

The effect of oxysterols on immune resistance in Triple Negative Breast Cancer

17/05/2024

Abstract

Triple Negative Breast Cancer (TNBC) is a highly immunogenic subtype of breast cancer untreatable with current hormonal or receptor-based therapies. Developments are needed in understanding the cause of immune resistance in TNBC to progress novel treatments. The present study was undertaken to determine the role of the oxysterols-LXR axis in inducing immune resistance in TNBC. The mRNA expression of three known immune checkpoint markers (PD-L1, CD80 and CD86) on MDA-MB-468 TNBC cells and two known immune checkpoint markers (PD-1 and CTLA-4) on Jurkat cells were measured using qPCR to determine the effect of three oxysterols (7α -OHC, 22(R)-OHC and 27-OHC) on the gene expression. The study demonstrates a role for oxysterols in regulating expression of immune biomarkers, potentially via activation of LXR. Generally, the results indicate that 27-OHC has the most predominant effect on mRNA expression of the immune checkpoint markers and may contribute significantly to immune resistance in TNBC. The study hopes to provide new evidence establishing the role of oxysterols in immune resistance and aid emerging immunotherapeutic strategies.

1. Introduction

Breast cancer is the most common form of cancer among women globally (Arnold et al., 2022). Approximately 10-20% of women with breast cancer have triple negative breast cancer (TNBC). TNBC is a highly immunogenic sub-type of breast cancer that, due to the lack of receptor present (estrogen receptor (ER), progesterone receptor (PR) or HER2), is untreatable with current hormonal or HER2 targeted therapies and therefore new therapeutic strategies are required to improve prognosis of patients with TNBC (Foulkes et al., 2010). In accordance with the most recent National Institute for Health and Care Excellence (NICE) guidelines, TNBC is currently treated with cytotoxic chemotherapy and immunotherapy (NICE, 2022). The most effective current immunotherapeutic strategy is the use of immune checkpoint inhibitors (ICIs) to block immunosuppressive receptors. ICIs enhance the cytotoxicity of tumour infiltrating lymphocytes (Keenan and Tolaney, 2020) and many immunotherapeutic drugs, such as pembrolizumab, have been developed to target immune receptors which are upregulated in TNBC cells. Immunotherapeutic drugs improve prognosis alongside chemotherapy, yet patients with TNBC typically relapse after approximately 2-3 years of treatment and have shorter total and post-reoccurrence survival rate (Liedtke et al., 2008) than non-TNBC patients. This clearly indicates a clinical need to identify underlying mechanisms of disease development and progression for an improved therapeutic approach.

T-cells play an essential role in immunosurveillance, the process by which pre-cancerous and cancerous cells are recognised by the immune system and the primary human defence against cancer cell onset and further advancement. Immune resistance is a mechanism of evasion from the immune system by cancer cells that develops in the early stages of cancer, persisting during the more advanced stages of the disease. This can be caused by a variety of mechanisms which enable the cancer cells to avoid detection by immune cells (Said and Ibrahim, 2023). Among these mechanisms is T-cell exhaustion, which is a form of T-cell dysfunction at the expense of a class of lymphocytes named CD8⁺ T cells (cytotoxic T lymphocytes). CD8⁺ T-cells are usually an effective immune response to several types of cancer cells, yet there is proof that tumour infiltrating CD8⁺ cells often lose their effector function in many cancer types, including TNBC cells (Ma et al., 2019b), which leads to cancer immune resistance.

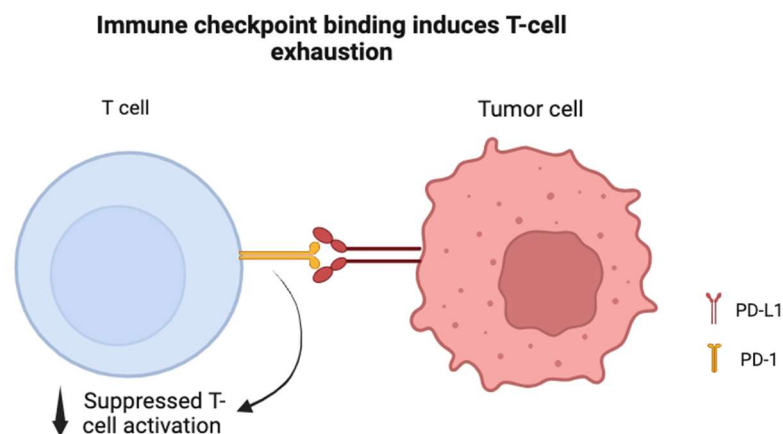


Figure 1. PD-1/PD-L1 binding induces T-cell exhaustion. PD-L1 on tumour cells bind to PD-1 on tumour specific T-cells, resulting in suppressed T-cell activity and consequently T-cell exhaustion. Created in Biorender.

Prolonged expression of programmed cell death protein 1 (PD-1) on T cells is a known marker of T cell exhaustion and when bound to programmed death ligand 1 (PD-L1) on TNBC cancer cells, PD-L1 inhibits the transcription and translation of genes required for T cell activation (Mittendorf et al., 2014), suggesting cancer cells directly inhibit activity of T cells via PD-L1/PD-1 binding (Fig.1). The expression of immune checkpoint receptor cytotoxic t-lymphocyte-associated protein 4 (CTLA-4) is also an indicator of T cell exhaustion in the tumour microenvironment. CTLA-4 counteracts costimulatory signals regulated by CD28 by competing for ligands CD80 and CD86 on the TNBC cells with increased affinity (Jiang et al., 2015). On tumour-specific T cells, CTLA-4 may increase the immune resistance of cancer cells as it downregulates CD80 and CD86, reducing the efficacy of the tumour cells to activate the T cells.

Oxysterols are cholesterol metabolites synthesised from the oxidation of cholesterol and have been identified in studies as natural ligands implicated in the regulation of several nuclear receptors such as estrogen receptors (ERs) and the retinoic acid-related orphan receptors (RORs). Among these nuclear receptors associated with oxysterol activity are liver X receptors (LXRs) (Xu et al., 2013). LXRs are nuclear receptors that, when activated, bind to promotor regions of genes involved in cholesterol metabolism and consequently increases their transcription and therefore expression. LXR α and LXR β are both activated by both synthetic and naturally synthesised ligands and physiologically, LXR α is imperative in maintaining function of the blood-brain barrier through up-regulation of p-glycoprotein (Wouters et al., 2019). It has also been found to be specifically active in the liver, adipose tissue, intestine, kidney, and macrophages, whereas LXR β is more widely expressed and has been demonstrated to have key roles in the nervous and immune systems (Spann and Glass, 2013). Oxysterols bind to and activate LXRs, inducing transcription of genes that encode proteins involved in cholesterol pathways, for instance transport, efflux, and absorption (Wang and Tontonoz, 2018). Hutchinson et al. (2021) investigated the effect of 7- α hydroxycholesterol (7 α -OHC) on TNBC chemoresistance, demonstrating a direct link between LXR activation by oxysterols in TNBC cells to induction of chemoresistance via reducing epirubicin response.

