Stratification of Respiratory Disease through Early Diagnostic Sampling

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Summary

Both lung cancer and Coronavirus Disease 2019 (COVID-19) present with a wide range of prognoses and eventual outcomes. In both conditions early diagnosis and commencement of treatment (where appropriate) can improve survival. Given the spectrums of disease seen in lung cancer and COVID-19, stratification of patients also aids in the selection of the most appropriate individual management options to further optimise patient outcome. The overall aim of this thesis is to examine the stratification of respiratory disease through early diagnostic sampling.

Chapter 2 describes the design and delivery of the multi-centre, prospective STRATIFY study (Staging by Thoracoscopy in potentially radically treatable Lung Cancer associated with Minimal Pleural Effusion). It has been established from retrospective data that those presenting with early stage, otherwise potentially radically treatable non-small cell lung cancer (NSCLC) and minimal pleural effusion do significantly worse in terms of survival than those without such effusions. Based on this previous retrospective data it has been hypothesised that this difference in survival is due to the presence of occult pleural metastases (OPM) not otherwise detected in the routine diagnostic work up of those with suspected NSCLC and minimal pleural effusion. STRATIFY was therefore designed as the first prospective, multicentre, observational study with the primary aim of determining the true prevalence of OPM in this cohort of patients through the addition of thoracoscopy to the diagnostic pathway.

Unfortunately, due to a multitude of issues many of which stemmed from the COVID-19 pandemic, recruitment to STRATIFY was slower than expected and therefore the decision to close the trial to recruitment was taken in May 2024. Primary outcome data including the prevalence of OPM and important safety data for the 27 recruited patients are however included here.

Patients with lung cancer (and many other common malignancies) often present with large pleural effusions which go on to be proven malignant through simple aspiration. The diagnostic yield of pleural fluid cytology is well documented at 60% on average but varies considerably by tumour type. The yield of predictive markers from effusion cytology, which are now mandated in the diagnostic work up of lung

and breast adenocarcinoma, is less well established. Chapter 3 of this thesis therefore aimed to assess the utility of pleural fluid cytology for the detection of predictive markers in lung and breast adenocarcinoma in a real-world setting. This multicentre, retrospective cohort study found that the full panel of predictive marker (PM) testing required by contemporaneous international cancer treatment guidelines was returned in only 20% of cases where these were requested on pleural fluid cytology. Performance differed by individual marker with yields for many markers improving over the time period of the study. No clinico-radiological factors were significantly associated with PM testing yield to guide pre-aspiration likelihood of success.

Perhaps even to a greater extent than lung cancer, outcomes from COVID-19 are markedly heterogeneous with some patients complaining of little to no symptoms while others go on to develop potentially fatal pneumonitis. Although risk factors for poor prognosis are well documented, less is understood about the individual immune response and how these immunological events affect disease outcome. In chapter 4 individual immune response at the time of COVID-19 diagnosis and how this relates to disease severity was examined. In keeping with previous work, severe COVID-19 (as defined by the need for supplemental oxygen) was associated with obesity and hypertension ($p= 0.0456$, $p= 0.0071$ respectively) in this cohort. Flow cytometry of baseline serum samples revealed lower activation (phosphorylation) of STAT 5 in response to stimulation across almost all immune cell subpopulations in those with severe disease. Interrogation of potential mechanisms linking metabolic syndrome and the altered STAT5 signalling observed are ongoing by collaborators at the time of writing.

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Acknowledgments

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A heart felt thank you goes to every patient who so selflessly took part in the research projects presented here without whom none of this work would have been possible.

And finally, to my husband Brendan. We hadn’t even met when I embarked on this research but he has been steadfast in his support and has kept me laughing every day and for that I will be forever grateful.

Declaration

The work presented in this thesis was undertaken during my time as a Clinical Research Fellow at the Glasgow Pleural Disease Unit, Queen Elizabeth University Hospital, Glasgow and at the School of Cancer Sciences, College of Medical and Veterinary Life Sciences, at the University of Glasgow. I was supervised by Professor Kevin Blyth and Dr Selina Tsim.

All of the work reported in this thesis was undertaken by me, with the assistance of a number of colleagues who have been formally acknowledged in the previous section and in each applicable chapter.

The work contained here has not been submitted for any other degree at the University of Glasgow or any other educational institution. The work contained in this thesis has however been published or written in preparation for submission and therefore this thesis is presented in alternative format.

The writing of this thesis constitutes my own work, prepared solely by me.

Signed ……

Jenny Ferguson, October 2024

List of Abbreviations

ΔMFI	Delta mean fluorescence intensity
ACE2	Angiotensin-converting enzyme 2
AE	Adverse event
ALK	Anaplastic lymphoma kinase
ARDS	Acute respiratory distress syndrome
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer gene
CBL	E3 ubiquitin-protein ligase
CD	Cluster of differentiation
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CRUK	Cancer research United Kingdom
CSO	Chief Scientist Office
CT	Computed tomography
CtDNA	Circulating tumour DNA
CTPA	Computed tomography pulmonary angiography
CTU	Clinical trials unit
CXR	Chest X-ray
DFS	Disease free survival
DNA	Deoxyribonucleic acid

EBUS	Endobronchial ultrasound
ED	Emergency Department
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EHR	Electronic health record
ER	Oestrogen receptor
ESMO	European Society for Medical Oncology
F/U	Follow up
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal bovine serum
FiO2	Fraction of inspired Oxygen
FISH	Fluorescent in situ hybridisation
FMO	Fluorescence minus one
FSC	Forward scatter
GA	General anaesthetic
GCS	Glasgow coma scale
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
ICI	Immune checkpoint inhibitor
ICU	Intensive care unit
ID	Identification
IFN	Interferon
IHC	Immunohistochemistry
IHD	Ischaemic heart disease

IL-6	Interleukin 6
IT	Information Technology
ITU	Intensive therapy unit
KG	Kilogram
KRAS	Kirsten rat sarcoma
LAT	Local anaesthetic thoracoscopy
LDH	Lactate dehydrogenase
LVSD	Left ventricular systolic dysfunction
MDT	Multidisciplinary team
MERS-CoV	Middle East respiratory syndrome coronavirus
Mini-PE	Minimal pleural effusion
ML	Millilitres
MPE	Malignant pleural effusion
MRI	Magnetic resonance imaging
NGS	Next generation sequencing
NHFO	Nasal high flow oxygen
NICE	National Institute for Health and Care Excellence
NK CELL	Natural Killer cell
NSCLC	Non-small cell lung cancer
O2	Oxygen
OPM	Occult pleural metastases
OR	Odds ratio
OS	Overall survival
PBS	Phosphate-Buffered Saline

PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-L1	Programmed cell death ligand 1
PE	Pulmonary embolism
PET-CT	Positron emission tomography-computed tomography
PI	Principal Investigator
PIS	Patient information sheet
PLC	Pleural lavage cytology
PM	Predictive marker
PPI	Personal and public involvement
PR	Progesterone receptor
PS	Performance status
PTX	Pneumothorax
PV	Pharmacovigilance
QEUH	Queen Elizabeth University Hospital
RFS	Recurrence free survival
RNA	Ribonucleic acid
ROS1	ROS proto-oncogene 1
RT	Radiotherapy
RT-PCR	Reverse transcriptase polymerase chain reaction
SABR	Stereotactic ablative body radiotherapy
SACT	Systemic anti-cancer therapy
SAE	Serious adverse event
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

SD	Standard deviation
SERM	Selective oestrogen receptor modulator
SIGN	Scottish Intercollegiate Guidelines Network
SMG	Study Management Group
SSC	Side scatter
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TB	Tuberculosis
TKI	Tyrosine kinase inhibitor
TMPRSS2	Transmembrane protease serine 2
TNM	Tumour Node Metastases
TUS	Thoracic Ultrasound
UK	United Kingdom
US	Ultrasound
VATS	Video-assisted Thoracoscopic Surgery
WHO	World Health Organisation

Publications Relating to this Thesis

Ferguson J, Tsim S, Kelly C, *et al*.

Staging by Thoracoscopy in Potentially Radically Treatable Lung Cancer Associated with Minimal Pleural Effusion (STRATIFY): Protocol of a Prospective, Multicentre, Observational Study. BMJ Open Respir Res. 2023 Nov 23;10(1):e001771.

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Presentations to Learned Societies

1. Staging by Thoracoscopy in Potentially Radically Treatable Non-Small Cell Lung Cancer Associated with Minimal Pleural Effusion (STRATIFY) Study: a prospective multicentre study

Ferguson J, Alexander L, Kelly C *et al*

Accepted for poster presentation at the British Thoracic Oncology Group Conference January 2020

2. Utility of pleural fluid for predictive marker testing in malignant pleural effusion

Ferguson J, Craig C, Randles V *et al*

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3. An update on the STRATIFY (Staging by Thoracoscopy in Potentially Radically Treatable Non-Small Cell Lung Cancer Associated with Minimal Pleural Effusion) study

Ferguson J, Alexander L, Kelly C *et al*

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4. Utility of pleural fluid predictive marker panels in metastatic lung and breast adenocarcinoma

Ferguson J, Craig C, Randles V *et al*

Accepted for poster presentation at the European Respiratory Society Congress September 2024

CHAPTER 1: INTRODUCTION

1 Chapter 1: Introduction

1.1 General Introduction

Respiratory disease encompasses a wide range of pathologies from those affecting the airways to disruption of the normal functioning of the lining of the lung. Two such conditions are lung cancer including malignant pleural effusion (MPE) and Coronavirus Disease 2019 (COVID-19). Despite their differences, early diagnosis and disease stratification are important first steps in the management of both conditions to ensure optimal patient outcome.

Diagnostic pathways must keep up with ongoing breakthroughs in the treatment of metastatic lung cancer and COVID-19 which often require increasingly detailed information about individual patients' disease over and above simply establishing a diagnosis.

In lung cancer, stage is the key stratification factor. MPE denotes stage 4 disease (as defined as M1a) which is a finding with considerable prognostic impact but can be hard to detect reliably. This task is particularly challenging in patients with small pleural effusions and otherwise potentially radically treatable disease. In patients with larger symptomatic effusions they may only have effusion cytology sent to obtain a tissue diagnosis placing increasing burdens on this material to direct systemic therapies. In COVID-19, several host features are associated with outcome, but the immunological basis of these associations remain uncertain.

As such, the aims of this thesis focus on examining novel methods for early diagnosis and improved stratification of respiratory disease to improve patient outcome by reducing time to diagnosis and to ensure patients receive optimal management based on specific features of their underlying disease. Specifically my projects involve (1) the early detection and stratification of MPE (within the STRATIFY study and MPE predictive marker study) and (2) early stratification of COVID-19. These three projects and this thesis as a whole are focused on improving stratification of both MPE and COVID-19 through early diagnostic sampling.

1.2 Stratification of Disease

A basic definition of stratification is “the arrangement or classification of something into different groups (1)”. By extension, disease stratification is the division of a cohort of patients into subgroups based on the presence or absence of specific disease characteristics (2). In clinical practice, stratification of disease can be based on anything from a measurement of inflammatory makers, cellular subtype of a cancer or even a patient’s likely response to treatment. In the majority of cases, such stratification aids in decision making around individual management to ensure patients receive optimal treatment to achieve the best outcome possible.

Depending on the pathophysiology and timeline of disease, stratification tools have different inputs and parameters to meet the needs of the patient group in question. For example, the TNM staging framework is used to quantify burden of disease in patients with proven malignancy in order to guide treatment strategies(3).

1.3 TNM Staging

Precise staging is necessary for prediction of prognosis and allocation to appropriate cancer treatment.

TNM staging, as introduced above, is an important aspect of cancer diagnosis and subsequent treatment planning. This is a system to describe the physical extent of disease in a standard and reproducible way and has been adopted globally since its introduction in France in the 1940s (4). In this classification system, T refers to tumour, N describes degree of lymph node involvement and M accounts for any distant spread, ie metastasis, of the primary disease (3).

Although the components of TNM staging are the same for all solid tumour types, the individual measurements included in each category vary (3). In order to keep up with advancements in diagnostics and treatment options, routine review of TNM staging was commenced in 2002 (5). During these reviews, TNM classifications are adjusted based on latest evidence so that they continue to best reflect prognostic groups as more knowledge is gained and advancements are made in cancer diagnosis and treatment.

1.3.1 Current TNM Staging of Lung Cancer

TNM staging of non-small cell lung cancer (NSCLC) is currently in its 8th edition (Table 1.1) and was introduced into clinical practice in 2017 (6). Lung cancer is the second most common malignancy and is the leading cause of cancer death worldwide (7). TNM classification of lung cancer is therefore one of the most commonly used applications of this tool.

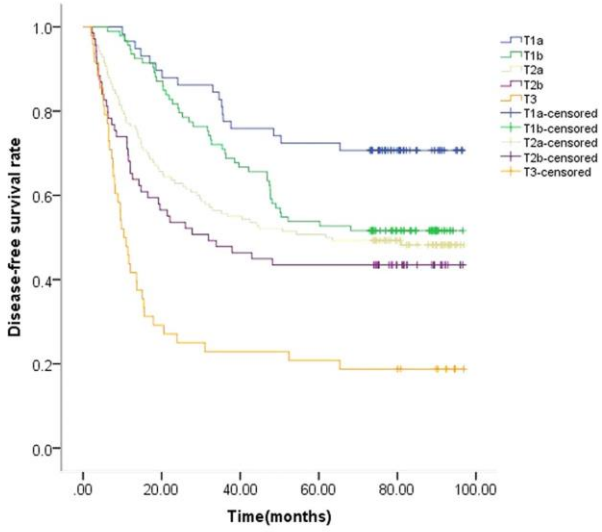
Table1.1 TNM 8th Edition for the staging of non-small cell lung cancer (6)

TUMOUR	
T0	No evidence of tumour
T1s	Carcinoma in situ
T1	≤ 3cm not involving a main bronchus
T1a	≤ 1cm
T1b	> 1cm to ≤ 2cm
T1c	> 2cm to ≤ 3cm
T2	> 3cm to ≤ 5cm (or involvement of main bronchus or invasion of visceral pleura)
T2a	> 3cm to ≤ 4cm
T2b	> 4cm to ≤ 5cm
T3	> 5cm to ≤ 7cm (or any size involving chest wall, pericardium, phrenic nerve or satellite nodules within the same lobe)
T4	> 7cm (or any invasion of mediastinum, diaphragm, heart, recurrent laryngeal nerve, trachea, oesophagus, spine or separate tumour in different lobe of ipsilateral lung)
NODES	
N1	Ipsilateral peribronchial and/or hilar or intrapulmonary nodes
N2	Ipsilateral mediastinal and/or subcarinal nodes
N3	Contralateral mediastinal/hilar or ipsi/contralateral scalene or supraclavicular nodes
MESTASTASES	
M1	Distant metastases
M1a	Tumour in contralateral lung, malignant effusion or pleural/pericardial nodule
M1b	Single extrathoracic metastasis including single non-regional lymph node
M1c	Multiple extrathoracic metastases in one or more organs

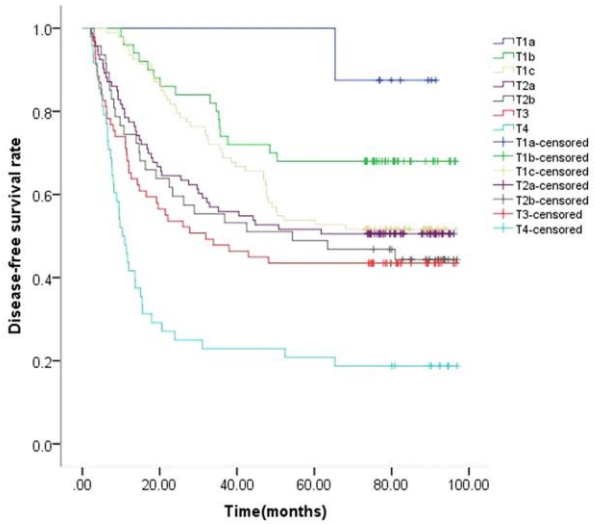
The main updates between TNM 7 and 8 for NSCLC were in the T and M descriptors with no changes to nodal staging (8). In version 8 additional T stages have been introduced with T1 now being subdivided into three (T1a, b and c) rather than two (T1a and b) (6). T1a now includes tumours up to only 1cm rather than 2cm as was the case in TNM 7. Tumours over 7cm which were previously classed as T3 are now given T4 status with T3 now describing tumours of 5-7cm in maximum diameter which would previously have been staged as T2b. Invasion of the diaphragm has also been upgraded to T4 rather than T3 as it was in the 7th edition (6). Categorisation

of metastases has also been adjusted to account for multiple distant metastases which are now classified as M1c while a single distant metastasis remains in the M1b category (6).

As you can see from the changes in TNM classification outlined above, even differences that may appear minor can have a significant impact on prognosis (Figure 1.1) (8). Accurate staging is crucial therefore not only in terms of prognosis but also to guide optimal management. With increasing treatment options available for NSCLC, accurate staging of the disease from the outset is crucial.



The 5-year DFS rates were 72%, 54%, 51%, 43% and 21% for patients with T1a (≤2 cm), T1b (>2 cm, ≤3 cm), T2a (>3 cm, ≤5 cm), T2b (>5 cm, ≤7 cm), T3 (>7 cm), respectively. P < 0.001.



The 5-year DFS rates were 100%, 68%, 54%, 52%, 49%, 43% and 21% for patients with T1a (≤1 cm), T1b (>1 cm, ≤2 cm), T1c (>2 cm, ≤3 cm), T2a (>3 cm, ≤4 cm), T2b (>4 cm, ≤5 cm), T3 (>5 cm, ≤7 cm), T4 (>7 cm), respectively. P < 0.001.

Figure 1.1 Kaplan-Meier estimates of disease free survival (DFS) according to the 7th (left) and 8th (right) edition T stage. (Graphs taken from Jin et al 2016 (9))

1.3.2 M1a Disease in NSCLC

As described above, M in the TNM staging classification stands for metastasis. By definition, anyone with distant spread of their cancer which fits the M category has incurable disease. In the current edition of NSCLC TNM staging the M category is divided into three subgroups: M1a, M1b and M1c (Table 1.1). M1a incorporates pleural involvement including malignant pleural effusion. It is therefore important to investigate pleural effusion, particularly in those with otherwise early stage,

potentially radically treatable disease given the significant impact this would have on staging (and therefore likely prognosis and appropriate treatment options) should MPE be confirmed.

1.4 Pleural Effusion

In the simplest terms pleural effusion is an excess volume of fluid within the pleural space or cavity.

1.4.1 Overview of the Pleural Cavity and Pleural Fluid

The area between the lung and the rib cage is known as the pleural cavity (10) which is lined by a double layered membrane called the pleura (Figure 1.2). One layer of this membrane lines the surface of the lung and its fissures (the visceral pleura) while the other lines the chest wall, diaphragm and mediastinum (the parietal pleura)(10). Although it is often described as a double layer, the pleura is in fact one continuous surface folded at the medial surface of the lower lobe forming the pulmonary ligament (11).

The parietal (rather than the visceral) pleura is largely responsible for fluid homeostasis the reasons for which are two fold (12). Firstly, the pleural vasculature is closer to the surface of the parietal pleura than that of the visceral layer resulting in a greater pressure gradient between the parietal pleura and the pleural cavity. Secondly the parietal layer contains lymphatic stomata (not present on the visceral pleura) through which the majority of pleural fluid drainage occurs (12).

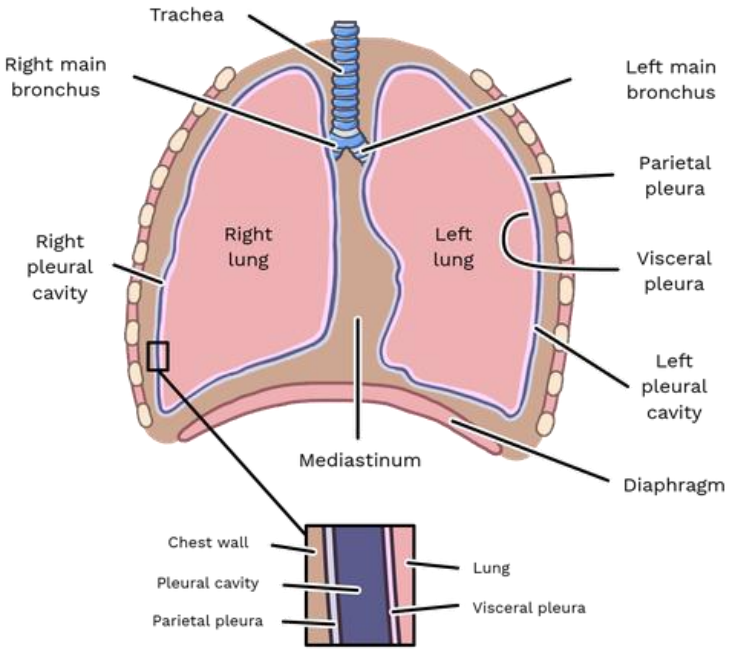


Figure1.2 Basic anatomy of the Pleural Cavity

1.4.2 Pathophysiology of Pleural Effusions

In health, the average adult has only around 0.3ml/kg of pleural fluid (13). Any process which leads to an imbalance between pleural fluid production and drainage where the volume of pleural fluid being produced is greater than the volume drained leads to a pleural effusion (12) (Figure 1.3). Prompt recognition and diagnosis of pleural effusion is clinically very important.

This is for two reasons: firstly depending on the size of effusion they can produce a high symptomatic burden in particular dyspnoea which often worsens as the effusion enlarges over time. Additionally given two major causes of pleural effusion globally are infection and malignancy, identifying and treating the underlying disease process is crucial to ensure the best outcome possible for the patient.

There are two broad categories of pleural effusion: transudative and exudative effusions. This is clinically important as identifying which category an effusion falls into is the first step towards a diagnosis. Light's criteria is used to determine if an effusion is transudative or exudative based on fluid protein and lactate dehydrogenase (LDH) (14). Light's criteria are as follows: pleural fluid protein/serum fluid protein ratio > 0.5, pleural fluid lactate dehydrogenase

(LDH)/serum fluid LDH ratio > 0.6, or pleural fluid LDH > 2/3 the upper limit of normal serum LDH (15). If any one of these criteria are met, the effusion is classed as an exudate.

Broadly speaking, transudates are caused by mechanisms which increase the hydrostatic pressure within the pleural vasculature such as congestive cardiac failure (16). Exudative, protein rich, effusions are caused by processes which increase the permeability of the pleural vasculature or disrupt the usual lymphatic drainage system, for example malignant invasion of the pleura by cancer cells (17).

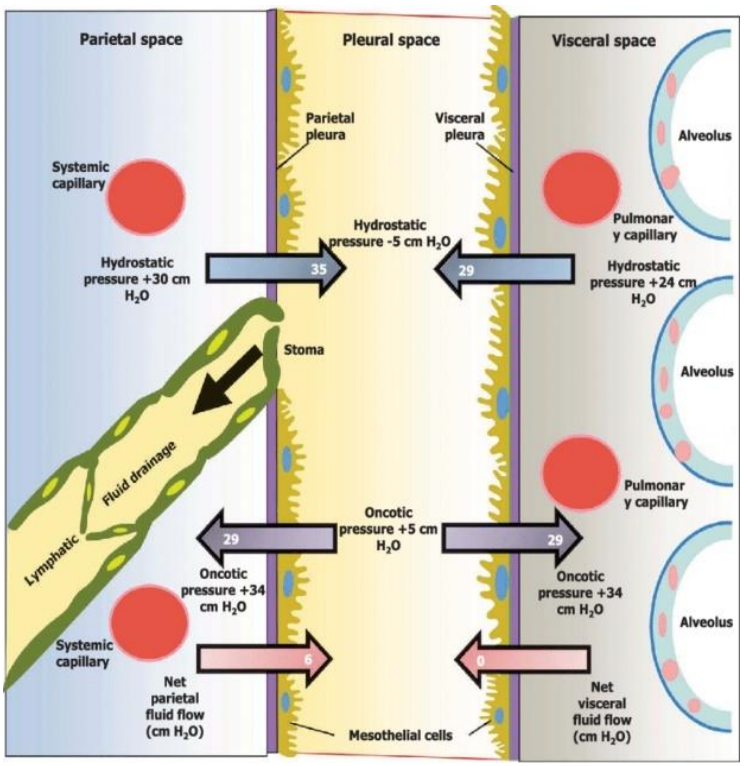


Figure 1.3 Physiology of Pleural Fluid Production (18)

1.5 Malignant Pleural Effusion

Malignant pleural effusion (MPE) is an accumulation of fluid in the pleural space caused by metastatic infiltration of the pleura(19) defined by the presence of malignant cells in fluid cytology as a surrogate.

MPE is a common finding in those presenting with cancer. Perhaps unsurprisingly, lung cancer is the leading cause of MPE accounting for around one third of all MPEs

with breast and ovarian cancer also commonly associated with effusion (25% and 5% of MPEs respectively)(20).

MPEs are clinically important as they often present with symptoms such as breathlessness requiring drainage. Additionally, as detailed in section 1.3.2, they are prognostically significant. In NSCLC (and other common adenocarcinomas such as breast cancer) the presence of MPE indicates metastatic, incurable disease (Table 1.1) and confers a survival of as little as 3-9 months on average (21).

1.6 Investigation of Suspected Malignant Pleural Effusion

The investigation of suspected MPE combines imaging and sampling modalities with the aim of reaching a diagnosis as quickly and in as few procedures as possible.

1.6.1 Chest X-Ray (CXR)

Due to the common presentation of shortness of breath with pleural effusion, chest x-ray (CXR) is often the first imaging test (22). Although this can be useful in pointing to a probable primary in the context of lung cancer, a CXR alone cannot diagnose MPE or accurately stage NSCLC (22). CXR can however allow the planning of pleural intervention while other tests are awaited in cases where a large, symptomatic effusion is discovered.

1.6.2 Computed Tomography (CT) Scanning

All patients with suspected MPE should go on to have a CT chest, abdomen and pelvis to identify the primary tumour as well as assess the extent of disease (7).

Despite all patients undergoing CT as standard during the diagnosis and staging of malignancy where MPE is suspected, it is not the optimum diagnostic test to assess for malignant involvement of the pleura (23). This is important as 15% of those with lung cancer present with an effusion (24) while 40% develop an effusion at some stage throughout the course of their disease (25). In a large retrospective study of those undergoing thoracoscopy for the investigation of new undiagnosed effusion who had CT prior to confirmatory biopsy, the sensitivity of CT for the detection of

malignant pleural changes was only 68% (26). The negative predictive value in this same study was 65% (26). Based on this data, around one third of all patients found to have effusion on CT with no other features suggestive of malignancy (for example reported as ‘indeterminate’ or ‘no cause identified’) will in fact have pleural malignancy(23).

1.6.3 Positron Emission Tomography Computed Tomography (PET-CT)

If there is no evidence of distant metastases on CT and radical (curative) treatment is felt to be an option after consideration of patient fitness, a whole body integrated positron-emission tomography/computed tomography (PET-CT) scan will be performed (27). This is to ensure there is no distant spread of disease not previously detected on CT. Again however PET-CT often also fails to reveal malignant involvement of the pleura. This has recently been confirmed by a meta-analysis looking at the ability of PET-CT to distinguish malignant from benign pleural effusion based on a combined cohort of 639 patients (28). On review of PET images this analysis found that PET-CT had a sensitivity of just 81% for the detection of malignant pleural involvement and therefore concluded that PET-CT alone should not be relied upon for accurate pleural staging of malignant disease (28).

Although CT and PET-CT are the gold standard imaging modalities as recommended by current guidelines in the diagnosis and staging of lung cancer (22), given the above findings, it is important to remember that the absence of malignant pleural features on imaging does not rule out pleural metastases in the context of effusion.

1.6.4 Cytology

As well as the importance of imaging in the investigation of suspected MPE, pathological confirmation of both primary and possible metastases is essential in order to plan the most appropriate treatment. This includes histological subtype where systemic therapies are planned.

Ideally tissue sampling is done with the aim of confirming a diagnosis as well as stage of disease where possible (27). Where MPE is suspected pleural fluid is

therefore commonly the first tissue to be sampled as fluid cytology can in some cases provide all of this information.

Pleural fluid can be obtained by simple needle aspiration which is a quick, safe and relatively non-invasive procedure. Unfortunately however, pleural fluid cytology has a diagnostic yield of on average only 60% (21) but this does vary widely depending on the underlying malignancy even down to the subtype of cancer(23, 29).

1.6.5 Thoracoscopy

In clinical scenarios where pleural biopsy is required thoracoscopy may be used. Thoracoscopy allows the direct visualisation of the pleural cavity with a thoracoscope (Figure 1.4) through which biopsy forceps can also be passed to obtain pleural tissue samples from areas of suspicion (30).

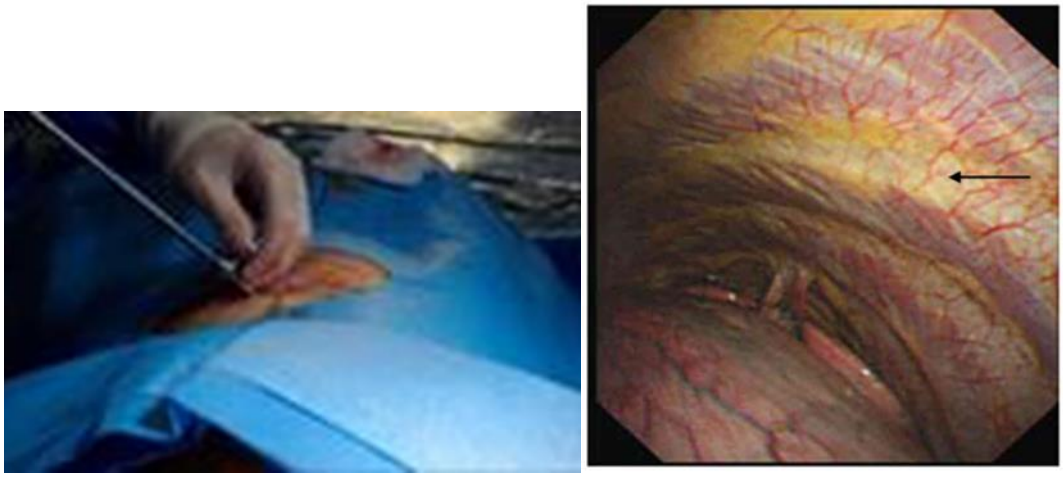


Figure 1.4 Images of local anaesthetic thoracoscopy showing thoracoscope entry via port site created between the ribs (right image) and thoracoscopic view of the pleural cavity and surfaces (left image)

Thoracoscopy itself is not new, with the first documented procedures taking place in 1910 (31) when the technique was used mainly in the treatment of TB (30). With lower levels of TB as well as cancer management becoming increasingly individualised with more detailed staging requirements, the role of thoracoscopy has shifted over the past 100 years. The investigation of undiagnosed pleural effusion is now its primary indication in the West (31).

Local anaesthetic thoracoscopy (LAT) is currently the gold standard in the diagnosis of suspected pleural malignancy (32). It has a diagnostic yield of > 90% (33) which is higher than both pleural aspiration (60%) or now outdated biopsy techniques such as Abraham's needle biopsy (<50%) (34). As well as its high diagnostic yield, LAT is also safe with a mortality rate of only 0.1% and major complications found to be rare (for example bleeding risk of just 1.6% with the majority of these being self-limiting) in a large meta-analysis of 4,705 patients (35).

LAT has an advantage over video assisted thoracoscopy (VATS) in that it does not require the use of general anaesthetic. This means LAT remains an option for those who would not be fit for VATS and importantly, in the context of lung cancer treatment planning, may not be fit for surgery where the risks of general anaesthetic are a contraindication.

One important area in which the role of thoracoscopy has not yet been established is as a pure staging tool. LAT may have particular utility in cases of lung cancer with small ipsilateral pleural effusions either too small to be amenable to aspiration or negative on cytology- otherwise known as minimal pleural effusions (mini-PE).

1.7 Minimal Pleural Effusion

Common to all reported definitions of minimal pleural effusion is that they are small effusions ipsilateral to the primary tumour that are either negative on pleural fluid cytology or too small to safely aspirate (25, 36).

