Myc linked to dysregulation of cholesterol transport and storage in non-small cell lung cancer

Running title: Cholesterol homeostasis in lung tumours

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Abstract

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related deaths. Whilst mutations in Kras and over-expression of Myc are commonly found in patients, the role of altered lipid metabolism in lung cancer and its interplay with oncogenic Myc is poorly understood. Here we use a transgenic mouse model of Kras-driven lung adenocarcinoma with reversible activation of Myc, in combination with surface analysis lipid profiling of lung tumours and transcriptomics, to study the effect of Myc activity on cholesterol homeostasis. Our findings reveal that activation of Myc leads to the accumulation of cholesteryl esters (CE), stored in lipid droplets. Subsequent Myc deactivation leads to further increases in CE, in contrast to tumours in which Myc was never activated. Gene expression analysis linked cholesterol transport and storage pathways to Myc activity. Our results suggest that increased Myc activity is associated with increased cholesterol influx, reduced efflux and accumulation of CE-rich lipid droplets in lung tumours. Targeting cholesterol homeostasis is proposed as a promising avenue to explore for novel treatments of lung cancer, with diagnostic and stratification potential in human NSCLC.

Key words: cholesteryl ester; liquid extraction surface analysis; mass spectrometry; lipid metabolism; adenocarcinoma
Introduction

Lung cancer is the leading cause of cancer-related mortality, with non-small cell lung cancer (NSCLC) the most common sub-type (1). Mutations in *Kras* are found in over 30% of NSCLC cases, while the RTK/RAS/RAF pathway is activated in 76% of cases (2). In addition, *Myc* is frequently over-expressed, focal amplifications occurring in over 30% of lung adenocarcinomas (3). *Myc* is a transcription factor with numerous functions in healthy cellular processes, including regulation of cell cycle and cell growth. Deregulation of *Myc* leads to uncontrolled cell proliferation in many tissues and is implicated in tumorigenesis of some, perhaps all, tumours (4, 5). Inhibition of *Myc* is therefore of interest as a cancer treatment (6), however its complex regulation of transcription factors and genes are not fully understood.

Enhanced lipid synthesis is recognised as a signature of cancer (7-9). Excess lipids are stored in lipid droplets, providing a source of energy for rapidly dividing cancer cells and structural components for building new membranes. Furthermore, lipids are increasingly being recognised as critical to signalling pathways in cancer. Cholesterol is a particularly important lipid messenger for signal transduction, control of membrane fluidity and regulation of the innate immune response (10-12), whilst cholesteryl ester (CE) accumulation has been implicated in prostate cancer aggressiveness (13). The link between oncogenic *Myc* and lipid metabolism is relatively underexplored. Previously, we showed that dysregulated *Myc* modulated the production of eicosanoids, critical for proliferation and cell survival, in lung adenocarcinoma (14). Other recent studies link *Myc* to increased lipogenesis in tumours (15, 16), whilst switching from a high to low-fat diet attenuates the *Myc* transcriptional program in prostate cancer (17). It is evident that lipids and their interaction with oncogenes, such as *Myc*, play a complex and elegant role in tumorigenesis and offer an underexploited therapeutic avenue.

Here we use a transgenic mouse model of Kras-driven lung adenocarcinoma, with reversible activation of *Myc*. We explore the effect of *Myc* activity on cholesterol homeostasis in lung tumours by integrating surface analysis mass spectrometry-based lipidomics, transcriptomics and quantitative
gene expression analysis. Our results reveal that increased Myc activity favours cholesterol influx over efflux in tumours and leads to accumulation of CEs, stored in lipid droplets. Deactivation of Myc triggers the clearance of cholesterol through increasing efflux, decreasing influx and further increasing cholesterol esterification. No accumulation of cholesterol occurred in tumours in which Myc was never activated. These findings provide new insights into the role of oncogenic Myc and dysregulation of cholesterol homeostasis in lung cancer.