Oxysterols have been previously studied regarding their relationship with various other nuclear receptors in addition to LXRs. The oxysterols investigated in the present study are all known ligands for LXR, however they vary in origin and activate several different nuclear receptors with varying affinities. 7 α -OHC is synthesised by CYP7A1 and CYP27A1 in the liver as a primary intermediate of bile acid metabolism. It acts as a weak activator of LXR α , LXR β and an agonist for both G-protein coupled receptor 183 (GPR183) and 17 (GPR17). Additionally, it has been recognised as an inverse agonist for ROR α and ROR γ (Mutemberezi et al., 2016). 22(R)-hydroxycholesterol (22(R)-OHC) is another commonly studied oxysterol involved in steroid hormone synthesis exhibits higher affinity for LXRs than 7 α -OHC (Mutemberezi et al., 2016). 22(R)-OHC has also been acknowledged as an agonist for ROR γ and GPR17. Another well-studied cholesterol metabolite is 27-hydroxycholesterol (27-OHC) which, akin to 7 α -OHC, is involved in bile acid synthesis but has been found to circulate in the body at elevated concentrations (Nelson, 2018) when compared with other endogenous oxysterols. The ligand activity of 27-OHC regarding nuclear receptor activation has been well researched and it has been recognised in literature as an agonist for GPR17 and ROR γ (Mutemberezi et al., 2016). It has also been identified as a partial agonist for LXR α and LXR β with similar affinity for the receptors as 22(R)-OHC and has been classified as a selective estrogen receptor modulator (SERM) for ER α , potentially also having a role in ER β signalling (Ma and Nelson, 2019). The difference in origin, structure and function of oxysterols may cause differences in their effect on TNBC cells and the mechanisms through which these effects are caused.

The role of cholesterol in T cell exhaustion has been previously studied by Ma et al. (2019a) who revealed that increased cholesterol in the tumour microenvironment induces expression of immune checkpoints on CD8⁺ T cells and therefore T cell exhaustion, potentially introducing a method for improving T cell immunotherapy through cholesterol reduction. It can be hypothesised that due to ligand similarity and previous research that increased LXR activation by oxysterols may induce immune resistance in TNBC cells in many ways, one primary mechanism of interest being through T-cell exhaustion. It has been shown that by binding to LXR β , 27-OHC alters the LXR pathway causing cholesterol deficiency of CAR-T cells, consequently leading to metabolic and signalling pathways that can drive T cell exhaustion (Yan et al., 2023). This indicates that the increase of LXR activation may lead to T cell exhaustion in the tumour microenvironment. However, it has not been demonstrated in published literature that LXR directly regulates transcription of immune checkpoint proteins, which the present study aims to investigate. The study conducted by Yan et al. (2023) demonstrates the ability of oxysterols to cause T-cell exhaustion using mouse melanoma and colorectal cancer tumour models, however, does not demonstrate using a breast cancer model so the mechanism may not apply to the TNBC cancer subtype. Additionally, the ER(+) variation of breast cancer has been studied closely in relation to oxysterol ligand function and their roles in breast tumorigenesis. In ER(+) breast cancer, 27-OHC behaved in a mitogenic manner (Dusell et al., 2008), whereas 22(R)-hydroxycholesterol suppressed proliferation and induced apoptosis (Nazih and Bard, 2020). Hutchinson et al. (2019) studied the difference in oxysterol signalling between ER(-) and ER(+) variations of breast cancer and it was found that ER(-) tumours had increased response to LXR ligands, which substantiates the concept that tumour receptor status alters the transcriptional activity of LXR. Previous studies have not been conducted investigating the effect of oxysterols on the triple negative subtype of breast cancer, however it can be suggested from current knowledge presented above that the oxysterols will cause an increase in immune resistance of the TNBC cells.

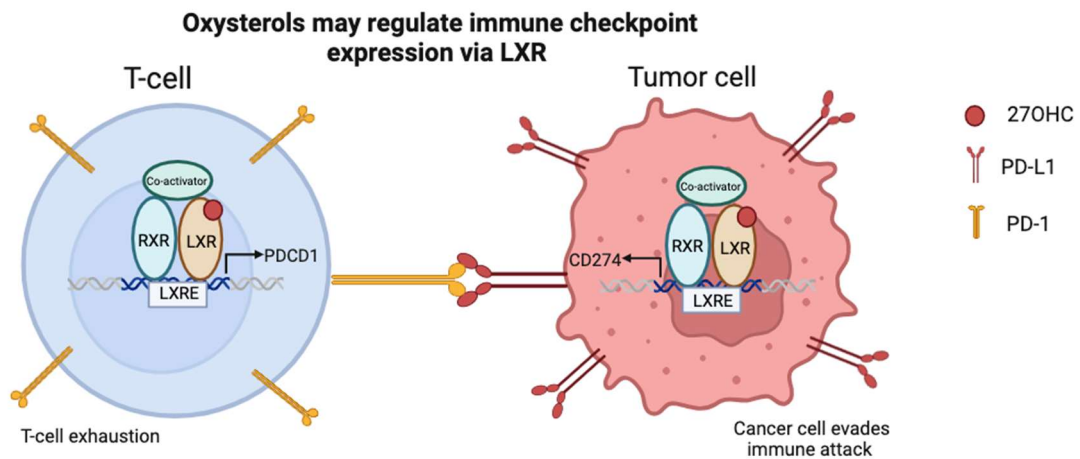


Figure 2. PD-1/PD-L1 upregulation may be caused by LXR activation via oxysterols. Oxysterols may cause upregulation of immune checkpoint markers PD-L1 on TNBC cells and PD-1 on T-cells. When PD-L1 binds to PD-1, T-cell exhaustion is induced, causing immune resistance. Created in Biorender.