1.7.1 Clinical Significance of Minimal Pleural Effusions

Due to the prognostic implications of confirmed MPE in NSCLC, minimal effusions are clinically important despite their lack of symptoms. They are found in up to 25% of those presenting with NSCLC (25) although half of these occur in patients with metastatic disease (25, 36). If a mini-PE is confirmed to be malignant in the context of NSCLC then this automatically signifies incurable disease (7).

Moreover, it has been shown repeatedly from retrospective data that the presence of mini-PE is associated with poorer survival than when compared to those with the

same stage of disease without mini-PE (36). Studies have highlighted mini- PE as a marker of particularly high recurrence risk, and excess mortality following radical treatment (25, 36). These studies suggest occult pleural metastases (OPM) may be responsible for up to 80% of mini-PEs in lung cancer (36-38), but agree other factors such as co-morbidities may also contribute to this observed difference in survival.

1.7.2 Current Approach to Mini-PE

There is no consensus approach to the management of mini-PE in the work up of those with suspected lung cancer, with the current NICE guidelines not formally addressing pleural staging (39). This means it is at the discretion of the treating team as to whether such effusions are further investigated or taken into consideration when staging and planning management. If so, multidisciplinary teams (MDTs) must then rely on imaging and pleural aspiration cytology (if possible), all of which are by definition negative in those with mini-PE.

1.8 Treatment of NSCLC

Lung cancer is the most common cause of MPE as well as the most common cause of cancer death in the UK (40) with NSCLC now accounting for around 90% of lung cancers (41). The current diagnostic pathway for NSCLC involves a combination of imaging and sampling techniques as outlined above with most patients undergoing a number of each to formulate the most appropriate treatment plan. This can put a significant emotional and physical burden on patients and therefore obtaining all of the information needed with as few tests, as quickly as possible, is paramount (42).

The core decision to be made in the management of NSCLC is whether the cancer is curable or not. In oncology, this is referred to as radically treatable and non-radically treatable disease respectively. Stage is the most important factor used to determine this and guide eventual treatment modality in confirmed NSCLC while taking into account patient fitness and wishes. Histopathological information to determine NSCLC subtype becomes important when deciding on systemic therapies where the advanced stage of disease precludes radical strategies.

1.9 Oncological Treatment of NSCLC Without Metastases

In general terms, when someone has early stage NSCLC without evidence of distant spread treatment will be with radical intent (27) often taking a multi-modality approach using a combination of surgery, radiotherapy and chemotherapy (27, 43).

All radical treatment options come with many risks and possible side effects some of which may be permanent and significantly impact a patient's quality of life. This underlines again how important accurate staging is. Not only so those with radically treatable disease are treated as such but also, crucially, to avoid putting those with no chance of cure through such invasive and potentially life altering treatments.

1.9.1 Surgery

Surgery remains the treatment of choice in those with radically treatable disease amenable to resection (27) and lobectomy should be offered where stage and fitness allow as per current guidelines. More extensive surgery (for example pneumonectomy) should only be considered if it is required to achieve clear margins. If lobectomy is contraindicated, sublobar resection can also be considered (27).

Despite being performed only in those with theoretically curable cancer, increasing disease stage is independently associated with poorer outcomes post-surgery (44). In a surgical cohort of 2449 patients, less than 50% of those with stage IIA and IIIA NSCLC survived to 5 years following surgery while the 5 year survival of those with surgically resected stage IIIB disease was just 13% (44). Although there will be many patient factors contributing to these deaths this does suggest that improvements could be made to the staging system and/or diagnostic pathway to improve these figures through more accurate staging and better selection of patients for surgery.

1.9.2 Radical Radiotherapy

Where surgery is contraindicated or declined by the patient, radical radiotherapy should be considered in those with radically treatable disease (27, 45). In such cases, particularly for those with stage 1 disease, stereotactic ablative body

radiotherapy (SABR) is associated with better outcomes when compared to conventionally fractionated radiotherapy regimens (46) and is now the treatment of choice in those with early stage, inoperable NSCLC (46, 47). Although surgery remains the radical treatment of choice where possible, there has yet to be a randomised controlled trial to assess outcomes in early stage lung cancer treated with lobectomy vs SABR (48).

SABR involves the delivery of a higher dose of radiotherapy over fewer sessions than conventional radiotherapy regimens. It is also a more precise method for delivery of radiotherapy meaning that less surrounding tissue is affected (49). Due to the high doses used per session however, if a tumour is too close to a sensitive structure prone to toxicity from radiotherapy (for example the heart) then the high fractions used in SABR may be contra-indicated in which case conventionally fractionated radiation therapy can be employed.

1.9.3 Chemoradiotherapy

As with radiotherapy, there is an ever increasing role for systemic therapy in the management of NSCLC both in the palliative setting as well as in combination with surgery or radiotherapy in those with locally advanced disease being treated with curative intent (43, 50).

Around one third of those with NSCLC present with stage III, locally advanced, disease with evidence of nodal or local invasion at presentation (51). In those with stage III NSCLC, radical treatment most commonly takes the form of chemoradiotherapy (50). Even in the select few with stage III disease who do undergo surgery, current guidance advises chemoradiotherapy in addition where patient fitness allows as this has been shown to improve progression-free survival (27).

1.10 Treatment of Lung Cancer with Metastases

If NSLC has metastasised or progressed beyond the possibility of cure (including those with malignant pleural effusion) then systemic anti-cancer therapy (SACT)

becomes the treatment modality of choice (27) and should be offered to all those deemed fit enough (7).

The choice of SACT is based on the presence (or not) of specific tumour cell characteristics the general term for which are predictive markers (PMs). If an individual's tumour displays no such PMs then traditional platinum based chemotherapy will be given (52). Otherwise a more targeted therapy based on pathological characteristics will be used.

1.11 The Role of Predictive Markers in Lung and Other Cancers

Predictive markers (PMs) are tumour cell characteristics that indicate, or predict, a likely response to a corresponding therapy. PMs are used to plan treatment in many of the commonest cancers including lung, breast and ovarian cancer.

This has been an important advancement in cancer treatment as in NSCLC it has been shown that those found to be positive for a predictive marker do better in terms of survival when given the corresponding PM directed therapy than when treated with traditional chemotherapy (53, 54) .

Not everyone with one cancer type will have tumour cells expressing the same, or in some cases any, PMs. Additionally in lung cancer, while PD-L1 may be seen alongside oncogenic driver mutations (55) the presence of the driver mutations currently used as PMs are thought to be largely mutually exclusive(56). It is therefore important to provide enough tissue or fluid to allow the testing of all markers of available therapies.

In light of this there now exist internationally mandated panels of PMs that must be tested for in the diagnosis of many major tumour groups including lung and breast cancer to direct the use of PM directed SACT(7, 54) (Table 1.2). Diagnostic sampling therefore must be sufficient to allow testing for each marker in addition to simply revealing the underlying tumour type.

Table 1.2 Summary of predictive markers, their associated adenocarcinomas and method of detection (PCR = Polymerase chain reaction, NGS = Next generation sequencing, IHC = Immunohistochemistry, FISH = Fluorescence in situ hybridisation)

Predictive Marker	Adenocarcinoma	Testing Modality	PM Directed Therapy
EGRF	Lung	PCR, NGS	Afatanib, Erlotinib, Gefitinib, Osimertinib
ALK	Lung	IHC, FISH, NGS	Alectinib, Brigatinib, Crizotinib
PD-L1	Lung	IHC	Atezolizumab, Cemiplimab, Pembrolizumab
ROS1	Lung	IHC, FISH, NGS	Crizotinib, Entrectinib, Lorlatinib
ER	Breast	IHC	Anastrozole, Exemestane, Letrozole, Tamoxifen
PR	Breast	IHC	Anastrozole, Exemestane, Letrozole, Tamoxifen
HER2	Breast	FISH	Pertuzumab, Trastuzumab

1.11.1 Therapies Based on Predictive Markers

As stated above, there are now mandated predictive marker panels included in the diagnostic pathways of lung, breast and ovarian cancer (7, 54, 57). A summary of the most common PMs currently in use and their associated therapies is available in table 1.2.

PD-L1

Over one quarter (28%) of those with stage 4 NSCLC are found to have programmed cell death ligand 1 (PD-L1) positivity of ≥ 50%. This rises to almost 70% when any level of PD-L1 positivity (≥1%) is included (58).Immunotherapy agents used in those with PD-L1 positivity (eg pembroluzumab) are known as immune checkpoint inhibitors. Inhibition of PD-L1 promotes T-cell activity against cancer cells to improve immune response to and subsequent destruction of malignant cells (59). It has been shown on multiple occasions that those with PD-L1 positivity treated with anti-PD-L1 monoclonal antibodies (eg pembrolizumab) have better progression free and overall survival than those treated with chemotherapy agents (60).

EGFR

An epidermal growth factor receptor (EGFR) mutation is found in around 10-15% of those in America and Europe with NSCLC (61) and is more common in those where there is no smoking history (62).EGFR is one of a group of enzymes called tyrosine kinases (TKs). TKs are involved in downstream cell signalling pathways that can

alter cell growth, differentiation and migration. Dysregulation of such pathways, for example caused by the oncogenic mutation of a TK such as EGFR can result in malignancy (63). Consequently, tyrosine kinase inhibitors (TKIs) have been developed which inhibit downstream aberrant TK signalling.

Again, it has been shown in many randomised control trials that those with an EGFR mutation receiving a PM specific tyrosine kinase inhibitor (eg afatanib or erlotinib or) have a significantly longer progression free survival when compared to those receiving traditional chemotherapy (64, 65).

ALK

Anaplastic lymphoma kinase (ALK) is another tyrosine kinase associated with lung cancer development due to oncogenic driver mutation (66). Compared to those with an EGFR mutation, ALK positive cancers are by comparison relatively uncommon accounting for only 4-5% of lung cancers (67). These are also treated with TKIs first line which in this setting work as ALK-inhibitors. TKIs used against ALK positive tumours are crizotinib and brigatinib (39). When compared with standard chemotherapy, patients treated with the ALK TKI crizotinib have a significantly longer progression free survival and a significant improvement in both symptoms and quality of life (68).

ROS1

ROS proto-oncogene 1 (ROS 1) is another druggable mutation now tested for in the diagnosis of NSCLC but is seen in only 1-2% of cases (69). Like those found to have EGFR mutations, ROS 1 alterations are most typically found in younger people with NSCLC who are often never or light smokers. Again, those with ROS 1 mutations respond to ROS 1 TKIs including crizotinib or entrectinib. Studies have shown an improved progression free survival with first line crizotinib when compared with traditional platinum based chemotherapy in those with ROS 1 positive disease (70).

Hormone Receptors (ER/PR)

In breast cancer, hormone receptor (namely oestrogen (ER) and progesterone receptor (PR)) expression is very common with around 70% of people positive for one if not both receptors (71).

Pre-menopausal women diagnosed with hormone receptor positive breast cancer are offered tamoxifen as a first line treatment (54). Tamoxifen is a selective oestrogen receptor modulator (SERM) which inhibits the growth of hormone receptor positive breast cancer through competitive binding to oestrogen receptors which disrupts downstream hormone signalling involved in cell growth (72).

In post-menopausal women aromatase inhibitors such as letrozole are the treatment of choice (73). Aromatase inhibitors prevent the enzyme aromatase converting androgens into oestrogens (a process called aromatisation) which again inhibits subsequent downstream signalling (74).

HER 2

The third PM routinely tested for in breast cancer is the human epidermal growth factor 2 receptor (HER 2). Much like oncogenic driver expression in lung cancer being mutually exclusive, the majority of hormone receptor positive breast cancers are HER2 negative (75). Around 15% of breast cancers are found to have an overexpression of the HER 2 receptor (75).

HER 2 is another TK involved in downstream signalling pathways that when overexpressed (as is the case in HER2 positive breast cancers) leads to increased cell proliferation (76). Trastuzumab is a monoclonal antibody which binds to HER-2 thereby inhibiting its role in this downstream signalling cascade. When used with more traditional chemotherapy the addition of trastuzumab, also known as Herceptin, improves patient outcome and where chemotherapy is contra-indicated, trastuzumab can be used first line (77).

1.12 Cancer and the Immune System

Immunotherapies are now a well-established treatment for many common malignancies including lung cancer (such as pembrolizumab as mentioned above which inhibits PD-L1) and it is known that there is a strong link between inflammation and carcinogenesis (78). The defining characteristic of cancer immunotherapies is their use of specific features of the immune response against cancer cells themselves (79). There is still however much to learn about the interplay between cancer and immune cell signalling.

One key signalling pathway which has been linked to both the immune response and malignancy is the Janus kinase /signal transducer and activator of transcription (JAK/STAT) pathway (80). JAK/STAT is a family of signalling molecules which upon activation relay extracellular signals across the membrane to the nucleus to produce an intracellular response (80). JAK/STAT signalling is involved in many aspects of the normal functioning of cells such as proliferation, apoptosis as well as being central to immune response signalling pathways (78). Consequently, JAK/STAT signalling is also involved in many of the processes necessary for tumour cell survival and proliferation including the evasion of immune detection by malignant cells (78).

Highlighting its wide-reaching influence, JAK/STAT signalling molecules can be activated by both inflammatory cytokines (such as interferons) as well as receptor tyrosine kinases (for example EGFR and HER) (78) and JAK/STAT signalling is currently being studied as a potential target for anti-cancer treatment in solid tumours (80). It is therefore imperative that we maximise or understanding of this pathway and its links between inflammation and cancer.

One way we can learn more about the immune system and its downstream signalling pathways such as JAK/STAT is through the study of the immune response to viruses such as SARS-CoV-2, the study of which has been at the forefront of respiratory disease research since its discovery in 2019.

1.13 COVID-19

In December 2019 there first emerged reports of a respiratory illness in the province of Wuhan in China. It was quickly discovered that this illness was caused by a novel coronavirus, eventually named SARS-CoV-2. This virus was found to cause an acute respiratory distress like syndrome which became known as COVID pneumonia (81).

SARS-CoV-2 is the seventh known coronavirus to have entered the human population (82) with the first such event discovered in the 1960s (83). Despite this, there were no proven treatments for any of the human coronaviruses including the potentially fatal SARS-CoV-1 or MERS-CoV that had come before SARS-CoV-2 (84).

By March 11th 2020, the WHO declared SARS-CoV-2 a pandemic (85). At the time of writing, there have been over 774 million reported cases of COVID-19 worldwide with just over 7 million deaths (86).

1.13.1 Virus Structure and Transmission

SARS-CoV-2 is a novel coronavirus. Coronaviruses get their name from the Latin corona meaning crown (87) because they all share a similar, crown like, shape (Figure 1.5). SARS-CoV-2 is an enveloped positive-sense single strand RNA virus and like other coronaviruses is able to infect humans, other mammals and birds (88).

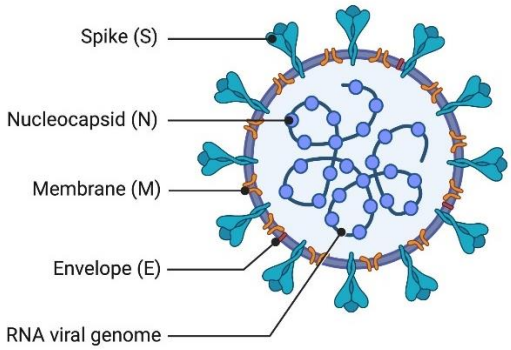


Figure1.5 Structure of a coronavirus

SARS-CoV-2 infects upper airway epithelial cells as well as deeper pneumocytes and bronchial cells. It achieves this by binding its surface spike protein (S-protein) to the angiotensin converting enzyme 2 (ACE2) receptor (83) (Figure 1.6).

To allow eventual entry of the virus into host cells after S-protein binding, fusion must occur. SARS-CoV-2 uses the host cell surface enzyme TMPRSS2 to achieve this (Figure 1.6). TMPRSS2 is present on the surface of both upper and lower respiratory tract cells (82).

One major difference between SARS-CoV-1 and SARS-CoV-2 is the propensity of SARS-CoV-2 to infect and replicate in upper as well as lower respiratory tract cells (89). This is one of the reasons for the significantly higher transmission rates seen in SARS-CoV-2 compared to its predecessor. Transmission occurs either via contact

spread between infected surfaces and vulnerable mucosa (for example of the nose or eye) or by droplet inhalation (89).

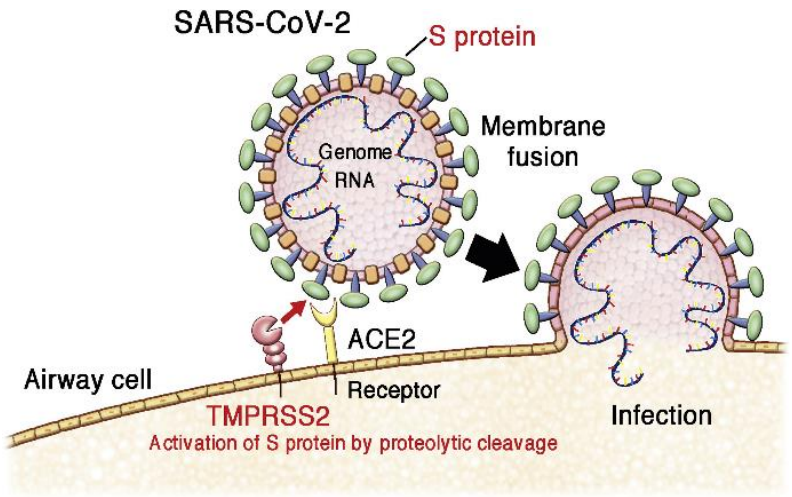


Figure1.6 SARS-CoV-2 structure and mechanism of entry into host cells

1.13.2 Presentation of COVID-19

Symptoms of COVID-19 take around 5 days to appear from the time of infection but this incubation period can be as long as 18 days(90). These symptoms may include fever, muscle ache and fatigue as well as cough, shortness of breath and a loss of taste and smell (91).

It became clear early in the pandemic that there is a broad spectrum of COVID-19 presentations, severities and outcomes. At the beginning of the pandemic, around one third of those admitted to hospital with COVID-19 eventually died of the disease with the major cause of death being from hypoxic respiratory failure (92).

Another early observation was that pulmonary embolism (PE) was not an uncommon finding in COVID-19. The rate of thrombotic events increases as disease severity increases (93) and it is now known that up to one third of people with COVID-19 will develop thrombus, most commonly in the form of a PE (93).

1.13.3 Diagnosis of COVID-19

The diagnosis of SARS-CoV-2 infection is necessary not only to commence treatment if required but also to help prevent further spread of the virus. This was of

particular importance at the outbreak of the pandemic. Multiple methods to diagnose SARS-CoV-2 infection are available such as lateral flow or rapid antigen testing. This testing modality uses an immunoassay to detect SARS-CoV-2 antigens from nasal or throat swabs and is simple enough to be performed at home (94). One other common method for the detection of SARS-CoV-2 which has an even higher sensitivity (88-96%) than lateral flow testing is reverse transcriptase polymerase chain reaction (RT-PCR) (90).

RT-PCR combines an initial reverse transcriptase reaction with the amplification process of PCR. Reverse transcription is the process by which DNA is produced from an RNA template allowing the resultant single strand DNA to become the basis of the subsequent PCR reaction (95). One of the advantages of PCR is its sensitivity - for example, gene expression of a single cell can be identified with the use of PCR (95).

The diagnosis of COVID pneumonia in those infected with SARS-CoV-2 is based on imaging appearances. Classically, COVID pneumonia is seen as widespread bilateral changes on CXR, or a combination of consolidation and ground glass changes when visualised on CT (81).

1.14 Risk Factors and Outcomes of COVID-19

It was acknowledged very early in the pandemic that there is a significant spectrum of disease and outcomes seen in those infected with SARS-CoV-2 (96). Patterns of disease started to emerge as well as the discovery of many risk factors for severity of disease. Recent evidence suggests an additive effect of comorbidity in patients with COVID-19, with increasing numbers of comorbidities associated with poorer outcomes (92, 97).

1.14.1 Outcomes and Survival COVID-19

During the first few months of the pandemic it was estimated that up to 10% of those infected required hospital admission (98) and from a large UK study of over 20,000 patients, 26% of those admitted died (99). The majority of deaths in COVID-19 are as a result of acute respiratory failure (92) often from an acute respiratory

distress syndrome (ARDS) like illness (100). With the discovery of therapeutic options and particularly with the introduction of the SARS-CoV-2 vaccine, COVID-19 outcomes have improved significantly since the outbreak of the pandemic.

Even from the earliest reports however it was clear there was significant heterogeneity in the outcome of those infected with the virus with around 80% having mild disease and some even being entirely asymptomatic (96).

1.14.2 Known Risk Factors for Poor Outcome

Initial data reported from the Lombardy region of Italy (which saw the highest rates of SARS-CoV-2 infections in Europe in the first few weeks of the pandemic) was able to identify risk factors associated with poor outcome. This data highlighted the predominance of men requiring ventilation (82% of 1591 patients)(101). The majority of people requiring intensive care unit (ICU) admission had at least one co-morbidity, most commonly hypertension. Furthermore, hypertension was also significantly more common in those who died in ICU than in those who survived their stay (101). As the pandemic continued, increasing age, type 2 diabetes mellitus and Black or Asian ethnicity were identified as risk factors for poorer outcomes (102, 103).

Knowledge of these risk factors was used to create mortality prediction scores such as ISARIC 4C (104) (Figure 1.7). This score uses a combination of co-morbidities and initial clinical data such as CRP and oxygen requirement to predict mortality from COVID-19. This information can then be used to aid decision making in the management of those with COVID-19.

CLINICAL CHARACTERISTIC	SCORE
Age (Years)	
18 – 49	0
50 – 59	+2
60 – 69	+4
70 – 79	+6
80+	+7
Sex at birth	
Female	0
Male	+1
Comorbidities	
0	0
1	+1
2+	+2
Respiratory Rate (breaths/min)	
< 20	0
20 – 29	+1
≥ 30	+2
Oxygen Saturation (% on air)	
≥ 92	0
< 92	+2
Glasgow Coma Scale	
15	0
< 15	+2
Blood Urea (mmol/L)	
< 7	0
≥ 7 - ≤ 14	+1
> 14	+3
CRP (mg/L)	
< 50	0
50 – 99	+1
≥ 100	+2

Score	Risk Group	In-hospital Mortality
0 – 3	Low	1.2 – 1.7%
4 – 8	Intermediate	9.1 – 9.9%
9 – 14	High	31.4 – 34.9%
15 - 21	Very high	61.5 – 66.2%

Figure 1.7 ISARIC 4 C Score and Associated In-hospital Mortality

1.14.3 Endotypes of COVID-19

An endotype is a subgroup of disease characterised by a specific pathophysiological response (105). This differs from disease phenotype which is any observable trait associated with the condition (106).

Through increasing knowledge of COVID-19 it became clear that there are distinct patterns or endotypes of disease. Two recognised endotypes being prothrombotic and immune activated COVID-19. Prothrombotic COVID-19 is characterised by a

procoagulant state with both macro and micro clots as a predominant pathological feature (93). Immune activated disease is more heavily associated with a cytokine storm leading to interstitial lung damage and resulting respiratory failure (107).

1.15 Immune Response to SARS-CoV-2 Infection

Although there are still major gaps in our knowledge, much work has been done to better understand the immune response to SARS-CoV-2 infection.

The innate immune system plays a key role in the individual response to SARS-CoV-2 infection. It has been established from the study of other viruses that antiviral cytokines released by cells of the innate immune system (eg immune cells including neutrophils, eosinophils, mast cells, natural killer cells, plus airway epithelial and endothelial cells) play a central role in the body's response to viral infection (108). Cytokine is an umbrella term for immune signalling molecules including interferons and interleukins (109). Such cytokine signalling by innate immune cells helps to limit viral replication alongside shaping the intensity and duration of the subsequent adaptive immune response mediated by T-cells, B-cells and antibodies (108).

It is thought that hyperactivation of this cytokine signalling (commonly referred to as a cytokine storm) is one of the main drivers of the ARDS picture seen in those with severe COVID pneumonia (107). Previous studies demonstrate that patients with severe COVID-19 disease have higher levels of pro-inflammatory cytokines than those with mild disease (110, 111). Study of the interferon response to other viral infection however has shown that both over and underactivity can have negative effects for the host (112).

This complexity in host immune response to SARS-CoV-2 has made it challenging to fully understand. One hypothesis is that there is likely a variation in immune response in COVID-19 throughout the course of the disease. It is postulated that those with severe disease have an exaggerated initial innate immune response with a consequently exaggerated (and delayed) adaptive immune response (113). This is one potential reason that promising therapeutic targets on paper, such as

enhancement of the interferon response as described in more detail below, have so far shown disappointing results (114).

1.16 Current Treatment of COVID Pneumonia

The discovery of effective therapies for those hospitalised with COVID-19 as well as the introduction of prophylactic vaccines dramatically altered the course of the pandemic for the better.

1.16.1 Supportive Treatment

At a time when there were no proven drugs for the treatment of COVID-19, medical teams were reliant on supportive therapies. It was shown early on that proning (positioning a patient face down on their front) helped with oxygenation in those with COVID-pneumonia. Studies have shown that proning while receiving high flow nasal oxygen reduces the need for intubation and mechanical ventilation (115).

There has also been much work done to determine the best ventilation modality for those requiring respiratory support due to COVID pneumonia. Within RECOVERY-RS, the largest trial of its kind so far, over 1200 patients were randomised to receive either continuous positive airway pressure (CPAP), nasal high flow oxygen (NHFO) or conventional oxygen therapy. This study revealed that the use of CPAP significantly reduced the need for intubation compared to the use of conventional oxygen therapy alone. No such difference was observed when NHFO was compared to conventional oxygen therapy (116).

1.16.2 Drug Treatments

Throughout the pandemic, many drugs were trialled based on hypotheses about drug action and pathogenesis of COVID-19 often with disappointing results. Continued work into the pathophysiology of COVID-19 is essential therefore to better understand which treatments work and why and to better target treatments at those who are most likely to benefit from them.

The RECOVERY trial was set up to look for potential drug therapies for COVID pneumonia using treatments already licensed for other indications. Through the enormous work put in by recruiting teams across the UK and latterly additional international sites, the first major breakthrough in the treatment of COVID pneumonia was discovered. It was found that the use of corticosteroids, specifically dexamethasone, significantly improved survival and decreased the need for mechanical ventilation in those with COVID-19 requiring supplemental oxygen (117).

Monoclonal antibodies known to target components of the immune response found to be involved in the pathogenesis of COVID pneumonia were also trialled. One such drug is tociluzumab which inhibits the action of the immune signalling molecule IL-6. Again through data from the RECOVERY trial it was shown that those admitted with COVID-19 requiring oxygen and with evidence of an inflammatory response to the virus (CRP ≥75) had improved outcomes with decreased 28 day mortality as well as increased likelihood of discharge at 28 days when given tocilizumab (118).

Other strategies to dampen the body's immune response and subsequent cytokine storm and resultant tissue damage seen in COVID pneumonia have not been so successful. One example of this is the use of interferon therapy. It had been shown from work during the SARS-CoV-1 outbreak that insufficient interferon response was a likely contributor to both the immune dysregulation and subsequent poor outcomes seen in those infected with SARS-CoV-1 (119).

This appeared to be the case during initial studies into COVID-19 with low levels of circulating interferon pointing again to an inadequate interferon response (120). As further work was undertaken however, exaggerated interferon responses have also been found in those with severe disease (114).

It is perhaps unsurprising then that there are many contradictory outcomes of studies looking into the use of therapeutic interferon for COVID-19 (114). Some data even suggests that depending on the timepoint in the disease at which such drugs are started, they could have a detrimental effect. One hypothesis is that a slow initial interferon response contributes to severe disease followed by increased and dysregulated interferon signalling which propagates the cytokine storm and resultant alveolar damage as the disease progresses (108). This theory is backed by

a multicentre cohort study which found that interferon therapy decreased mortality when used early in the disease while the same treatment worsened survival when used later in the disease course (121).

All of the above highlights the need for a better understanding of the immune response to COVID-19, how this relates to outcome and crucially, how we can harness this knowledge to better manage COVID-19 and improve patient outcome further.

1.17 Summary

Despite this thesis consisting of three studies in different clinical settings, all three share the common goal of improving early diagnosis and stratification of respiratory disease.

- Minimal pleural effusion: There are a subgroup of patients with suspected NSCLC who present not with malignant effusion but with minimal pleural effusion which are either small and cytology negative or too small to safely aspirate at all. Previous retrospective studies show that those with early stage lung cancer and such mini-PE do significantly worse than their stage matched counterparts however these minimal effusions are not formally accounted for in lung cancer staging. The addition of thoracoscopy to the diagnostic pathway of those with suspected early stage lung cancer and mini-PE could assess for the presence of occult pleural metastases (hypothesised as a likely cause of mini-PE) allowing for the more accurate staging.
  Malignant pleural effusion: Larger pleural effusions are often present from the outset in advanced malignancy and in such cases are routinely used as a first source of tissue to allow diagnosis and staging, signifying metastatic disease when cytology is diagnostic. Predictive marker testing is now mandated in the diagnostic pathway of most common cancers as PMs predict response to individual therapies to direct optimal treatment decisions.
- COVID-19: The need for accurate diagnosis and prognostic information from the outset is also crucial in those with COVID-19. A better understanding of the immune response and mechanisms which drive poor outcomes from

COVID-19 could enable more targeted management strategies with the aim of further improving individual outcome from SARS-CoV-2 infection. Additionally, this better understanding could aid in the planning of resources at times of limited availability such as the need for critical care beds or ventilatory support and even potentially improve the recruitment of patients to clinical trials of potential new treatments.

1.18 Aims of Thesis and Hypotheses Tested

The overall aim of this thesis is to examine novel methods to improve the stratification of respiratory disease from the time of diagnosis. This in turn would be with the aim of improving patient outcome either by minimising the time to treatment or by directing patients towards the most appropriate treatment. In order to accomplish this, I have performed three separate studies each with their own distinct hypotheses and primary outcomes all with an aim of improving early diagnosis and stratification of respiratory disease as outlined below.

CHAPTER 2: Staging by Thoracoscopy in potentially radically treatable Lung Cancer associated with Minimal Pleural Effusion (STRATIFY)

Previous retrospective studies have reported worse survival in those with otherwise potentially curable lung cancer found to have a minimal pleural effusion at presentation than their stage matched counterparts without such an effusion. From these retrospective studies, it has been proposed that the main reason for the presence of these minimal effusions and subsequent poorer outcomes is the spread of the cancer to the lining of the lung not otherwise detected by current routine diagnostic tests. The hypothesis of STRATIFY is therefore that occult pleural metastases (OPM) account for 70-80% of minimal effusions in early stage lung cancer and that this could be detected through the addition of thoracoscopy to the diagnostic pathway to those with early stage disease presenting with mini-PE. The primary objective of STRATIFY is therefore to prospectively determine the prevalence of OPM in patients with suspected or confirmed stage I-III lung cancer and mini-PE through the addition of thoracoscopy.

CHAPTER 3: Pleural Fluid Predictive Marker Testing in Metastatic Lung and Breast Adenocarcinoma

Pleural fluid aspiration has long been considered the standard first test in the diagnosis of malignancy when an effusion is present as it provides an opportunity to gain both diagnostic and staging information in one relatively non-invasive test.

Pleural fluid cytology is known to have a diagnostic yield of only 60% on average however and additional predictive marker testing is now mandated for a number of the most common malignancies to direct optimal management. As such there is a need to re-evaluate the role of pleural fluid cytology in the diagnosis of those with malignant pleural effusions. The hypothesis of this study was that in many cases pleural fluid cytology is insufficient for full predictive marker testing in common malignancies. The primary endpoint of this retrospective study is therefore to determine the success rate of complete predictive marker assessment in metastatic pleural adenocarcinomas for which established panels of predictive markers exist. Secondary outcomes were to determine if there were any pre-test clinical or radiological features which could better predict the likelihood of PM test success.

CHAPTER 4: Exploring Baseline Immune Response to COVID-19 Infection and its Association with Disease Severity

Finally, it is well established that there is a wide spectrum of presentations and outcomes from those infected with SARS-CoV-2. What there is less known about is the individual immune response responsible for these differing outcomes. An increased understanding of COVID-19 and how each individual's immune response to the virus from the time of diagnosis affects outcome is essential to improve management strategies and survival of this disease.