Materials and Methods

Tumour models

Mice were maintained on regular diet in a pathogen-free facility on a 12 hr light/dark cycle with continuous access to food and water. Lung tumours were generated in adult Kras\textsuperscript{G12D} or LSL-Kras\textsuperscript{G12D}, R26\textsuperscript{LSL-CAG-c-MycER/LSL-CAG-c-MycER} (R26\textsuperscript{LSL-CMER}) mice as previously described (5). Briefly, mice were anesthetised (isoflurane) and intranasal instillation of adenoCre virus (7x10\textsuperscript{8} – 3.5x10\textsuperscript{9} plaque-forming units, University of Iowa Viral Vector Core) was performed by placing virus droplets on the nose of the mouse. This resulted in expression of cre-recombinase removing the stop element sporadically in lung epithelium of both the Kras and Rosa26 alleles. Consequently, these lung epithelial cells express oncogenic Kras\textsuperscript{G12D}, driving the formation of lung adenocarcinomas. Tamoxifen added to the diet of LSL-Kras\textsuperscript{G12D}, R26\textsuperscript{LSL-CMER} mice results in activation of c-MycER\textsuperscript{T2} protein specifically within the Cre-deleted tumour tissues (18). Subsequent removal of tamoxifen results in rapid deactivation of Myc\textsuperscript{ER}.

Twelve adeno-Cre-infected LSL-Kras\textsuperscript{G12D}, R26\textsuperscript{LSL-CMER} were maintained on a tamoxifen-containing diet for one month. Tamoxifen was removed from the diet for 24 (n=3) or 72 (n=3) hours to deactivate Myc before culling (“Myc inactive”). The remaining mice were continuously fed a tamoxifen-containing diet (“Myc activated”). LSL-Kras\textsuperscript{G12D} were also fed a tamoxifen-containing diet, however
Myc expression is unaffected (“Kras only”, n=4). Lung samples were collected, snap-frozen in liquid nitrogen and stored at – 80 °C. All animal procedures were approved by the UK Home Office and the University of Cambridge.

Oil red staining for neutral lipids was performed according to an established protocol (19). Fresh frozen unfixed tissue sections were incubated with working oil red O solution (Sigma Aldrich) for up to 10 min, quickly rinsed in tap water following by counterstaining with Mayer’s Haematoxylin (VWR) and prolonged wash in tap water. Slides were then mounted with glycerol and imaged immediately.

Microarray

Lung cells from adeno-cre infected \( R_{26}^{\text{LSL-CMER} \; \text{and LSL-Kras}^{G12D}} ; \ R_{26}^{\text{LSL-CMER}} \) mice were isolated as previously described (20), 14 days post treatment with \( (\text{LSL-Kras}^{G12D} ; \ R_{26}^{\text{LSL-CMER}}) \) or without \( (R_{26}^{\text{LSL-CMER}}) \) tamoxifen. Single cells were flow sorted for positivity of GFP directly into Trizol (Thermo Fisher Scientific). To increase RNA yield, cells from two mice of the same genotype and treatment were combined, resulting in two biological replicates for each condition. RNA was extracted with Trizol following the manufacturer’s protocol. RNA was amplified using the Ovation Pico WTA v2 kit (NuGEN Technologies, Leek, Netherlands) and subsequently labelled using the BiotinIL kit (NuGEN). RNA was assessed for concentration and quality using a SpectroStar (BMG Labtech, Aylesbury, UK) and a Bioanalyser (Agilent Technologies, Cheadle, UK). The concentration, purity and integrity of the resulting cDNA was measured using the Nanodrop ND-1000 (Thermo Fisher Scientific) and by Bioanalyser. cDNA was hybridised to the MouseWG-6 v2 BeadChip overnight followed by washing, staining and scanning using the Bead Array Reader (Illumina, Chesterford, UK).

Raw data was loaded into R using the lumi package (21) and divided into subsets according to the groups being compared. Subsets were then filtered to remove any non-expressed probes using the detection \( P \)-value from Illumina. Across all samples, probes for which the intensity values were not
statistically significantly different \( (p > 0.01) \) from the negative controls were removed (~25,000 probes remained). Following filtering, the data was transformed using the Variance Stabilization Transformation (22) from lumi and then normalised to remove technical variation between arrays using quantile normalisation. Comparisons were performed using the limma package (23) with results corrected for multiple testing using False Discovery Rate (FDR) correction.