The hypothesis of the present study is that differential activation of the oxysterols-LXR axis will cause changes in the gene expression of immune markers associated with immune resistance in TNBC and effector T-lymphocyte model of immune cells (Fig.2). The study aims to demonstrate that the oxysterols-LXR axis induces immune resistance on both cancer and immune cells. The results of this investigation may assist in identifying patients at risk of immune resistance through recognition of biomarkers and novel targeted cancer therapy developments.

2. Materials and Methods

2.1 Sub-culturing and cell viability

Human triple negative breast cancer cell line MDA-MB-468 (ATCC, Cat. No. HTB-132) was cultured in Dulbecco's Modified Eagle Medium (DMEM) Glutamax (cat no. 10569010) with 10% Fetal Bovine Serum (FBS) (cat no. S12550H) and split every three/four day as per laboratory protocol. Cells were washed prior to splitting using phosphate buffered saline (PBS) and detached using 0.5% Trypsin (T2) (cat no. 15400054). Cell viability was determined using trypan blue (cat no. 15250061) to stain the cells for counting using a hemocytometer. Cells were then plated into a TC75 flask with 1×10^6 total cells for subculture and maintained at 37°C in 5% CO₂.

Jurkat human cell line cells were kindly donated by Dr Robert Salmond, Faculty of Health and Medicine, University of Leeds. Jurkat cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Cat. No. 21875034) with 10% FBS and split every three/four day as per laboratory protocol. Cell viability was determined using trypan blue to stain the cells for counting using a hemocytometer. The cells were sub-cultured at 0.5×10^6 cells/T25 flask and maintained at 37°C in 5% CO₂.

2.2 Oxysterols

Oxysterols used to treat the cells were 7 Alpha-Hydroxycholesterol (Avanti Polar Lipids, cat No. 700034P), 22(R)-Hydroxycholesterol (Avanti Polar Lipids, cat No. 700058P) and 27-Hydroxycholesterol (Avanti Polar Lipids, Cat No 700021P).

2.3 RNA extraction, Reverse Transcription, and qPCR

Total RNA was extracted from MDA-MB-468 cells and Jurkat cells at 24hr and 48hr using RNeasy RNA Cell Miniprep System (Promega, cat no. Z6012) according to manufacturer instructions. Briefly, cells were lysed in BL +TG buffer and RNA was then prepared using RNA wash solution and then extracted using a master mix of yellow core buffer, manganese chloride and DNase I enzyme. RNA was then purified using column wash solution and RNA wash solution was then used to remove traces of ethanol. Nuclease-free water was used to complete RNA extraction and RNA yield and integrity was determined using BMG Labtech CLARIOstar microplate reader.

Reverse transcription was completed on ice using Random Primer (Thermo Fisher, cat no. 48190011) and Reverse transcription Master Mix containing GoScript™ 5X Reaction Buffer (Promega, cat no. A5003), Magnesium Chloride (Promega, cat no. A5003), PCR Nucleotide Mix (Promega, cat no. C1145), GoScript™ Reverse transcriptase (Promega, cat no. A5003) to obtain DNA samples. The samples were placed in a controlled-temperature heat block equilibrated at 25 °C for 5 min, at 42 °C for 59 min, then 70 °C for 15 min before being chilled. 60uL nuclease free water was added to each sample.

During all the above procedures, 1% Chemgene and 7% Isopropanol were used for cleaning purposes.

2.4 Experimental Procedure

10mM stock concentration was used for both the 7 α -Hydroxycholesterol and 22(R)-Hydroxycholesterol. 5mg 7 α -Hydroxycholesterol was dissolved in 1.24ml ETOH and 1mg 22(R)-Hydroxycholesterol was dissolved in 0.24ml ETOH to make 10mM stock solutions for treatment for vehicle control (VC).

MDA-MB-468 cells were treated in 6 well plates suspended in DMEM with 10% FCS with 3 x 10⁵ cells per well. Jurkat cells were treated in T25 flasks suspended in RPMI with 10% FCS 1.5 x 10⁶ total cells. All cells were treated on day 1. For the 7 α -Hydroxycholesterol treatment, 2uL stock solution was used to make 10uM treatment concentration (0.1% solvent). For the 22(R)-Hydroxycholesterol treatment, 2uL 10mM stock solution was used to make 10uM treatment concentration (0.1% solvent). For the vehicle control, cells were treated with 2uL 100% ethanol (0.1% solvent).

The PCR Reaction Mix was prepared using Taqman™ Fast Advance Master Mix (Thermo Fisher, cat no. 4444557) and appropriate Taqman Gene Expression Assays (Thermo Fisher, FAM cat no. 4331182; VIC cat no. 4448484). The PCR reaction plate was prepared by the addition of 5uL cDNA and 5uL appropriate master mix to each well (some replicates were prepared using 10uL cDNA and 10uL master mix), the plate was then sufficiently centrifuged to mix the components and qPCR was run on Applied Biosystems QuantStudio7. HPRT 1 was used as a reference housekeeping gene (Thermo Fisher, VIC cat no. 4448484).

Samples of cDNA for both MDA-MB-468 and Jurkat cells treated with 27-OHC were kindly given by Giorgia Ciccoloni (University of Leeds, School of Food Science and Nutrition).

2.5 Statistical Analysis

Comparative CT values on an amplification plot were used to obtain final gene mRNA relative expression data ($2^{-\Delta\Delta C_t}$). Analysis of the qPCR results was performed using ordinary one-way ANOVA. P value ≤ 0.05 was considered significant. Assuming equal homoscedasticity, a Dunnett's multiple comparisons test was performed to compare all treatments at once against the control.

