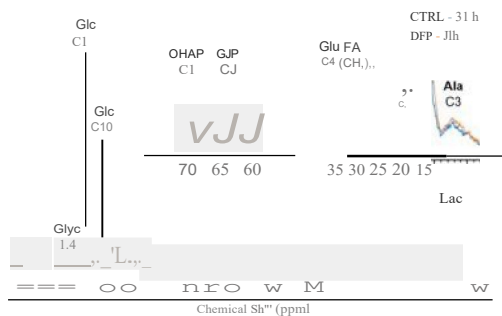
cell mass and cellular energy ratio



1D MRS results for DFP-treated and untreated MDA-MB-231 cells.

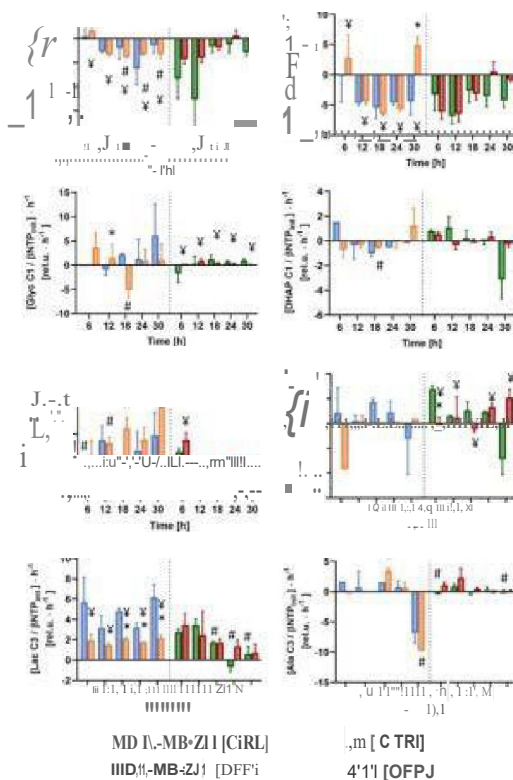
B C D E

B C D



Effect of DFP on live MDA-MB-231 cell metabolism over time, as detected by ¹³C MRI. A

B



Intracellular Iron Chelation affects Cellular Metabolism in Triple Negative Breast Cancer Cells: Comparison between Human and Murine Models

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Synopsis

Impairment of cell growth in the human MDA-MB-231 and murine 4T1 triple-negative breast cancer models.

Introduction

Triple-negative breast cancers (TNBC) are aggressive tumours associated with poor prognosis and lacking in effective therapies. Cellular iron is essential for cell proliferation and vitality and its cellular level is often related to cancer progression.^{3,4} Here, we investigated the effect of Deferiprone (DFP), an iron chelator clinically used for non-cancer related diseases, on two TNBC cell lines with the goal to better understand the mechanism underpinning the metabolic changes following DFP exposure. Metabolism of both cell lines was monitored over time using an MR-compatible cell bioreactor, and the changes, following DFP exposure, were compared between both cell lines. Findings on quantitative mitochondrial parameters were investigated for both cell lines and compared with ¹³C MRS results. The effect of DFP on cell migration was studied in relation to changes of cell doubling times due to DFP treatment.

Methods

Human MDA-MB-231 and murine 4T1 cells were grown in high glucose Dulbecco's Modified Essential (DME) medium, supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO₂ humidified atmosphere. For the MR bioreactor studies, 6×10⁵ MDA-MB-231 cells or 3×10⁵ 4T1 cells were seeded on microcarriers (0.5 ml; 125-212 µm diameter; PP3772, Corning, New York, NY) in six deep-form bacteriological Petri dishes until reaching approximately 70% confluence. Live cells were studied in an MR-compatible bioreactor on a Bruker AVANCE III 11.7T MRI system (Bruker BioSpin, Billerica, MA) equipped with a 10-mm multi-nuclear broad-band probe, tunable to ¹H and ³¹P/¹³C nuclei. For each cell line, three independent experiments were performed in regular medium conditions (CTRL) and three in medium containing 100 µM DFP (DFP-treated). After 1 h setup, the unlabelled medium was replaced with medium containing 25 mM of 99% D-Glucose-1-¹³C to monitor the perfused cells by ¹³C-MRS. For each experiment, ¹H-decoupled, Nuclear Overhauser Effect-enhanced ¹³C MR spectra (TR = 1200 s, 45° flip angle, 30 kHz sweep width, 8 k points, 1000 averages, 20 min acquisition time) were acquired repeatedly every six hours interspersed by ³¹P MRS for a total of 30 h. Quantification of MR spectral peaks was performed in the time domain using AMARES (jMRUI v6.0). Metabolites were normalized to the b-NTP signal of the initial ³¹P MR spectrum (bNTP_{mit}).⁵ A two-way ANOVA followed by Tukey's post hoc test was used to compare metabolite ¹³C-labelling rates. The cell doubling times were determined for untreated and DFP-treated MDA-MB-231 and 4T1 cells respectively. The effect of DFP on the migration of confluent MDA-MB-231 and 4T1 cells was assessed by wound healing assay. The mitochondrial response of both cell lines to DFP exposure was assessed from the oxygen consumption rates (OCRs) using the Seahorse XF Cell Mito Stress Test Kit on the Seahorse XFe96 flux analyser (Agilent, Santa Clara, CA).