Pathway enrichment analysis was performed on differentially expressed genes \( (P < 0.05, \ -1.5 > \text{fold change} > 1.5) \) using GSEA (24) to identify over-represented gene ontology (GO) terms for Biological Process. Pathways were ranked according to their normalised enrichment scores (NES) and FDR \( (q) \).

Network analysis was performed using Cytoscape (25). Transcription factor analysis was carried out with TRRUST database in the EnrichR platform (26, 27), to identify potential upstream regulators influencing expression of dysregulated genes. These were ranked according to \( P \) value and predicted activation status.

**Lipid profiling**

Samples were embedded in Tissue-Tek OCT\textsuperscript{TM} and 12 \( \mu \text{m} \) tissue sections prepared on glass microscope slides using a cryostat. Sections were dried in a vacuum desiccator for 30 minutes prior to analysis. Adjacent sections were stained with haematoxylin and eosin (H&E). Lipids for mass spectrometry (MS) were extracted using liquid extraction surface analysis (LESA) at user-defined points \( (1 \text{ mm}^2) \) across the tissue surface by dispensing 0.8 \( \mu \text{L} \ 1:2:4 \text{ chloroform: methanol: isopropanol with 10 mM ammonium formate and incubating for 2.5 seconds. Analytes extracted from the surface were directly infused by a Triversa Nanomate (Advion BioSciences, UK), with capillary voltage 1.2 kV, capillary temperature 200 °C, 0.3 gas flow, for 1 minute into an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). Mass spectra were acquired in positive ion mode, from 200 to 1,000 \text{ m/z} \) at 60,000 mass resolution.
Data were converted to mzML format and features extracted using an in-house R script. Lipid annotation was performed by accurate mass using the LipidMaps database (<5 ppm) (28). Metaboanalyst software was used to further analyse the data (29). Features with >50% missing values were removed and remaining missing values were imputed using k-nearest neighbours (KNN) method (30). The features with the lowest 10% mean signal intensity were removed; data were then normalised to the total ion count, mean-centred and scaled by dividing by the standard deviation of each variable. Principal components analysis identified outliers which were subsequently excluded. Volcano plot represents a combination of fold change and unpaired t-test P values for individual variables. Heatmap was generated in Metaboanalyst using the Ward clustering method, with Euclidean distance measure. Features were centred and scaled using the “Autoscale” function, and the top 25 hits based on their significance (ANOVA) calculated.

**Transcript quantification**

Total RNA was purified from tumour-containing lung tissue using RNeasy Mini Kit (Qiagen). Approximately 20 mg of each tissue sample was lysed and homogenized in Trizol (1 mL) using a TissueLyzer (Qiagen). The samples were centrifuged at 12,000 g for 15 minutes after the addition of chloroform (200 µL) and the RNA-containing aqueous phase combined with 1 volume of 70% ethanol. Samples were loaded on spin columns and the procedure was performed according to the manufacturer’s guidelines. Purified RNA concentration was quantified (260 nm) using a NanoDrop 100 (Thermo Fisher Scientific). Genomic DNA contamination was eliminated using RT² First Strand Kit (Qiagen) and complimentary DNA was produced using an RT² First Strand Kit (Qiagen). Relative abundance of transcripts of interest was assessed using quantitative polymerase chain reaction in RT² SYBRgreen Mastermix (Qiagen) with a StepOnePlus detection system (Applied Biosciences, Warrington, UK). RT² primer assays for mouse *Rn18s* (endogenous control); *Ldlr, Olr1, Scara1, Hmgcr, Soat1, Apoc1, Abca1, Abca3, Abcg1, Srebfl, Srebf2, Ppara, Pparg, Nr1h3* were obtained from Qiagen.
Thermocycler (PTC-200, MJ Research) parameters were as follows: incubation, 95 °C for 10 minutes; elongation, 95 °C for 15 seconds; and cooling, 60 °C for 1 minute. Elongation and cooling were performed in 40 cycles. Expression levels were normalized to endogenous controls using the ΔΔCT method and fold changes reported relative to the “Kras only” group. Statistical significance was determined using one-way ANOVA (α = 0.05) and adjusted for multiple comparisons using the Holm-Sidak method.