3. Results

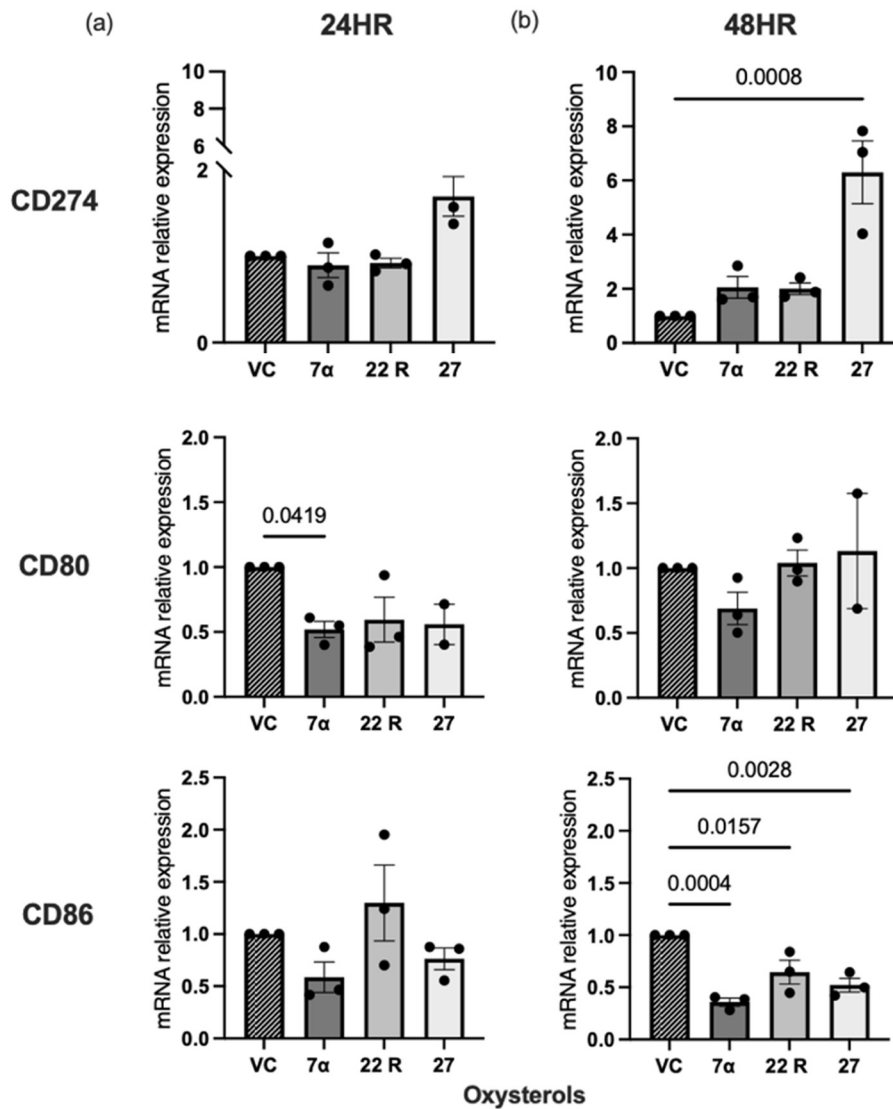


Figure 3. Effect of 7α-OHC, 22(R)-OHC and 27-OHC on gene expression of CD274, CD80 and CD86 after (a) 24hrs and (b) 48hrs in MDA-MB-468 cells. HPRT 1 was used as a housekeeping gene. mRNA relative expression was calculated as $2^{-\Delta\Delta C_t}$, treatment results were compared with control sample. Data are expressed as the mean $\pm$ SEM of independent experiments performed in duplicate or triplicate. Statistical difference was determined as $p \leq 0.05$.

3.1 Effect of treatment with oxysterols on mRNA expression in TNBC cells

To establish if oxysterols induce upregulation of immune checkpoint proteins in the TNBC cells, qPCR was performed on the cDNA samples to determine relative gene expression levels of CD274 (PD-L1). The relative mRNA expression of CD274 after 24 hours after treatment with 7α-OHC and 22(R)-OHC was not statistically significant against the vehicle control (Fig. 3a; $p > 0.05$). However, after 48 hours an upregulation of CD274 was observed for both treatment with 7α-OHC and 22(R)-OHC but no statistically significant difference was found (Fig 3b; $p > 0.05$). Treatment with 27-OHC caused an upregulation of the gene at both 24 hours and 48 hours, however statistical significance was only found at 48 hours ($p = 0.0008$), shown in Fig. 3b.

The relative mRNA expression of CD80 was also investigated using qPCR to aid determination of the effect of oxysterols on immune checkpoint proteins to further establish the role of oxysterols in immune resistance

of the TNBC cells. Notably, the qPCR results show that there was statistically significant down regulation of the gene after treatment with 7α -OHC ($p=0.0419$) at 24 hours (Fig. 3a). 22(R)-OHC and 27-OHC also caused downregulation of the gene at 24 hours however no statistically significant difference was found upon analysis (Fig. 3a; $p>0.05$). At 48 hours, as shown in Fig. 3b, treatment with 7α -OHC downregulated the gene however expression of the gene after treatment with 22(R)-OHC and 27-OHC was not statistically significant against the vehicle control.

To further aid the hypothesis that oxysterols alter gene expression of immune checkpoint proteins, the mRNA relative expression of CD86 was determined using qPCR. As hypothesised, treatment of the TNBC cells with 22(R)-OHC caused upregulation of the gene at 24 hours (Fig 3a). Treatment of the TNBC cells with 7α -OHC and 27-OHC caused downregulation of the gene at 24 hours, however no statistically significant differences were found (Fig 3a; $p > 0.05$). Notably, at 48 hours (Fig. 3b), statistically significant downregulation of the gene was observed after treatment with 7α -OHC ($p = 0.0004$), 22(R)-OHC ($p = 0.0157$) and 27-OHC ($p = 0.0028$).