The hypothesis of my fourth chapter is that varying levels of immune cell activation at the time of diagnosis of COVID-19 are associated with disease trajectory and eventual outcome. The primary endpoint of this study is to assess the level and activation status of common immune cell signalling molecules in those presenting to hospital with COVID-19. I will then determine if there is a difference in these levels between those who require supplemental oxygen and those who do not.

CHAPTER 2:
STAGING BY THORACOSCOPY IN POTENTIALLY RADICALLY TREATABLE LUNG CANCER ASSOCIATED WITH MINIMAL PLEURAL EFFUSION (STRATIFY): PROTOCOL OF A PROSPECTIVE, MULTICENTRE, OBSERVATIONAL STUDY

2 Chapter 2 Staging by Thoracoscopy in Potentially Radically Treatable Lung Cancer Associated with Minimal Pleural Effusion (STRATIFY): Protocol of a Prospective, Multicentre, Observational Study

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2.1 Introduction to this Paper

The central theme of this thesis is early diagnosis and stratification of respiratory disease. One such condition which encapsulates the importance of both of these principals is lung cancer. Accurate staging from the time of diagnosis is fundamental to the management of lung cancer to guide patients onto the most appropriate treatment pathway. One key element of lung cancer staging is to confirm or rule out the presence of distant spread of disease. As outlined in chapter 1, this is most commonly achieved through imaging such as PET-CT and biopsy of any suspicious lesions if required.

There is a subgroup of patients however in whom there is no definite evidence of metastatic disease on imaging but who present with a small pleural effusion. Some such effusions are too small to attempt sampling by aspiration while others may be just large enough to sample but with no proven malignancy on cytology. This leaves treating teams with a decision to make about whether to give these patients the benefit of the doubt and proceed to treatment with curative intent or to consider the effusion metastatic without any confirmatory evidence. There is however

retrospective data to suggest that the majority of such effusions are due to occult pleural metastases.

I therefore undertook a prospective study to define the true prevalence of occult pleural metastases in those with otherwise early stage lung cancer with minimal effusion. The protocol for this study, entitled STRATIFY (Staging by Thoracoscopy in Potentially Radically Treatable Lung Cancer Associated with Minimal Pleural Effusion), is presented here.

This protocol paper is published in BMJ Respiratory Research and the protocol has been presented at both the British Thoracic Society Winter Meeting and British Thoracic Oncology Group Conference as an abstract. The final results following closure to recruitment are currently being analysed and prepared for publication but are not available at the time of submission.

2.2 Abstract

2.2.1 Introduction

Recurrence rate following radical therapy for lung cancer remains high, potentially reflecting occult metastatic disease, and better staging tools are required. Minimal pleural effusion (mini-PE) is associated with particularly high recurrence risk and is defined as an ipsilateral pleural collection (<1/3 hemithorax on chest radiograph), which is either too small to safely aspirate fluid for cytology using a needle, or from which fluid cytology is negative. Thoracoscopy (local anaesthetic thoracoscopy (LAT) or video-assisted thoracoscopic surgery (VATS)) is the gold-standard diagnostic test for pleural malignancy in patients with larger symptomatic effusions. Staging by Thoracoscopy in potentially radically treatable Lung Cancer associated with Minimal Pleural Effusion (STRATIFY) will prospectively evaluate thoracoscopic staging in lung cancer associated-mini-PE for the first time.

2.2.2 Methods and Analysis

STRATIFY is a prospective multicentre observational study. Recruitment opened in January 2020. The primary objective is to determine the prevalence of detectable

occult pleural metastases (OPM). Secondary objectives include assessment of technical feasibility and safety, and the impact of thoracoscopy results on treatment plans, overall survival and recurrence free survival. Inclusion criteria are (1) suspected/confirmed stages I–III lung cancer, (2) mini-PE, (3) Performance Status 0–2 (4), radical treatment feasible if OPM excluded, (5) ≥16 years old and (6) informed consent. Exclusion criteria are any metastatic disease or contraindication to the chosen thoracoscopy method (LAT/VATS). All patients have LAT or VATS within 7 (±5) days of registration, with results returned to lung cancer teams for treatment planning. Following an interim analysis, the sample size was reduced from 96 to 50, based on a lower-than-expected OPM rate. An MRI substudy was removed in November 2022 due to pandemic-related site setup/recruitment delays. These also necessitated a no-cost recruitment extension until October 2023.

2.2.3 Ethics and Dissemination

Protocol approved by the West of Scotland Research Ethics Committee (Ref: 19/WS/0093). Results will be published in peer-reviewed journals and presented at international meetings.

2.2.4 Trial Registration Number

ISRCTN13584097.

2.3 Citation

BMJ Open Respir Res. 2023 Nov 23;10(1):e001771. doi: 10.1136/bmjresp-2023-001771. PMID: 37996118; PMCID: PMC10668291.

2.4 Author Contributions

JF, ST, CK, LA, GC, EB, ME, NR, NM contributed to the conception or design of the work; data acquisition, analysis and interpretation of data for the work. SS, SG, JC, ND, AS contributed to data analysis or interpretation of data for the work. JF, BZ, MS, MN and MT were involved in patient screening and recruitment. JF, ST, CK, LA,

SS, SG, JC, ND, AS, GC, EB, ME, NR, NM were involved in revising the work critically for important intellectual content, final approval of the version to be published, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. KGB provided principal contribution to the conception and design of the work; data acquisition, analysis and interpretation of data for the work; drafting the work; final approval of the version to be published; and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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2.5 Manuscript

2.5.1 Introduction

Lung cancer is the most common cause of cancer-related death. Despite major advances in staging and potentially curative treatments (surgery and radiotherapy (RT)), recurrence rates remain unacceptably high. In patients with stages I, II and IIIA, non-small cell lung cancer (NSCLC) 2-year mortality is currently 15%, 30% (44) and 50% (122), respectively. A likely reason for this is radiologically occult metastatic disease and novel staging tools are urgently required. Recent studies have highlighted minimal pleural effusion (mini-PE) as a marker of particularly high recurrence risk, and excess mortality following radical treatment (25, 36).

Mini-PE has been defined as a small pleural collection ipsilateral to the primary tumour, which is either too small to safely aspirate for a cytology sample, or one that has been aspirated and initial fluid cytology is negative (see figure 2.1). Mini-PE affects up to 25% of patients presenting with NSCLC (25), although half of these occur in patients with metastatic disease (25, 36). Since 2014, two large retrospective series have described clear association between excess mortality following radical treatment for stages I–IIIA NSCLC and mini-PE on diagnostic (pretreatment) CT imaging. These series conclude that mini-PE reflects occult pleural metastases (OPM) in up to 80% of patients (25, 36). However, this is based on indirect evidence and supportive follow-up imaging. In both series, it is acknowledged that other factors may have contributed to the adverse survival observed, including benign pleuritis, systemic comorbidities (123, 124) and undertreatment due to the suspicion of OPM.

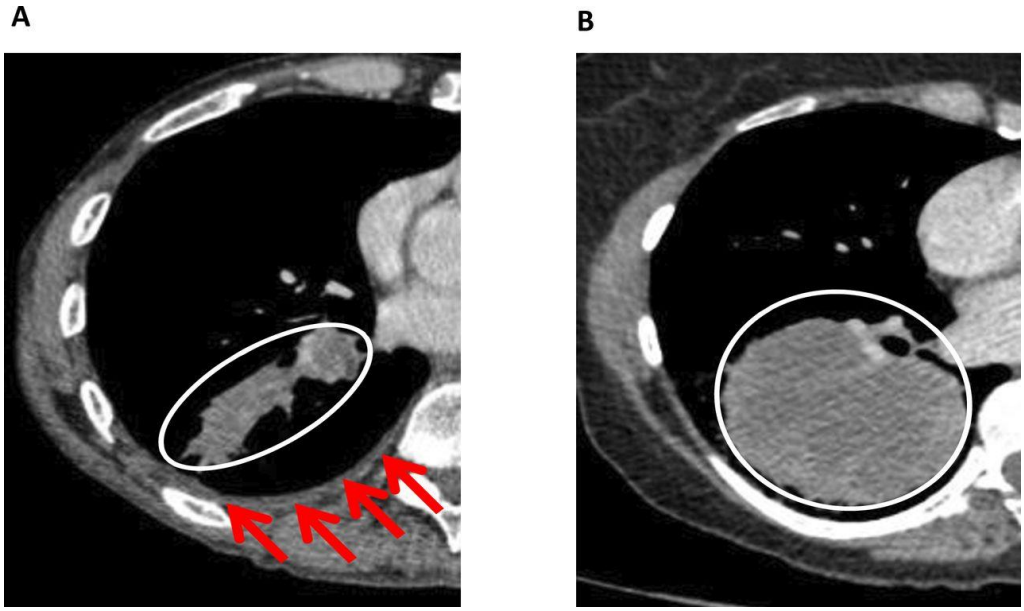

Figure 2.1 Minimal pleural effusion (mini-PE) examples. Both panels show axial plane CT images in patients with non-small cell lung cancer (NSCLC). *(A) A T2b N1 M0 (stage 2A) NSCLC with associated Mini-PE (red arrows). Based on retrospective data, the HR for death in this case is 2.24 relative to T2b N1 without Mini-PE[25]. (B) A T3 N1 M0 (stage 2B) NSCLC without Mini-PE. Both patients have potentially radically treatable disease (circled).*

In 2015, 441 consecutive patients, who presented with NSCLC to Glasgow centres over 6 months in 2009 were reviewed retrospectively. Overall, 167/441 had radically treatable NSCLC (stages I–IIIA) and of these 26/167 (16%) had Mini-PE (20/127) (125). In this study, a marked survival disadvantage was found in patients with mini-PE, as shown in previous series (25, 36). In the Glasgow cohort, more conservative treatment was delivered (more supportive care/palliative RT, less surgery, no radical RT, less chemotherapy) in patients with mini-PE, even though they had apparently radically treatable disease (125). Mini-PE, therefore, appears to confer excess mortality risk, but there is a notable tail on the survival curves reported in all three prior retrospective series with 10%–20% of patients surviving for several years (25, 36, 125). Some mini-PE cases may, therefore, be receiving overly cautious therapy because of inaccurate staging. Precise pleural staging would, therefore, protect patients with OPM from toxicities associated with radical treatments that cannot cure them and encourage radical treatment in patients who can benefit.

Lung cancer staging guidelines that were current at the time of the current study's design either do not specifically address pleural staging (National Institute for Health and Care Excellence CG 121, 2011), or suggest 'a pleural biopsy should be considered' in patients with an effusion, without specifying a modality or biopsy technique (American College of Chest Physicians (2013)) and Scottish Intercollegiate Guidelines Network (SIGN) guidelines (SIGN 137, (2014)). Lung cancer teams are, therefore, reliant on CT, Positron Emission Tomography (PET)-CT and pleural aspiration cytology (if this can be performed), all of which are negative by definition in patients with mini-PE. Even in patients with larger, symptomatic PE, CT is limited by a sensitivity of 68% (95% CI 62% to 75%), with a low negative predictive value (65% (95% CI 58% to 72%)) (26, 126). With regard to semiquantitative PET-CT, a recent meta-analysis concluded this should not be used for pleural staging, based on a pooled sensitivity of 81% (specificity of 74%), and recommended further studies, particularly in mini-PE (28). Using current methods, pleural staging is, therefore, an overly subjective process, with treatment decisions based on incomplete data. Instinctively, clinicians have tended to give patients 'the benefit of the doubt', preferring to risk missed metastatic disease than deny a patient 'potentially' radical treatment. However, the adverse prognosis recently associated with mini-PE demands a more objective strategy, particularly considering the toxicities of radical treatment. Additional data are particularly needed regarding the utility and safety of staging thoracoscopy, since it is plausible that most patients could be staged by this technique, ideally local anaesthetic thoracoscopy (LAT), without recourse to video-assisted thoracoscopic surgical (VATS) thoracoscopy, which requires general anaesthesia (GA).

Pleural Staging by Thoracoscopy

VATS thoracoscopy under GA is likely to be a highly sensitive staging tool for mini-PE (38). In previous studies it has also been combined with pleural lavage cytology (PLC, which involves saline irrigation during surgery in patients without an effusion) (127, 128). However, VATS is not a practical option for all patients, in whom non-surgical treatments are frequently required due to comorbidities or patient choice. In addition, the significance of PLC results is not clear, since positive results might not necessarily preclude surgical resection (127). By contrast, LAT is the gold-

standard diagnostic test for patients with larger, symptomatic effusions and offers diagnostic performance to equivalent to VATS (sensitivity 93% (95% CI 91% to 94%)) and a low major complication rate (2.3% (95% CI 1.9% to 2.8%) (34). LAT can be performed as a day-case in patients with mini-PE/no PE, but its performance and safety profile may differ in mini-PE, and this has never been prospectively evaluated. Staging by Thoracoscopy in potentially radically treatable Lung Cancer associated with Minimal Pleural Effusion (STRATIFY) will determine the true prevalence of OPM using either LAT or VATS, with sites encouraged to offer LAT when it is technically feasible. This will be assessed at a dedicated screening visit when LAT is the method preferred by the local team.

2.5.2 Methods and Analysis

Study Design and Setting

STRATIFY is a multicentre observational trial, which will be performed according to the UK Policy Framework for Health and Social Care Research. The overall study design is summarised in figure 2.2. Sample size and associated assumptions are reported under ‘sample size and statistical analysis plan’ section. Eight UK sites will recruit participants. Site selection was based on the availability of a dedicated pleural disease service offering LAT, or ready access to VATS thoracoscopy as an alternative. All sites required integration with their local lung cancer team.

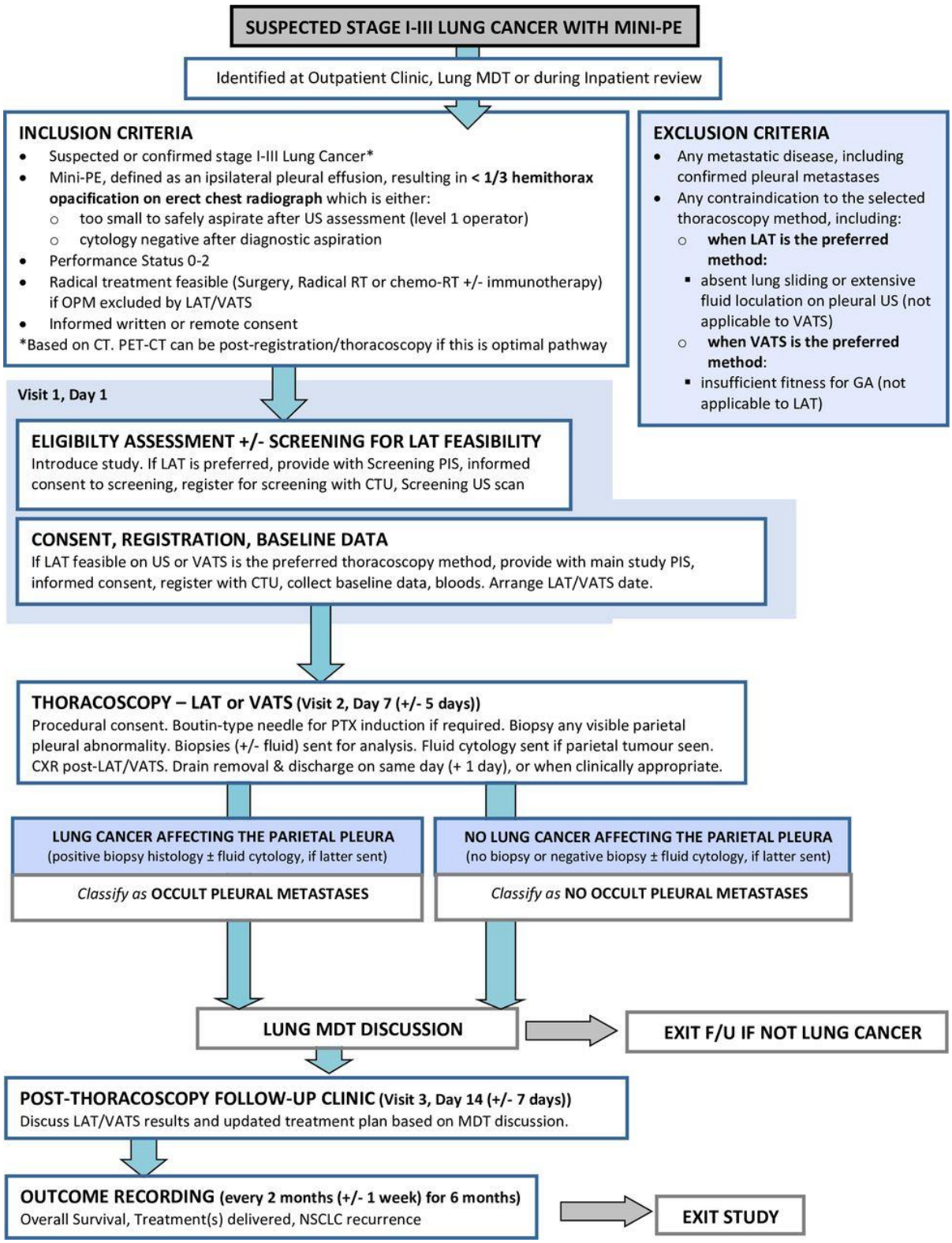

Figure 2.2 Study flow chart summarising the design and major study interventions

CT, Computed Tomography; LAT, local anaesthetic thoracoscopy; MDT, multidisciplinary team; mini-PE, minimal pleural effusion; NSCLC, non-small cell lung cancer; OPM, occult pleural metastases; PET-CT, Positron Emission Tomography-Computed Tomography; RT, radiotherapy; VATS, Video Assisted Thoracoscopic Surgery

Study Objectives and Endpoints

Objectives and their associated endpoints are summarised in Table 2.1.

Table 2.1Study objectives and associated endpoints

	Objective	Associated endpoint(s)
Primary	To determine the prevalence of detectable OPM in patients with suspected or confirmed stages I–III lung cancer and mini-PE	The prevalence of detectable OPM, as defined by the by the proportion of patients with lung cancer affecting the parietal pleura, based on thoracoscopic sampling (LAT or VATS).
Secondary	To determine the impact of thoracoscopy results on recurrence free and overall survival (RFS and OS) in patients with stages I–III lung cancer and mini-PE	► Thoracoscopy results, recorded as: OPM demonstrated/not demonstrated ► RFS, defined as the time from completion of lung cancer treatment to recurrence or death from any cause ► OS, calculated from thoracoscopy to death from any cause
	To determine whether staging thoracoscopy is feasible and safe in patients with stages I–III lung cancer and mini-PE	► LAT feasibility will be recorded as LAT complete/incomplete/not feasible ► VATS feasibility will be recorded as complete /incomplete/not performed Safety will be defined by adverse event (AE) and serious AE rates
	To determine the impact of thoracoscopy results on oncological treatment plans in patients with stages I–III lung cancer and mini-PE	► Thoracoscopy results, recorded as: OPM demonstrated/not demonstrated ► The treatment plan prior to registration The treatment plan following LAT/VATS
Exploratory	To determine the diagnostic performance of blood/pleural fluid biomarkers for OPM and/or adverse outcomes in subsequent studies	Venous blood and pleural fluid samples will be collected but not analysed in this study

LAT, local anaesthetic thoracoscopy; Mini-PE, minimal pleural effusion; OPM, occult pleural metastases; VATS, video-assisted thoracoscopic surgery.

Eligibility Assessment

All patients will be subject to the following eligibility criteria. There will be no exception to the eligibility requirements at the time of registration. Patients are eligible for the trial if all the inclusion criteria are met and none of the exclusion criteria apply.

Inclusion criteria

- Suspected or confirmed stages I–III lung cancer, as defined by at least contrast-enhanced CT*.
- Mini-PE, defined as an ipsilateral PE, resulting in <1/3 hemithorax opacification on erect chest radiograph which is either:

(1) Too small to safely aspirate after US assessment (level 1 ultrasound operator judgement).

(2) Cytology-negative after diagnostic aspiration.

- Performance Status 0–2.
- Radical treatment feasible (surgery, radical RT or chemo-RT±immunotherapy) if OPM excluded by thoracoscopy (local principal investigator (PI) judgement).
- ≥16 years of age.
- Informed written or remote consent.

*All participants will have contrast-enhanced CT prior to registration, and it is expected that PET-CT will also occur preregistration and pre-thoracoscopy. However, PET-CT can be completed after registration and after thoracoscopy if this is considered the optimal pathway for the patient. There are no previous data regarding potential false positive PET-CT pleural findings following thoracoscopy (excluding previous reports related to pleurodesis, which will not be performed here). Nevertheless, this is considered sufficiently unlikely to allow the sequencing of these tests to be decided on a per participant basis.

<u>Exclusion Criteria</u>

- Any metastatic disease, including confirmed pleural metastases.
- Any contraindication to the selected thoracoscopy method, including:

(1) When LAT is the preferred method: absent lung sliding or extensive fluid loculation on pleural ultrasound (not applicable to VATS); this will be assessed at a dedicated screening visit only applicable when LAT is the preferred approach.

(2) When VATS is the preferred method: insufficient fitness for GA (not applicable to LAT).

- Uncorrectable bleeding disorder (applicable to both LAT and VATS).

Note that patients with bilateral PE are not excluded but there should be sufficient suspicion of OPM to justify thoracoscopy (in the opinion of the PI), for example, a larger effusion ipsilateral to the primary disease.

Identification of Participants and Consent

Potentially eligible patients will be identified and assessed by the respiratory physician/site PI coordinating their care or delegated members of the research team. The study can be introduced at earlier clinic visits if eligibility is likely, and this discussion is clinically appropriate. Potential participants will be given sufficient time (in their own judgement) to consider the commitment required to fulfil trial requirements, and to decide whether to participate. Due to the nature of the trial, and since some patients will be attending 'one-stop' clinics, same-day consent is permissible. Patients may choose to defer consent if they require additional time and will be offered a follow-up telephone call with a member of the study team for this purpose. This call will occur no later than 48 hours after visit 1. In addition, all patients will be made aware that participation is voluntary, and they may withdraw at any time without their standard care being affected. No screening activities related to the trial will be undertaken until informed consent has been obtained. Consent can be obtained face to face or remotely. For remote consent, the patient information sheet (PIS) can be posted or emailed to the patient and then remote consent sought, via telephone or videoconference. The study must have been adequately explained to the patient and the patient must have had had the opportunity to ask questions. This must be fully documented in the patient notes. When the subject attends for the first on site clinical visit, consent must be reaffirmed, and signatures of the subject and PI/designee obtained on the consent form. Eligibility will be confirmed by a medical practitioner.

Screening and Registration

If the site PI selects LAT as the optimal thoracoscopy method, formal screening by thoracic ultrasound (TUS) is required as part of visit 1. This is essential to confirm the absence of sonographic exclusion criteria, including absent lung sliding (a surrogate marker of a fixed pleural space not amenable to pneumothorax induction)

(129) or extensive fluid loculation, with specific guidance provided in a dedicated Ultrasound Manual (see online supplemental appendix 1). Either of these features will preclude LAT, but are not relevant for VATS thoracoscopy, where they can be overcome by the surgeon. Therefore, if LAT is the preferred method, an initial screening PIS will be provided, followed by written consent to screening by TUS and allocation of a screening number. The protocol also allows patients to defer formal screening and subsequent consent and registration until the day of LAT (visit 2) if this provides the optimal pathway for the patient or is more practical for the study team. This may be particularly useful if the first study contact is via a remote (eg, video) consultation and/or the patient wishes additional time to consider involvement. Once screening has been completed, eligible patients will be provided with the main study PIS. Those who wish to participate will be registered and a trial number will be allocated. If VATS is selected as the preferred thoracoscopy method, participants are provided with the main PIS immediately, with subsequent consent and registration and without prior TUS screening.

Study Procedures

Baseline data collection

At visit 1, baseline data collected will include lung cancer diagnosis status (lung cancer suspected or confirmed), histological subtype, radiological stage and current (pre-thoracoscopy) treatment plan. Mini-PE laterality, results of any pleural fluid aspiration (if attempted), comorbidities, performance status and staging investigations will also be captured. Baseline organ function and demographics will be recorded.

Local anaesthetic thoracoscopy

LAT is performed under conscious sedation, with the patient in the lateral decubitus position. It allows complete visualisation of the parietal and visceral pleural surfaces and directed biopsies. In larger, symptomatic PEs, LAT is the established gold standard diagnostic test, being well-tolerated and feasible as a day-case. In that setting, LAT offers high diagnostic accuracy (sensitivity 92.6%, specificity 100% n=1369 cases) with a low complication rate (34). The technical feasibility, safety

profile and diagnostic performance of LAT in mini-PE will be recorded in the current study since certain procedural modifications are necessary in this setting. The primary adaptation needed is use of a Boutin-type pneumothorax induction needle (34, 130) following TUS marking of a suitable entry point, sterile field creation and local anaesthetic infiltration at the chosen access site. This introduces a small volume of air into the pleural space, allowing the lung to drop away from the chest wall under conditions of atmospheric (rather than physiologically negative) pleural pressure and subject to gravity. This ensures the lung is not immediately adjacent to the chest wall during the next stage of the procedure which involves blunt dissection and placement of a 7 mm port to act as conduit for the thoracoscope. A dedicated thoracoscopy manual is provided for sites, outlining this and other study specific procedures (see online supplemental appendix 2). These include directions to biopsy only visible abnormalities on the parietal pleura surface; visceral pleura is not sampled during LAT due to the risk of air-leak. LAT operators are required to complete the thoracoscopy worksheet, included in the thoracoscopy manual and an LAT report form for review by the lung multidisciplinary team (MDT). This latter item (see online supplemental appendix 3) is uploaded onto the electronic health record system (EHR), ideally immediately after the procedure. During LAT, pleural fluid is only sent for routine cytological assessment if pleural biopsies have been taken. This is to maximise diagnostic yield in patients with visible parietal pleural lesions, while avoiding unhelpful uncertainty in patients with macroscopically normal pleura. This uncertainty arises from previous studies of PLC, which although not directly equivalent to LAT fluid cytology, suggested that positive PLC did not dramatically reduce survival in patients who had surgical resection despite this observation (127). In this setting, therefore, pleural fluid will be banked for later analysis only.

VATS thoracoscopy

VATS thoracoscopy offers similar high diagnostic sensitivity to LAT and is also safe with a low complication rate (30, 131). However, the procedure requires GA, intubation and single lung ventilation and is therefore not suitable for all patients, including those with major comorbidities. Study-specific guidance for VATS is provided in the thoracoscopy manual (see online supplemental appendix 2),

including instructions to only send fluid for cytology if parietal pleural lesions are sampled, as per LAT, for the same reason described above. During VATS, the operator can sample visceral pleural lesions if clinically indicated, since this is standard practice, with routine options available to manage any resulting air-leak. Participants with positive visceral pleural biopsies will not be classified as OPM positive as per the prespecified definition of the primary endpoint.

Translational research samples

A single blood draw for later translational research will be collected at either visit 1 (eligibility assessment±screening, consent and registration) or visit 2 (LAT/VATS). Pleural fluid samples will be collected during LAT or VATS (visit 2). All samples will be processed and stored in a −80°C freezer within 2 hours of collection. Serum, plasma and pleural fluid samples will all be centrifuged at 2200 g for 15 min at room temperature prior to freezing while whole blood will be frozen immediately without prior processing. Detailed guidance is provided in the sample handling manual (online supplemental appendix 4).

Post-thoracoscopy results and MDT feedback

Site teams will upload the LAT report form to the EHR and ensure the patient is listed for the next Lung MDT meeting. This records whether parietal pleural biopsies±pleural fluid samples were sent for routine pathology assessment. The primary endpoint (OPM positive or OPM negative) will be recorded in the study case report form. The final staging and post-thoracoscopy management plan will also be recorded using EHR, but the study team will have no direct input to this decision-making process. A single post-thoracoscopy visit (visit 3) will occur 7 (±7 days) days after thoracoscopy (visit 2). This visit can be virtual or face to face depending on local arrangements. Subsequent study follow-up will involve 2-monthly remote recording of adverse events (AEs), survival, treatment(s)±recurrence.

Survival

Overall survival (OS) will be recorded from date of registration until death from any cause. Participants alive at 6 months will be censored. Recurrence-free survival

(RFS) will be recorded from treatment completion date to disease recurrence or death from any cause.

2.5.3 Statistical Considerations

Sample size

The target sample size of 50 patients will allow estimation of the prevalence of OPM, AE rate and the impact on treatment plans with 95% CI bounds not exceeding 10% if the OPM prevalence is ≤15%. This represents a change in estimated prevalence, which was initially set at 70% (requiring a minimum sample size of 96). The initial OPM estimate of 70% reflected solely the retrospective data previously reported (25, 36). The updated estimate of OPM prevalence and sample size calculation acknowledges data from the first 12 recruits to STRATIFY, of whom only one case of OPM has been observed (8.3% OPM rate) (132). The reduction in the sample size from 96 to 50 cases means the trial will no longer have adequate power (80%) to detect an OS HR of 0.5 as planned in previous iterations of this protocol. This original HR corresponded to data from previous retrospective studies, which reported a median OS in OPM positive 6.32 months vs 12.65 months in OPM negative cases (36). OS differences between OPM positive and OPM negative groups will nevertheless be reported. Post hoc power calculations taking account of the observed prevalence will be performed.

Statistical Analysis Plan

Primary efficacy analysis: The estimate of the proportion of cases demonstrating OPM (OPM positive) and the associated 95% CI will use standard statistical methods. The CI will be based on the Clopper-Pearson exact approach.

Secondary efficacy analyses: The estimate of the proportions of OPM demonstrated/not demonstrated and LAT complete/LAT incomplete and the associated 95% CIs will use standard statistical methods. The CI will be based on the Clopper-Pearson exact approach. The comparison of the RFS and OS between OPM positive and OPM negative patients will be illustrated with Kaplan-Meier curves; the

HR will be estimated using Cox regression. AE data and the impact on oncological treatment plans will be summarised in tables and listings.

Exploratory Analysis: The number of recruits with banked samples suitable for later analysis will be reported but no other analysis will be performed under this protocol.

Safety Analysis: AE data will be summarised in tables and listings.

2.5.4 Patient and Public Involvement

The study has benefited from patient and public involvement (PPI) input throughout the design stage, including input to the original funding application, the study protocol and the content and language used in all patient facing materials, for example, PIS/consent forms. EB (our PPI representative) was a coapplicant on the study funding application in 2018 and has remained involved since. This has included attendance at monthly study management group (SMG) meetings and input to all protocol amendments and any updated patient facing materials.

2.5.5 Changes to Protocol

The protocol described here reflects the current version 5.0, dated 16 November 2022. The following changes were made in previous versions:

V.2.0, dated 26 June 2020

- The definition of the primary endpoint (OPM) was clarified to make it clearer that pleural fluid samples could be sent for routine cytology assessment, alongside parietal pleural biopsies if these were taken

V.3.0, dated 25 February 2021

- The maximum size used to define mini-PE in the inclusion criteria was changed from <40 mm maximum depth on axial CT images to an effusion occupying <1/3 of the hemithorax on erect chest radiograph. The original definition (drawn from previous retrospective mini-PE studies)(25, 36) proved difficult to deploy reliably in practice due to variation in where the user could make this measurement.

- Schedule of assessments updated to include a COVID swab prior to thoracoscopy (Visit 2), in line with COVID-19 guidance at that time.

At this point, a major protocol amendment was undertaken to address significant recruitment challenges, including (1) a change in the diagnostic pathway for lung cancer prompted by COVID-19, with a move to virtual consultations in many centres, (2) low lung cancer referral rates, which dropped by 60% in some networks and (3) significant delays in opening sites due to UK-wide prioritisation of Urgent Public Health-badged studies. One-to-one sessions with our current sites revealed a series of changes to patient flow and visit scheduling that would make the current protocol, which assumed as series of sequential face to face visits, undeliverable. These discussions also identified other recruitment barriers including the handling of tiny contralateral effusions (currently an exclusion criterion) and use of surgical thoracoscopy (under general anaesthetic) which has become more available in some centres since the original protocol design. Based on this feedback and following PPI review, v 4.0, dated 19 November 2022 was deployed implementing the following changes:

V.4.0, dated 16 November 2022

- Introduction of remote verbal consent as an option, with subsequent written consent at next contact.
- Allowing completion of screening, consent, registration and baseline data collection on the day of thoracoscopy if this aligns better with local pathways and patient preference.
- Allowing recruitment earlier in the diagnostic pathway so that STRATIFY pleural staging can be completed without prior histological confirmation of NSCLC. This reduces the burden of invasive tests and necessarily broadens the eligibility criteria to ‘suspected or confirmed stages I–III lung cancer’.
- Allowing STRATIFY pleural staging to be performed by surgical thoracoscopy (ie, under general anaesthetic); this opens the study to centres without access to LAT.