Results

Figure 1 shows the differences between representative ¹³C MR spectra of untreated (Fig. 1A) and DFP-treated (Fig. 1B) MDA-MB-231 cells, both acquired at the start (1 h) and end (31 h) of the perfusion experiment. The quantitative evaluation of ¹³C spectral metabolites (Fig. 2) showed higher glucose consumption in DFP-treated MDA-MB-231 cells than DFP-treated 4T1 cells reaching significant levels at 6 and 24 hours of treatment (Fig. 2B). In addition, glutamate and lactate synthesis showed higher levels in DFP-treated MDA-MB-231 cells compared with DFP-treated 4T1 cells reaching statistical significance at 24 hours (Fig. 2B). The doubling time of both cell lines was significantly increased with the treatment (Fig. 3) Wound healing was significantly reduced in DFP-treated 4T1 cells at 24 and 36 h exposure, delaying wound closure to about 48 h compared to the 36 h in untreated and 50 µM DFP-treated 4T1 cells (Fig. 4B). Conversely, cell migration was higher in DFP-treated than untreated MDA-MB-231 cells (Fig. 4A) reaching significance at about 24 hours while at later time points control cells catch up in the wound closure. Conversely, cell migration was higher in DFP-treated cells than in untreated MDA-MB-231 cells (Fig. 4A) reaching significance at about 24 hours, while control cells reached wound closure at the later time points. These findings indicate that wound closure is most affected by DFP exposure when at least 1 cell doubling time has passed. In both TNBC cell lines, DFP exposure decreased basal respiration, ATP production, maximal respiration, spare respiratory capacity (SRC), and % spare respiratory capacity (% SRC).

Discussion

Our findings on ¹³C metabolic changes due to DFP treatment (Fig. 2) along with those related to the mitochondrial parameters (Fig. 5B) suggest that human MDA-MB-231 cells are more affected by the DFP treatment than the murine TNBC 4T1 cells. In comparison to MDA-MB-231 cells, this might be due to an enhanced reliance of 4T1 cells on glycolytic energy metabolism and/or its higher plasticity to switch between glycolytic and mitochondrial metabolism, as previously observed.

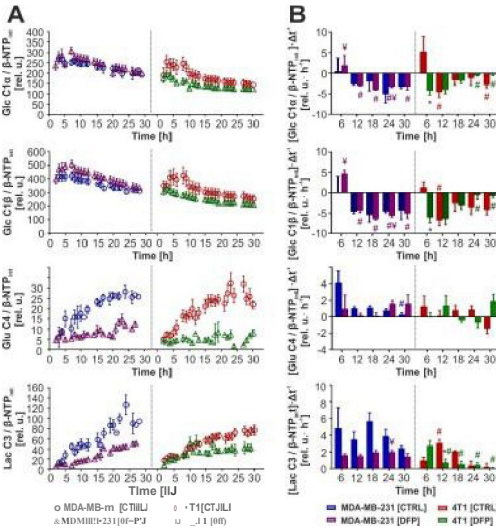
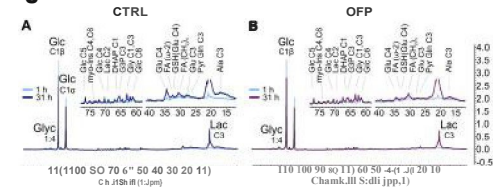
Conclusion

This study shows that DFP exposure to TNBC cell models has a stronger effect on the human MDA-MB-231 cells than on the murine 4T1 cells, as confirmed by ¹³C metabolic results and mitochondrial respiration findings.