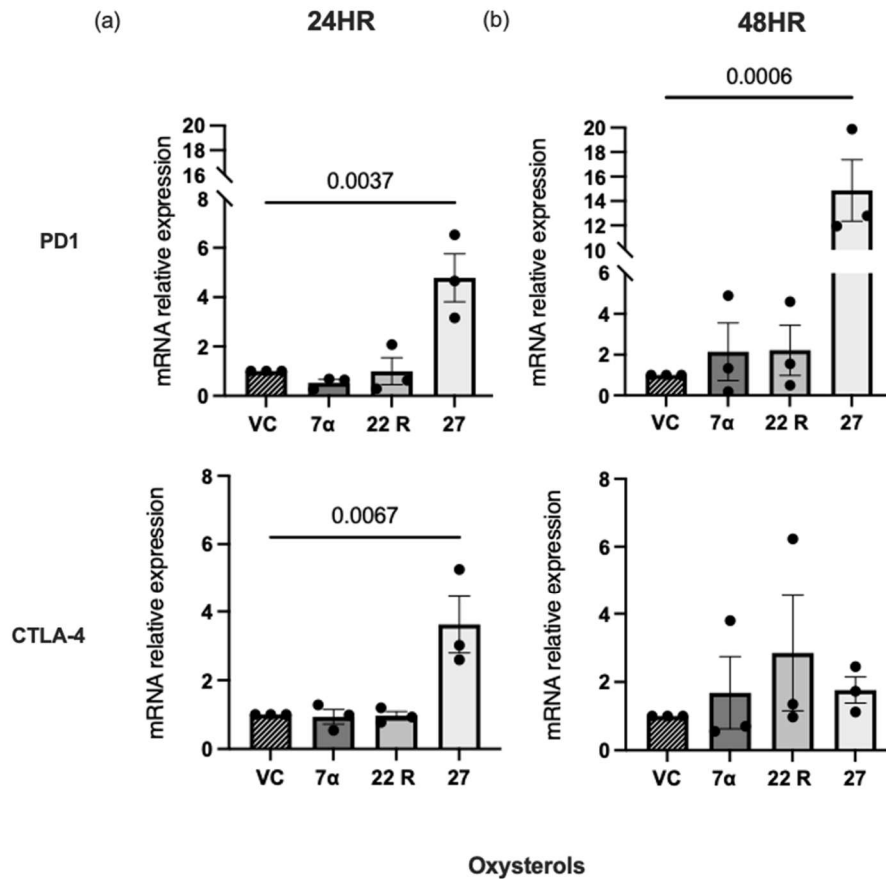


Figure 4. Effect of 7α -OHC, 22(R)-OHC and 27-OHC on gene expression of PD-1 and CTLA-4 after (a) 24hrs and (b) 48hrs in Jurkat cells. HPRT 1 was used as the housekeeping gene. mRNA relative expression was calculated as $2^{-\Delta\Delta Ct}$, treatment results were compared with control sample. Data are expressed as the mean $\pm$ SEM of experiments performed in triplicate. Statistical difference was determined as $p \leq 0.05$.

3.2 Effect of treatment with oxysterols on mRNA expression in Jurkat cells

To establish if oxysterols influence mRNA expression of immune checkpoint proteins on T-cells, Jurkat cells were used as an effector T-lymphocyte model. Relative mRNA expression levels were obtained using qPCR method. Treatment with 27-OHC caused statistically significant upregulation of PD-1 at 24hrs and 48hrs (Fig 4a; $p=0.0037$ and Fig 4b; $p=0.0006$ respectively). Treatment with 7α -OHC and 22(R)-OHC also caused upregulation of PD-1 at 48 hours (Fig. 4b), however the upregulation was not statistically significant in comparison to the upregulation observed with the treatment of 27-OHC. Treatment with 7α -OHC caused slight downregulation of PD-1 at 24hrs, however no effect was observed at 24hrs of treatment with 22(R)-OHC (Fig 4a).

The mRNA relative expression of the CTLA-4 gene was also measured to further assess the effect of oxysterols on immune marker gene expression, which may aid determination as to whether oxysterols induce immune resistance as CTLA-4 is a known marker of T-cell exhaustion. The results were obtained using qPCR. Figure 3 demonstrates that a statistically significant upregulation of CTLA-4 was observed after treatment with 27-OHC at 24hrs (Fig 4a; $p=0.0067$). Slight upregulation after treatment with 27-OHC was also noted after 48hrs (Fig 4b), however this was weak and not statistically significant ($p>0.05$). Very little variation from the vehicle control was seen after treatment with 7α -OHC and 22(R)-OHC at 24 hours (Fig. 4b), however a weak, not statistically significant upregulation was seen for both 7α -OHC and 22(R)-OHC after 48hrs ($p>0.05$).

4. Discussion

The purpose of the present experiment was to demonstrate the effect of oxysterols on the expression of immune marker genes to determine if the oxysterols-LXR axis induces immune resistance in TNBC cells. Immune marker mRNA expression data was obtained for known markers of T-cell exhaustion on both MDA-MB-468 TNBC cells and Jurkat cells to establish if oxysterols influence the mRNA expression, which then demonstrates the effect of oxysterols on immune resistance. The results are suggestive that oxysterols, primarily 27-OHC, do influence mRNA expression of immune marker genes, however each oxysterol used in the present experiment has individual effects on each gene and the effect cannot be generalised to all oxysterols as they behave differently in the tumour microenvironment. Most current immunotherapy research is based on the PD-L1/PD-1 axis, however the CTLA-4:CD80/CD86 axis shows promise as an immunotherapeutic target to improve anti-tumour response.

One observation to be noted is the effect of 27-OHC on PD-L1 expression 48hrs post treatment. A slight upregulation of the gene at is observed at 24hrs followed by a significant upregulation at 48hrs. As discussed previously, overexpressed PD-L1 binds to PD-1 on T-cells and impairs T-cell activation (Yi et al., 2021), linking PD-L1 expression to immune resistance in tumour cells. Current knowledge demonstrates that 27-OHC is an endogenous ligand that acts by many pathways (Kim et al., 2022), therefore the upregulation of PD-L1 instigated by treatment with 27-OHC may not be due to increased LXR activation as hypothesised. This is further aided by the lack of upregulation of PD-L1 post treatment with 22(R)-OHC, which is a strong agonist for LXR with similar affinity for the receptor as 27-OHC, suggesting that if LXRs mediated the upregulation, treatment with 22(R)-OHC would have similar or equal effect. This lack of upregulation after treatment with 22(R)-OHC suggests that other nuclear receptors may be activated by 27-OHC to induce transcription of PD-

L1, however existing research to establish this is very limited and is a critical aspect for future research. Despite underlying mechanisms needing further research to be fully understood, the upregulation of PD-L1 caused by 27-OHC may assist in identifying patients that may benefit from immunotherapy via immune checkpoint inhibitors and aids in establishing the hypothesis that 27-OHC causes immune resistance in TNBC cells.

