Breast Cancer UK Final Report

Oxysterol signalling in triple negative breast cancer

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General Summary

Results 1: Oxysterols can induce chemotherapy resistance in TNBC

- Certain oxysterols were shown in vitro to cause resistance to a chemotherapy drug (termed chemoresistance) used to treat triple negative breast cancer (TNBC).
- Biochemical and molecular biology studies showed that these oxysterols cause an increase in synthesis of a protein (known as the Pgp efflux pump) which exports many chemotherapy drugs out of breast cancer cells.
- Reducing synthesis of Pgp prevented oxysterols from causing chemoresistance in TNBC cells.
- Oxysterol levels within tumours correlate with expression of Pgp in TNBC samples donated by patients.
- Expression of oxysterol producing enzymes correlate with expression of Pgp in TNBC samples donated by patients.

Results 2: Non-cancer cells in TNBC tumours produce the oxysterols that cause chemotherapy resistance

- Cellular markers of fibroblasts, a type of support cell found mixed in with TNBC cells in tumours, positively correlate oxysterol activity.
- Fibroblasts produce higher levels of oxysterols than many other cell types, including TNBC cells.
- Fibroblasts activate oxysterol pathways within TNBC cancer cells in vitro when they are in direct contact with each other, and via unidentified compounds released by fibroblasts into their surroundings.
- Reducing synthesis of oxysterol producing enzymes in fibroblasts reduces fibroblast induced oxysterol activity in cancer cells.
- High levels of fibroblast-like regions within TNBC tumours positively associates with expression of Pgp in the cancer cells and a greater risk of disease relapse.
- High levels of oxysterol producing enzymes in fibroblast-like regions also associate with high levels of Pgp in the tumour cells and these patients have a higher risk of relapse.

Results 3:

- A systematic review of the scientific literature was performed to determine the level of evidence that supported the hypothesis that cholesterol storage inside cells could be linked to more rapid tumour initiation and growth .
- Preventing cholesterol storage by the addition of an ester molecule to cholesterol reduced tumour growth.
- This is achieved through boosting the immune response against cancer, increasing cancer self-destruction and reducing tumour growth rate.

Background and Aim

Patients with breast tumours that are negative for the oestrogen, progesterone and HER2 receptors, known as triple negative breast cancers (TNBC), have worse prognosis than patients with other subtypes. Targeted therapies such as Tamoxifen do not work so these patients often have chemotherapy instead. Chemotherapy targets all dividing cells, not just cancer cells, meaning side-effects can be severe. Furthermore, as not all cancer cells are always dividing, chemotherapy may not always kill all the tumour cells, meaning the cancer may return.

The pilot data for this project suggested that early products during cholesterol metabolism, known as oxysterols, may enable TNBC to resist chemotherapy. Cholesterol is converted into oxysterols during the synthesis of hormones, some vitamins, and other important cellular components. Oxysterols are toxic at high levels and can cause damage to proteins, so their levels inside cells need to be carefully controlled. *The hypothesis tested during this project is that the biological mechanisms that prevent toxic levels of oxysterols from accumulating, are the same as those that detoxify anti-cancer drugs. If a tumour has developed in the presence of high cholesterol and therefore high oxysterols, then that tumour is better prepared to survive the chemotherapy drugs that are given to treat breast cancer.* The aim of this project was therefore to understand if cholesterol metabolism is linked to chemotherapy resistance in TNBC patients.

Preventing secondary breast cancer (cancer that started in the breast and spreads to other parts of the body) would have a dramatic impact on survival rates. Interestingly, the pilot data suggested that this oxysterol-chemotherapy pathway may be restricted to TNBC, indicating a new 'targeted' method to prevent recurrence may work particularly well for these patients. Better understanding of how oxysterols are altered in TNBC may help prevent patients suffering a cancer recurrence or help find a way to reduce risk of primary disease.

Results

1. Oxysterols induce chemotherapy resistance in TNBC

To explore if oxysterols could cause chemotherapy resistance the role of a well-known drug efflux pump was examined. Chemotherapy resistance is caused by these pumps in TNBC and many other cancers. We found that increased cellular production of a particular chemotherapy efflux pump, known as P-glycoprotein (Pgp), was linked to the activity of a protein that should be regulated cholesterol metabolism instead, namely liver x receptor (LXR). The activity of LXR is controlled by oxysterols and in non-cancer cells LXR ensures that each cell has access to just the right amount of cholesterol, not too much and not too little. Within this experiment, a synthetic oxysterol substitute (GW) was used as it is more stable. TNBC cells that should be killed by epirubicin, a common drug used to treat TNBC patients, were protected from epirubicin if the GW-LXR pathway was first activated (Figure 1A: compare bars 3 and 4). This told us that LXR links excessive cholesterol levels to chemotherapy detoxification pathways. Then, to demonstrate that LXR induced chemotherapy resistance needed the Pgp efflux pump, Pgp was removed from the cells (using gene knockdown technology) and the experiment repeated. Now the GW-LXR pathway, which remained intact, could not rescue the cells from the killing effect of epirubicin (Figure 1A: compare bars 7 and 8). This part of the project proved that the LXR protein can protect TNBC cells from chemotherapy by utilising the drug efflux pump, Pgp.