- Allowing inclusion of cases with bilateral PEs, assuming the collection ipsilateral to the primary is judged to be suspicious, for example, asymmetrically large, with a small contralateral effusion.

A further, final protocol amendment was then made to ensure the study could report on the primary endpoint within its original funding envelope by extending the original recruitment period to 31 October 2023 via a no cost extension from the funder (CSO). This involved the following additional changes:

V.5.0, dated 16 November 2022

- Removal of the MRI substudy, which involved perfusion MRI after registration and prior to thoracoscopy. Overall, 3/42 recruits had completed MRI by this time and these data will be reported in the results publication.
- Reduction of the sample size from 96 to 50. This was based on review of the OPM prevalence in the first 12 participants (see the Sample size section) and will allow the primary endpoint to be reported with the same precision as originally intended, but with a more realistic recruitment target.

2.5.6 Definition of End of Study

The end of study definition will be the date of last data capture, which will be met when all outstanding data has been returned from all sites, all required data queries have been resolved and the database is finalised for analysis.

2.5.7 Monitoring, Data Management and Quality Assurance

No routine site or telephone monitoring will be performed. If issues arise, an on-site visit or telephone monitoring call will be arranged. The Cancer Research UK (CRUK) Clinical Trials Unit (CTU) will regularly chase outstanding data and queries. Routine requests for missing or queried data will occur quarterly.

2.5.8 Safety Considerations

All AEs and serious AE (SAEs) thought to be related to study procedures will be recorded. This includes AEs resulting from ultrasound, chest radiographs, venous

blood sampling, LAT or VATS. Although the MRI substudy has been removed from the current protocol, any AEs or SAEs related to the MRI in the three patients recruited to the substudy prior this point will be reported. This will include any events related to image acquisition, including administration of gadolinium contrast, or the X-ray of orbits (if required) which were the only additional AEs recorded. Safety reporting is overseen by the Pharmacovigilance Department of the CRUK CTU Glasgow as delegated by the trial sponsor.

2.5.9 Dissemination

Study results, including those related to the MRI substudy removed from the current protocol version, will be presented at national and international scientific meetings and published in full in a peer-reviewed journal.

2.5.10 Study Management

STRATIFY will be coordinated from the CRUK Glasgow CTU. The SMG, comprising the chief investigator, selected coinvestigators, project manager, statistician, trial coordinator, PV coordinator, PPI representative and IT programmer meet monthly to oversee the study.

2.5.11 Data Availability Statement

Data are available on reasonable request. Study results, including those related to the MRI substudy removed from the current protocol version, will be presented at national and international scientific meetings and published in full in a peer-reviewed journal.

2.5.12 Ethics Statements

Patient Consent for publication

Not applicable.

Ethics Approval

This study involves human participants and was approved by West of Scotland Research Ethics Committee (Ref: 19/WS/0093). Participants gave informed consent to participate in the study before taking part.

2.5.13 Acknowledgements

The authors acknowledge CSO (Scotland) as the study funder, participating sites and staff, and the patients involved.

2.6 Results

Following closure of the study and analysis of the data, the results of STRATIFY are presented here in addition to the above published protocol paper.

2.6.1 Study Cohort

A total of 27 patients were recruited to STRATIFY (Figure 2.3)

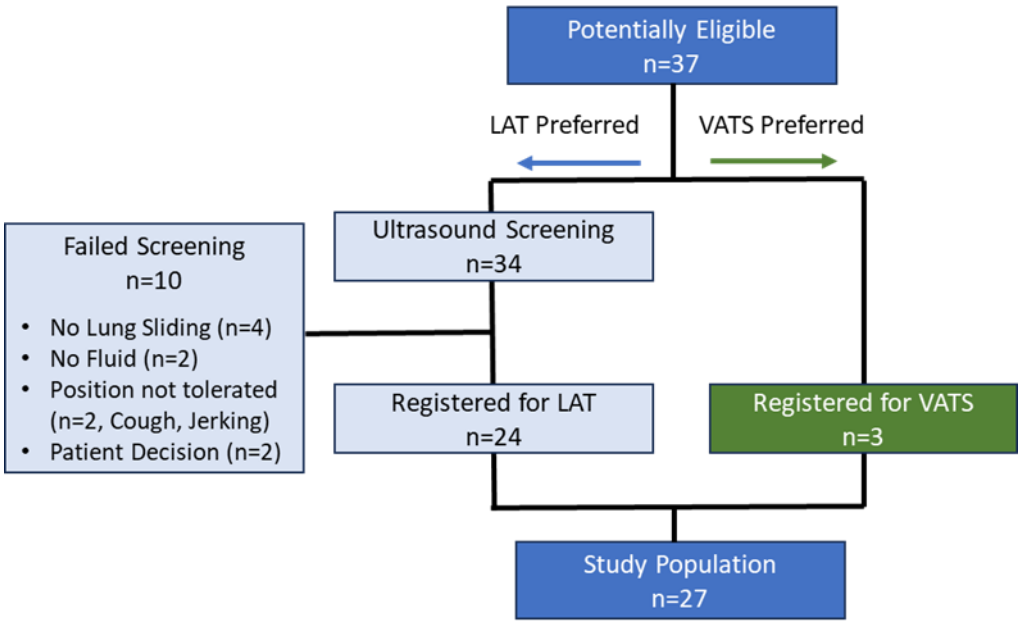

Figure 2.3 Study recruitment flowchart

The average age of participants was 69 (SD 9.1) years and 70% (19/27) were male. A full breakdown of baseline characteristics can be found in table 2.2

Table 2.2 STRATIFY cohort characteristics

Age	69.2 (9.1) years
Male	19/27 (70.4%)
Current smoker Ex-smoker	11/27 (40.7%) 16/27 (59.3%)
Performance Status 0 1 2	 7/27 (25.9%) 13/27 (48.1%) 7/27 (25.9%)
MRC Dyspnoea Scale 0 1 2 3 4 N/A	 0/27 (0%) 6/27 (22.2%) 12/27 (44.4%) 5/27 (18.5%) 1/27 (3.7%) 3/27 (11.1%)

2.6.2 Pleural Effusion Characteristics

19/27 (70%) of mini-PEs in STRATIFY were too small to safely aspirate and the remaining 8/27 (30%) were cytology negative. The median depth of mini-PE on ultrasound was 2cm (IQR 1-5cm).

2.6.3 Pre-thoracoscopy histology and staging

Pre-thoracoscopy histology and staging are reported in table 2.3.

Table 2.3 Pre-thoracoscopy histology and staging

Histology	
Adenocarcinoma	4/27 (14.8%)
NSCLC NOS	3/27 (11.1%)
Squamous cell	14/27 (51.9%)
N/A	6/27 (22.2%)
Stage	
IA	1/27 (3.7%)
IB	2/27 (7.4%)
IIA	2/27 (7.4%)
IIB	2/27 (7.4%)
IIIA	4/27 (14.8%)
IIIB	3/27 (11.1%)
IIIC	1/27 (3.7%)
Incomplete	7/27 (25.9%)
N/A	5/27 (18.5%)

2.6.4 Primary Outcome

4 out of the 27 patients recruited were found to have pleural metastases. This gives an OPM prevalence of 14.8% with a 95% confidence interval of 4.2 – 33.7%.

Despite the reduced precision of this confidence interval due to the under-recruitment to STRATIFY (27 of the 50 target), this prevalence remains significantly lower than those previously suggested by retrospective data (25, 36).

2.6.5 Secondary Outcomes

Our results show that staging thoracoscopy is technically feasible and safe. Thoracoscopy was technically possible in 96% (25/26) of those attempted within STRATIFY (Figure 2.4). Only two adverse events were reported (2/27, 7.4%) – one case of post-procedure subcutaneous emphysema and one case of post-procedure pain. Both of these occurred post-LAT and neither were significant adverse events.

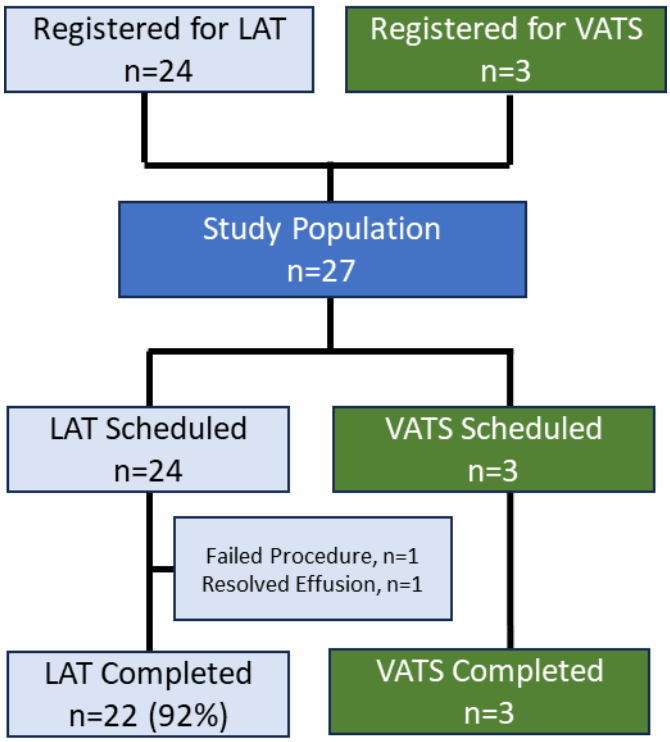

Figure 2.4 Feasibility of staging thoracoscopy within STRATIFY

2.6.6 Clinical Impact of Staging Thoracoscopy

In addition, we found that staging thoracoscopy is clinically useful. In our cohort, post-thoracoscopy staging changed in 19% (5/27) of cases while the MDT treatment plan post-thoracoscopy was changed in 30% (8/27) of cases (Figure 2.5).

Post-LAT staging changed in 5/27 (19%)

LAT	T4 N3 M0 → T2b N0 M0
LAT	T4 N1 M0 → T4 N1 M1a
LAT	T4 N2 M0 → T4 N2 M1a
LAT	T4 N1 M0 → T4 N2 M1b
LAT	T2a N0 M0 → T2a N0 M1b
VATS	No change
VATS	No change
VATS	No change

MDT treatment plan changed in 8/27 (30%)

Surgery	Radical RT
Radical RT	Radical Chemo-RT +/- IO
Radical Chemo-RT +/- IO	Palliative Chemotherapy
Radical RT	Best Supportive Care
Surgery	Palliative Chemotherapy
Surgery	Radical RT
Surgery	Radical RT
Surgery	Radical Chemo-RT +/- IO

Figure 2.5 Changes to staging and treatment plans at post-thoracoscopy MDT

2.7 Reflections on This Paper

The STRATIFY study faced many challenges during its recruitment period, primarily as a result of the outbreak of COVID-19. After opening for recruitment in Glasgow and enrolling the first patient, we had to pause further recruitment due to the emergent pandemic. This also delayed other sites opening as research focus and resources shifted to COVID-19 trials. In total, STRATIFY recruitment was paused for 11 months.

In addition, the COVID-19 pandemic led to many changes to the diagnostic pathway for lung cancer patients as well as a significant drop in lung cancer referral rates (of up to 60% in some centres). After discussions and feedback from sites we made multiple amendments to the STRATIFY protocol as outlined in the 'Changes to Protocol' section above to keep study activity in line with routine clinical care wherever possible. We also made the decision to focus solely on the intended primary outcome of STRATIFY (to determine the true prevalence of occult pleural metastases in suspected early stage lung cancer with mini-PE) which based on updated sample size calculations according to our preliminary results allowed us reduce our target recruitment from 96 to 50 patients (132).

As well as working on the protocol, site set up and recruitment for STRATIFY, I developed my thoracoscopy skills and personally performed a number of the STRATIFY thoracoscopies under supervision from Professor Blyth. I was also involved

in all protocol amendment decisions throughout STRATIFY from opening to study closure.

The first amendment made was to clarify the role of pleural fluid cytology in STRATIFY. To maximise diagnostic yield where pleural biopsies were taken it was clarified within the protocol that in such cases, pleural fluid cytology could be sent alongside these biopsies. This clarification was made after I received repeated queries from sites regarding pleural fluid sampling. This amendment was introduced as fluid samples would be sent routinely in standard of care thoracoscopies where biopsies are taken in patients who had visible pleural abnormalities at LAT.

In version 3.0 we altered the definition of mini-PE. I realised after talking to other members of the local team and to those from other sites that measurement of effusion depth on CT was not being performed uniformly between individuals or sites. The decision was made therefore to base effusion size on erect CXR to minimise variability between recruiters. The decision to define mini-PE as <1/3 of the hemithorax was taken pragmatically to maximise potential recruits after discussion with sites.

Protocol version 4.0 was devised during COVID at a time of very low recruitment. The changes outlined in version 4.0 above (namely, allowing remote consent, a condensed face to face visit schedule and the inclusion of bilateral effusions where the ipsilateral fluid was felt to be suspicious) were all introduced based on my own experience from screening and after I met with all other recruiting sites. Each site gave their own suggestions as to what would work best for them given how significantly lung cancer pathways had changed since the outbreak of the pandemic. I took all of these suggestions to the next STRATIFY TMG and wherever possible all suggestions by sites were included as long as it did not impact negatively on the patients or affect the ability to meet our primary endpoint. In version 4.0 of STRATIFY we also opened recruitment to sites with access to VATS again as a way of further maximising recruitment while still being able to report the primary outcome as set out in the original protocol.

The final amendments in version 5.0 of the STRATIFY protocol were firstly the decision to remove the MRI sub-study and secondly to reduce our recruitment target

from 96 to 50. The decision to remove the MRI sub-study was a financial one as we had been given a no-cost extension by the CSO and did not have the budget to further recruit to the sub-study while extending recruitment to the main study.

The last amendment to reduce the sample size was based on interim review of the first 12 recruits. Of these patients, only one (1/12, 8.3%) was found to have OPM. Based on work by Arya et al (132). it was therefore calculated that with a prevalence of 10% a target of 50 patients would allow us to report the primary outcome with the same level of precision as our previously planned target of 96 based on a hypothesised OPM prevalence of 70%. Given the primary outcome of STRATIFY was of course the reason for undertaking the study I agreed that this change was necessary even at the expense of some of our secondary and exploratory outcomes in order to meet our primary endpoint within the limits of our no-cost extension.

As well as protocol development and study set up, my work on STRATIFY showed me the importance of continually assessing progress while being adaptable and open to change. If we had kept STRATIFY as it was in version 1.0, we would not have been able to achieve the recruitment we did. The challenges I encountered during STRATIFY showed me that you must always put the research first rather than any ego or sentimentality about the original protocol. This was shown to me by the fact that many of the amendments were made as a result of suggestions from other sites and team members. It was only by embracing any and all suggestions and evaluating them in the context of the study that these amendments (and subsequent further recruitment) was possible.

CHAPTER 3: PLEURAL FLUID PREDICTIVE MARKER TESTING IN METASTATIC LUNG AND BREAST ADENOCARCINOMA

3 Chapter 3 Pleural Fluid Predictive Marker Testing in Metastatic Lung and Breast Adenocarcinoma

Full author list: Jenny Ferguson, Christopher Craig, Victoria Randles, Louise Brown, Duncan Fullerton, Matthew Evison, S. Tsim, Kevin G Blyth

3.1 Introduction to this Paper

Once metastatic malignancy is proven, the focus then shifts to selecting the most appropriate systemic therapy for those patients who are willing and fit for treatment.

Pleural fluid where present is often the first tissue sampled in the diagnosis of suspected malignancy. Where malignant effusion is proven this signifies metastatic disease and therefore systemic therapies become the mainstay of treatment. There now exist a variety of such therapies over and above traditional chemotherapy agents. Many of these newer therapies target specific characteristics of the underlying tumour cells which therefore must now be tested for in the routine diagnostic work up of advanced malignancy. The presence of these characteristics predicts response to corresponding therapies and are therefore known as predictive markers.

Although the overall diagnostic yield of pleural fluid is known, what is not so well established is the utility of pleural fluid for predictive marker testing. Building on the work of STRATIFY outlined in the last chapter which was focussed on the diagnosis of malignant pleural involvement in lung cancer, the next chapter moves beyond establishing a diagnosis of malignant pleural effusion and shifts focus to the use of pleural fluid in determining the most appropriate SACT through the testing of pleural fluid for predictive markers.

This paper is currently under review for publication in Respirology and an abstract for this work was recently presented as a poster at the 2024 European Respiratory Society Congress in Vienna.

3.2 Abstract

3.2.1 Introduction

Malignant pleural effusion (MPE) is common in lung and breast adenocarcinoma. Effusion cytology is often diagnostic, but multiple predictive markers (PM) are needed to optimally plan systemic anticancer therapy (SACT).

3.2.2 Methods

We performed a multicentre, retrospective cohort study at 4 UK centres. Data were retrieved for patients with lung and breast adenocarcinoma on pleural cytology. PM success (%) was defined as completion of all markers required by international guidelines. Associations between PM success and demographics, effusion and imaging features were evaluated by chi square test +/- multivariate regression. PM success, individual marker yield and effusion PM marker-directed SACT rate were compared between 2016-2018 and 2018- 2021.

3.2.3 Results

PMs were evaluated in 327 patients (222 Lung, 105 Breast) using individual Polymerase Chain Reaction (PCR), Immunohistochemistry (IHC) or Fluorescence In-situ Hybridization (FISH) tests. All PMs were available in only 20% (66/327; 19% Lung, 22% Breast). Individual marker yields were: Anaplastic Lymphoma Kinase (ALK): 84%, Epidermal Growth Factor Receptor (EGFR): 62%, Programmed Cell Death-Ligand 1 (PD-L1): 36%, ROS proto-oncogene 1 (ROS-1): 34%; Estrogen Receptor (ER): 93%, Progesterone receptor (PR): 88%, Human Epidermal Growth Factor Receptor 2 (HER2): 57%. Clinico-radiological features were not associated with PM success. PD-L1 (IHC), ROS-1 (IHC +/- FISH), ER (IHC) yield, and effusion PM marker-directed SACT increased over time (25% to 57%, p=0.002). Median reporting time was 19(9-24) days, overlapping with time to SACT initiation (31(4-45) days).

3.2.4 Conclusion

MPE PM testing is frequently incomplete. Direct-to-biopsy stratification may be appropriate in selected patients. Genomic sequencing may improve future effusion utility.

3.3 Author Contribution

KGB and ST conceived the study. JF, CC, VR, LB, DF, ME collected and cleaned study data. JF and KGB performed the data analysis and interpretation. JF created the first draft of the manuscript. All authors reviewed and approved the final manuscript.

JF Jenny Ferguson

ST Selina Tsim

CC Christopher Craig

VR Victoria Randles

LB Louise Brown

DF Duncan Fullerton

ME Matthew Evison

KGB Kevin G Blyth

3.4 Manuscript

3.4.1 Introduction

Malignant pleural effusion (MPE) is common in advanced malignancy, with most cases attributable to lung or breast adenocarcinoma(133). In this setting, prompt completion of the diagnostic process and rapid access to therapy is essential given the adverse prognostic impact of metastatic pleural disease. In lung cancer-associated MPE, median survival is <3 months in some series(134), with a one-year mortality of 80%(21). For this purpose, pleural fluid aspiration provides ready access

to tumour cells, resulting in an average diagnostic yield of 60%, with performance varying by tumour cell type(29). Simple aspiration is therefore not only convenient but may obviate the need for more invasive histological sampling in many patients. However, modern oncological treatment pathways also require predictive markers (PMs), in addition to diagnostic information, typically in the form of a panel of markers, which are all needed to inform selection of optimal systemic anticancer therapies (SACT).

This requirement for PM testing has been formalised in international guidelines, including those from the European Society for Medical Oncology (ESMO) for metastatic lung and breast cancer(7, 54). Use of effusion PM testing in this way may have a strong positive effect on outcomes by helping clinicians select the most effective SACT regime from an increasingly complex list of options. These may include targeted therapies, such as tyrosine kinase inhibitors (TKIs) for Epidermal Growth Factor Receptor (EGFR)-mutant and Anaplastic Lymphoma Kinase (ALK)/ROS proto-oncogene 1 (ROS-1) translocated lung cancers(7) , monoclonal antibody therapy for Human Epidermal Growth Factor Receptor 2 (HER2)- expressing breast cancers(54), immune-check point inhibitors (ICIs) for Programmed Cell Death Ligand 1 (PD-L1)-expressing lung cancers(7) and hormonal therapies (e.g. aromatase inhibitors) for Estrogen Receptor (ER)-expressing breast cancers(54). For this purpose, having complete PM panel results is important since some agents take precedent over others when both are expressed (e.g. a TKI would commonly be used first for an EGFR-mutant lung cancer also expressing PD-L1)(7). Having only the PD-L1 result, with a failed EGFR assay would therefore not be optimal.

Reliance on effusion PM markers also brings additional burdens and risks, including delay in the time to treatment initiation, which can be critical in some patients with metastatic MPE. The additional time needed to complete effusion PM panels is clearly worth it if yields from the tests involved are high. However, if many of the markers fail and subsequent histological sampling is frequently needed, it may be more effective to proceed to direct-to-biopsy. This was evident in a recent UK National Lung Cancer Audit report, in which, patients who underwent pleural aspiration were most likely to require additional sampling(135). Data supporting a direct-to-biopsy approach for diagnostic purposes was recently reported by Tsim et

al. In that study, the yield of effusion cytology was extremely low in patients with asbestos exposure and a malignant-looking computed tomography (CT) scan (negative predictive value 9%), supporting direct-to-biopsy in patients with these features at first clinic review(23). Similar data has yet to be reported in a sufficiently sized study regarding the predictive effusion markers but could theoretically allow further stratification of the pleural diagnostic pathway. These data might for example direct omission of pleural fluid PM testing (saving the time taken to return these results) in patients with baseline features associated with a high pre-test probability of incomplete PM findings. Tsim et al previously reported that lung and breast adenocarcinoma were associated with increased risk of incomplete PM results (Odds Ratio (OR 9.7 (95% CI 3.14–29.97) and OR 22.43 (95% CI 4.86– 103.55))(23). However, only 92 suitable cases were studied, precluding any rigorous analysis of baseline features as predictors of incomplete PM results. For this purpose, the cancer type found in the effusion cytology sample cannot be considered a baseline feature since it is only known after the sample has been taken. A similar earlier study by Mercer et al also reported that effusion PM results were insufficient to guide management in most cases(136).

We performed the current study to describe the success rate of effusion PM panels in a large, multi-centre cohort drawn from 4 UK centres. We collected baseline phenotyping information, including demographics, CT appearances, and effusion size and laterality. Importantly, we defined PM marker success as completion of all markers recommended by contemporaneous ESMO guidelines (since all markers are needed by treating oncologists). We also examined the tests used to record each marker, the yield of individual makers, the time taken to report these results and how that interval compares with time to SACT initiation. We intentionally focused exclusively on lung and breast adenocarcinoma, which in addition to ovarian cancer, cause the majority of secondary MPE cases(136). We excluded ovarian cancer because suitable assays were not available in the centres involved over the period of data collection. However BRCA 1/2 mutation testing is recommended by ESMO guidelines(57), and suitable assays have since been validated in effusion samples(137), making this an important topic for future work.

3.4.2 Materials and Methods

Study Design

A retrospective, multi-centre cohort study was performed at four UK sites (Queen Elizabeth University Hospital Glasgow, North Manchester General Hospital, Manchester Royal Infirmary, Mid-Cheshire Hospital Trust). The study was classified as service evaluation; therefore the protocol was approved by the local Caldicott Guardian at each site.

Study Objectives and Outcome Measures

The primary objective was to determine the success rate of pleural fluid PM panels in metastatic lung and breast adenocarcinoma. This was defined as completion of all markers (whether positive or negative) required by contemporaneous ESMO guidelines(7, 54) and reported as a simple proportion(%). A sub-group analysis of cases with PS 0-2, in whom SACT would typically be feasible, was also performed. Successful completion rate was also reported for each individual marker, i.e. EGFR, ALK, PD-L1 and ROS-1 for lung cancer and ER, PR and HER-2 for breast cancer.

Secondary objectives included determination of:

(1) the time taken to report PM results, measured from the date of pleural fluid aspiration in days.

(2) clinico-radiological features associated with incomplete PM testing results. Potential features for this analysis included demographics, cancer type, effusion size (≤1/3, 1-3 to 2/3, ≥2/3 opacification of erect chest radiograph prior to aspiration), effusion laterality, the presence of previously reported computed tomography (CT) imaging features of malignancy and pleural effusion aspiration volume (ml).

(3) any change in the overall PM success rate and the yield of individual makers over time. For this analysis, the cohort was divided into two sequential time periods (2016-2018 and 2018-2021).

(4) any change in the rate of SACT receipt, including pleural effusion PM marker-directed SACT receipt over the same two sequential time periods

(2016-2018 and 2018-2021). This analysis was restricted to Glasgow cases in whom this data was available.

Secondary objectives (3) and (4) were analysed post hoc based on observed patterns during the initial analysis of the data.

Identification of Cases and Eligibility Criteria

All cases were identified using electronic local pathology department records. Cases were potentially eligible if they had adenocarcinoma diagnosed on pleural fluid cytology at any of the 4 study centres between January 2016 and October 2018. Glasgow cases were also potentially eligible if diagnosed between October 2018 and December 2021. All pathology reports were reviewed, and the following exclusion criteria applied: (1) any adenocarcinoma other than lung or breast adenocarcinoma (including ovarian) (2) fluid sample not sent for predictive marker testing (3) clinical data not available in electronic record (4) fluid sample only sent for T7090M testing. At the point of data collection, this marker was being selectively used in the study centres involved to select patients with EGFR mutant lung adenocarcinoma for potential second-line treatment with Osimertinib and was not part of routine predictive marker testing as outlined by ESMO guidelines(7, 54).

Data Collection

The following data were extracted retrospectively for each included case using electronic health records at each site: sex, age and performance status at pleural fluid aspiration, date of aspiration, volume of fluid sent for analysis and analysis method. The result of each pre-specified PM was recorded, alongside the laboratory method used where this was stated and the date of PM reporting. The date of first oncology review was recorded as was receipt of any SACT and effusion PM-directed SACT (e.g. TKI where the effusion EGFR result was positive). In Glasgow cases only, data regarding effusion size, effusion laterality and the presence of previously reported CT imaging features of malignancy were also recorded. This data was not available at other sites.

Statistical Analysis

Data are reported as mean (standard deviation) or median (interquartile) based on distribution. PM success rate was reported as a simple proportion (%), including a sub-group analysis in cases with PS 0-2. Potential univariate associations between clinico-radiological features and PM success (all PMs successful vs some PMs unsuccessful) were tested using chi square or Fisher's exact test for categorical values. Continuous data were compared using unpaired t-test or Mann Whitney test depending on distribution. Multivariate logistic regression was planned for any significantly associated features (defined by $p<0.1$). Since the focus of the project was service evaluation, a sample size calculation was not performed.

3.4.3 Results

Study Population

702 potentially eligible cases were identified of which 327 met all eligibility criteria and were included (see Figure 3.1). Patient characteristics are summarised in Table 3.1.

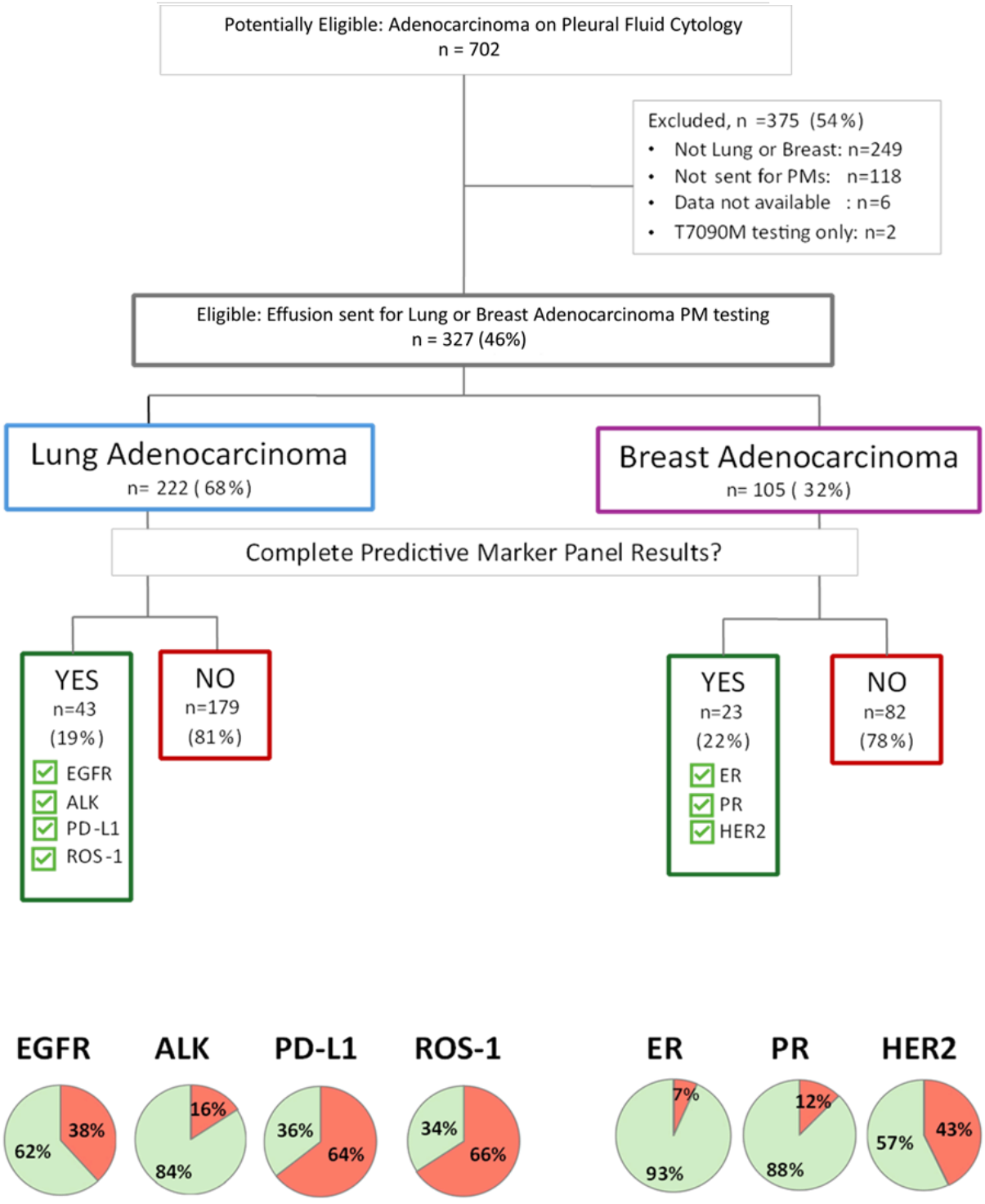

Figure 3.1 Study flow chart summarising selection of eligible cases, the success rate of predictive marker (PM) testing, as defined by completion of all assays specified in international guidelines. *The individual success rate of each individual marker testing is also shown in pie charts with green representing successful testing of the PM.*

Table 3.1Clinico-radiological characteristics of 327 patients with lung and breast adenocarcinoma, in whom pleural fluid was sent for predictive marker analyses.