Treatment of the Jurkat cells with 27-OHC caused significant upregulation of PD-1 at 24hrs and 48hrs, which is also noteworthy in determining the role of oxysterols in immune resistance. Increased PD-1 expression on T-cells is a known marker of T-cell exhaustion (Ma et al., 2019a), and increased cholesterol has been shown to induce T-cell exhaustion in CD8⁺ T-cells. Initial PD-1 expression on the surface of T-cells is typical of T-cell activation, but prolonged expression is indicative of T-cell exhaustion (Catakovic et al., 2017). As PD-1 expression was significantly upregulated at 24hrs and upregulation was increased at 48hrs, this is suggestive that 27-OHC induces T-cell exhaustion via upregulation of PD-1. This indicates that 27-OHC may aid immune resistance of TNBC cells by inducing T-cell exhaustion in tumour specific T-cells, weakening the anti-tumour immune response. The mechanism via which 27-OHC induces this significant upregulation cannot be determined with the results of the present study alone and current literature on plausible mechanisms is limited. It has been suggested in studies focused on LXR-inverse agonism that LXR ligands may influence PD-1 expression (Carpenter et al., 2019), however PD-1 has not been previously demonstrated to be transcribed by LXR therefore alternative nuclear receptors that regulate PD-1 expression must also be investigated in further research. 22(R)-OHC has a similar affinity for LXR when compared to 27-OHC which would present the hypothesis that they would have similar effect on PD-1 expression if the upregulation was instigated by the activation of LXR. However, due to the lack of upregulation caused by 22(R)-OHC, it should be considered that 27-OHC is acting via alternative pathways. Involvement of 27-OHC in PD-1 upregulation via activation of LXR cannot be established from the present study, however it can be determined that 27-OHC increases T-cell exhaustion via upregulation of PD-1, which would aid immune resistance. Further research using gene expression-based experiments to identify pathways involved are needed to confirm the underlying mechanism of PD-1 upregulation via treatment with 27-OHC.

CD80, like PD-L1, has been found to show increased expression in TNBC when compared to non-TNBC (Zhang et al., 2022). However, when treated with 7 α -OHC, a significant downregulation of the gene is observed after 24hrs. Current knowledge of CD80 suggests that it plays a role in T-cell activation through CD28:CD80/86 co-stimulatory pathways on tumour specific T-cells (Papadimitriou et al., 2021) and loss of CD80 alone has been found to increase ability of cancer cells to evade immune attack (Zhang et al., 2022). It has also been demonstrated to promote memory responses in cytotoxic T lymphocytes (CTLs) (Wang et al., 2013), further indicating a role of CD80 in aiding efficient anti-tumour immune responses. Current literature suggests CD80 may play a vital role in the regulation of T-cell immune functions against TNBC cells and therefore the downregulation of CD80 may result in reduced ability of the cancer cells to activate T-cells and initiate immune responses. LXR agonistic activity has been demonstrated to play a role in upregulation of CD80/CD86 monocytes (Kim et al., 2021) and whilst this does not provide solid evidence for the involvement of LXR in CD80 expression in TNBC, it supports the hypothesised potential mechanism involving LXR in regulating CD80 expression. However, 7 α -OHC caused significant downregulation of CD80, which is indicative of an alternate pathway not facilitated by LXR as 7 α -OHC has poor affinity for LXRs. This is further insinuated by the lack of effect produced by treatment with 22(R)-OHC, which has a much higher affinity for LXRs when compared to 7 α -OHC (Mutemberezi et al., 2016). The pathway by which 7 α -OHC causes the downregulation of CD80 cannot be established from the study or interpreted from current literature, therefore further research is required to determine the underlying mechanisms. However, the present study does establish

a definite role for 7 α -OHC in the immune resistance of TNBC cells by suppressing genes involved in costimulatory T-cell activation, reducing the efficacy of the tumour cells to activate the T-cells and induce an immune response therefore aiding immune evasion. CD80 may be a promising target for immunotherapy in TNBC patients.

Similarly, CD86 plays a costimulatory role in T-cell activation via the CD28:CD80/86 axis (Papadimitriou et al., 2021). Suppression of the gene may therefore also reduce ability of tumour cells to activate T-cells. Treatment with all 3 oxysterols used in the current experiment caused significant downregulation of CD86 at 48hrs, which is strongly suggestive of a role of LXR in CD86 downregulation. This is implied by the results of the present study as 22(R)-OHC and 27-OHC, both which have similar high affinities for LXRs, both cause a significant downregulation of the gene, indicating activation of LXR is the most likely mechanism involved. As hypothesised, it is likely oxysterol mediated LXR activation causes a suppression in CD86 expression which may indicate LXR activation by oxysterols causes transcriptional repression of CD86 or may be suggestive of inhibition by LXR of other pathways that transcribe. It is important to note that CD86 and CD80 both bind to CD28 and CTLA-4 with similar affinities, both binding to CTLA-4 with higher affinity. CD28:CD80/CD86 pathways activate T-cells, whilst CTLA-4:CD80/CD86 pathways result in T-cell attenuation (Papadimitriou et al., 2021). The CD80:CD28 axis has been found to be impaired in the presence of CTLA-4, suggesting CD86 is the primary costimulatory ligand for T-cell activation (Halliday et al., 2020). This suggests that downregulation of CD86 aids immune evasion by reducing tumour cell ability to activate the T-cells. The mechanism by which CD86 is downregulated in TNBC by oxysterols is undetermined, however activation of LXR is a strong candidate.

Treatment of the Jurkat cells with 27-OHC also significantly increased mRNA expression of CTLA-4 after 24hrs, however no significant upregulation was observed after 48hrs. CTLA-4 can prevent the stimulatory signal typically provided by the binding of CD28 to CD80/86 by competing for the ligands with higher affinity (Buchbinder and Desai, 2016). This results in reduced T-cell survival and weakens the anti-tumour immune response. The statistically significant upregulation of CTLA-4 with treatment of 27-OHC after 24hrs indicates a role for oxysterols in dampening T-cell activation. Herold et al. (2017) demonstrated that in vivo LXR activation results in induction of regulatory T-cells (Treg), which is associated with upregulation of CTLA-4. Treg suppress activation of effector T-cells, therefore induction of these cells weakens anti-tumour immune response and demonstrates a link between increased CTLA-4 expression and enhanced tumour immune resistance. This suggests LXR is a plausible mechanism for the observed increase in mRNA expression of CTLA-4 via activation by 27-OHC as activation of LXR has been previously associated with upregulation of CTLA-4. However, alternative novel mechanisms should also be considered as 22(R)-OHC, which has similar affinity for LXR as 27-OHC, does not demonstrate similar effect. A direct relationship between CTLA-4 upregulation and activation of nuclear receptors by 27-OHC has not been documented in literature and therefore further research is required to establish this. CTLA-4 is a promising immunotherapeutic target to reduce immunosuppression via blockade and upregulation of the gene by 27-OHC aids in identifying patients that may benefit from these potential immunotherapeutic strategies. The results of the study demonstrate a definite role for 27-OHC in immune resistance via upregulation of CTLA-4.