When looking at tumours donated by TNBC patients, expression of oxysterol producing enzymes (OPEs) in cancer cells correlated with the expression of Pgp in TNBC tumours (Figure 1B). Furthermore, the actual concentration of oxysterols themselves within the TNBC tumours also correlated with the expression of the Pgp gene (Figure 1C). These results performed in cells in the laboratory (Figure 1A) and obtained from

the primary tumour from patients (Figure 1B and 1C) support our hypothesis that if cholesterol is metabolised into oxysterols at high enough levels, the tumour is prepared to respond to chemotherapy and causes drug resistance in these patients. This section of results was published in a peer reviewed journal article: https://www.nature.com/articles/s41388-021-01720-w.

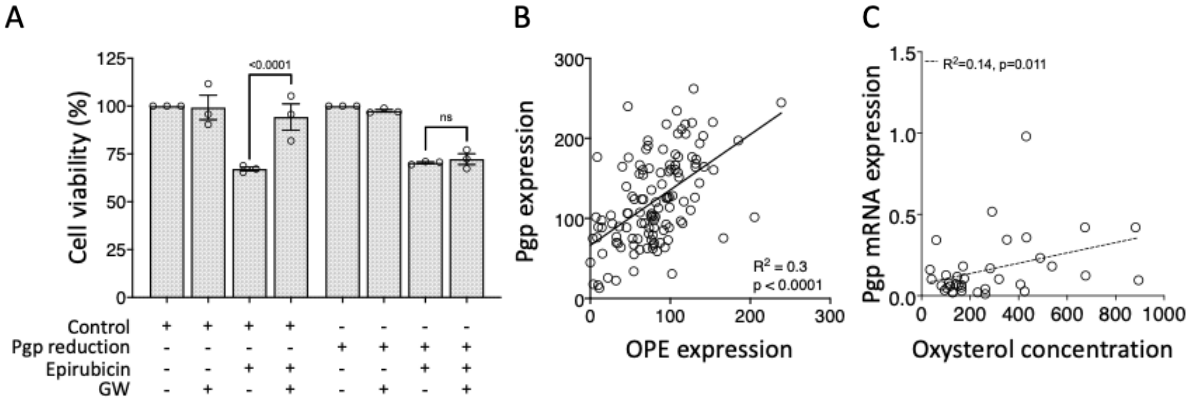

Figure 1. The drug efflux pump Pgp mediates chemotherapy resistance caused by oxysterol. (A) Reduction of Pgp expression in cancer cells prevents oxysterol substitute-mediated chemoresistance to epirubicin. (B) Expression of oxysterol producing enzymes (OPEs) in cancer cells positively associates with Pgp expression (C) Intratumour oxysterol content positively associates with Pgp mRNA expression. P-values less than 0.05 were considered to be statistically significant.

2. Non-cancer cells in TNBC tumours produce the oxysterols that cause chemotherapy resistance

After showing oxysterols could induce chemotherapy resistance in TNBC, it was then important to determine where the oxysterols came from. The tumour itself is usually a mixture of many cell types, not just cancer cells, and may include fat cells, cells of the immune system, and support cells (termed here fibroblasts). Previous work had found that many of these cell types were highly specialised to convert cholesterol into oxysterols.

First, seven genes were identified that if present in a tumour sample meant that the fibroblast support cells were present at high levels. These are hereafter referred to as “fibroblast marker genes”. Expression of these fibroblast marker genes was measured in TNBC tumour samples and tested to see if they correlated with a set of genes usually controlled by LXR. The rationale was that if the fibroblast and LXR genes were positively correlated then the presence of fibroblasts was linked to greater activity of LXR. The fibroblasts would likely be releasing oxysterols into the tumour mass, and perhaps contributing to chemoresistance. Some, but not all, of the fibroblast marker genes did indeed strongly correlate with LXR target genes in samples from TNBC patients. Oxysterol levels in fibroblasts grown in the lab were then measured and found to produce almost twice the amount of oxysterols than cancer cells were able to (Figure 2A). Furthermore, fibroblasts grown in the same culture flask as TNBC cells could activate LXR in the adjacent cancer cells (Figure 2B). This suggested the fibroblasts were indeed releasing oxysterols that were taken up by the cancer cells. When the oxysterol producing enzymes (OPEs) in fibroblasts were removed (gene knockdown), the fibroblasts could no longer activate the LXR pathways in nearby TNBC cells (Figure 2C). Fibroblast secretions were also able to activate LXR signalling and induce expression of LXR target genes (including Pgp) in TNBC cells (Figure 2D) even if the two cell types had not been in direct contact.