Age	69 (12.3)
Female Sex	217/327 (66.4%)
Performance Status	
0	31/327 (9.5%)
1	79/327 (24.2%)
2	59/327 (18%)
3	59/327 (18%)
4	18/327 (5.5%)
Not Available	81/327 (24.8%)
Cancer Cell Type	
Lung	222/327 (68.2%)
Breast	105/327 (32.7%)
Effusion Laterality*	
Right	113/211 (53.6%)
Left	98/211 (46.4%)
Effusion Size*	
≤ 1/3	52/211 (24.6%)
1-2/3	95/211 (45%)
≥2/3	61/211 (28.9%)
Not Available	3/211 (1.4%)
Malignant CT Features*	
Yes	105/211 (49.8%)
No	89/211 (42.2%)
Not Available	17/211 (8.1%)
Volume of pleural effusion sent for analysis	60 (30-120) ml

***** Effusion laterally and size, and CT features were only available for Glasgow cases (n=211).

Primary Outcome: PM Success Rate

In 66/327 cases (20%), all PMs required by international guidelines (7, 54) were completed. As summarised in Figure 3.1, this overall success rate of 20% reflected successful testing of all PM assays indicated in 43 of 222 lung cases (19%) and 23 of

105 (22%) breast cases. Individual marker yield was highest in lung for ALK (187/222, 84%), followed by EGFR (137/222, 62%), PD-L1 (79/222, 36%) and ROS-1 (76/222, 34%), and highest in breast for ER (98/105, 93%) followed by PR (92/105, 88%) and HER2 (60/105, 57%).

As summarised in Table 3.2, overleaf, the laboratory tests used for each marker included Polymerase Chain Reaction (PCR), Immunohistochemistry (IHC) and Fluorescence In-situ Hybridization (FISH) assays. Table 3.2 also reports individual marker success in 2016-2018 compared with 2018-2021. Across all markers, PM success rate was significantly higher during 2018-2021 than during 2016-2018 (31/228 (14%) v 35/99 (35%) respectively, ($p<0.0001$). This improvement reflected higher success rates for PD-L1 (IHC) and ROS 1 (IHC +/- FISH) in lung cancer and ER (IHC) in Breast Cancer.

Table 3.2 Laboratory test methods used for predictive markers and comparison of success rate (%) over two consecutive time periods in 327 patients with lung and breast adenocarcinoma. *For the time period data, the values shown are numbers successful/total samples sent for that marker (%). Markers with significantly higher success rate in the later time period are highlighted in bold italics.*

			PM Testing Success Rate		
Marker	**Test Method(s) Recorded**		**2016 – 2018**	**2018 – 2021**	**p value**
EGFR	PCR	136/137 (99%)	91/155 (58.7%)	47/68 (69.1%)	0.178
	N/R	1/137 (1%)			
ALK	IHC	84/187 (45%)	89/155 (57.4%)	46/68 (67.6%)	0.181
	FISH	23/187 (12%)			
	IHC + FISH	27/187 (14%)			
	N/R	53/187 (28.3%)			
PD-L1	IHC	78/79 (99%)	38/155 (24.5%)	***46/68 (67.6%)***	***<0.0001***
	N/R	1/79 (1%)			
ROS 1	IHC	69/76 (91%)	33/155 (21.3%)	***38/68 (55.9%)***	***<0.0001***
	IHC + FISH	6/76 (8%)			
	N/R	1/76 (1%)			
ER	IHC	91/98 (93%)	62/73 (84.9%)	***31/31 (100%)***	***0.031***
	N/R	7/98 (7%)			
PR	IHC	84/92 (91%)	56/73 (76.7%)	27/31 (87.1%)	0.292
	N/R	8/92 (9%)			
HER2	IHC	52/60 (87%)	23/73 (31.5%)	3/4 (75%)*	0.109
	IHC + FISH	4/60 (7%)			
	N/R	4/60 (7%)			

EGFR: Epidermal Growth Factor Receptor; ALK: Anaplastic Lymphoma Kinase; PD-L1: Programmed Cell Death Ligand 1; ROS-1: ROS proto-oncogene 1; ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor; IHC: Immunohistochemistry; FISH: Fluorescence In-situ Hybridization; N/A: Not Recorded; ****** *Of the 31 samples in this cohort, only four were sent for HER2 testing.*

Sub-group analysis of PM Success Rate in PS 0-2

169/327 (52%) cases were PS 0-2. In this subgroup, all PMs were completed in 43/169 cases (25%), comprising 31/122 lung cases (25%) and 12/47 breast cases (26%). The overall success rate was not significantly different to the entire cohort (25% v 20%, p=0.18). Individual marker success rates were also similar in PS 0-2 cases, including in lung cancer: ALK 86% (105/122) EGFR 66% (81/122), PD-L1 43% (52/122) and ROS-1 36% (44/122) and in breast cancer: ER 89% (42/47), PR 87% (41/47) and HER2 68% (32/47).

Secondary Objectives

Time to PM Reporting and SACT initiation

The median time from pleural aspiration to PM reporting was 19 (9-24) days. In 110/327 cases (34%), date of SACT commencement was recorded. In this subgroup, the median time from pleural aspiration to SACT initiation was 31 (4-45) days.

Baseline Features Associated with PM Success

As summarised in Table 3.3, overleaf, only female sex met the prespecified p-value threshold ($p<0.1$) for inclusion in subsequent multivariable logistic regression models (p=0.07). However, since this was a single value, this modelling was not performed.

Change in SACT Receipt over Time

Of the 211 Glasgow patients in whom SACT data was available, 99 (47%) received SACT. Of these, 41/99 (41%) received pleural fluid PM-directed therapy. The proportion of cases receiving SACT did not change significantly between 2016-2018 and 2018-21 (43% v 52%, respectively (p=0.217)). However, significantly more patients received pleural fluid PM-directed SACT in the later period (25% v 57%, respectively, p=0.002).

Table 3.3 Univariate analysis examining potential associations between successful pleural predictive marker testing and baseline clinic-radiological features. Factors associated with a p<0.1 (bold italics) were eligible for inclusion in subsequent multivariable logistic regression

Baseline Feature	**All PM Successful**	**Some PM unsuccessful**	**p value**
Age	69 (12.8)	69 (12.1)	0.867
Female Sex	50/66 (75.8%)	168/261 (64.4%)	0.076
Performance Status			
0	11/66 (16.7%)	20/261 (7.7%)	0.164
1	17/66 (25.8%)	62/261 (23.8%)	
2	15/66 (22.7%)	44/261 (16.9%)	
3	9/66 (13.6%)	50/261 (19.2%)	
4	2/66 (3.0%)	16/261 (6.1%)	
Not Available	12/66 (18.2%)	69/261 (26.4%)	
Cancer Type			
Lung	43/66 (65.2%)	180/261 (69.0%)	0.552
Breast	23/66 (34.8%)	81/261 (31.0%)	
Volume	50 (29-120) ml	60 (26-104) ml	0.992
Effusion Laterality			
Right	22/37 (59.5%)	91/174 (52.3%)	0.428
Left	15/37 (40.5%)	83/174 (47.7%)	
Effusion Size			
≤1/3	10/37 (27%)	42/174 (24.1%)	0.402
1-2/3	12/37 (32.4%)	83/174 (47.7%)	
≥2/3	15/37 (40.5%)	46/174 (26.4%)	
Not Available	0/37 (0%)	3/174 (1.7%)	
Malignant CT Features			
Yes	23/37 (62.2%)	81/174 (46.6%)	0.301
No	13/37 (35.1%)	30/174 (17.2%)	
Not Available	1/37 (2.7%)	63/174 (36.2%)	

***** Effusion laterally and size, and CT features were only available for Glasgow cases (n=211)

3.4.4 Discussion

In this retrospective, multi-centre cohort study of 327 patients with metastatic lung and breast adenocarcinoma, pleural fluid PM testing by multiple individual assays was only complete in 20% of samples. This overall performance reflected a PM success rate of 19% in lung (43/222) and 22% in breast (23/105). Individual marker yield was highest in lung for ALK (84%), followed by EGFR (62%), PD-L1 (36%) and ROS-1 (34%), and highest in breast for ER (93%) followed by PR (88%) and HER2 (57%). No clinico-radiological feature was reliably associated with subsequent PM success rate on univariate analysis, precluding multivariate testing. PD-L1 (by IHC), ROS-1(by IHC +/- FISH) and ER (by IHC) success rates increased over time, as did the rate of effusion PM marker-directed SACT (25% v 57%, p=0.002). Although increasing, the modest rates of pleural PM marker-directed SACT observed may reflect the median reporting time of 19 (9-24) days, which may be too long to wait in some patients with MPE-associated metastatic disease whose prognosis can be as little as three months depending on their primary tumour type. The failure of effusion PMs to effectively direct SACT choices in many patients is reflected in overlapping confidence intervals when comparing reporting time and median time to SACT initiation (19 (9-24) v 31 (4-45), respectively).

The rate of SACT receipt in the current study (47% in the 110 cases with this data available) is broadly comparable to that reported by Varatharajah et al (30%, with 57% receiving pleural fluid PM-directed SACT between 2015-17)(138). The latter figure is nearly identical to the pleural PM-directed SACT rate observed in the current study between 2018-2021. Previous studies have reported higher success rate for individual markers than those reported here. For example, in recent conference proceedings, Navarra et al reported successful EGFR testing in 76/93 (82%) samples collected between 2013-21(139). While this rate is higher than the EGFR rate for even our later cohort (62% (2018-21)), this study was considerably smaller and excluded samples from patients not deemed fit enough for treatment, which may remove confounding elements such as concomitant pleural infection, bleeding or heart failure. A small study of 9 cases from a single lung cancer clinic in Wales also reported 90% EGFR testing success, but the size of this cohort limits meaningful conclusions(140).

Mercer et al previously reported that effusion cytology results were ‘sufficient to guide subsequent therapy’ in 45/71 (63.4%) cases with positive initial cytology at a single centre(136). This value is considerably higher than the figures reported here, however the definition of ‘sufficiency’ used included cases who were deemed not fit for SACT (PS>2) and had only positive cytology (without PMs). In addition, this study did not report the completeness of the PM panel results, meaning samples could presumably be labelled sufficient if only one marker was positive (e.g. PD-L1) and that therapy was given. This approach does not capture circumstances when all other assays fail, meaning alternative therapies could not be considered. (e.g. a TKI if EGFR-mutant). This is particularly important in lung cancer where TKIs would be preferred over ICI if both EGFR and PD-L1 were positive(7). The definition of PM success as return of a definitive result for all PM needed is therefore an important strength of the current study, since it replicates the clinical requirement for these samples.

The relatively low overall PM success rate observed likely reflects several real-world weaknesses in the processing and analytical pipelines used for clinical samples based on multiple individual marker tests. Acknowledging these, and either correcting them, or avoiding the delay involved in waiting for a test with a low success rate is clinically important. In a previous prospective study, Wu et al performed prospective sampling of MPE in 872 Asian patients with metastatic lung adenocarcinoma, followed by immediate processing and RNA sequencing for driver mutation analysis. Using this approach, 747 (86%) had samples suitable for analysis of 12 different driver genes(141). While not addressing key phenotypes, including PD-L1 status, these data suggest that alternative processing and/or different analytical methods may result in enhanced outcomes. Developments in circulating tumour DNA (ctDNA), which can be recovered from effusions for molecular stratification may also affect future pathway design(142, 143). However, this technology is not routinely available in most centres and recent reports of reduced sensitivity in patients with disease confined to the thorax(142), including MPE, suggest further optimisation may be needed for use with effusion samples.

Clinical Implications

Based on our data it was not possible to define reliable baseline features for prediction of low PM success rate. This is concordant with previous studies reported by Tsim et al and Mercer et al(23, 136). It was hoped that such features could serve as additional criteria for pathway stratification in a similar manner to those previously associated with low diagnostic cytology yield (asbestos exposure and malignant CT features)(23). Nevertheless, until improved laboratory methods are routinely available (e.g. ctDNA in pleural effusion samples) a PM success rate for all markers needed to plan oncological therapy of only 20.2% (19.4% in lung and 21.9% in breast) may be sufficient for some clinicians to consider omitting pleural fluid sampling and moving direct to biopsy (e.g. LAT). This may be particularly appropriate in fit patients, for whom the full range of SACT options would be feasible. By contrast, in less fit patients, particularly those with breast cancer, it may be reasonable to proceed initially to pleural fluid aspiration given the high yield of ER and PR testing, which will be adequate to stratify patients to low-risk, well-tolerated hormone therapies. Only if these tests are negative might one need to consider histological sampling for HER2 status, knowledge of which is required by ESMO breast cancer guidelines(54). The techniques deployed in a stratified 'direct-to-biopsy' pathway may also depend on the skills available in individual centres. Going 'direct-to-LAT' is attractive since this would allow simultaneous effusion palliation. However, combining fluid aspiration for diagnostic material and palliation with EBUS for histological sampling may be a reasonable alternative in centres without LAT access.

Limitations

It is important to acknowledge that the current study does not include ovarian cancer, which was associated with an OR for incomplete PM results of 83.09 (95% CI 11.26–613.12) in the earlier study from Tsim et al(23).This is because, although BRCA testing is recommended in relevant international guidelines(57) pleural fluid was not being sent for PM analyses in the participating centres over the study period. Nevertheless, ovarian cancer is the third most common cause of MPE in women after lung and breast cancer(144), and BRCA mutation status is a critical factor in treatment planning and access to trials. As the feasibility of BRCA testing

in pleural effusion evolves, this will be an important area for future study. In the interim, we advocate for histological sampling in ovarian patients with MPE if BRCA status cannot be tested on effusion samples. The current study also does not address PMs that have come into practice recently, including as KRAS and BRAF in lung cancer(145, 146). This reflects the retrospective design, which is also associated with important limitations including recall and omission bias related to missing data including the 118 cases in which fluid was not sent for PM testing.

3.4.5 Conclusions

The current study is the first, to our knowledge, to describe the real-world performance and associated limitations of pleural fluid PM analysis in lung and breast adenocarcinoma. A complete PM analysis, which is needed for rational planning of licensed SACT was available in only 20% of 372 cases from 4 UK centres (19% in lung and 22% in breast). We could not define reliable baseline features of PM testing failure, but the low overall success rate may be sufficiently low to support ‘direct to biopsy’ stratification in selected patients. Use of pleural PM-directed therapy increased over the study period but remained modest at 57% by 2018-21. This may, in part at least, reflect the time taken for PM reporting 19 (9-24) days, which overlapped with the time to SACT initiation (31 (4-45) days).

3.5 Acknowledgements

Guarantor Statement

KGB takes responsibility for the content of the manuscript, including the data and analyses.

Conflicts of Interest Statement

The authors declare no known conflicts of interest.

Funding

No funding was involved in the current study.

3.6 Reflections on this Paper

This retrospective data shows that pleural fluid cytology was not sufficient for complete predictive marker testing in the majority of patients. Our data found that all necessary PMs were successfully tested in only 20% of patients from pleural fluid cytology. This is of clinical relevance as pleural fluid sampling remains the mainstay of diagnostic sampling where a patient presents with suspected malignancy and a pleural effusion with treating teams often only embarking on other diagnostic procedures once cytology has been found to be insufficient. Given the importance of PMs in guiding optimal patient management, direct-to-biopsy stratification may be appropriate in selected patients.

Out of all three studies presented here, this work has impacted on my day to day practice the most in that I am now more vocal in my argument to pursue tissue biopsy from the outset rather than relying on pleural fluid cytology for diagnostics. Access to pleural aspiration is however safe and often quicker than biopsy. Therefore I do believe there is still a place for pleural fluid aspiration (even where drainage is not required for symptomatic benefit) in cases where there will be a delay in getting tissue from another source.

My main takeaway from this study is the importance of assessing the utility of pleural fluid aspiration for each individual rather than using it universally as a first test. This of course may change once the use of cell free DNA and next generation sequencing technology comes into wider use. Currently their expense, limited availability and associated limited expertise in analysing data outputs from such technology means they are not currently a viable option to be used on pleural fluid samples within the NHS. If such techniques do become the norm then the role of pleural fluid sampling must again be reassessed.

CHAPTER 4 EXPLORING BASELINE IMMUNE RESPONSE TO COVID-19 INFECTION AND ITS ASSOCIATION WITH DISEASE SEVERITY

4 Chapter 4 Exploring Baseline Immune Response to COVID-19 Infection and its Association with Disease Severity

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4.1 Introduction to this Paper

While lung cancer remains a large part of the clinical workload for many respiratory physicians, in late 2019 there emerged a new respiratory disease which became the focus of the world's attention as well as accounting for the majority of respiratory admissions to hospital in the early part of 2020. This of course was COVID-19.

Much like lung cancer it became quickly apparent that there was a wide spectrum of presentations and outcomes of those infected with SARS-CoV-2 and that early diagnosis was key, initially in aiding to slow the spread of the virus and eventually to ensure patients were commenced on the appropriate treatment as quickly as possible.

Risk factors for poor outcome from COVID-19 were also soon recognised. Patients with comorbidities associated with poor metabolic health (for example hypertension or obesity) are known to have poorer outcomes while there are immunological markers such as lymphopenia, raised CRP or raised IL-6 which have also been associated with poorer outcomes. What is less clear is the mechanisms that link these predisposing comorbidities to these immunological responses and whether there are immunological markers at first presentation that could allow better prediction of disease severity.

My last project, outlined in chapter 4, therefore continues on the theme of early diagnosis and stratification by examining individual immune response to COVID-19 to identify any immune changes which could be identified at the time of diagnosis of COVID-19 to better predict eventual disease outcome and to gain insight into the immune mechanisms underlying these differing disease trajectories.

This project has not yet been prepared for publication as there are additional analyses ongoing via University of Glasgow bioinformaticians the results of which I plan to include in the final submission for publication.

4.2 Abstract

4.2.1 Introduction

It became clear soon after the outbreak of SARS-CoV-2 that there was a wide spectrum of disease severity and outcomes in those infected with the virus. The immunological changes responsible for this spectrum of disease remain poorly characterised and it is uncertain how these differing immune responses relate to individual outcome.

This study therefore aims to increase our understanding of the immune response to COVID-19 and how this relates to eventual outcome. Clinical features associated with varying disease severity will also be assessed. An improved understanding of how individual immune response affects disease severity and eventual outcome would not only aid in management decisions for the individual but could lead to improved therapeutic strategies.

4.2.2 Methods

In this study, 60 patients presenting to the Queen Elizabeth University Hospital in Glasgow with COVID-19 were recruited between January and July 2021. All participants had baseline blood samples and clinical data collected and their illness trajectory was recorded. These baseline samples were then analysed using flow cytometry to gain insight into immune cell subpopulations including the activation status of intracellular signalling molecules to determine what differences, if any, exist between those with mild disease and those who went on to develop severe COVID pneumonia.

4.2.3 Results

This study has found that there is significantly lower activation (phosphorylation) of STAT 5 in those who go on to develop severe COVID-19 requiring supplemental oxygen than in those with non-severe disease who do not require oxygen. Severe disease was also associated with obesity and hypertension.

4.2.4 Conclusions

In this cohort, there is a lower level of activation (phosphorylation) of STAT5 in those with severe COVID-19 than those with non-severe COVID-19 as defined by the need for supplemental oxygen. Increased disease severity in this cohort was also associated with obesity and hypertension.

4.3 Author Contribution

KGB and CG conceived the study. JF and MT screened for and recruited study participants.SM conducted sample processing for storage. JF and ATV performed flow cytometry and subsequent flow cytometry data analysis. JF performed statistical analysis. JF ATV, CG and KGB performed data interpretation. JF created the first draft of the manuscript.

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MT Matthew Tate

SM Samantha Miller

ATV Aysin Tulunay Virlan

CG Carl Goodyear

KGB Kevin G Blyth

4.4 Manuscript

4.4.1 Introduction

In December 2019 in the Hubei Province of China, Wuhan, the first cases of a novel betacoronavirus were documented(90). This virus was named SARS-CoV-2 and spread rapidly, with COVID-19 (the disease caused by the virus) being officially classed as a pandemic by the WHO in March 2020(147). It quickly became clear even from the first patients exposed to SARS-CoV-2 that this virus produced a wide range of illness severities with some people experiencing mild or even no symptoms while others developed life threatening and often fatal COVID pneumonia(81).

Risk factors associated with disease severity were soon described including older age, male sex, hypertension, obesity and diabetes(99). Validated mortality scores, such as ISARIC 4C were developed using these risk factors to aid clinicians in their decision making based on likely prognosis(104). While such risk factors were quickly established, and differing immune responses postulated as a reason for the spectrum of outcomes seen with COVID-19, little was understood about individual immune response to the virus and how this related to risk factors and eventual disease outcome.

It has been hypothesised that those with severe disease mount an exaggerated immune response resulting in a ‘cytokine storm’(107). Previous authors have suggested that this cytokine storm, rather than the direct effects of the virus itself, results in immune-mediated acute lung injury(82) and respiratory failure- one of the commonest causes of death from COVID-19(148). COVID-19 related ARDS is associated with a mortality of up to 40%(107) and found to be present in up to 80% of those admitted to ITU with COVID-19 during the first wave of the pandemic(149).

The finding of ARDS in COVID-19 is well established, with certain predisposing features and an exaggerated immune response both frequently observed in those with severe COVID-19. The specific mechanisms connecting these predisposing features and acute immunological response have been challenging to define however. This lack of knowledge is likely one of the reasons that potential therapies with a seemingly solid evidence base for their use in viral illness often did not

produce the expected benefits in COVID-19. One such therapy were interferons (IFN) which largely failed to show any significant improvement in COVID-19 outcomes despite being based on previous knowledge of increased IFN levels in response to viral infection(150). Given the likelihood of varying immune response between individuals, with some work even showing how this immune response changes within the same person over time(151), one explanation for this failure may therefore be poor patient selection into trials.

One factor which may play a part in this differing immune response and spectrum of disease seen in COVID-19 is the activation status of immune cell subpopulations and subsequent impact on immune signalling. The JAK/STAT signalling pathway allows interaction between cells and their environment through transmembrane signalling and is known to be involved in immune response and inflammation(152).Overactivity of the JAK/STAT axis has been observed in patients with metabolic syndrome and could potentially drive dysergulated immune responses to SARS-CoV-2 and therefore adverse outcomes(153).

The STAT family of signalling molecules must be phosphorylated in order to allow their movement into the nucleus where they can then perform their function as transcription factors(154). It follows then that differences in the phosphorylation status of such molecules will have a knock-on effect for downstream immune signalling and function.

At the time of this study's design, there has been very limited work undertaken on the activation status of cell signalling molecules in response to COVID-19. Only a single study based on a cohort of 30 patients has looked at this in any detail and focusses on the transcription factor STAT1(155). This is known to be a crucial molecule in the immune response to many viruses as it activates the transcription of many pro-inflammatory genes as well as having a role in interferon signalling (155). It was shown in this previous study that those with COVID-19 had a higher total level of serum STAT1 compared to the levels detected in control subjects. Interestingly however, while the overall level of STAT1 was higher in those with mild COVID-19, those with severe COVID-19 had a higher level of phosphorylated, or activated, STAT1(155).

The aim of this study is to assess the levels of activated immune signalling molecules in those infected with SARS-CoV-2 at the time of hospital admission and to determine if there are any differences in the patterns of activation seen between those with mild and severe COVID-19. This is the first paper, to our knowledge, to assess individual immune response in terms of activation status of important immune mediators at the time of diagnosis.

4.4.2 Materials and Methods

Study Design

This is a prospective, observational, single centre study. A study flowchart can be seen in figure 4.2 outlining screening and recruitment numbers as well as reasons for exclusion. Ethics approval was granted by North of Scotland Research Ethics Committee (Ref: 20/NS/0093). All participants gave informed consent to participate in the study before taking part. This study was funded by the Chief Scientist Office Scotland (COV/GLA/20/07) and University of Cambridge.

Study Recruitment

60 patients presenting to the Queen Elizabeth University Hospital (QEUH) in Glasgow with suspected or confirmed COVID-19 were prospectively recruited from January to August 2021 (Figure 4 2). I recruited patients to this study with support from CRF (clinical research facility) nurses. For any days I was unable to personally recruit, I created a rota for the CRF nurses to ensure attendance in all acute receiving wards within the QE as well as the high dependency unit. Additionally, I screened in-patient wards electronically which allowed me to identify anyone with COVID-19 who had been transferred to a ward but were still within 24 hours of admission and therefore potentially eligible for recruitment. Finally, I created a poster which was displayed throughout the Emergency Department (ED) to advertise the trial with contact details for myself and the CRF team so that our ED colleagues could make us aware of anybody they reviewed who was potentially eligible for recruitment.

Once written consent to participation was obtained, baseline blood samples were collected. Participants were recruited as early as possible (within 24 hours of their

hospital admission) to obtain baseline samples as close to their COVID-19 diagnosis as possible.

The inclusion and exclusion criteria for this study were kept deliberately broad to gain as representative a study cohort as possible. Inclusion criteria were: ≥ 18 years of age, ≤24 hours since admission, suspected or confirmed COVID-19 and able to give written informed consent.

Data Collection

Clinical data was recorded at the point of recruitment including patient demographics, co-morbidities, smoking status and COVID-19 illness features. The latter included initial observations (peripheral oxygen saturations, respiratory rate, blood pressure, heart rate, temperature and neurological status as defined by the Glasgow Come Scale(GCS)), maximum administered oxygen dose (as denoted by the fraction of inspired oxygen (FiO2)) and the use of respiratory support including continuous positive airway pressure (CPAP), high flow nasal oxygen (HFNO) and invasive mechanical ventilation. The occurrence of pulmonary complications was also documented including COVID pneumonia where its presence was reported on imaging and pulmonary thromboembolism as defined by the finding of a pulmonary embolism (PE) on computed tomography pulmonary angiography (CTPA). Data on COVID-19 treatments received (including vaccination status) was also recorded.

Co-morbidities (specifically any chronic respiratory or cardiovascular conditions, diabetes (type 1 or type 2), obesity and smoking status were recorded based on information from admission documentation or electronic records (General Practitioner summary notes, clinic letters or if documented during a previous admission). Cardiovascular disease was defined as the presence of ischaemic heart disease (IHD); hypertension; left ventricular systolic dysfunction (LVSD) (there were no other cardiovascular diseases documented for any study participant). Chronic respiratory disease was defined as a diagnosis of chronic obstructive pulmonary disease (COPD) or asthma (again in our cohort, these were the only two chronic respiratory conditions identified). Obesity was defined as either having a documented BMI of >30 or if it was described by the examining physician in the

admission documentation. Each data point was anonymously linked to individual participants through their unique trial ID.

Sample Collection and processing

4ml of whole blood was collected into sodium citrate tubes from all participants at the time of recruitment. These samples were transported as soon as possible on the day of collection for downstream processing in Carl Goodyear's lab at the University of Glasgow.

All whole blood samples were processed prior to storage at -80˚C. First each sample was divided equally in half. One half was stimulated with a cell stimulation cocktail (Table 4.1) while the other half was not. This latter half became the control (unstimulated) sample. The stimulated samples were incubated with the cell stimulation cocktail for 15 minutes in a water bath at 37˚C. The control samples were also placed in the water bath at 37˚C for 15 minutes. All samples were then incubated for 10 minutes at room temperature with proteomic stabilisation buffer before being divided into labelled 1.6ml aliquots for storage at -80 ˚C.

Table 4.1 Cell Stimulation Cocktail

Cytokine/stimulant	Final Conc. per 1 ml	Storage Temp	Volume per 1ml	Master mix Volume Per 2ml +10%	Cat Number	Manufacturer
IFN – Alpha	40,000 units	-80°c	10	22	PHC4814	ThermoFisher
IL-6	100ng	-20°c	1	2.2	PHC0066	ThermoFisher
IL-2	200ng	-20°c	2	4.4	PHC0027	ThermoFisher
Cell Stimulation Cocktail	80nM PMA/1.3 Ionomycin	-20°c	2	4.4	00-4970-93	ThermoFisher
T cell activation beads	N/A	4°c	5	11	130-091-441	Miltenyi Biotech
INF Gamma	25ng	-20°c	2.5	5.5	PHC4031	ThermoFisher
IL-4	20ng	-20°c	2	4.4	766202	Biolegend
Total volume to add to 2ml Blood				**49ul**		

Flow Cytometry

To allow analysis of immune cell populations and signalling molecule activity within these samples, I performed flow cytometry on all patient whole blood samples. This was done under the guidance and supervision of Aysin Tulunay Virlan in Professor Carl Goodyear's lab at the University of Glasgow (School of Infection and Immunity). I received in person training from Aysin on the entire process allowing me to then work independently.

Thawing and Erythrocyte Lysis:

Whole blood samples from each trial participant were first thawed in a cold-water bath then transferred into 50ml test tubes. The thawed samples were mixed with 10ml Thaw-Lyse buffer and incubated for 10 minutes at room temperature. Samples were then centrifuged with the resulting cell pellet resuspended in a further 25ml Thaw-Lyse buffer and left to incubate at room temperature for another 10 minutes. The samples were centrifuged again with the cell pellet this time being resuspended in 25ml of Thawing Buffer (10% FBS in PBS). This process was repeated a final time with the cell pellets finally being suspended in 300 µl Thawing Buffer.

Cell Staining:

Each patient sample in its 50 ml tube was then split into four 5ml tubes labelled with the corresponding patient ID and either stimulated/ unstimulated and mixed/ FMO (fluorescence minus one). For example: 001 US Mix, 001 US FMO, 001 Stim Mix, 001 Stim FMO. The FMO tubes acted as controls. **1**µl Fc block was added to each tube along with 50µl surface staining cocktail (Table 4.2) before being incubated at room temperature for 30 minutes protected from light. The surface staining cocktail contained a combination of surface markers corresponding to different immune cells (eg CD4, CD8, B cell – Table 4.3) to allow distinct subpopulations to be identified from the serum samples during flow cytometry.

Table 4.2 Cell surface staining cocktail

Marker	Color	Panel volume for 1 test (µl)	Panel volume for 1 patient (4 Surface)	Panel volume for 4 patients (16 Surface)+1 extra	Cat No	Company
CD4	eFluor 506	1	4	17	69-0049-42	Thermo Fisher
CD3	AF700	2	8	34	56-0038-42	Thermo Fisher
CD8a	APC-Cy7	3	12	51	557834	BD
CD14	BV711	3	12	51	563372	BD
CD56	BV605	3	12	51	562780	BD
CD19	PE-Cy7	5	20	85	25-0199-42	Thermo Fisher
Thawing buffer		33	132	561		
	Total	50	200	850		

Table 4.3 Cell type by corresponding surface marker

Surface Marker	Cell Population
CD4	CD4 (Helper) T cells
CD3	T cells
CD8a	CD8 (Cytotoxic) T cells
CD14	Monocytes
CD56	NK cells
CD19	B cell

Next, the samples were washed in ice-cold FACS buffer before being permeabilised (to allow intracellular staining) with the addition of 500µL of chilled Perm Buffer II before being incubated again for 30 minutes protected from light, this time on ice.

Once permeabilised, the samples were re-washed in ice-cold FACS buffer before 50µl of the intracellular staining cocktail (Table 4.4) was added to the 'mix' tubes only. Intracellular staining was used to identify important immune cell signalling

molecules and in the case of the STAT family, specifically the activated from of these molecules. Again, the samples were then incubated for 30 minutes on ice protected from light. Finally, the samples were washed once more in ice-cold FACS buffer before finally being suspended in 300µl FACS buffer ready for analysis.

Table 4.4 Intracellular staining cocktail

Marker	Color	Panel volume for 1 test (µl)	Panel volume for 1 patient (2 Intracellular)	Panel volume for 4 patients (8 Intra)+1 extra	Cat No	Company
pSTAT3	PE-CF594	2	4	18	562673	BD
pSTAT5	BV421	2	4	18	562984	BD
pSTAT6	PerCP-Cy5.5	5	10	45	686010	Biolegend
Syk	PE	5	10	45	12-6696-42	Thermo Fisher
c-Cbl	AF647	5	10	45	558103	BD
pSTAT1	AF488	5	10	45	612596	BD
FACS Buffer		26	52	234		
	Total	50	100	450		

Data Analysis

Flow cytometry output was analysed and gated to identify specific peripheral blood mononuclear cell (PBMC) subpopulations as per Figure 4.1.

First doublet cells were excluded based on cell height and area (Figure 4.1 **(a)**) to ensure subsequent cell sorting was as accurate as possible. Next any non-PBMCs (such as platelets or remaining red cell fragments) were excluded by gating for cell size, as measured by forward scatter (FSC) and granularity (ie complexity) based on the degree of side scatter (SSC) (Figure 4.1 **(b)**).

The identified PBMC population was then further divided into lymphocyte and monocyte populations again based on their size and granularity (Figure 4.1 **(c)**). Monocytes were further identified by the presence of the extracellular marker CD14 (Figure 4.1 **(d)**). The identified lymphocytes were then differentiated into NK cells, T cells and B cells based on the presence of extracellular CD56, CD3 and CD19 respectively (Figures 4.1 **(e)** and **(f)**). Finally, T cells were further divided into cytotoxic or helper T cell subpopulations based on the presence of extracellular CD8 or CD4 respectively (Figure 4.1 **(g)**).