Results

Enrichment of lipid-related pathways in lung cells expressing high Myc and oncogenic Kras

First, we determined pathways modulated by Myc and Kras by studying the gene expression of lung cells from tamoxifen-treated LSL-KrasG12D; R26LSL-CMER mice compared to untreated R26LSL-CMER mice. Using FACS, we sorted lung cells based on GFP positivity from the R26LSL-CMER allele. We compared the gene expression profiles for lung cells expressing both active (tamoxifen treated) MycER and oncogenic KrasG12D with those that had inactive (untreated) MycER. Pathway analysis of differentially expressed genes (P < 0.05; -1.5 > fold change > 1.5) revealed that biological processes relating to cell cycle, DNA replication and repair, ribosome biogenesis and RNA processing were significantly over-represented (P < 0.05; NES >1) in the MycER/KrasG12D expressing cells as expected (Supplemental Table S1, Figure 1A). Interestingly, pathways pertaining to immune signalling, inflammation, lipid metabolism and transport were also significantly enriched (P < 0.05; NES < 1). Of the lipid-related pathways, key terms included: lipid metabolic process, lipid localisation, cellular response to lipid, steroid metabolic process, lipid catabolic process and fatty acid metabolic process (Supplemental Table S1, Figure 1B). Transcription factor analysis identified several upstream regulators significantly (P < 0.05) associated with the input gene expression datasets (Figure 1C; full list in Supplemental Table S2). As expected, there was predicted activation of upstream regulators associated with cancer (MYC, TP53), regulation
of cell cycle and division (MYC, MYCN, E2F1, E2F4), and response to hypoxia (HIF1A). Predicted inhibition of key transcriptional regulators of lipid metabolism were found. These included the peroxisome proliferator-activated receptors (PPARA, PPARD, PPARG), retinoic acid receptor (RARA) and sterol regulatory element-binding proteins (SREBP1, SREBP2; regulators of cholesterol biosynthesis and uptake).

**In situ extraction of lipids from lung tumours**

Given the changes to lipid metabolic pathways, we carried out lipid profiling on $LSL-Kras^{G12D}$, $R26^{CME}$ and $LSL-Kras^{G12D}$ mice. Both sets of mice express oncogenic $Kras^{G12D}$, which drives the formation of lung adenocarcinomas. Using tamoxifen-inducible activation of MycER, lung tumours in $LSL-Kras^{G12D}$, $R26^{CME}$ mice expressed high levels of MycER in its active or inactive form. Myc expression in lung tumours from $LSL-Kras^{G12D}$ mice was unmodified (“Kras only”). LESA-MS was used to extract lipids from the surfaces of tissue sections. Sampling locations could be precisely defined and were guided by adjacent H&E stained tissue sections. LESA-MS involves a liquid microjunction being formed when a droplet of solvent makes contact with the tissue surface (31, 32). Lipids were dissolved in the solvent droplet, and directly infused into a high-resolution mass spectrometer. Over five hundred features were detected and annotated by their accurate mass and database searching. These covered multiple lipid classes, including phosphatidylcholines, phosphatidylethanolamines, triacylglycerides (TAG), plasmalogens, diacylglycerides, lysophosphatidylcholines, lysophosphatidylethanolamines, sphingomyelins, ceramides and CEs.