Finally, the amount of non-cancer regions within the TNBC tumours was measured as it was expected that the greater the number of cells able to produce oxysterols that were present, the more likely the tumour was to be drug resistant. Our first observation was that tumours with greater amounts of non-cancer regions within them had higher levels of the drug efflux pump Pgp in the cancer cells (Figure 2E).

Additionally, expression of the OPEs within the non-cancer region positively correlated with expression of Pgp in cancer cells (Figure 2F). Finally, we tested if there was a link between relapse or survival and the presence of oxysterol producing cells or OPEs. The patients with high levels of the cells or OPEs were more likely to suffer relapse or die from their disease (Figure 2G). These results are currently being prepared for publication.

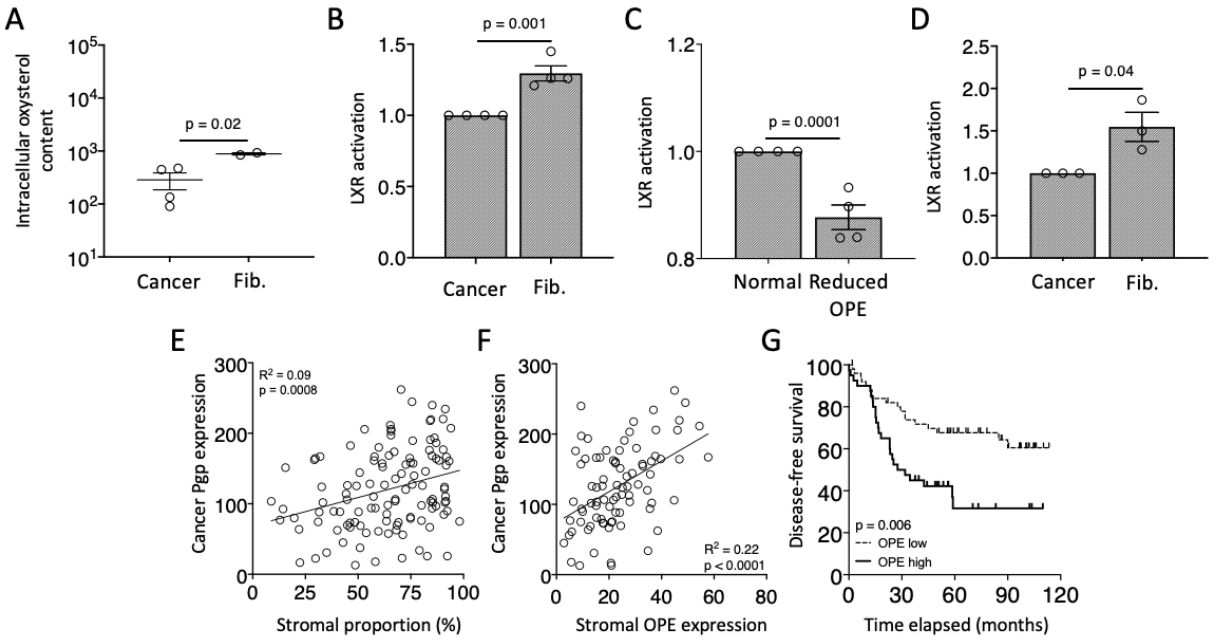

Figure 2. Non-tumour cells produce oxysterols and are linked to chemotherapy resistance in TNBC patients. (A) Fibroblasts (Fib.) contain more oxysterols than TNBC cells. (B) Growing fibroblasts alongside cancer cells activates LXR in cancer cells. (C) Reducing the synthesis of oxysterols in fibroblasts reduces their ability to activate LXR in cancer cells. (D) Fibroblast conditioned media activates LXR more than cancer cells. (E) The amount of stroma within a tumour positively correlates with Pgp expression in cancer cells. (F)The expression of OPEs in tumour stroma positively correlates with Pgp expression in cancer cells. (G) High expression of OPEs in tumour stroma associates with reduced disease-free survival. P-values less than 0.05 were considered to be statistically significant.