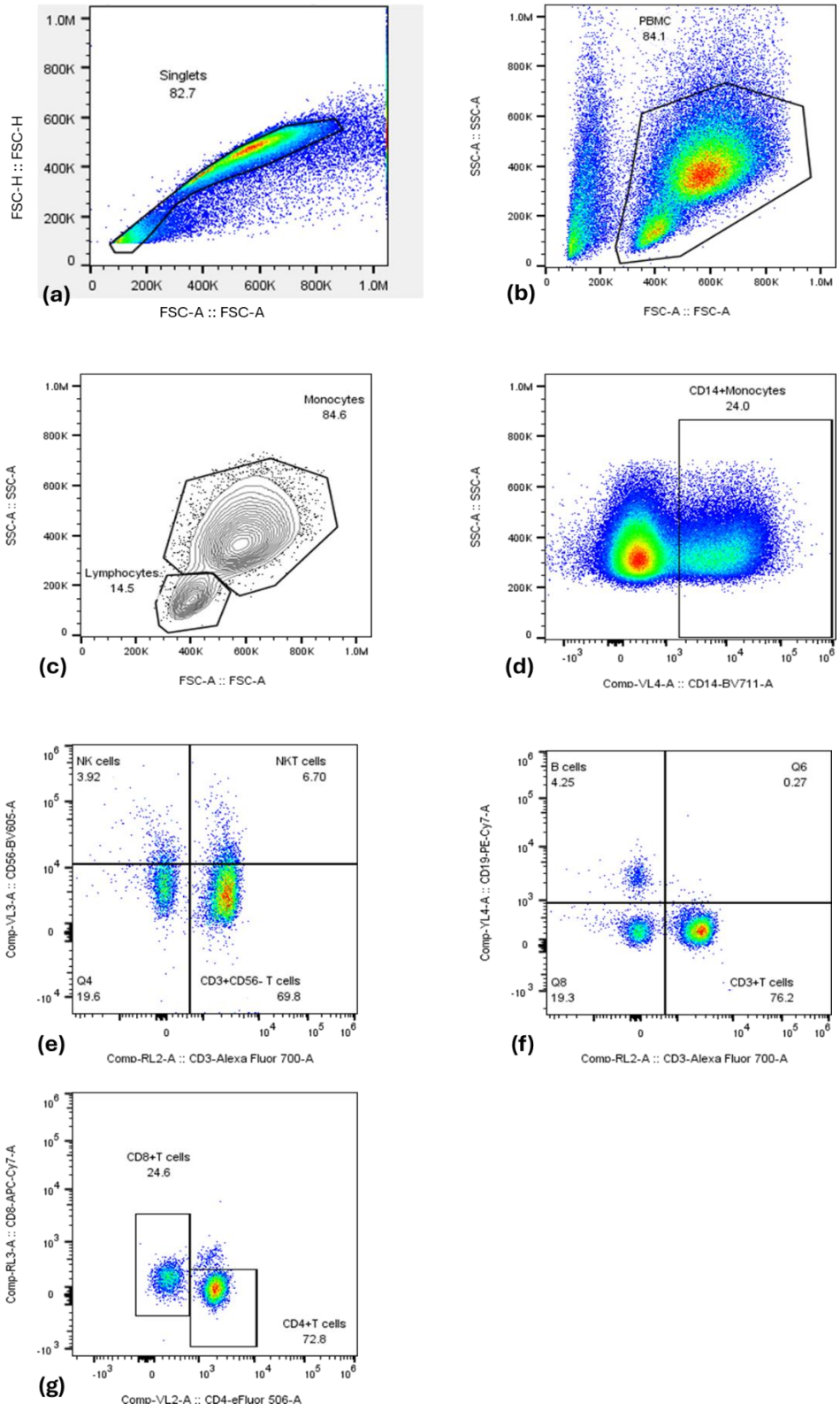

Figure 4.1 Gating of flow cytometry outputs for the identification of immune cell subpopulations: (a)Singlets, (b)PBMCs, (c)Monocytes and Lymphocytes, (d) CD14 monocytes, (e)NK cells, (f) B cells, (g) CD8 and CD4 T cells

Group Comparisons

For the purposes of analysis, the cohort was divided into two subgroups based on oxygen requirement (those that required oxygen during their admission and those who did not) to allow comparison of disease severities based on the objective outcome of supplemental oxygen usage. This resulted in two distinct groups: a non-severe group (who did not require oxygen), n=20 and a severe group (who did require oxygen during their admission), n=29 (Figure 4.2). Baseline characteristics such as age and gender as well as co-morbidities (Table 4.5) as well as immune cell subpopulations (Figure 4.3) were then compared as outlined in the statistical methods section below.

Intracellular signalling activity was assessed in three ways:

1) Intracellular signalling activity in unstimulated cells was compared between the severe and non-severe groups
2) intracellular signalling activity in the stimulated cells was compared between the severe and non-severe groups
3) the change in intracellular signalling induced by stimulation (as defined by stimulated-unstimulated measurements in signalling activity) was calculated and compared between the severe and non-severe groups.

The results for the above analysis of immune cell signalling activity is only reported for pSTAT 5 here as this was the only signalling molecule in which there was a significant difference in activity across all immune cell subpopulations (other than monocytes) between the severe and non-severe groups. The results of analyses of all other intracellular signalling molecules analysed can be found in Appendix 5.

Statistical Methods

Differences in baseline characteristics (other than age) between the severe and non-severe group were assessed using fisher's exact test due to the small numbers involved in some of these categorical variables. A difference in age between the groups was examined using a t-test as this data was both quantitative and normally distributed.

Mann-Whitney U testing was employed to identify any significant differences between the proportions of individual immune cell subpopulations in the serum between the severe vs non-severe groups. The activity levels of intracellular signalling molecules between the severe and non-severe groups within these cell subpopulations were also analysed using Mann-Whitney U tests as this data was non-parametric.

GraphPad Prism (Version 10) was used to perform all statistical analysis.

4.4.3 Results

Of the 141 patients screened, 60 patients were recruited into the study of which 50 baseline serum samples were used for further analysis (Figure 4.2). 10 samples had insufficient cellularity after processing to allow for meaningful flow cytometric analysis. On reviewing the flow cytometry results there was one further sample which was a significant outlier across all immune cell subpopulations for which there were no clinical features to account for this difference. This sample was therefore also excluded from further analysis resulting in a final cohort of 49 patients (Figure 4.2). The average age of this cohort was 51 years (24-79) and 57% were male.

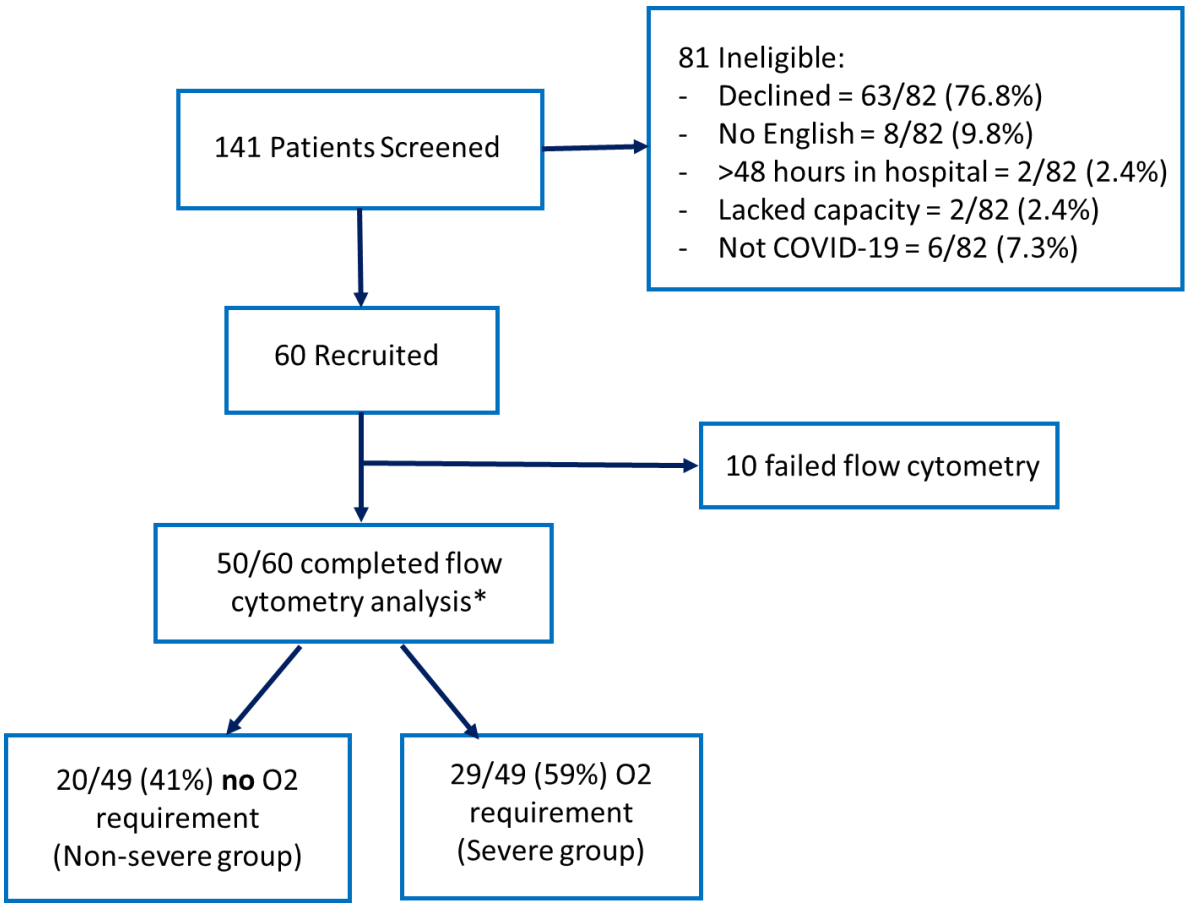

*Figure 4.2 Study flowchart (*1 flow cytometry sample was an outlier and therefore excluded from classification by COVID-19 disease severity)*

Study Population and Baseline Features

Baseline features of the cohort and comparisons of clinical characteristics and co-morbidities between the two groups are detailed in Table 4.5. There was no significant difference in gender, age, ethnicity, or vaccination status between our two cohorts however there were significant associations between obesity (p= 0.0456) and cardiovascular co-morbidity (p= 0.0242) and the need for supplemental oxygen.

As defined in the methods section, cardiovascular comorbidity was defined by the presence of any of the following: IHD; hypertension; LVSD. All severe cases in which cardiovascular comorbidity was recorded had hypertension. In a post hoc analysis, hypertension alone was more strongly associated with disease severity than CV comorbidity more broadly (p= 0.0071 vs p=0.0242 respectively).

Although there was no significant difference in the numbers of those with a formal diabetic diagnosis between the groups, there was a significant difference in median baseline glucose levels between those who required oxygen and those who did not at 6.2 mmol/l (IQR 5.9-9.1 mmol/l) in the severe group vs 5.7 mmol/l (IQR 51-6.2 mmol/l) in the non-severe group (p= 0.0493). These glucose levels are from bloods taken on admission before any COVID treatment was commenced and so is not confounded by dexamethasone use.

There was no difference in median CRP between the two groups at 73 mg/l (IQR 35-120 mg/l) in the severe group and 51 mg/l (IQR 19-79 mg/l) in the non-severe group (p= 0.1080).Level of vaccination was also no different between the severe (24%) and non-severe (45%) groups (p= 0.2145).

*Table4.5 Cohort characteristics of non-severe vs severe groups - significant p-values are highlighted in **bold** (multiple comparison testing was not performed)*

	Non-Severe Disease (n=20)	Severe Disease (n=29)	P Value
Age	Mean 48 (Range: 29 - 72)	Mean 53 (Range: 28 - 79)	0.1475
Gender	Male: 12/20 (60.0%)	Male: 16/29 (55.2%)	0.7768
Ethnicity	Caucasian: 14/20 (70.0%) Other: 6/20 (30.0%)	Caucasian: 26/29 (89.7%) Other: 3/29 (10.3%)	0.1328
Smoking Status	Ever-smoker: 8/20 (40.0%)	Ever-smoker: 8/29 (27.6%)	0.5362
Findings at Presentation:			
COVID Pneumonia	10/20 (50.0%)	25/29 (86.2%)	**0.0096**
Pulmonary Embolism	1/20 (5.0%)	3/29 (10.3%)	0.6359
Co-morbidities:			
Respiratory	7/20 (35.0%)	6/29 (20.7%)	0.3314
Cardiovascular	2/20 (10.0%)	12/29 (41.4%)	**0.0242**
Diabetes	2/20 (10.0%)	8/29 (27.6%)	0.1674
Obesity	7/20 (35.0%)	19/29 (65.5%)	**0.0456**

COVID-19 Disease Course

As summarised in Table 4.5, COVID pneumonia at presentation (as defined by the presence of COVID pneumonia as reported on CXR or CT) was significantly more

common in those who required oxygen during their admission than those who did not (p= 0.0096).

Of those in the severe group, 26/29 (89.7%) were already on supplemental oxygen at the time of blood sampling while only 3/29 (10.3%) were not. These three patients subsequently developed an oxygen requirement 3, 9 and 38 hours after their study blood samples were taken.

4.4.3 Flow Cytometry

Immune Cell Proportions by Severity Group:

Flow cytometry data was used to determine the makeup of the immune cell populations found in each patient's baseline blood sample. As summarised in Figure 4.3, patients with severe disease had a lower proportion of lymphocytes (p= 0.0085) and a higher proportion of monocytes (0.0088) in their peripheral blood immune cells. Lymphocytes and monocytes are presented as proportions of the whole blood cell count. B cells and T cells (CD 3 cells) are presented as proportions of lymphocytes and CD4 and CD8 are presented as proportions of T cells.

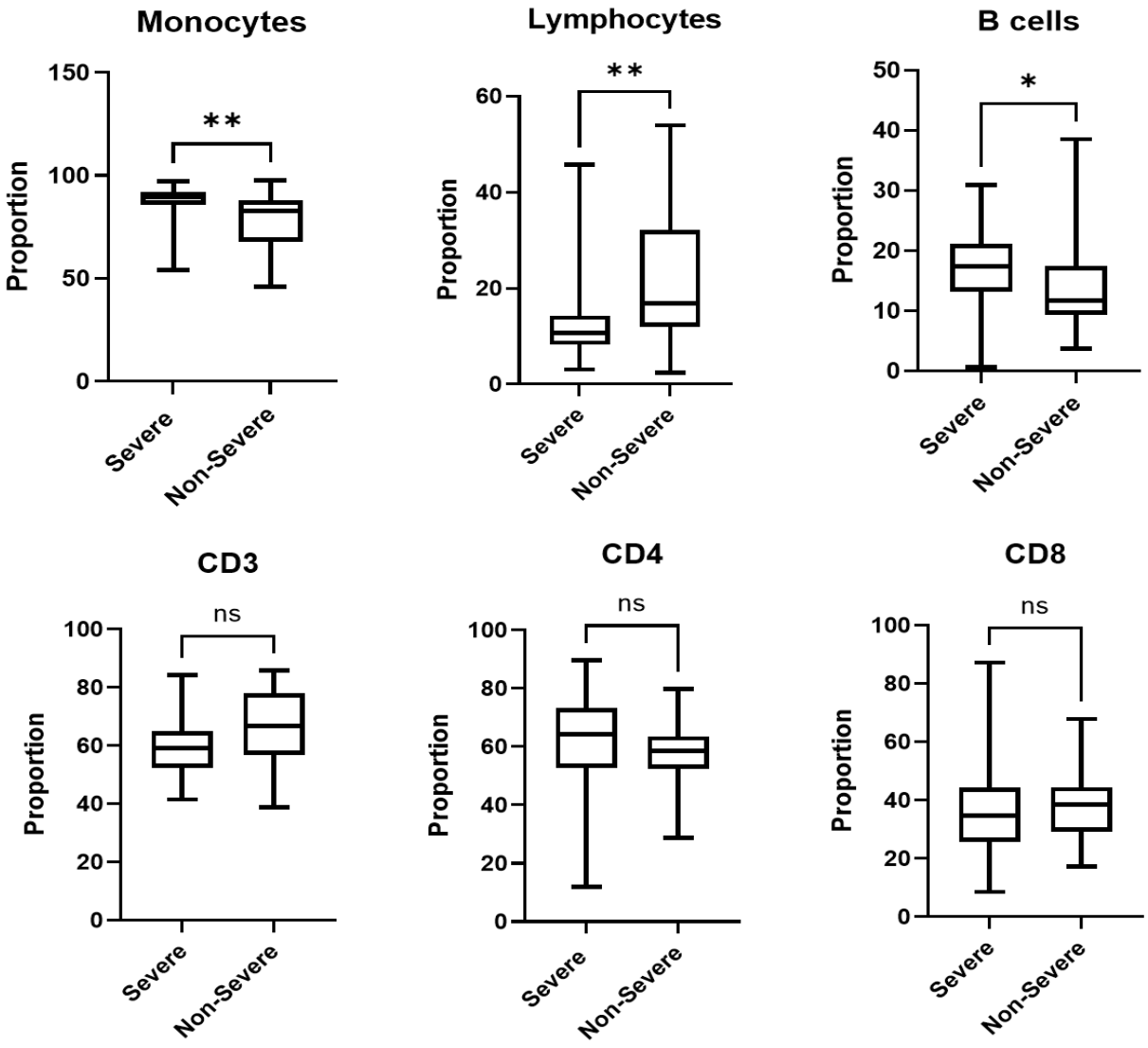

Figure 4.3 Immune cell subpopulation proportions between severe vs non-severe groups

Intracellular Signalling by Severity Group

Over and above simply comparing the numbers within cell subpopulations between each group, the activation status of intracellular signalling molecules was also assessed. Levels of the intracellular markers as listed in Table 4.4 were analysed. Although there was no significant difference in the level of pSTAT5 between the groups after stimulation (Figure 4.5), there was a nonsignificant trend towards a higher baseline level of STAT5 activity in the unstimulated samples in the severe group compared to the non-severe group (Figure 4.4). Across all immune cell subtypes (other than monocytes) there was a significantly smaller increment in STAT

5 activation after stimulation in the severe group when compared to the non-severe group (Figure 4.6). As stated in the methods section, although all of the markers as listed in Table 4.4 were analysed, only STAT5 activity levels were significantly different between the severe and non-severe groups and are therefore the only results reported here. Results for all other signalling molecules can be found in Appendix 5.

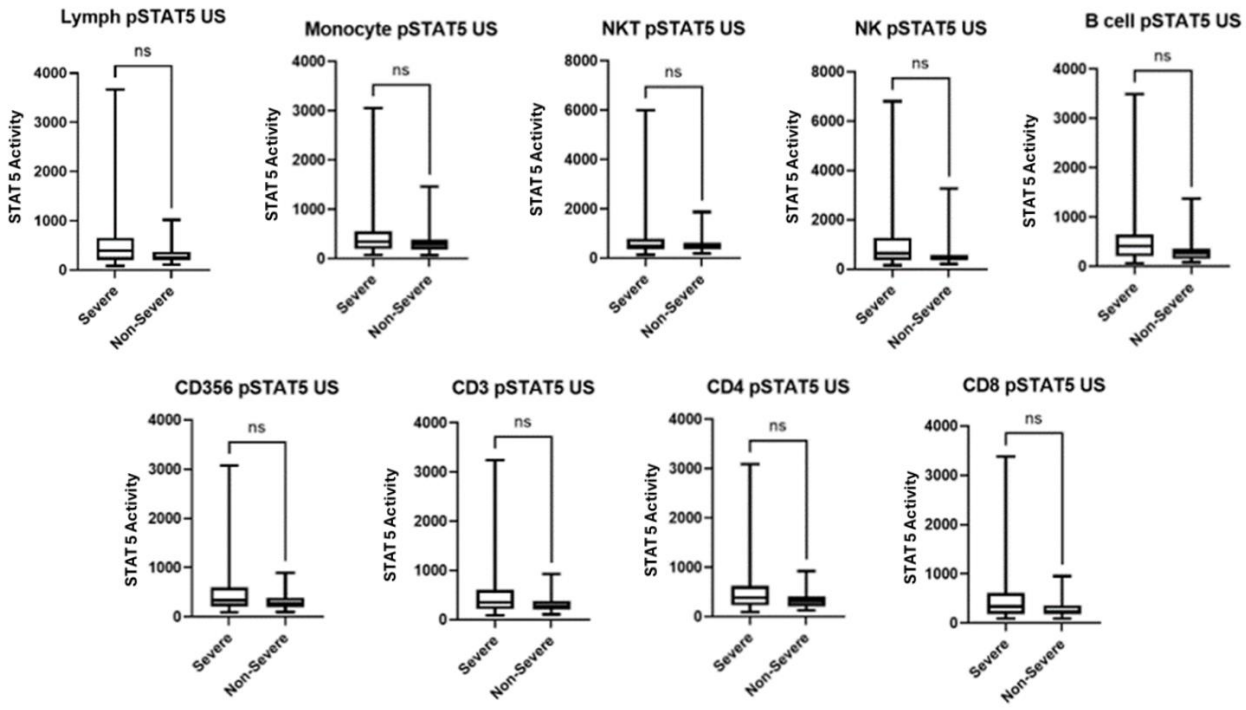

Figure 4.4 pSTAT5 levels in unstimulated peripheral blood immune cell subpopulations collected at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)

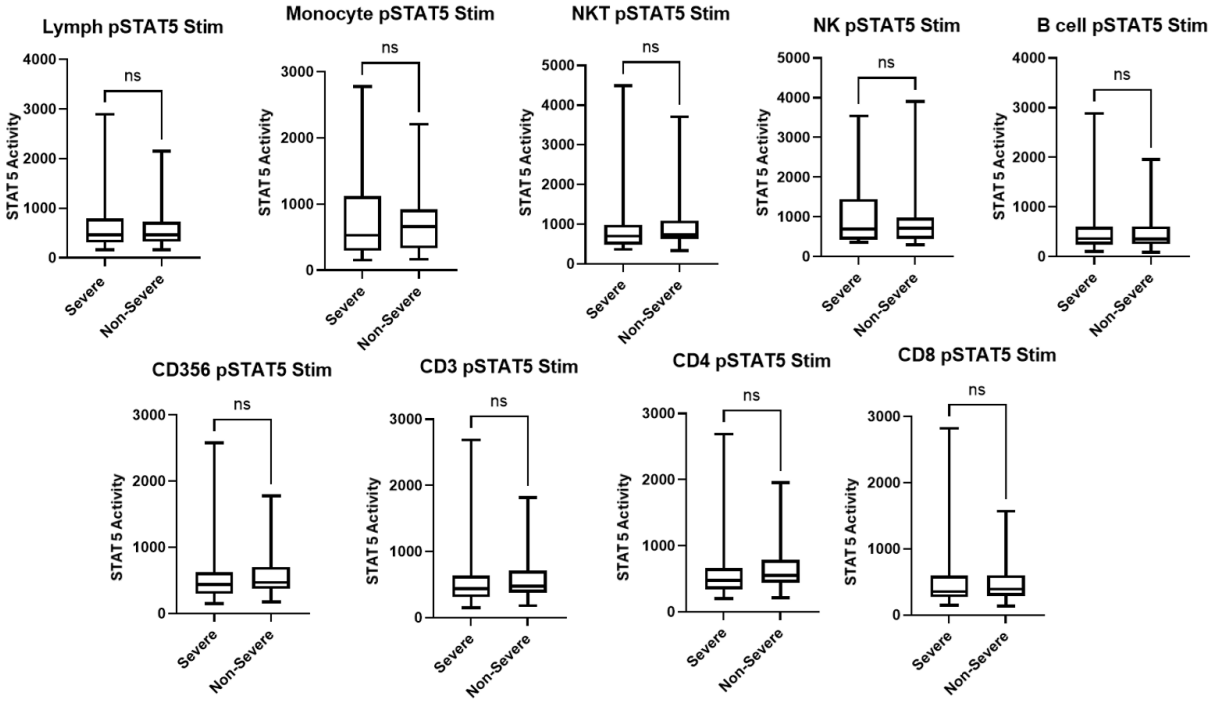

Figure 4.5 pSTAT5 levels in stimulated peripheral blood immune cell subpopulations collected at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 Requirement)

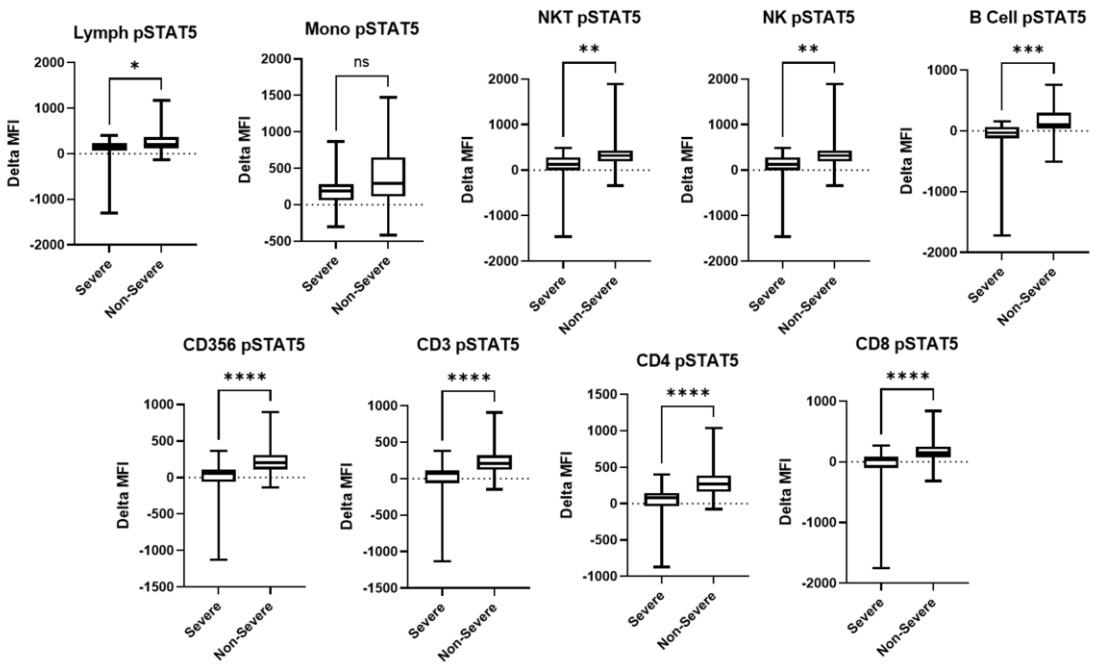

Figure 4.6 Increment in pSTAT 5 activity (Delta MFI, delta mean fluorescence intensity) after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement).

4.4.5 Discussion

In this prospective, single centre study of 60 patients admitted to hospital with COVID-19, it was possible to complete immunological characterisation by flow cytometry in 49 patients (81.7%). There was no difference in the level of STAT5 activity between severity groups after stimulation but we did find a nonsignificant trend towards higher STAT5 activity prior to stimulation in those with severe disease (figure 4.4). There was however a lower increment in STAT 5 activation after stimulation across all immune cell types except monocytes in those with severe disease compared to those with non-severe disease. Those with severe disease also commonly had features of metabolic syndrome (namely significantly greater levels of both hypertension and obesity).

The cohort presented here is a majority male cohort with an average age of 51 years, which was a representative cohort of hospitalised COVID-19 patients at the time of recruitment(104). Hypertension is a well-established risk factor for poor

outcome in COVID-19 and has been shown to remain a risk factor for severe COVID-19 independent of other factors such as male sex and obesity(156). Our findings of increased disease severity in those with obesity and hypertension are therefore in agreement with previous findings (99, 101).

This suggests that patients with severe disease, who frequently had features of metabolic syndrome (hypertension, obesity, resting hyperglycaemia but not diabetes mellitus), may have chronically higher baseline (pre-SARS-CoV-2 infection) peripheral blood immune cell STAT5 activation. Such patients may therefore be less able to mount an additional pSTAT5 response to SARS-CoV-2 resulting in adverse clinical outcomes. Previous work has suggested an association between metabolic syndrome and chronic activation of STAT signalling (153, 157).

Our cohort had a male predominance (57%) with a mean age of 51 years (SD 12.5). Clinical characteristics associated with severe disease in this cohort were obesity ($p= 0.0456$) and cardiovascular disease ($p= 0.0242$). When cardiovascular disease was broken down into individual conditions, this association was strongest for hypertension ($p= 0.0071$). Although gender, ethnicity and age have previously been reported as risk factors for poor prognosis and increased mortality in COVID-19, there was no significant difference in any of these features between our severity groups ($p= 0.7768$, $p= 0.1328$ and $p= 0.1279$ respectively). Unsurprisingly, those in the severe group had a significantly higher incidence of COVID pneumonia than those in the non-severe group($p= 0.0096$). We therefore believe the cohort we recruited is reasonably representative of the general population of COVID-19 admissions. As such, we conclude that the results generated regarding immunological characterisation are likely to be externally valid.

Additionally, although the STAT5 finding is novel, other features of the study cohort's immune response are in keeping with well documented findings in COVID-19. One such example is that of lymphopenia. It has been demonstrated previously that increasing lymphopenia correlates with increasing severity of COVID-19 and worsening outcome(158, 159). Our findings showed that those in the severe group had a significantly lower proportion of lymphocytes when compared to those in the non-severe group ($p= 0.0085$). One possible reason for this finding (ie lower peripheral blood lymphocytes in those with severe disease) is that lymphocytes may

be preferentially trafficked to the lungs mediating the pneumonia and lung injury commonly associated with severe COVID-19(160).

Most of what we know about the immune response to SARS-CoV-2 infection so far comes from serum analysis. As discussed above, given one of the major causes of death from COVID-19 is respiratory failure from COVID pneumonia(148) one area for future work would be to analyse bronchial lavage samples to gain a better understanding of the local immune response within the lungs themselves. One recent review describes differing intra-pulmonary T-cell responses to SARS-Cov-2 infection for example (113). Certainly it has been shown that there is lymphocytic infiltration in post-mortem sampling of the lungs of those who have died from COVID-19 (159, 161). It may therefore be that the key to understanding the immune response to SARS-CoV-2 infection involves both the systemic and local responses to the virus.

Another potential consequence of an improved understanding of the immune response to COVID-19 would be the discovery of a prognostic biomarker to aid further in the prediction of outcome from SARS-CoV-2 infection. Although the serum examined here was taken at baseline within 24 hours of admission, over three quarters (24/29 (83%)) of those in the severe group were already receiving oxygen at the time of sampling. As such, from this data, it is not possible to use STAT 5 activation status as a predictor of the need for oxygen but rather a marker of severe disease.

Study Limitations

Although recruited within 24 hours of admission, study participants were on average 8.8 days into their illness at the time of hospital presentation which matches data coming from China at the very start of the pandemic(81). A study of some of the very first people to contract COVID-19 found that dyspnoea developed around 8 days into the illness with the mean time to hospital admission being seven days(81).

It is important to acknowledge that the cohort examined here is relatively small with only 49 patients included in the final analysis. Additionally, all participants were recruited from a single centre. As explained however, our results certainly fit

with much of what is already known about COVID-19 and who develop severe disease(104, 156) suggesting our study population should be generalisable. Further work in a larger cohort is however warranted to assess if our findings are true of the wider population.

Another aspect to highlight about the results presented here is that they represent the immune response at one point in time. This may be important as it has been hypothesised that the immune response to COVID-19 likely changes throughout the course of the disease(151). In our cohort, our baseline samples were taken on average at almost day 9 of disease and therefore it is impossible to know from our data when this change in STAT 5 activation happens or indeed if it is maintained throughout the disease course. Further work is therefore needed to assess if this finding of a decreased level of activation of STAT5 in those with severe disease is present and maintained throughout the illness.

Clinical Implications and Future Work

There are many reasons to continue to improve our understanding of the individual immune response to COVID-19 and there are important outstanding questions generated from this work and the results reported here that could be studied to build on this knowledge.