A comparison of global lipid profiles for Kras and Myc tumours suggested a relative increase in CEs and plasmalogens when Myc was activated (**Figure 2A, Supplemental Figure S1**) compared to the Kras control. In addition, there was a relative decrease in DAGs and TAGs (particularly those containing shorter fatty acyl chains, **Supplemental Figure S1**). Examining the LESA-MS spectra, a clear increase in the peak at $m/z$ 668.6348 was observed when Myc was activated compared to “Kras only” tumours.
This peak was assigned as the ammonium adduct of CE(18:1). We next carried out an in-depth analysis of all the lipid features across Myc activated/deactivated tumours and their paired non-tumour tissue, and Kras control tumours. Of the top 25 features, almost half corresponded to CEs (Figure 2C, Supplemental Table S3). These were increased in tumours where Myc was activated compared to Kras control, and further increased following Myc deactivation. The observed changes were specific to tumours.

Cholesteryl esters accumulate in lung tumours with high Myc

Intra-tumour CE accumulation has been implicated in prostate, breast and pancreatic cancers (13, 33, 34). However, it has not been reported in NSCLC, and neither has it been linked to Myc. Our LESA-MS results point to an increase in CE upon Myc activation, and a further increase following Myc deactivation (Figure 3A). This was confirmed by LC-MS/MS analysis of bulk tissue extracts (Supplemental Figure S2). Analysis of the CE fatty acyl chain compositions showed that CEs containing a monounsaturated fatty acid chain (MUFA) were disproportionately increased, compared to their saturated and polyunsaturated counterparts (Figure 3A).

Accumulation of free cholesterol can induce an apoptotic response, whilst the biologically-inert esterified form is preferred for storage. Measurement of free cholesterol with LESA-MS has limitations: since CEs fragment readily during the ionisation process, it is difficult to reliably distinguish free cholesterol from CE fragments. We mitigated this by using a low capillary temperature, tuning the source parameters to minimise CE fragmentation. Furthermore, isobaric species of cholesterol cannot be ruled out and thus we refer to the species measured at m/z 369.3519 as “cholesterol-like backbone” (CB). We calculated the ratio of bound (CE) to free (CB) cholesterol in tumours, finding an increase on Myc activation compared to Kras, with further increase over time with Myc deactivation (Figure 3B). These changes were not recapitulated in the corresponding non-tumour tissue. This
highlights that dysregulation of cholesterol storage is limited to the tumour environment and
demonstrates the importance of spatially-resolved metabolic analysis over bulk tissue measurements.

Cholesterol and CEs may be incorporated into lipid membranes or stored in lipid droplets. In order to
establish whether there was any change in lipid droplet content with Myc, we carried out oil red O
staining for neutral lipids. There was significant lipid droplet staining in the adjacent non-tumour
tissue, which did not vary noticeably between the different groups (Figure 3C). These lipid droplets
may arise from presence of pulmonary surfactant, of which cholesterol is a major component (35). In
contrast, lipid droplet staining in the tumour region was low in the “Kras only” group, and substantially
greater in tumours with high Myc activity (Figure 3C). Consistent with the MS results, lipid droplet
staining was further increased following the deactivation of Myc. Since lipid droplets are primarily
composed of TAGs and CEs, we calculated the ratio of CE to TAG, to test whether the increased CE
content was the result of increased storage of neutral lipids more generally (Figure 3D). Interestingly,
this ratio was substantially increased in tumours with Myc activation compared to Kras and to paired
non-tumour tissue, suggesting that changes to cholesterol/CE are not purely the result of increased
lipid storage per se.

**Cholesterol transport and storage linked to Myc activity**

Transcriptomics and lipidomics experiments point to alterations in cholesterol homeostasis in the lung
with Myc activity. To investigate this further, we extracted lung RNA from the different groups and
carried out transcript quantification. We specifically targeted genes involved in cholesterol influx and
efflux, as well as those involved in cholesterol synthesis and esterification. No significant differences
were noted in expression for *Hmgcr*, the rate-determining step in cholesterol synthesis (Figure 4). On
the other hand, expression for genes governing cholesterol influx (*Ldlr*, *Olr1* and *Scara1*) increased
with Myc activation and decreased with time after Myc deactivation (Figure 4). The reverse trend was
established for those genes whose main role is cholesterol efflux out of the cell. These transcripts,
which included Abca1, Abca3, and Abcg1, decreased with Myc activity (Figure 4). Expression of Soat1, responsible for esterification of cholesterol, increased with time following deactivation, in-line with the increased CE species noted by lipid profiling (Figure 4). APOC1, which plays a major role in lipoprotein metabolism and cholesterol transport, has been proposed as diagnostic/prognostic marker in lung cancer (36). Here, gene expression for Apoc1 was markedly increased by Myc activation (Figure 4).