4.1 Limitations

The study has several limitations which are important to consider when interpreting the results. Notably, when measuring mRNA expression of CD80 after treatment with 27-OHC only two replicates were obtainable due

to the number of cycles exceeding 40 each time a third replicate was attempted. This meant the mRNA expression was not measured in triplicate and comparison of these results with the others of the study must take this into consideration. The restricted time frame of the study must also be considered as with extended length of time, a third replicate may have been obtainable. Availability of samples during the study also did not allow for a positive control to be measured, which may have assisted in the validation of the experimental conditions and enabled the quality of the results to be assessed.

The study also uses simplified models of TNBC and T-cells, which may not represent the complexity of the tumour microenvironment *in vivo*. The TNBC cell line used is also not representative of all TNBC subtypes and therefore these results may not be typical of other TNBC cell lines used *in vitro*. It must also be noted that for several of the replicates obtained there are noticeable outliers which may be due to several possible factors, for instance excess sample use or variance in cell viability.

5. Conclusion

The results of the current experiment suggest a potential role for oxysterols in aiding immune resistance in TNBC. It can be concluded that 27-OHC has the most predominant effects on immune checkpoint marker expression, primarily PD-L1, PD-1 and CTLA-4, which may be due to the activation of LXRs however this is a critical aspect for future research. The upregulation of PD-L1, PD-1 and CTLA-4 after treatment with 27-OHC is highly suggestive that 27-OHC weakens immune response as increased expression of these genes are known markers of T-cell exhaustion, therefore demonstrating that 27-OHC induces immune resistance in TNBC cells. The downregulation of CD86 by all 3 oxysterols used in the present study also establishes a role for oxysterols in immune resistance as it suggests that the oxysterols dampen T-cell activation, therefore causing immune resistance of the TNBC cells. Significant upregulation of CTLA-4 also demonstrates the role of 27-OHC in impairing immune response due to the known role of CTLA-4 in preventing T-cell activation. These key findings reveal a definite role for oxysterols in inducing immune resistance via upregulation of known immune checkpoint markers. It cannot be concluded from the study if the pathway by which oxysterols regulate immune response is LXR mediated and further research involving LXR activation by oxysterols and immune checkpoint marker expression is needed to fully understand the underlying mechanisms. The results of the study also may aid in identifying patients at risk of immune resistance and assist in the development of targeted immunotherapy by demonstrating the role of endogenous oxysterols in immune resistance.

Acknowledgements

I would like to thank my supervisor, Giorgia Cioccoloni, for her unwavering support, kindness, patience, and incredibly valuable feedback throughout the project. Her expertise and scientific understanding have provided me with invaluable skills and knowledge for the future and I will always have the upmost gratitude. Thank-you for everything.

I would also like to thank my family and friends for all their encouragement, advice and constant support during the project and my time at university, I could not have completed it without them.

I would also like to express my gratitude to Lourdes-Callum Shaw, who I spent countless hours in the lab with and who has been a great support to me during the project.

I would also like to personally thank Dr Robert Salmond as without his kind donation the project would not have been possible.

I would lastly like to thank the University of Leeds for the opportunity to complete this valuable research.

References

- Arnold, M., Morgan, E., Rumgay, H., Mafra, A., Singh, D., Laversanne, M., Vignat, J., Gralow, J. R., Cardoso, F., Siesling, S. & Soerjomataram, I. 2022. Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast*, **66**, pp. 15-23.
- Buchbinder, E. I. & Desai, A. 2016. Ctlα-4 and pd-1 pathways: Similarities, differences, and implications of their inhibition. *American Journal of Clinical Oncology*, **39** (1), pp. 98-106.
- Carpenter, K. J., Valfort, A.-C., Steinauer, N., Chatterjee, A., Abuirqeba, S., Majidi, S., Sengupta, M., Di Paolo, R. J., Shornick, L. P., Zhang, J. & Flaveny, C. A. 2019. Lxr-inverse agonism stimulates immune-mediated tumor destruction by enhancing cd8 t-cell activity in triple negative breast cancer. *Scientific Reports*, **9** (1), article no: 19530.
- Catakovic, K., Klierer, E., Neureiter, D. & Geisberger, R. 2017. T cell exhaustion: From pathophysiological basics to tumor immunotherapy. *Cell Communication and Signaling*, **15** (1), pp. 1-16.
- DuSell, C.D., Umetani, M., Shaul, P.W., Mangelsdorf, D.J. and McDonnell, D.P., 2008. 27-hydroxycholesterol is an endogenous selective estrogen receptor modulator. *Molecular Endocrinology*, **22**(1), pp.65-77.
- Foulkes, W. D., Smith, I. E. & Reis-Filho, J. S. 2010. Triple-negative breast cancer. *New England Journal of Medicine*, **363** (20), pp. 1938-1948.
- Halliday, N., Williams, C., Kennedy, A., Waters, E., Pesenacker, A. M., Soskic, B., Hinze, C., Hou, T. Z., Rowshanravan, B. & Janman, D. 2020. Cd86 is a selective cd28 ligand supporting foxp3⁺ regulatory t cell homeostasis in the presence of high levels of ctla-4. *Frontiers in Immunology*, **11**, p. 600000.
- Herold, M., Breuer, J., Hucke, S., Knolle, P., Schwab, N., Wiendl, H. & Klotz, L. 2017. Liver x receptor activation promotes differentiation of regulatory t cells. *PLOS ONE*, **12** (9), article no: 0184985.
- Hutchinson, S.A., Lianto, P., Moore, J.B., Hughes, T.A. and Thorne, J.L., 2019. Phytosterols inhibit side-chain oxysterol mediated activation of LXR in breast cancer cells. *International Journal of Molecular Sciences*, **20** (13), p. 3241.
- Hutchinson, S. A., Websdale, A., Cioccoloni, G., Røberg-Larsen, H., Lianto, P., Kim, B., Rose, A., Soteriou, C., Pramanik, A., Wastall, L. M., Williams, B. J., Henn, M. A., Chen, J. J., Ma, L., Moore, J. B., Nelson, E., Hughes, T. A. & Thorne, J. L. 2021. Liver x receptor alpha drives chemoresistance in response to side-chain hydroxycholesterols in triple negative breast cancer. *Oncogene*, **40** (16), pp. 2872-2883.
- Jiang, Y., Li, Y. & Zhu, B. 2015. T-cell exhaustion in the tumor microenvironment. *Cell Death & Disease*, **6** (6), article no: 1792