Acknowledgements

References

Figures



A Time- course effect of DFP on cell metabolism of human MDA-MB- 231 (left panels) and murine 4T1 (right panel) cells, compared to control (CTRL) cells. **B** Six-hour average ^{11}C -labeling rates of untreated and DFP-treated MDA-MB-231 (left panels) and 4T1 (right panels) cells. * Significantly different from CTRL ($p < 0.05$); ‡ Significantly different from the corresponding value in DFP-treated 4T1 ($p < 0.05$); # Significantly different from the value at the 6 h (first) time point for the same cell line and treatment condition ($p < 0.05$). Colour coded for cell line and condition.

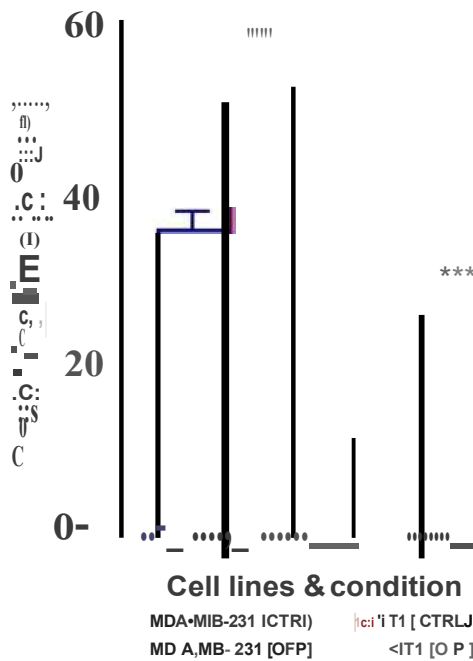


Figure 3. Mean doubling times for untreated (CTRL) and Deferiprone (DFP)-treated MDA-MB-231 (left panel) and 4T1 (right panel) cells. *** ($p < 0.001$) and * ($p < 0.01$): Significantly different from CTRL (one-tailed, unpaired t-test).

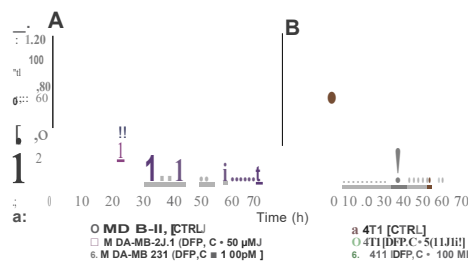
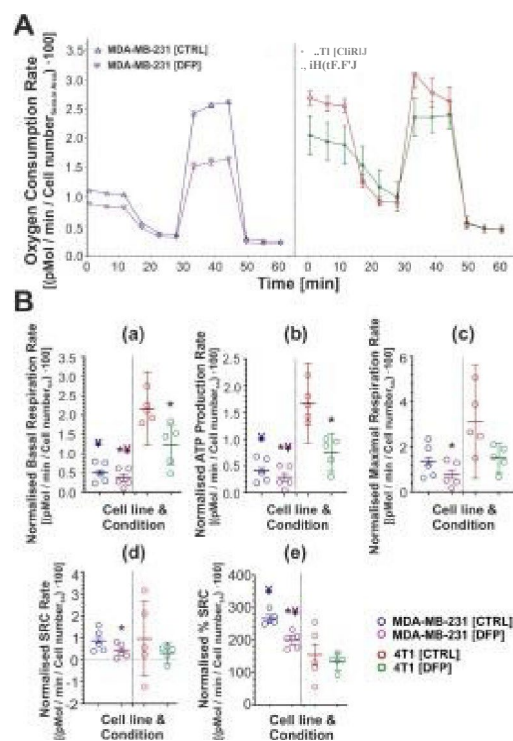


Figure 4. Effect of Deferiprone (DFP) exposure on cell migration of MDA-MB-231 (A) and 4T1 (B) cells. The wound closure is shown as a function of no DFP exposure (CTRL; A, blue circle; B, maroon circle), 50 μM of DFP (A violet square; B, lime square), and 100 μM of DFP (A purple triangle; B, green triangle). Data in A and B are averaged over 2-6 independent experiments with 3-6 replicates each and reported as mean ± SD. * Significantly different from CTRL ($p < 0.05$, two-way ANOVA followed by Tukey's post hoc test).



A Representative time course of oxygen consumption rates in untreated (control, CTRL) or DFP-treated MDA-MB-231 (left graph) and 4T1 (right graph) cells. Values were normalised to the cell number in the sensor area (SA) of each well. B. Summary graphs of five quantitative mitochondrial parameters for MDA-MB-231 (left) and 4T1 (right) cells.

* Significantly different from CTRL ($p < 0.05$);

¥ Significantly different from the corresponding value in DFP-treated 4T1 ($p < 0.05$);
¥ Significantly different from the corresponding value in CTRL 4T1 ($p < 0.05$).