Finally, we examined gene expression for nuclear receptors and transcription factors considered important for regulating cholesterol homeostasis. Srebf1 expression decreased when Myc was activated (Figure 4), whilst no link to Myc was found for Srebf2 or PPAR (Ppara, Pparg) gene expression (Supplemental Figure S3). Nr1h3, which codes for the alpha subunit of the liver X receptor (LXR), was also decreased when Myc was activated, increasing following Myc deactivation (Figure 4). LXRs form heterodimers with the retinoid X receptor (RXR) and PPARs to regulate lipid homeostasis, in particular acting as cholesterol sensors, promoting transcription of genes that protect from cholesterol overload (37). Its decrease with Myc activation is consistent with the downregulated transcription of cholesterol efflux transporters and the predicted suppression of upstream regulator PPAR (Figure 1C).

To assess whether changes to cholesterol transport and storage were recapitulated in human NSCLC, we mined two datasets from the GEO repository (Figure 5A). Tumour and non-tumour samples from a population of non-smoking women with NSCLC were compared in GSE19804 (38, 39). We found significantly increased expression for SOAT1, and significantly decreased expression of the ABC transporters ABCA1, ABCA3, ABCG1. Interestingly, in a separate study (40) comparing the two main sub-types of NSCLC, expression of SOAT1 was increased in adenocarcinoma compared to squamous cell lung cancer (GDS3627; Figure 5B).

Lastly, survival data representing months of disease-specific survival were downloaded from the CBioPortal (41) and applied to the lung adenocarcinoma data set (N = 514) from the TCGA PanCancer atlas (42). Based on the mRNA expression z-score threshold relative to all samples, patients were
classified into two expression groups per gene of interest. The correlation between expression level and patient survival were examined using Kaplan-Meier survival curves. Interestingly, we found that low ABCA3 expression was associated with a significantly lower 5-year survival rate (log rank $P$ value = 0.0008; Figure 5C).

Overall, we have shown that pathways regulating cholesterol transport and storage are modulated at the gene expression level in lung tumours, are linked to oncogenic Myc, and have potential as diagnostic markers or patient stratification in human NSCLC.

**Discussion**

Through a combination of transcriptomics, lipidomics and quantitative gene expression analysis, we show that Myc activity in lung tumours is linked to a disruption in cholesterol homeostasis. Transcriptomics and pathway analysis revealed dysregulation of lipid metabolism in MycER/ KRasG12D-positive cells, compared to control. By extracting lipids directly from tumour surfaces, we found a dramatic increase in CE s when Myc was activated, compared to when Myc was unmodified. Finally, we showed that the expression of genes relating to cholesterol influx, efflux and esterification were linked to Myc activity.

There was a striking correlation between the expression of ABC transporters and Myc activity. These transporters are expressed in both alveolar epithelial cells and macrophages and regulate cholesterol clearance out the cell to circulation via high density lipoproteins (HDL). A decrease in their expression leads to increased cholesterol loading and inflammation in cells (43-45). It is therefore plausible that accumulation of cholesterol in high Myc tumours is a result of the predicted deactivation of PPAR/LXR which promotes cholesterol efflux through the induction of transporter expression.

Interpretation of the link between Myc and cholesterol uptake is less clear. Increased expression for several genes pertaining to cholesterol uptake was found in tumours with high Myc activity. On the
other hand, SREBP a key regulator of cholesterol uptake and biosynthesis, was predicted to be deactivated in the upstream regulator analysis of oncogenic MYC/KRAS lung cells. One possible explanation is that SREBP is suppressed by the high levels of cholesterol in tumour cells, which could also account for the corresponding decrease of short-chain TAGs in MYC-activated tumours.