- Keenan, T. E. & Tolaney, S. M. 2020. Role of immunotherapy in triple-negative breast cancer. *Journal of the National Comprehensive Cancer Network*, **18** (4), pp. 479-489.
- Kim, B. Y., Son, Y., Cho, H. R., Lee, D., Eo, S. K. & Kim, K. 2021. 27-hydroxycholesterol induces macrophage gene expression via lxr-dependent and -independent mechanisms. *Korean J Physiology Pharmacology*, **25** (2), pp. 111-118.
- Kim, D., Lee, K. M., Lee, C., Jo, Y. S., Muradillaevna, M. S., Kim, J. H., Yoon, J. H. & Song, P. 2022. Pathophysiological role of 27-hydroxycholesterol in human diseases. *Advances Biological Regulation*, **83**, p. 100837.
- Liedtke, C., Mazouni, C., Hess, K. R., André, F., Tordai, A., Mejia, J. A., Symmans, W. F., Gonzalez-Angulo, A. M., Hennessy, B. & Green, M. 2008. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *Journal of Clinical Oncology*, **26** (8), pp. 1275-1281.
- Ma, L. & Nelson, E. R. 2019. Oxysterols and nuclear receptors. *Molecular and Cellular Endocrinology*, **484**, pp. 42-51.
- Ma, X., Bi, E., Lu, Y., Su, P., Huang, C., Liu, L., Wang, Q., Yang, M., Kalady, M. F. & Qian, J. 2019a. Cholesterol induces cd8+ t cell exhaustion in the tumor microenvironment. *Cell Metabolism*, **30** (1), pp. 143-156.
- Ma, X., Bi, E., Lu, Y., Su, P., Huang, C., Liu, L., Wang, Q., Yang, M., Kalady, M. F., Qian, J., Zhang, A., Gupte, A. A., Hamilton, D. J., Zheng, C. & Yi, Q. 2019b. Cholesterol induces cd8(+) t cell exhaustion in the tumor microenvironment. *Cell Metabolisms*, **30** (1), pp. 143-156.
- Mittendorf, E. A., Philips, A. V., Meric-Bernstam, F., Qiao, N., Wu, Y., Harrington, S., Su, X., Wang, Y., Gonzalez-Angulo, A. M., Akcakanat, A., Chawla, A., Curran, M., Hwu, P., Sharma, P., Litton, J. K., Molldrem, J. J. & Alatrash, G. 2014. Pd-11 expression in triple-negative breast cancer. *Cancer Immunol Res*, **2** (4), pp. 361-70.
- Mutemberezi, V., Guillemot-Legriss, O. & Muccioli, G. G. 2016. Oxysterols: From cholesterol metabolites to key mediators. *Progress in Lipid Research*, **64**, pp. 152-169.
- National Institute for health Care and Excellence (NICE). 2022. *Pembrolizumab for Neoadjuvant and adjuvant treatment of triple-negative early or locally advanced breast cancer*. Available: <https://www.nice.org.uk/guidance/ta851/chapter/1-Recommendations>. [Accessed 20th February 2024].
- Nazih, H. and Bard, J.M., 2020. Cholesterol, oxysterols and LXRs in breast cancer pathophysiology. *International Journal of Molecular sciences*, **21**(4), p.1356.
- Nelson, E.R., 2018. The significance of cholesterol and its metabolite, 27-hydroxycholesterol in breast cancer. *Molecular and Cellular Endocrinology*, **466**, pp.73-80.
- Papadimitriou, M., Liakouli, Z. & Papadimitriou, C. 2021. The role of immune checkpoint inhibitors in triple-negative breast cancer: Recent developments and future perspectives. *Journal of Cancer Metastasis and Treatment*, **7**, p. 63.
- Said, S. S. & Ibrahim, W. N. 2023. Cancer resistance to immunotherapy: Comprehensive insights with future perspectives. *Pharmaceutics*, **15** (4), p. 1143.
- Spann, N. J. & Glass, C. K. 2013. Sterols and oxysterols in immune cell function. *Nature Immunology*, **14** (9), pp. 893-900.
- Wang, B. and Tontonoz, P., 2018. Liver X receptors in lipid signaling and membrane homeostasis. *Nature Reviews Endocrinology*, **14** (8), pp.452-463.
- Wang, L. X., Mei, Z. Y., Zhou, J. H., Yao, Y. S., Li, Y. H., Xu, Y. H., Li, J. X., Gao, X. N., Zhou, M. H., Jiang, M. M., Gao, L., Ding, Y., Lu, X. C., Shi, J. L., Luo, X. F., Wang, J., Wang, L. L., Qu, C., Bai, X. F. & Yu, L. 2013. Low dose decitabine treatment induces cd80 expression in cancer cells and stimulates tumor specific cytotoxic t lymphocyte responses. *PLOS ONE*, **8** (5), article no: 62924.
- Wouters, E., De Wit, N. M., Vanmol, J., Van Der Pol, S. M. A., Van Het Hof, B., Sommer, D., Loix, M., Geerts, D., Gustafsson, J. A., Steffensen, K. R., Vanmierlo, T., Bogie, J. F. J., Hendriks, J. J. A. & De Vries, H. E. 2019. Liver x receptor alpha is important in maintaining blood-brain barrier function. *Front Immunology*, **10**, pp. 1811.
- Xu, P., Li, D., Tang, X., Bao, X., Huang, J., Tang, Y., Yang, Y., Xu, H. & Fan, X. 2013. Lxr agonists: New potential therapeutic drug for neurodegenerative diseases. *Molecular Neurobiology*, **48** (3), pp. 715-728.
- Yan, C., Zheng, L., Jiang, S., Yang, H., Guo, J., Jiang, L.-Y., Li, T., Zhang, H., Bai, Y., Lou, Y., Zhang, Q., Liang, T., Schamel, W., Wang, H., Yang, W., Wang, G., Zhu, Z.-J., Song, B.-L. & Xu, C. 2023. Exhaustion-associated cholesterol deficiency dampens the cytotoxic arm of antitumor immunity. *Cancer Cell*, **41** (7), 1276-1293.
- Yi, M., Niu, M., Xu, L., Luo, S. & Wu, K. 2021. Regulation of pd-11 expression in the tumor microenvironment. *Journal of Hematology & Oncology*, **14** (1), p. 10.
- Zhang, Q., Gao, C., Shao, J., Zhang, S., Wang, P. & Wang, Z. 2022. Molecular and clinical characterization of cd80 expression via large-scale analysis in breast cancer. *Front Pharmacology*, **13**, article no: 869877.