3. The addition of an ester to cholesterol leads to tumour growth

Due to lab closures during lockdown a systematic review and meta-analysis (SRMA) of model organisms was designed and carried out. The aim was to investigate the level of evidence to support the hypothesis that cholesterol esterification was important for cancer initiation and growth. Cholesterol esterification changes how effectively cells can store and utilise cholesterol during periods of rapid growth (for example during cancer initiation and progression). The systematic review process is designed to identify every research article that has been published within a specific topic (i.e., cholesterol and oxysterol esterification in cancer model organisms). The meta-analysis process then amalgamates all the data from these multiple studies to give a very powerful statistical analysis of a research question. Typically, if a SRMA gives a conclusive answer regarding an effect or lack of effect of an intervention (inhibition of cholesterol esterification) and outcome (tumour initiation and growth), those types of experiments are no longer needed and evidence is sufficient for the next stage of clinical research. Amalgamated data from such studies are presented as a summary analysis called a forest plot where each row shows the finding of an individual study, and a diamond shows the average response in the intervention group compared to the control group (see Figure 3).

The data shown indicates that across the 40 different experiments, there was a significant reduction in tumour size. Further analyses were performed to determine what was causing reduced tumour volume (not presented in report). Prevention of esterification was found to lead more cancer cells undergoing

self-destruction, more invading immune cells present in tumours that were better at attacking cancer cells, and cancer cells grew slower. Breast cancer was examined in one study (Figure 3; see "Other cancer tumour site": Lei J. 2019) and inhibiting esterification was found to reduce tumour growth and metastasis. These data are published here: www.sciencedirect.com/science/article/pii/S0006295221003476.

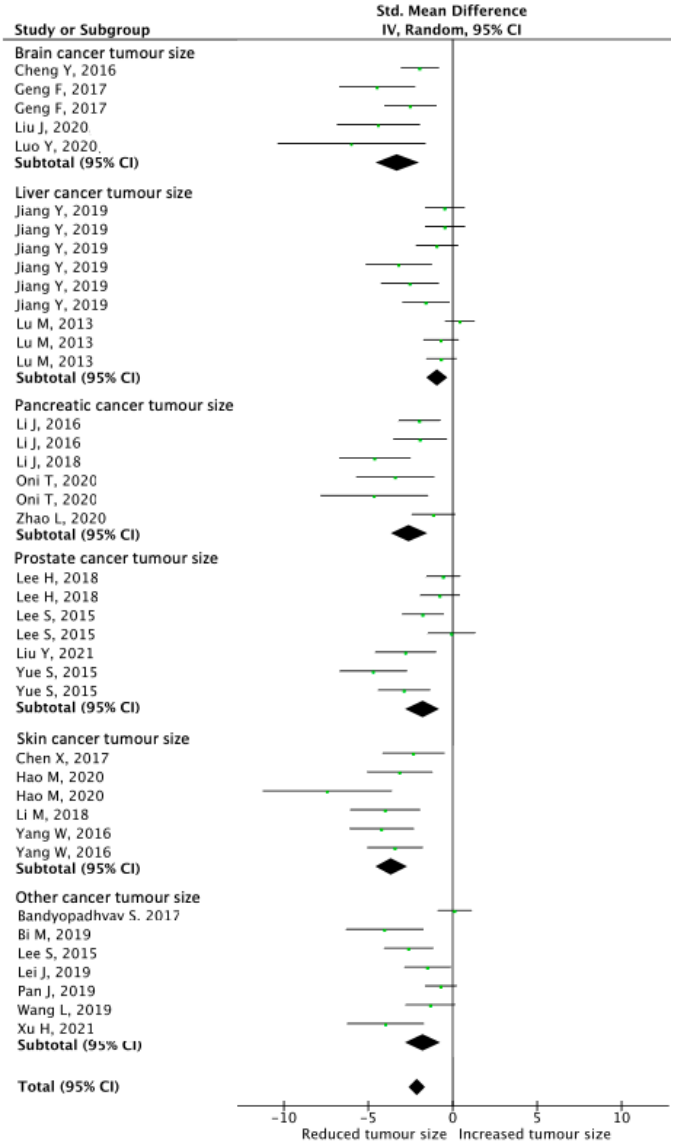

Figure 3. If the enzymes that cause cholesterol esterification are inhibited then tumours are less likely to form and are smaller in size. P=0.0002.

Conclusions

This work highlights a mechanism through which cholesterol can lead to TNBC recurrence by increasing resistance to chemotherapy. This mechanism is initiated by cholesterol metabolites, known as oxysterols, that activate LXR-mediated gene regulation, leading to increased production of chemotherapy efflux pump, Pgp. Expression of OPEs and the concentration of oxysterols inside tumours correlate with cancer cell expression of Pgp, showing that this relationship exists in patients. Within TNBC tumours, fibroblasts are a major source of oxysterols and are capable of both activating LXR signalling and inducing expression of Pgp in cancer cells through fibroblast secretions. Therefore, TNBC patients may benefit from therapies that will reduce the rate of oxysterol production inside tumours, such as statin therapy to reduce circulating cholesterol that can be converted into oxysterols.