Firstly, as alluded to above, it is important to confirm if the pattern of intracellular signalling molecule activity seen in the peripheral blood immune cells of the 49 subjects in this study is reproducible in a larger cohort. One possible way to achieve this would be to access the large biobank of over 4000 samples collected in the ASTERIX study. This resource includes surplus blood samples taken during the routine care of COVID patients across NHS Greater Glasgow and Clyde and could therefore allow analysis of samples from a considerably larger cohort taken at admission from patients with confirmed COVID-19.

Moreover, the link between poor outcomes from COVID-19 and those with features of metabolic syndrome has been widely demonstrated and this finding is reproduced in our cohort. Another question raised by our findings is of a potential connection between metabolic syndrome and altered STAT signalling in peripheral blood. Work

to look at the possibility of immune cell reprogramming mediated by excess adipocytes is currently being planned. This hypothesis will be tested by measurements of a panel of adipokines and other metabolic markers from additional banked samples taken within COLLECT at the time of recruitment. This work and the analysis of its results is being performed by the team at the Goodyear Lab.

This line of research is of particular importance given that JAK/STAT pathway modulators are now licenced and widely used in the treatment of COVID (for example tocilizumab and baricitinib)(118, 162). If there is a pattern of immune dysregulation involving JAK/STAT signalling associated with the metabolic syndrome phenotype then this raises a question as to whether such therapies may be less effective in patients with metabolic syndrome. One possible way of testing this theory would be to use samples banked from other trials. The COVACTA trial for example which recruited in NHS Greater Glasgow & Clyde was a randomised controlled trial to evaluate the efficacy of Tocilizumab in patients with severe COVID-19 pneumonia. This trial incorporated exploratory biomarker objectives specifically to identify and evaluate potential biomarkers associated with a response to tocilizumab. It may be possible therefore to access this bank of stored samples to test STAT activity in this cohort to evaluate the potential of STAT5 activity as a predictive marker of response to JAK/STAT modulators such as tocilizumab based on the findings presented here. The RECOVERY trial which incorporated a tocilizumab arm did also store patient samples in some cases which again may be a source for future research into the immune profiles of COVID-19 patients. Depending on the results of future work, there is a possibility that baseline STAT activity level could act as a predictive marker for the use of JAK/STAT modulators.

4.4.6 Conclusions

The current study is the first, to our knowledge, to describe a difference in individual STAT5 response to SARS-CoV-2. We report a novel finding of lower activation of STAT5 in those with severe COVID-19 requiring oxygen than in those with non-severe disease. Additionally, we found that in this cohort (as has been

widely reported in previous studies) severe COVID-19 was significantly associated with obesity and hypertension. These findings are from preliminary work based on a small cohort of just 49 people therefore future work is still required with a larger sample size to assess these findings further.

4.4.7 Reflections on this Paper

Developing this research protocol and recruiting to an exploratory COVID-19 trial at the height of the pandemic came with many challenges such as coordinating patient recruitment at a time when both clinical and research staff were extremely busy. I also had to learn many new skills such as flow cytometry and the analysis of its subsequent output in order to deliver the results outlined above.

This data shows a significantly smaller increase in STAT5 activation in all peripheral blood immune cells other than monocytes in response to stimulation in those with severe COVID-19 who require oxygen compared to those with mild disease and no oxygen requirement. As has been previously documented we also showed a link between poor COVID-19 outcome and metabolic syndrome with severe disease being significantly more common in those with obesity and hypertension in this cohort.

As such, further work is now ongoing by the Goodyear Lab at the University of Glasgow to look at adipokines from the patients in this cohort to assess for a potential association between altered STAT signalling in peripheral blood immune cells and the presence of metabolic syndrome. The results from this additional work will be included in the final publication of this project.

Future research based on the above findings may allow a better insight into the management of COVID-19. It may be for example that those with metabolic syndrome are less likely to benefit from therapies which alter JAK/STAT signalling with this increased knowledge potentially leading to improved patient selection for specific therapies.

CHAPTER 5: SUMMARY

5 Chapter 5 Summary

5.1 Summary of thesis

Both COVID-19 and lung cancer encompass a wide spectrum of disease severity and prognosis. Given COVID-19 can worsen over days while those with malignant effusion have a prognosis of as little as 3 months in some cases(21), prompt diagnosis and stratification of both conditions is important to ensure patients are optimally managed from the outset.

The work in this thesis was undertaken to examine the clinical utility of established diagnostic pathways in the real-world context of advanced lung cancer as well as the potential for improved staging of the disease through the addition of thoracoscopy in the diagnostic work up of those with otherwise early stage lung cancer and mini-PE. In addition, individual immune response to SARS-Cov-2 infection was explored to assess for any significant differences between those with mild vs severe disease and which immunological changes may drive these differing disease outcomes from the time of diagnosis.

5.2 Staging by Thoracoscopy in Potentially Radically Treatable Lung Cancer Associated with Minimal Pleural Effusion (STRATIFY): Protocol of a Prospective, Multicentre, Observational Study

The prospective, multi-centre, observational STRATIFY study was designed to address the uncertainty surrounding mini-PE in otherwise radically treatable lung cancer by prospectively evaluating the thoracoscopic staging of mini-PE for the first time. The primary endpoint was to determine the true prevalence of occult pleural metastases in those with mini-PE but otherwise radically treatable lung cancer.

STRATIFY recruited from 8 UK centres between January 2020 and May 2024. The inclusion criteria were kept deliberately broad to capture a real-world cohort of patients. Recruitment was suspended after the enrolment of just one patient due to

the outbreak of COVID-19 and accrual was slower than expected on re-opening. I worked with recruiting sites to amend the protocol to streamline the recruitment process and bring it in line with new diagnostic pathways which had been altered out of necessity during the pandemic. Changes included allowing LAT assessment and written consent to be performed on the same day as the LAT itself to minimise patient visits to hospital. We also opened the study up to include VATS to maximise possible recruiting centres.

37 patients were formally screened and of these, 27 were recruited (54% of the target sample size). 24 underwent LAT while VATS was performed on the remaining 3 participants. The mean age of those recruited was 69 (SD 1.7) years and 19/27 (70%) participants were male. The prevalence of OPM in this cohort was significantly lower than previously thought at just 14% (4/27) and importantly we showed that thoracoscopy is feasible, safe and clinically useful in this patient population.

Despite the protocol amendments we could not meet our recruitment targets. Some of this was down to delayed site opening however pre-screening data suggests that many patients with mini-PE were unfit for radical therapy or had metastatic disease elsewhere. We therefore made the decision to close STRATIFY to recruitment in May 2024.

5.2.1 Lessons Learned from STRATIFY and future work

I learned many valuable lessons from STRATIFY. In future I may think more closely about the benefits of a feasibility study for example. STRATIFY recruitment timelines and targets were however based on feedback and data from all recruiting sites prior to protocol finalisation and opening.

Despite the amendments I made to STRATIFY, none were able to entirely overcome the impact of the COVID pandemic. Having to adapt during that time however taught me how to be flexible and responsive when things are not going as planned. Through STRATIFY I learned how crucial it is to get ongoing feedback from sites in order to improve the protocol to facilitate recruitment. The challenges of STRATIFY also showed me that difficult decisions often have to be made in order to achieve

the primary endpoint of a study. This has given me the confidence to be able to make such decisions in the future if necessary.

Moving forward, given the prevalence of OPM was lower than predicted, further work into other reasons for the poor outcomes seen in those with mini-PE is warranted. Factors such as nutritional status, sarcopoenia and cardiac function are all areas for further investigation as factors that could both contribute to the presence of mini-PE as well as impacting on survival.

5.3 Pleural Fluid Predictive Marker Testing in Metastatic Lung and Breast Adenocarcinoma

To examine the utility of pleural fluid cytology in the assessment of predictive markers (PMs) for those with advanced lung and breast cancer I conducted a retrospective analysis of PM testing from pleural fluid cytology of proven lung and breast adenocarcinoma across 4 UK sites between 2016 – 2021. This is the first study, to our knowledge, to describe the real-world performance of pleural fluid PM analysis in lung and breast adenocarcinoma.

Of the 327 patients included in this analysis, PM testing was successful (with results for all mandated PMs) in only 20% of cases. This reflected a success rate of 19% for lung cancer and 22% in breast cancer.

PM success rate was significantly higher between 2018-2021 than between 2016-2018 (31/228 (14%) v 35/99 (35%) respectively, ($p<0.0001$)). This improvement reflected significantly improved success rates for the testing of PD-L1 (38/155 (24.5%) v 46/68 (67.6%), ($p<0.001$)) and ROS 1 (33/155 (21.3%) v 38/68 (55.9%), ($p<0.0001$)) in lung cancer and ER (62/73 (84.9%) v 31/31 (100%), ($p= 0.031$)) in breast cancer.

The median time from pleural fluid sampling to the reporting of full PM results was 19 (9-24) days. This confidence interval overlapped with time to SACT commencement with a median of 31 (4-45) days. This delay in result availability may in part explain the low PM directed SACT use in this cohort which accounted

for only 41% of those who received SACT. No pre-aspiration clinico-radiological factors were associated with the ability to test for PMs in pleural fluid.

These data suggest that pleural fluid cytology is insufficient for complete PM testing in the majority of cases and supports an argument for a direct-to-biopsy approach in select patients where full PM testing would potentially alter optimal management.

5.3.1 Lessons Learned and Future Work into Predictive Markers

My work on predictive markers has given me a better understanding of retrospective data and how to use it. I now better appreciate the work that must go into preparing for data collection. Firstly the need to be clear in your intentions when creating the data points to avoid wherever possible having to go back to collect more data at a later date while also minimising time spent collecting data that is not ultimately of use. Secondly, as this was a multi-centre study it taught me the importance of ensuring data collection is in a coded format wherever possible (eg 1-5, yes/no). This ensures the data collected is uniform and subsequently allows easier interpretation and analysis. Since completing the PM study, I have already used this experience to advise on others' projects and will bring this knowledge into any future retrospective work I undertake.

Building on this work, a prospective study into the role of pleural fluid for PM testing is warranted given that there are now new markers in routine use as well as our finding that successful testing of many markers significantly increased over the time period of my retrospective analysis. Unlike a retrospective study, this would capture the current utility of such tests as well as providing more information about how this information is used and the decision making around PM directed SACT from contemporaneous discussions.

Additionally, work looking at the real-world utility of cell free DNA, next generation sequencing and how to implement these into the NHS will be vital as routine use of this technology is likely to become the next major advancement in cancer diagnosis.

5.4 Exploring Baseline Immune Response to COVID-19 Infection and Its Association with Disease Severity

The aim of my final study was to examine the immune response in those infected with SARS-CoV-2 to assess any differences between those with mild vs severe COVID-19. This is the first paper, to our knowledge, to assess individual immune response in terms of activation status of important immune mediators at the time of diagnosis.

The average age of those included in the final analysis was 51 years with a slight male predominance making up 57% of the cohort. Severe disease was defined by the need for supplemental oxygen (n=29) compared to those with mild disease who had no oxygen requirement (n=20). In agreement with previous studies, obesity and hypertension were associated with severe disease in our cohort (p= 0.0456 and p= 0.0071 respectively).

Those with severe disease had a significantly higher level of monocytes (p = 0.0088) but a significantly lower proportion of lymphocytes (p = 0.0085) when compared to those with mild disease.

There was significantly less activation (phosphorylation) of STAT 5 in peripheral blood immune cells in response to stimulation in those with severe disease compared to those with mild COVID-19. There was no difference in the absolute levels of phosphorylated STAT5 before or after stimulation when comparing those with severe vs mild disease. There was however a trend towards higher baseline levels of STAT5 activation in those with severe disease. Our findings therefore raise the possibility of a connection between altered STAT signalling and the metabolic syndrome.

5.4.1 Future work into the Immune Response to COVID-19

The main lesson I have taken away from my work surrounding the immune response to COVID-19 is the value of an exploratory study. It has shown me how much can be learned from analysing a small cohort for potential signals in order to best plan future work involving larger numbers.

Based on my findings, the association between STAT 5 signalling activity, disease outcome from COVID-19 and its possible link to metabolic syndrome should be examined further in a larger cohort. There is a large biobank of samples in Glasgow taken from COVID-19 patients at the time of diagnosis that could allow for such further work. Additionally, research to look at how the immune response to SARS-CoV-2 changes over time as well as how the peripheral immune response compares to that of the lungs would both be valuable avenues for future research.

Finally, additional analysis of my exploratory cohort looking at the adipokines of these patients to further examine a possible link between the metabolic syndrome and altered JAK/STAT signalling in COVID-19 is currently underway.

Appendix 1 STRATIFY Ultrasound Manual

Ultrasound Manual

STRATIFY (L190)

Staging by **T**horacoscopy in potentially **Ra**dically **T**reatable Lung Cancer associated w**i**th Minimal Pleural E**f**fusion

Protocol No: STRATIFY2018

Sponsor Ref: GN16ON040

Version 1.1, 22 March 2023

Contents

1 Overview

Patients entered to the Screening stage of the STRATIFY study will undergo a thoracic ultrasound to determine their eligibility for the main STRATIFY study. The aim of this manual is to provide instruction for performing the thoracic ultrasound. This guidance should be followed for all study patients.

2 Contact Information

Chief Investigator: **Dr Kevin Blyth**
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Queen Elizabeth University Hospital

Clinical Research Fellow: **Dr Jenny Ferguson**
Department of Respiratory Medicine
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Project Manager: **Laura Alexander**
Level 0, Cancer Research UK Clinical Trials Unit, Beatson West of Scotland Cancer Centre

When contacting, please include the following information:

- Trial name (STRATIFY)
- Your name, email address and telephone number
- Your centre details
- Patient trial number (if applicable)

1 Pre-LAT Thoracic Ultrasound Assessment

1. Position patient comfortably in the lateral decubitus position lying on the opposite side to their effusion.

2. Position yourself in front of the patient.

3. Set ultrasound machine to B mode (2D)

4. Identify effusion and ipsilateral hemi-diaphragm.

5. Optimise image with appropriate depth and gain settings

4. Assess effusion:
   Maximum depth
   Height in number of rib spaces
   Extent of loculation

5. Assess for the presence of lung sliding:
   It is recommended this be assessed at multiple points not just within the safe triangle at the proposed access point
   Do this at three points

6. Identify a suitable point of entry within the safe triangle.

7. The final decision regarding feasibility of LAT should be made by the local Principal Investigator (PI) or a suitably trained assessor delegated by the PI.

Appendix 2 STRATIFY Thoracoscopy Manual

CHIEF SCIENTIST OFFICE

SCOTLAND
NHS RESEARCH SCOTLAND

STRATIFY

Thoracoscopy Manual

STRATIFY (L190)

Staging by **T**horacoscopy in potentially **Ra**dically **T**reatable Lung Cancer associated w**i**th Minimal Pleural E**f**fusion

Protocol No: STRATIFY2018

Sponsor Ref: GN16ON040

Version 1.2, 22nd Mar 2023

CANCER RESEARCH UK

Glasgow Pleural Disease Unit

University of Glasgow

CaCTUS

Contents

1 Overview

The aim of this manual is to provide instruction for the technical procedures related to Local Anaesthetic Thoracoscopy (LAT) and Video Assisted Thoracoscopy Surgery (VATS) thoracoscopy in STRATIFY. This guidance is not meant to replace existing protocols and it is acknowledged that practices vary considerably. Nevertheless, the following are minimum requirements that should apply to all study participants. Areas expected to vary between sites are *italicised*.

2 Contact Information

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Project Manager: **Laura Alexander**
Level 0, Cancer Research UK Clinical Trials Unit, Beatson West of Scotland Cancer Centre

When contacting, please include the following information:

- Trial name (STRATIFY)
- Your name, email address and telephone number
- Your centre details
- Patient trial number (if applicable)

1 Local Anaesthetic Thoracoscopy (LAT)

1.1 Immediate Pre-LAT Assessment and Safety Procedures

A detailed assessment regarding the safety and feasibility of LAT will have been performed at the formal screening visit. At that visit, an ultrasound scan will have been performed and appropriate blood tests sent in preparation. The following guide is not meant to replace existing pre-LAT checklists that exist in participating units, but should serve as a minimum standard for the immediate pre-LAT assessment, performed on the day of the procedure:

- Review blood results, including full blood count, coagulation screen, renal function and Group and Save (G&S) results. Ensure the G&S is valid and in date. *Note that a second valid result may be required in some centres before provision of blood, if required*.
- Update ECG as appropriate
- Review the patient's medication list. Note:
    - Warfarin should have been stopped at least 3 days pre-procedure and a normalized INR must be confirmed before LAT
    - Clopidogrel must be stopped at least 7 days pre-procedure
    - All DOACs must be stopped at least 2 days pre-procedure
    - Aspirin can be continued
- Ensure the patient has been appropriately fasted (at least 6 hrs prior to procedure time)
- Review the patient's understanding of procedure, answer any questions and complete procedural consent form (*this may be done pre-admission if this is local policy*)
- Secure IV access
    - minimum calibre 20G (Pink); ideally 18G (Green)
    - ideally distal to the elbow on the same side as the effusion
    - flush the venflon once sited to ensure patency
- Prescribe post-procedure analgesia, thromboprophylaxis +/- sedation
- Nursing staff should record routine observations pre-procedure

1.2 Location and Staffing

Local anaesthetic thoracoscopy should be performed in a suitable location *as per local arrangements* (ideally an endoscopy suite or theatre) to ensure sterile conditions can be maintained throughout the procedure. Minimum staffing should include:

- **Primary operator**: Suitably trained, independent operator should be present at all times. This operator should have received formal training and be experienced in Level II Thoracoscopy, including use of a Boutin needle for pneumothorax induction in cases with minimal or no pleural fluid
- **Scrub nurse**: One suitably trained nurse to assist the first operator during the procedure
- A third **adequately trained member** of the team to administer IV sedation and analgesia where necessary is also required. This member of staff may be medical or nursing and

may need to deliver the duties with the second nurse, see below

- A *second nurse* acting as a 'runner' is also recommended. This team member should be available to assist with any non-sterile duties, e.g. performing regular observations, changing fluids, opening equipment packs

1.2 Instruments and Equipment

All LAT procedures should be carried out using existing *instruments and equipment at each site*. Minimum requirements include:

- Sterile gowns and gloves
- Sterile needles and syringe for local anaesthetic administration
- Surgical cut down kit including, scalpel and blunt forceps
- Rigid or semi-rigid thoracoscope
- Boutine-type needle
- Port with conical tip trocar and cannula
- Cold light source
- Optical biopsy forceps (double spoon) for use with rigid thoracoscope or appropriate disposable biopsy forceps for use with semi-rigid kit
- De-mister. Either via thoracoscope warmer or suitable sterile de-misting solution
- Chest drain (20F), tubing and bottle
- Sutures
- Chest drain dressing

1.3 Positioning, Ultrasound & Site Preparation

Positioning

The patient should be positioned in the lateral decubitus position with the affected side lying superiorly. The patient should be made as comfortable as possible. It is recommended that at least one pillow is placed under the head and a further pillow placed underneath the dependant ribcage to avoid unhelpful rib crowding on the affected side. The patient's arms should be flexed and rested in front of their face or extended straight using an arm support.

Ultrasound

In addition to the ultrasound performed as part of the eligibility assessment for entry into STRATIFY, on-table ultrasound must be performed after positioning and before access. This is to ensure the anatomy, including the extent of loculation has not changed since screening and to facilitate surface marking, including marking of the optimal site of safe access. On-table US should be focused in the safe triangle. If no fluid is visible, lung sliding in at least one position should be confirmed by a suitably trained operator before proceeding. The diaphragm should

then be identified and its position marked. On the left side, the cardio-pleural angle should also be identified by a surface marking. Finally, a suitable entry site within the safe triangle should be marked in such a way that it remains visible after site clearing and preparation.

Site preparation

Once the patient has been suitably positioned and a safe site of access marked, a sterile field must be created. The operator must wash their hands using a standard surgical scrub technique before donning sterile gloves and gown. The patient's skin at the access site should be thoroughly cleaned using an *iodine-based solution or equivalent* as per local protocol. The site should be dressed using sterile drapes.

1.1 Access

Local anaesthetic

1-2% lidocaine (+/- adrenaline) should be used to anaesthetise the skin and subcutaneous tissues down to the parietal pleura. The maximum dose of 3mg/kg should not be exceeded.

Incision

Once the skin and underlying tissue have been adequately anaesthetised, an incision in the same plane as the underlying rib should be made. This should be just deep enough to expose underlying subcutaneous fat and just long enough to allow blunt dissection and subsequent entry of the thoracoscopy port.

Induction of pneumothorax

Given the nature of the STRATIFY study, it is likely that pneumothorax induction will frequently be required and should not dissuade proceeding with LAT. This can be done *with or without direct ultrasound guidance* (*as per Corcoran et al, Thorax 2015*), at the discretion of the operator, and based on their current practice. A Boutin-type needle should carefully be inserted into the pleural cavity. A detailed description of the method is not required but this should include initial shallow penetration using the sharp obturator, which should not be inserted into the intercostal space. Prior this depth, the blunt obturator should be swapped in, allowing safe access to the pleural cavity. Once the parietal pleural has been punctured, the blunt obturator should be removed to allow entrainment of air into the pleural cavity. Ten breaths should be counted to allow a sufficient volume of air to enter the space before replacing the blunt obturator, screwing it in place and removing the entire needle. Blunt dissection should then be performed to create a tract suitable for placement of the thoracoscopy port. This should be done with blunt forceps as per standard practice. Following blunt dissection, it should be possible to insert the port with no (or minimal) resistance.

1.1 Inspection & Pleural Fluid Sampling

Insertion of the thoracoscope for visual inspection should be preceded by removal of any pleural effusion using a flexible suction catheter. Note that samples of this pleural fluid should be collected and processed for storage and use in future research as per the STRATIFY Sample Handling Manual. **However, pleural fluid samples should not be sent for cytology analysis unless biopsies are also sent given the uncertain significance of positive results in this clinical context.** Systematic visual inspection of the whole hemi thoracic cavity (apex, costal surface, diaphragm, lung surface) should then be performed. Any abnormalities should be documented on the corresponding STRATIFY Thoracoscopy Worksheet (Appendix 1). Where the lung fails to deflate sufficiently to allow full inspection, operators are advised to:

a) ensure any fluid sitting on the mediastinal surface of the lung has been completely removed by reinserted a flexible suction catheter around the posterior and anterior borders of the lung
b) Remove the thoracoscope and allow a larger volume of air to enter the pleural cavity during free breathing: to facilitate this the tip of the port should be directed upwards, so that it lies within the volume of air already within the space

1.2 Biopsy Sampling

Once inspection is complete, up to 5 biopsies should be taken from sites of **visible parietal pleural abnormality**. IV analgesia should be administered prior to biopsy. Biopsy sites should be chosen by the primary operator but **should not include areas of visceral pleura.** The sampling of diaphragmatic sites is permitted since this a parietal surface, but given the increased pain and risk associated with this technique, caution is advised unless easily sampleable disease is identified. All biopsies should be collected and processed as per local policy for pleural pathology samples.

1.3 Drain Placement and Use of Pleurodesis

Once the operator is satisfied that all necessary biopsies have been taken, a final visual inspection of the pleural cavity should be performed. As per standard practice, this is to ensure haemostasis has been achieved at each biopsy site and to plan drain placement. Care should be taken to minimise the time between removal of the thoracoscope and associated port and insertion of the chest drain, given the potential for the lung to re-expand during this interval. An intercostal drain (*20F Argyle-style is recommended but can be as per local policy*) should be inserted using the stiffened trochar provided at the original access site and directed to the lung apex, if possible. Operators may also choose to direct the drain using a guidewire inserted via the port prior to its removal, e.g. in cases where blunt dissection was technically challenging. Once the drain is in place, it should be connected to an underwater seal or electronic drainage system (e.g. Thopaz®) depending on local policies. The drain should be secured, ideally by 2 sutures,which should also close the wound around the tube. The site should be cleaned of any blood before the application of a suitable dressing.

1 Post-LAT Procedures

1.1 LAT Report

All LATs should be documented as per local policy in patient notes. In addition, the STRATIFY Thoracoscopy Worksheet (Appendix 1) should be completed, including recording of any biopsies taken marked on the map provided. **This worksheet should be uploaded onto local electronic health records to act as source data for the procedure that will be common to all sites. The completed worksheet data must also be inputted to the STRATIFY MACRO® database.**

1.2 LAT Pleural Fluid Sample Processing and Storage

Pleural tissue biopsies should be processed and analysed as per normal local policy. Pleural fluid samples should be handled and stored as described in the STRATIFY Sample Handling Manual. Note**: Pleural fluid samples should not be sent for cytology analysis unless biopsies are also sent given the uncertain significance of positive results in this clinical context.**

1.3 Electronic transfer system

Linked anonymised LAT reports should be transferred as .pdf files, annotated by the participant's unique study ID and the date of LAT using the University of Glasgow Transfer Service (https://transfer.gla.ac.uk/). This is a secure system with all files transferred in an encrypted format and access strictly controlled and logged. Data files will be uploaded to the service in a password-protected encrypted archive format. When the recipient collects the transferred file a notification is emailed to the sender who will then provide the recipient with the password to unlock the file. See Appendix 2 for full instructions on the transfer process.

1.4 Drain Removal, Discharge from Hospital and Follow-up

The intercostal drain should be removed once maximum lung re-expansion has been achieved on a pre-removal chest radiograph. This radiograph should occur at least 1 hour after completion of LAT, and no later than 12 hours after LAT completion. Ideally, drain removal should occur on the same day as the procedure. A further chest radiograph following drain removal is not required, unless clinically indicated. 1-2 stitches should be placed after drain removal and an occlusive dressing applied. The patient should be discharged as soon as clinically appropriate, ideally on the same day as LAT**.** *Where clinically indicated, or for logistical reasons, patients may be admitted to hospital overnight after LAT.*

Patients should be provided with written details of their follow-up appointment (venue, date, time) to discuss LAT results (Study Visit 4) prior to discharge home. Patients should be discharged with adequate analgesia, a supply of replacement dressings, appropriate clinical worsening advice and contact details for the clinical team.

1 Video-Assisted Thoracoscopy Surgery (VATS)

1.1 Location and Staffing

VATS should be performed in an operating theatre to ensure sterile conditions can be maintained throughout the procedure. Minimum staffing should include:

- **Primary operator**: Suitably trained, independent operator should be present at all times.
- **Anaesthetist:** Responsible for induction and maintenance of general anaesthesia.
- **Scrub nurse**: One suitably trained nurse to assist the first operator during the procedure
- **A *second nurse*** acting as a 'runner'; available to assist with any non-sterile duties, e.g., performing regular observations, changing fluids, opening equipment packs

1.2 Instruments and Equipment

All VATS procedures in STRATIFY should be carried using existing *instruments and equipment at each site*. Minimum Instrument and Equipment requirements should include:

- Sterile gowns and gloves
- Sterile needles and syringe for local anaesthetic administration
- Surgical cut down kit including, scalpel and blunt forceps
- Rigid or semi-rigid thoracoscope and appropriate biopsy forceps
- Port with Conical tip trocar and cannula
- Cold light source
- Chest drain (20F), tubing and bottle, dressings
- Sutures

1.3 Positioning and Site Preparation

The patient should be positioned in the lateral decubitus position with the affected side lying superiorly. A sterile field must be created, including use of an *iodine-based solution or equivalent* as per local protocol. The site should be dressed using sterile drapes applied.

1.4 Access

Access should use standard VATS methodology. One or two ports may be inserted.

1.5 Inspection & Pleural Fluid Sampling

Insertion of the thoracoscope for visual inspection should be preceded by removal of any pleural effusion using a flexible suction catheter. Note that samples of this pleural fluid should be collected and processed for storage and use in future research as per the STRATIFY Sample Handling Manual. **Note: Pleural fluid samples should not be sent for cytology analysis unless biopsies are also sent given the uncertain significance of positive results in this clinical context.**

Systematic visual inspection of the entire pleural space (including costal surface, diaphragm, apex, lung surface) should then be performed. Any abnormalities should be documented on the STRATIFY Thoracoscopy Worksheet (Appendix 1).

1.1 Biopsy Sampling

Once inspection is complete, up to five biopsies should be taken from different sites of visible pleural abnormality, in addition to clinical biopsies. The number of clinical biopsies taken should be at the discretion of the primary operator in line with their usual clinical practice. Biopsy sites should be chosen by the primary operator and may include areas of visceral pleura. Biopsies for clinical diagnostic use should be collected and processed as per existing local policies.

1.2 Drain Placement and Use of Pleurodesis

Once the operator is satisfied that all necessary biopsies have been taken, a final visual inspection of the pleural cavity should be performed. As per standard practice, this is to ensure haemostasis has been achieved at each biopsy site and to plan drain placement. An intercostal drain (at least *20F Argyle-style is recommended but should be as per local policy*) should be inserted using the stiffened trochar provided at the original access site and directed to the lung apex, if possible. Once the drain is in place, it should be connected to an underwater seal or electronic drainage system (e.g., Thopaz®) depending on local policies. The drain should be secured, ideally by 2 sutures, which should also tighten the wound around the tube. The site should be cleaned of any blood before the application of a suitable dressing.

2 Post-VATS Thoracoscopy Procedures

2.1 Thoracoscopy Report

All thoracoscopies should be *documented as per local policy* in patient notes. In addition, the STRATIFY Thoracoscopy Worksheet (Appendix 1) should be completed, including recording of any biopsies taken on the map provided. **This worksheet should be uploaded onto local electronic records to act as source data for the procedure that will be common to all sites. The completed worksheet data must also be inputted to the STRATIFY MACRO® database.**

2.2 Pleural Fluid Sample Processing and Storage

Pleural tissue biopsies should be processed and analysed as per normal local policy. Pleural fluid samples should be handled and stored as described in the STRATIFY Sample Handling Manual. Note**: Pleural fluid samples should not be sent for cytology analysis unless biopsies are also sent given** the uncertain significance of positive results in this clinical context.

2.3 Electronic transfer system

Linked anonymised thoracoscopy reports should be transferred as .pdf files, annotated by the participant's unique study ID and the date of thoracoscopy using the University of Glasgow Transfer Service (https://transfer.gla.ac.uk/). This is a secure system with all files transferred in an encrypted format and access strictly controlled and logged. Data files will be uploaded to the service in a password-protected encrypted archive format. When the recipient collects the

transferred file a notification is emailed to the sender who will then provide the recipient with the password to unlock the file. See Appendix 2 for full instructions on the transfer process.

1.1 Drain Removal, Discharge from Hospital and Follow-up

The intercostal drain should be removed once maximum lung re-expansion has been achieved, confirmed by a chest radiograph 1-12 post-VATS. Ideally drain removal should occur on the same day as the procedure unless talc poudrage performed. The patient should be discharged as soon as clinically appropriate, ideally on the same day if possible. *Where clinically indicated, or for logistical reasons, patients may be admitted to hospital overnight after VATS Thoracoscopy*.