Taken together our data shows that upon activation of Myc, there is a net accumulation of cholesterol, resulting from dysregulation of influx/efflux pathways. Esterification of free cholesterol and storage in lipid droplets avoids cholesterol-induced apoptotic death of cancer cells. When Myc is subsequently deactivated, there is a return towards homeostasis. Influx of cholesterol decreases, efflux increases, and to process accumulated cholesterol, further increases to cholesterol esterification occur (Figure 6).

The accumulation of non-adipocyte lipid droplets more generally has been associated with aggressive cancers and linked to inflammation, mitochondrial dysfunction and oxidative stress (12, 46, 47). These lipid droplets maintain a store of excess fatty acids packaged into TAGs and CEs. Hydrolysis of these neutral lipid-containing droplets releases fatty acids and free cholesterol which may be then used by cancer cells for altering membrane fluidity, fuelling cellular proliferation and downstream cellular signalling (48, 49). A recent study has shown that Myc and Ras cooperate in NSCLC to reprogram inflammation and immune response. Deregulation of Myc in lung epithelia triggers the release of specific cytokines, which recruit macrophages. These macrophages in turn stimulate angiogenesis, inflammation and clearance of specific immune cells (50). Interestingly, cholesterol imbalance has been implicated as a contributor to immune dysfunction, for instance cholesterol loading in macrophages promotes Toll Like Receptor (TLR) signalling and activation of the inflammasome (11). Undoubtedly, dysregulation of cholesterol homeostasis will have an important effect on many cellular functions, including inflammatory and immune responses. The measurement of oxysterols may provide a missing link to further understand these complex mechanisms in future studies. These
understudied bioactive metabolites of cholesterol act as ligands for LXR, suppress SREBP (51) and have been linked to modulation of immune response and cancer (52).

This study furthermore suggests new potential drug targets for lung cancer therapy. Treatment with statins, which reduce serum cholesterol levels and pro-inflammatory signalling, are already being explored in preclinical trials for a variety of cancers. Statins have also been demonstrated to benefit a variety of pulmonary diseases in which inflammation plays a role, as well as improving survival rates for patients with stage IV NSCLC (10, 53, 54). Another intriguing prospect for NSCLC treatment is by the inhibition of SOAT1. This is currently being explored to treat aggressive cases of prostate cancer, whilst studies have shown SOAT1 inhibition to prolong survival, suppress tumour growth and metastasis in mouse models of different cancers (33, 55, 56). Lastly, since Western diets are typically high in cholesterol, with obesity a risk factor for developing a variety of cancers, dietary intervention in combination with drug therapy may be a fruitful area for future clinical trials (57).

To conclude, a murine model of lung adenocarcinoma was used to link changes in cholesterol transport and storage to Myc activity, pointing to dysregulation of cholesterol homeostasis in NSCLC. Accumulation of cholesterol may play a role in downstream signalling or used to fuel cellular proliferation. Targeting cholesterol metabolism is therefore proposed as a promising avenue to explore novel treatments of lung cancer.

Acknowledgements

Microarray experiments were performed at Cambridge Genomic Services, University of Cambridge. Oil red O staining was performed by the Wellcome-MRC Cambridge Stem Cell Institute. The Kaplan-Meier survival estimate is based upon data generated by the TCGA Research Network: [https://www.cancer.gov/tcga](https://www.cancer.gov/tcga). Z.H. is funded by the Royal Society of Chemistry (Analytical Chemistry Trust Fund / CAMS-UK partnership) and the Royal Society. J.L.G. and Z.H. are supported by the Imperial
Biomedical Research Centre, NIHR and the Medical Research Council, UK (MC UP A90 1006). G.I.E, C.H.W and D.L.B are funded by Cancer Research UK (Programme Grant A12077).

Conflict of interest: The authors declare no conflict of interest.

Data availability statement: All data can be found in the manuscript and supplementary material. Raw microarray data has been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-9131.

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Figure 1. Pathway analysis for differentially expressed genes in Kras\textsuperscript{G12D}/Myc\textsuperscript{ER} positive lung cells.

Gene expression was compared (N=2) between GFP-positive FACs sorted cells from LSL-Kras\textsuperscript{G12D}; R26\textsuperscript{LSL-CMER} mice treated with tamoxifen and untreated R26\textsuperscript{LSL-CMER} mice (each biological replicate represents combined cells from two mice). Gene set enrichment based on gene ontology (GO; Biological Process), with subsequent network analysis (P < 0.05; q < 0.1; A). Size of circles (nodes) reflect the P value. Thickness of lines (edges) reflect degree of overlap. Gene sets are colour coded according to their broad function. Selected enriched gene ontologies are shown (q < 0.1; B), with the full pathway list in Supplemental Table S1 (NES = normalised enrichment score). Activation status of upstream regulators (full list in Supplemental Table S2). Intensity of colour scale reflects magnitude of \(-\log_{10}(P)\); red and blue colours show predicted activated and deactivated transcription factors, respectively (C).
Figure 2. LESA-MS analysis of lung tumours. Liquid extraction surface analysis (LESA) was used to extract lipids directly from tissue surfaces at user-defined points. Direct comparison of lipid profiles for “Kras only” and “Myc activated” tumours (4 biological replicates per group; A). Representative spectra from “Kras only” and “Myc activated” lung tumours (B). Highlighted peak is the feature corresponding to cholesteryl oleate, CE(18:1) at m/z 668.6348. Heatmap analysis of lipid features across the tumour groups (“Kras only” = 4, “Myc activated” = 4, “Myc deactivated” = 6) and paired non-tumour (NT) tissue from the Myc activated/deactivated groups. The top 25 features (based on ANOVA) are shown (C).
Figure 3. Cholesteryl esters differentiate lung tumours with varying Myc activity. Comparison of individual CE species across the tumour groups revealed that activation of Myc resulted in a large global increase in the abundance of CEs and free cholesterol, with further increases upon Myc deactivation. The inset shows changes in CEs across different fatty acid compositions (A). The bound to free cholesterol ratio (CE/CB) was calculated in tumours, and in the non-tumour tissue from the “Myc activated” group (B). Oil red O staining of neutral lipids was performed. Representative images (scale bar shows 50 µm) are shown for each group (C). The CE/TAG ratio was calculated in tumours, and in the “Myc activated” non-tumour tissue (D). Data show mean +/- SEM; * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 4. Cholesterol transport and storage linked to Myc activity. Transcript quantification in lung tissue for genes related to cholesterol synthesis (Hmgcr), influx (Ldlr, Olr1, Scara1), efflux (Abca1, Abca3, Abcg1), esterification (Soat1), transport (Apoc1) and regulation (Nr1h3, Srebpf1). Data show mean +/- SEM; P values were calculated using ANOVA (* P < 0.05, ** P < 0.01, *** P < 0.001).
Figure 5. Dysregulation of cholesterol efflux and storage in human NSCLC. Gene expression for ABC transporters was decreased and SOAT1 increased in lung tumours from a population (N = 60) of non-smokers with NSCLC (compared to paired non-tumour tissue; GSE19804; A). SOAT1 expression was also increased in lung adenocarcinoma (AdC; N = 40) compared to squamous cell carcinoma (SCC, N = 18; GDS3627; B). Kaplan-Meier survival estimates for two expression groups of ABCA3 mRNA in lung adenocarcinoma patients (N = 514, TCGA; C).
Figure 6. Proposed mechanism for control of cholesterol transport by Myc. The activation of Myc in Kras-driven lung tumours results in increased cholesterol influx and reduced cholesterol efflux, whilst synthesis of cholesterol appears largely unaffected at gene expression level. Subsequent deactivation of Myc results in decreased cholesterol influx and increased efflux. Esterification prevents the accumulation of excess free cholesterol in the cell and is increased with Myc deactivation.