Patients should be provided with written details of their follow-up appointment (venue, date, time) to discuss VATS results (Study Visit 4) prior to discharge home. Patients should be discharged with adequate analgesia, a supply of replacement dressings, appropriate clinical worsening advice and contact details for the clinical tea

1 Appendix 1: Thoracoscopy Worksheet

STRATIFY
THORACOSCOPY WORKSHEET

L190
ISRCTN13584097

Staging by Thoracoscopy in potentially Radically Treatable Lung Cancer associated with Minimal Pleural Effusion

Patient Initials: (F) ____ (S) ____	Date of Birth: DD / MON / YYYY	Study Number:

GENERAL

Side: ☐ Right ☐ Left **Procedure Type** ☐ LAT ☐ VATS

Septations: ☐ Yes ☐ No **Volume drained:**_____ **ml**

PROCEDURE DETAILS

DRUG	OPTION (please ✓)	DOSE
Pre-medication	☐ Oramorph	_____mg
	☐ Atropine	_____mg
	☐ Sevredol	_____mg
	☐ Other, specify *(incl unit)*: _____	_____
Sedation	☐ Midazolam	_____mg
	☐ Propofol	_____mg
	☐ Other, specify *(incl unit)*: _____	
	☐ General Anaesthesia	
Local anaesthetic	☐ Lidocaine ☐ 1% ☐ 2% _____ml	☐ Adrenaline inclusion
	☐ Other, specify *(incl unit)*: _____	_____
Analgesia	☐ Alfentanyl	_____micrograms
	☐ Fentanyl	_____micrograms
	☐ Morphine	_____mg
	☐ Other, specify *(incl unit)*: _____	_____

US on table: ☐ Yes ☐ No **Boutin with US:** ☐ Yes ☐ No

Fluid on US: ☐ Yes ☐ No ☐ N/A **Talc:** ☐ Yes ☐ No **If yes, dose given:**_____ g

Boutin induction: ☐ Yes ☐ No **Drain size:** _____F

IMMEDIATE COMPLICATIONS: IF NONE TICK HERE ☐

Haemorrhage requiring transfusion: ☐ Yes ☐ No **Failure of procedure:** ☐ Yes ☐ No

Hypotension requiring intervention: ☐ Yes ☐ No **Other** ☐ **Yes specify:-** _____

BIOPSY DETAILS

SITE	ABNORMALITY	NO. OF BIOPSIES
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		

RECORD ABNORMALITY:

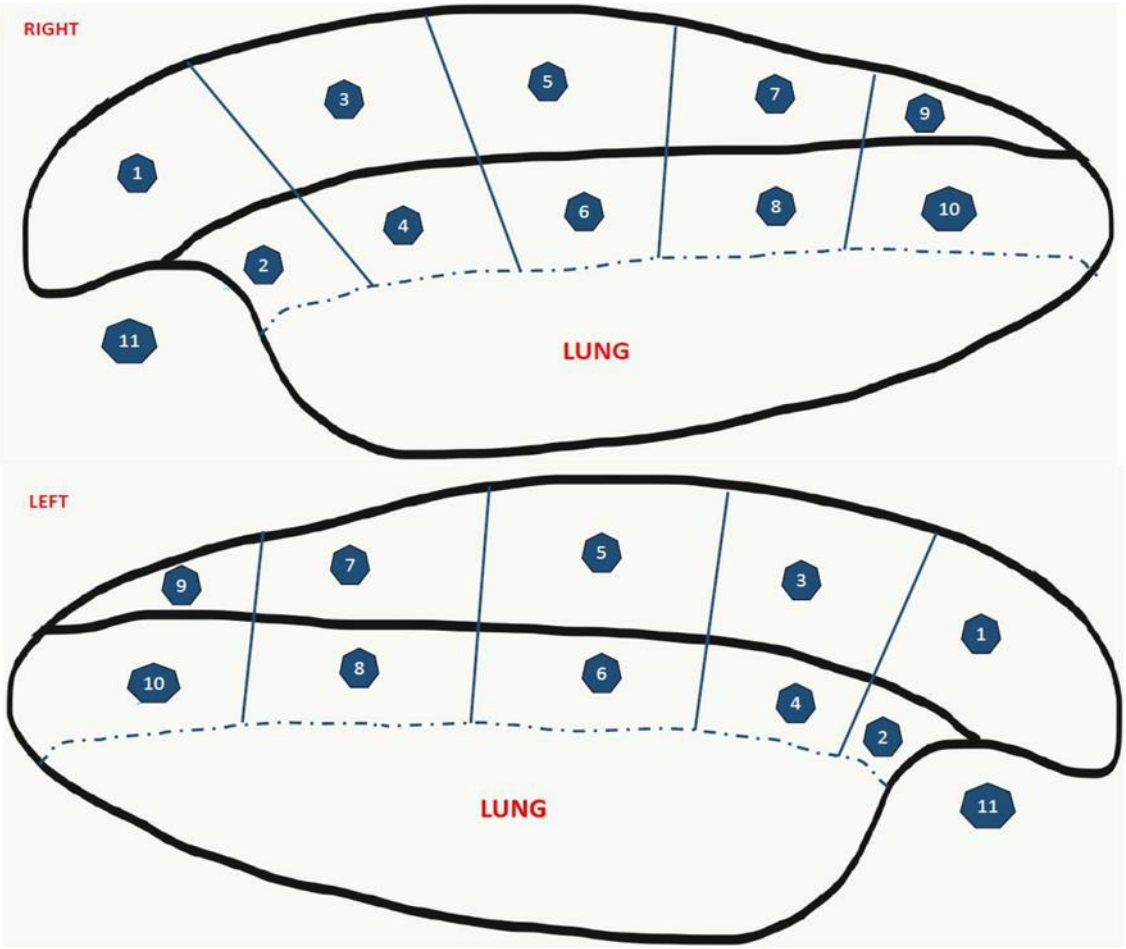
- MACRO - *macronodularity*
- MICRO - *micronodularity*
- THICK - *pleural thickening*
- NORMAL – *where site appears normal, but a biopsy is taken**

**If no abnormality is present in a numbered site and no biopsy is taken, leave site row blank*

RECORD BIOPSIES:

- Number taken – 1-5
- Unsuccessful - *attempted but unsuccessful*
- N/A - *Not attempted*

FOR REFERENCE:



INVESTIGATOR SIGNATURE: _____ **DATE:** DD / MON / YYYY

1 Appendix 2 - Glasgow University Transfer Service User Instructions

1.1 Access the service at https://transfer.gla.ac.uk/

1.2 Types of User

There are two kinds of users that can access the transfer system:

- Internal: University of Glasgow staff who are allowed to create a drop-off that can be delivered to one or more individuals (whether they are internal or external to the University)

- External: anyone else, anywhere on the Internet, who are only allowed to create a drop-off that is to be delivered to University of Glasgow staff members

1.3 Drop off and pick up

- A drop-off is one or more files uploaded to Transfer as a single entity for delivery to a specified person

- A pick-up allows a person to collect the dropped-off files

1.4 Creating a drop-off

- When creating a drop-off you:
    - enter identifying information about yourself by logging in or providing you name, organisation and email address
    - enter identifying information about the recipient (name and email address)

      choose which files should be uploaded to the drop-off

- If the files are successfully uploaded, an email is sent to the recipient explaining that a drop-off has been made with a link to access the drop-off.

- Other information (the internet address and/or hostname from which the drop-off was created, for example) is retained, so that the recipient can verify the identity of the sender

- The recipient has **14 days** to pick-up the files. Each night, drop-offs that are older than 14 days are deleted from the system. If your recipient(s) have not picked them up in that time, you will have to repeat the drop-off

Appendix 3 STRATIFY Thoracoscopy Report Form

STRATIFY LAT REPORT FORM – MDT SUMMARY

Patient Trial number:

Patient Initials:

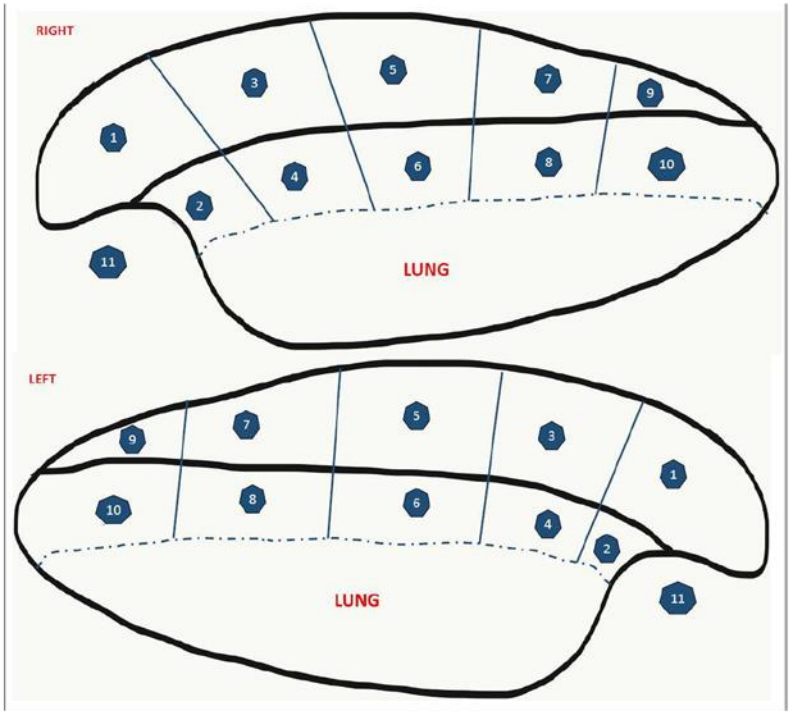
Trial Site:

Resp Consultant:

LAT Date:

LAT 1st Operator:

LAT 2nd Operator



Samples Taken: (NB all sent for urgent processing)

Biopsies taken? Y/N If Y, specify all sites sampled:

Were biopsies taken from visible parietal pleural tumour? Y/N

Fluid sent for cytology: Y/N Volume:

NOTE: Fluid is only sent for cytological analysis in patients in whom parietal pleural tumour is visualised. This is to maximise the diagnostic yield of sampling in that context.

The prognostic significance of positive fluid cytology results in patients without parietal pleural tumour is uncertain and may not exceed that of pleural lavage cytology, which would not preclude radical treatment [1, 2].

1. **Lim et al:** Impact of positive pleural lavage cytology on survival in patients having lung resection for non-small cell lung cancer: An international individual patient data meta-analysis (JTCVS, June 2010, Vol 139, Issue 6, Pages 1441-1446)
2. **Lim et al:** Intraoperative pleural lavage cytology is an independent prognostic indicator for staging non-small cell lung cancer (JTCVS, April 2004, Vol 127, Issue 4, pages 1113-1118)

Appendix 4 STRATIFY Sample Handling Manual

Sample Handling Manual

STRATIFY (L190)

Staging by **T**horacoscopy in potentially **Ra**dically **T**reatable Lung Cancer associated w**i**th Minimal Pleural E**f**fusion

Version 2.1: 22 March 2023

Protocol No: STRATIFY2018

Sponsor Ref: GN16ON040

Contents

1. Contact Information

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Clinical Research Fellow: **Dr Jenny Ferguson**
Department of Respiratory Medicine
Queen Elizabeth University Hospital

Glasgow Biorepository Contacts: **Alexandra Bell or Clare Orange**
NHS GG&C Biorepository
Level 3 Laboratory Medicine /FM Building
Queen Elizabeth University Hospital

When contacting, please include the following information:

- Trial name (STRATIFY)
- Your name, email address and telephone number
- Your centre details
- Patient trial number (if applicable)

1. Introduction

The purpose of this manual is to describe the collection, processing, storage and transportation of blood samples for patients who have consented to take part in the translational research aspect of STRATIFY.

2. Scope

This manual covers handling of blood and pleural fluid samples at clinical centres.

3. Responsibilities

The clinical staff at participating centres are responsible for ensuring that samples are collected, handled, processed and stored at their clinical centre in accordance with these instructions.

Please read this manual carefully and contact the Clinical Research Fellow or Project Manager with any questions. Please ensure that you complete and return the declaration at the end of this document stating that you have received, read and understood this manual.

4. Related Documents

- Clinical Trial Protocol: STRATIFY Staging by Thoracoscopy in potentially Radically Treatable Non-Small Cell Lung Cancer associated with Minimal Pleural Effusion
- STRATIFY Local Anaesthetic Thoracoscopy Manual

1. Consumables and Equipment

1.1. **Equipment**

To be provided by the Clinical Site

- Centrifuge (refrigerated)

1.2. **Consumables**

The CRUK CTU will provide the following items:

Item
EDTA tube: VACUETTE® TUBE 6 ml K2EDTA, lavender capped
EDTA K3 tube, 9 ml Lavender capped
SST Clot activator 5ml tube, yellow capped
1.5ml cryovials
Yellow cryovial caps
Red cryovial caps
30ml universal containers for pleural fluid
5.0ml cryovials
Cryolabels
Pipettes
Sample bags (mini grip) 15x20cm
Needles for research blood draw
Syringes for research blood draw
Cryoboxes for cryovials
Cryoboxes for whole blood tubes
Padded envelopes

The clinical site will provide the following items:

Item
Bubble wrap
Indelible marker pen

1. Sample Collection Schedule

Whole blood, plasma, serum, and pleural fluid samples will be collected from patients according to the schedule of assessments outlined below.

Study Procedure	Visit 1 *or* Visit 2 *or* Visit 3*	Visit 3 (LAT)
Plasma sample	X	
Serum sample	X	
Whole blood sample	X	
Pleural fluid sample		X

**per protocol, if plasma and serum sample not taken at Visit 1, should be taken at Visit 2 for MRI sub-study patients, or at Visit 3 for patients not participating in MRI sub-study*

2. Research Blood Sample Processing, Storage and Shipment

As outlined above, research bloods will be collected and processed at Visit 1 *or* 2 *or* 3* to generate the following samples:

Sample Type	Blood Collection Volume /Tube Type	Manual
Whole blood	10ml of blood collected into 2 x 6ml EDTA tubes (5ml/tube)	Section 8.1
Serum	4ml blood collected into a 5ml Serum Vacuette tube, processed into 4-5 microfuge tubes	Section 8.2
Plasma	10ml blood collected into 2 x 9ml EDTA tubes (8ml/tube), processed into 10-15 microfuge tubes	Section 8.3

**per protocol, if plasma and serum sample not taken at Visit 1, should be taken at Visit 2 for MRI sub-study patients, or at Visit 3 for patients not participating in MRI sub-study*

2.1. **Whole Blood Sample Processing Method**

- Samples to be collected at either Visit 1, 2 or 3 as described
- Check expiration date on 6ml EDTA tube; if expired replace with new one
- Collect approximately 5mls of venous blood into a 6ml EDTA tube
- Gently invert the tube 8-10 times
- Complete the STRATIFY Whole Blood label with an indelible pen and place label firmly onto tube, ensuring that the bottom of the label is twisted at the base of the tube.

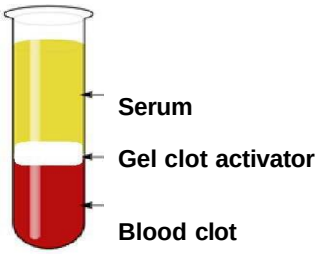
<u>Please ensure the label is placed on the tube before it is frozen otherwise it will not adhere</u>

- Immediately place the tube into a small sample storage bag labelled using an indelible pen with the following information:
  - Trial name (STRATIFY)
  - Recruiting Centre
  - Patient Trial Number

  - Patient initials
  - Whole blood
- Place bag into -80ºC (+/- 10ºC) freezer until ready to ship (see section 9 for shipping instructions)
- Complete the whole blood worksheet with the time sample was frozen and the details of the operator.

1.2. **Blood collection for isolation of serum**

Centrifugation of clotted blood causes separation of blood cells from the serum. Serum moves to the top of the tube and forms the supernatant. The gel layer in the vacutainer serves to separate the blood clot from the serum after centrifugation (see diagram). This top layer of serum can then be carefully removed with a pipette and stored at -80ºC.



Centrifugation should occur as soon as possible after blood has clotted and all specimens should be processed and frozen within **2 hours** of venepuncture.

Method

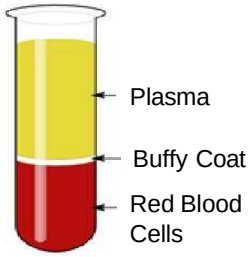
- Samples to be collected at either Visit 1 or 2 or 3 as described
- Check expiration date on yellow vacutainer; if expired replace with new one
- Collect approximately 4ml of venous blood into 1 yellow vacutainer tube containing SST clot activator
- Gently invert sample 5-6 times
- Record sample collection date and time on serum worksheet
- Allow the sample to clot for 30 minutes at room temperature before centrifugation
- Centrifuge at 2200g for 15 minutes at room temperature
- Record centrifugation time on serum worksheet
- Carefully withdraw the top layer using a pipette and dispense 500µl aliquots into the DNase/RNase free microfuge tubes. There should be enough serum for 4-5 microfuge tubes. Do not overfill these tubes
- Place a red cap on each tube
- Complete the STRATIFY Serum labels with an indelible marker and stick them securely onto tubes, ensuring that the bottom of the label is twisted around the base of the tube. Please ensure the label is placed on the tube before it is frozen otherwise it will not adhere
- Label the top of the tubes using an indelible marker with the patient trial number, S (serum) and time-point (baseline).
- Place into cryobox labelled using indelible marker with the following information:
  - Trial Name (STRATIFY)
  - Recruiting Centre
  - Patient trial number
  - Patient initials

- Place cryobox into -80ºC (+/- 10ºC) freezer until ready to ship (see section 5 for shipping instructions)
- Complete the serum worksheet with the time serum samples were frozen, the number of microfuge tubes and the details of the operator.

1.1. **Plasma Sample Processing**

IMPORTANT: Blood samples must be centrifuged within 1hr of collection to avoid fragmentation, degradation and leukocyte lysis.

- Centrifugation of un-clotted blood causes separation of blood cells from plasma. A clear layer of plasma will form the supernatant and can then be carefully removed using a pipette.
- The white cells and platelets will form a layer underneath the plasma - this is known as the buffy coat layer. The red blood cells form a layer underneath the buffy coat (see diagram).



Method

- Samples to be collected at either Visit 1, 2 or 3 as described
- Check expiration date on 9ml EDTA tubes; if expired replace with new ones
- Collect 8mls of venous blood into 2 x EDTA tubes (approximately 16mls in total)
- Gently invert samples 8-10 times and leave upright prior to centrifugation
- Record the sample collection time on the plasma laboratory worksheet
- Centrifugation should be done **immediately** with these samples as they do not need to clot
- Centrifuge at 2200g for 15 minutes at room temperature
- Record the time of centrifugation on the plasma laboratory worksheet
- Carefully withdraw upper plasma layer using a pipette Transfer 500µl aliquots of plasma into 1.5ml DNase/RNase free microfuge tubes and discard the pellet and any remaining plasma. There should be sufficient plasma for 8-10 microfuge tubes. Do not overfill the tubes
- Place a yellow cap on each of these tubes (these can be re-used from previous step)
- Complete the STRATIFY Plasma labels using an indelible pen and stick them onto the tubes, ensuring that they are secure and the bottom of the label is twisted around the end of the microfuge tube. Please ensure the label is placed on the tube before it is frozen otherwise it will not adhere
- Label the top of the tubes using an indelible marker with the patient trial number, P (plasma) and time-point:
- Place tubes into a cryobox labelled using indelible marker with the following information:
    - Trial name (STRATIFY)
    - Recruiting Centre
    - Patient trial number
    - Patient initials
- Place cryobox into -80ºC (+/- 10ºC) freezer until ready to ship (see section 5 for shipping instructions)
- Complete the plasma worksheet with the time plasma samples were frozen, the number of microfuge tubes and the details of the operator.

1.1. **Pleural Fluid Sampling and isolation of supernatant**

At Visit 3 pleural fluid will be collected during the LAT and processed to generate the following samples:

Sample Type	Blood Collection Volume /Tube Type
Pleural fluid	1pleural fluid collected in a 30ml universal containers and processed into 7-8 x 5ml cryotubes.

- Further information regarding the LAT procedure itself can be found in the accompanying handbook. Once the fluid samples are obtained they should be processed and frozen within 2 hours as follows:
- Centrifuge at 2200 x g for 15 minutes at room temperature
- Record centrifugation time on pleural fluid worksheet
- Carefully withdraw the supernatant using a pipette and dispense 4ml aliquots into the 5ml tubes. There should be enough serum for 7-8 x 5ml tubes. Do not overfill these tubes
- Securely fasten the cap on each tube
- Complete the STRATIFY pleural fluid labels with an indelible marker and stick them securely onto tubes, ensuring that the bottom of the label is twisted around the base of the tube.

Please ensure the label is placed on the tube before it is frozen otherwise it will not adhere

- Label the top of the tubes using an indelible marker with the patient trial number
- Place into cryobox labelled using indelible marker with the following information:
    - Trial Name (STRATIFY)
    - Recruiting Centre
    - Patient trial number
    - Patient initials

- Place cryobox into -80ºC (+/- 10ºC) freezer until ready to ship (see section 5 for shipping instructions)
- Complete the pleural fluid worksheet with the time serum samples were frozen, the number of microfuge tubes and the details of the operator.

1. Handling and Transport of Processed Samples

- At the end of the trial, each patient should have the following cryo-boxed samples:
  - 2 x 6 ml tubes with whole blood
  - Up to 5 Serum samples with red lids in 1.5ml tubes
  - Up to 15 Plasma samples with yellow lids in 1.5ml tubes
  - Up to 8 pleural fluid samples in 5.0ml tubes

- Sample tubes must be stored in the cryo-boxes provided by the CTU. Box number and tube position within the cryo-box will require to be completed on the provided STRATIFY sample submission form, prior to shipping.

  Samples should be kept at local sites in -80ºC (+/-10ºC) storage conditions and will be transferred to the Glasgow Biorepository on dry ice when study recruitment is completed at all sites. The Cancer Research UK Clinical Trials Unit will contact each site to advise when samples are to be shipped and will provide courier instructions

- Samples must be packed securely to avoid breakage during transit and with sufficient dry ice to prevent thawing for at least 2 days to allow for any delays in transport or delivery (2.3 – 4.5 kg per 24 hours). Dry ice and transportation box will be provided by the courier at the time of sample collection. Completed worksheets, and sample submission forms should be packaged with the samples. A receipt will be included in the paperwork for Glasgow Biorepository to record receipt of the samples (see worksheets).

- For queries relating to the transfer of samples to the Glasgow Biorepository, please contact Laura Alexander at Cancer Research UK Clinical Trials Unit, Glasgow (page 3). Please include the trial ID (STRATIFY) in all communications.

1. Worksheets

1.1. **STRATIFY Whole Blood Worksheet**

Patient Study Number:_____ Patient Initials:_____

Centre Name:_____

Time Point	Date and Collection Time	Time Frozen	Operator (Print Name and Sign)
Baseline			

Record whether blood was drawn using peripheral venous access device (e.g. butterfly) or central venous access device (CVAD) here: _____

Please describe any deviations from the laboratory manual or issues below:

_____

_____

Dispatch Details for Whole Blood

Number of tubes sent:_____ Date:_____

Staff Responsible:_____ _____

(print name) **(signature)**

Whole Blood Sample Receipt (for Glasgow Biorepository use)

Date/time received:_____ Number of samples received:_____

Condition of samples on arrival:_____

_____

Staff responsible:_____ _____

(print name) **(signature)**

1.1. **STRATIFY Serum Worksheet**

Patient Study Number:_____ Patient Initials:_____

Centre Name:_____

Time Point	Date and Collection Time	Centrifugation start time	Time Frozen	No of Tubes	Operator (Print Name and Sign)
Baseline					

Record whether blood was drawn using peripheral venous access device (e.g. butterfly) or central venous access device (CVAD) here: _____

Please describe any deviations from the laboratory manual or issues below:

_____

_____

Dispatch Details for Serum

Number of tubes sent:_____ Date:_____

Staff Responsible:_____ _____

(print name) **(signature)**

Serum Sample Receipt(for Glasgow Biorepository use)

Date/time received:_____ Number of samples received:_____

Condition of samples on arrival:_____

_____

Staff responsible:_____ _____

(print name) **(signature)**

1.1. **STRATIFY Plasma Worksheet**

Patient Study Number:_____ Patient Initials:_____

Centre Name:_____

Time Point	Date and Collection Time	Centrifugation start time	Time Frozen	No of Tubes	Operator (Print Name and Sign)
Baseline					

Record whether blood was drawn using peripheral venous access device (e.g. butterfly) or central venous access device (CVAD) here: _____

Please describe any deviations from the laboratory manual or issues below:

_____

_____

Dispatch Details for Plasma

Number of tubes sent:_____ Date:_____

Staff Responsible:_____ _____

(print name) **(signature)**

Plasma Sample Receipt (for Glasgow Biorepository use)

Date/time received:_____ Number of samples received:_____

Condition of samples on arrival:_____

_____

Staff responsible:_____ _____

(print name) **(signature)**

1.1. **STRATIFY Pleural Fluid Worksheet**

Patient Study Number:_____ Patient Initials:_____

Centre Name:_____

Time Point	Date and Collection Time	Time Frozen	Operator (Print Name and Sign)
Visit 3			

Please describe any deviations from the laboratory manual or issues below:

_____

_____

Dispatch Details for Pleural Fluid

Number of tubes sent:_____ Date:_____

Staff Responsible:_____ _____

(print name) **(signature)**

Pleural Fluid Sample Receipt (for Glasgow Biorepository use)

Date/time received:_____ Number of samples received:_____

Condition of samples on arrival:_____

_____

Staff responsible:_____ _____

(print name) **(signature)**

1. Labels

1.1. Labels for EDTA whole blood collection tubes

STRATIFY Baseline Whole blood
(Genomic DNA)
Pt No:_____Initials_
Centre:_____
Date:_____Time:_____

1.2. Labels for 1.5ml microtubes

STRATIFY Baseline Serum
Pt No:__ Initials__ Centre:
_____
Date:_____Time:____

STRATIFY Baseline Plasma
Pt No:_____ Initials_____
Centre:_____
Date:_____Time:_____

1.3. Labels for 5ml cryovials

STRATIFY Pleural Fluid
Pt No:__ Initials__ Centre:
_____
Date:_____Time:_
Timepoint: Visit 3

1. Declaration

I confirm that I have received, read and understood this manual

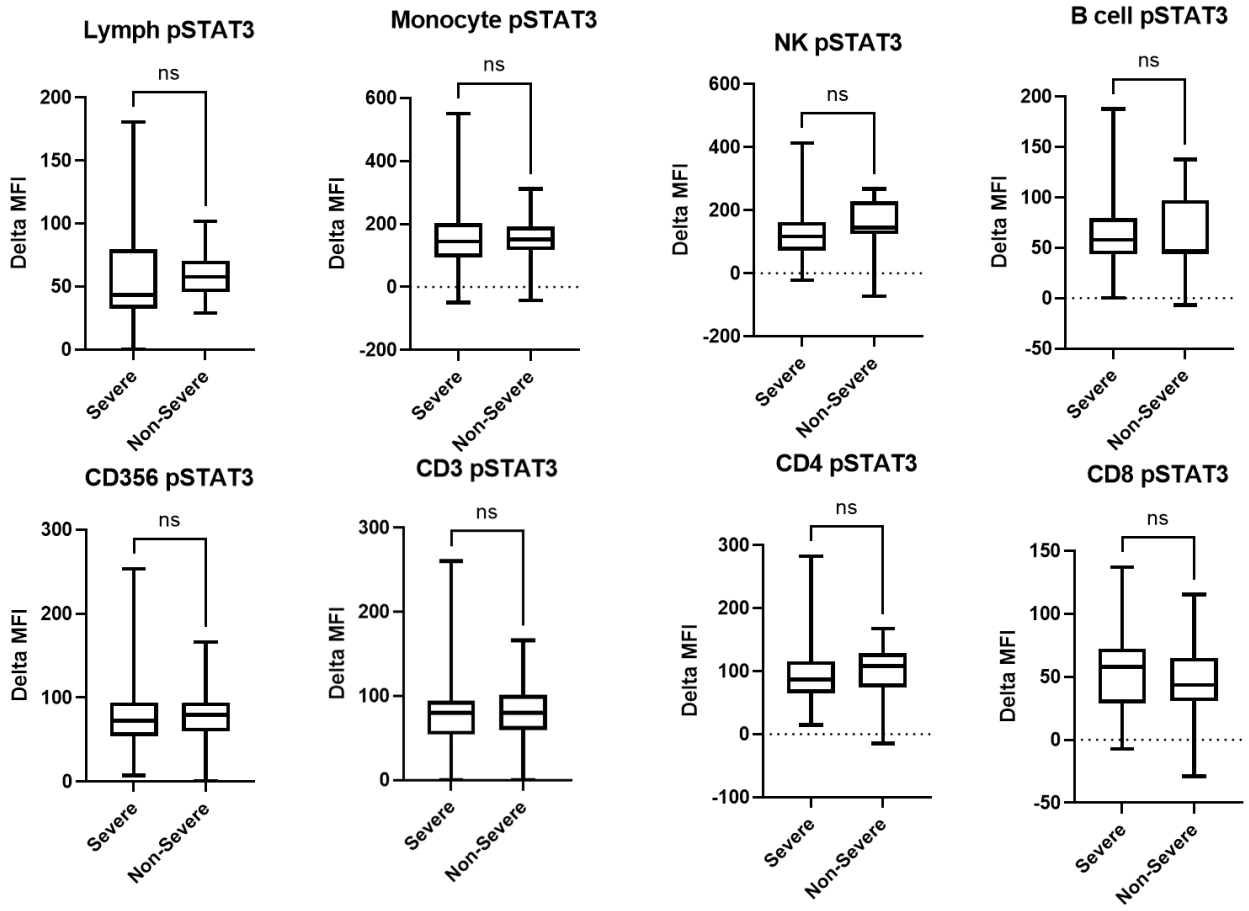
Name:_____

Signature:_____

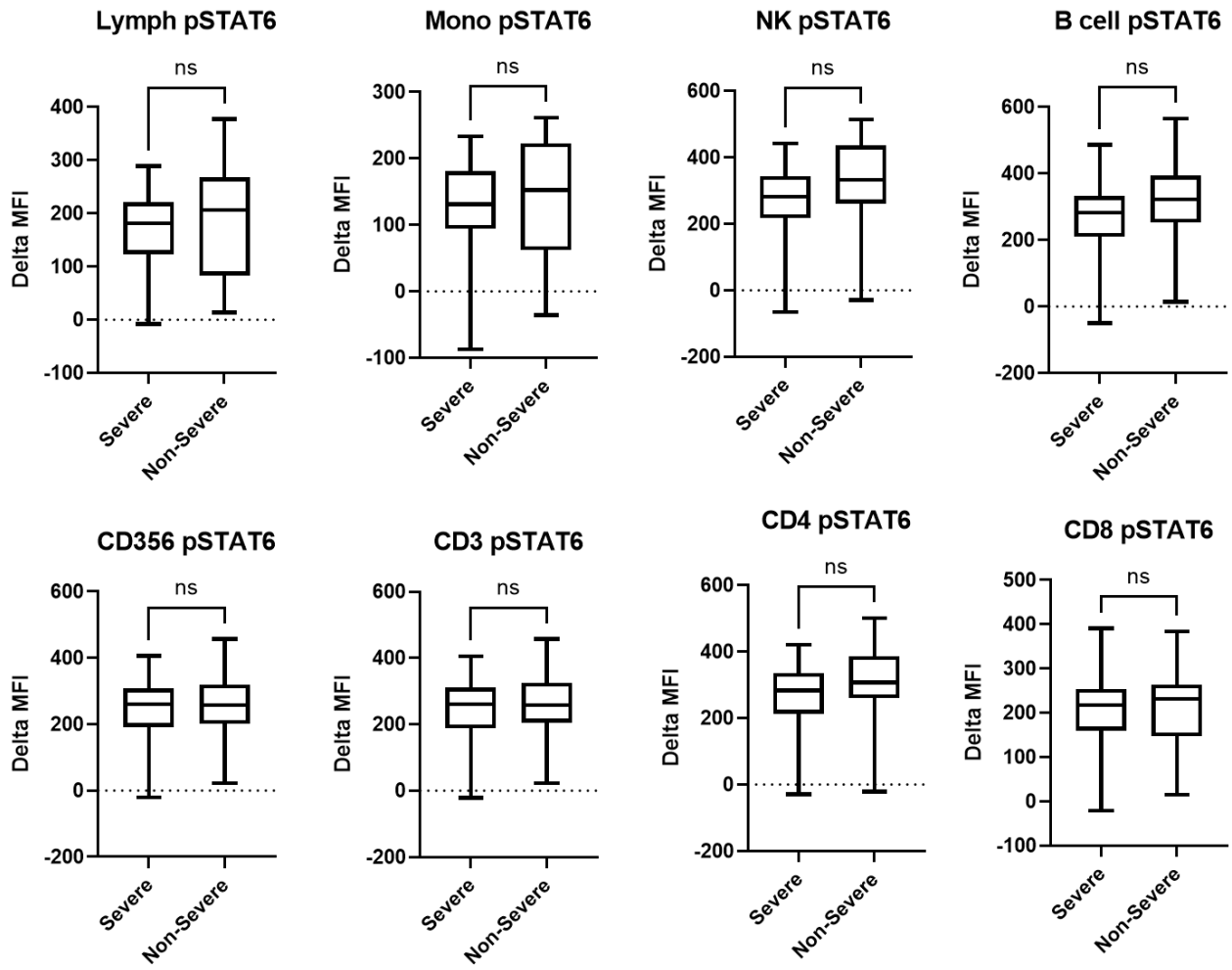
Date:_____

Please return this declaration to the Project Manager, CTU Glasgow (see section 2).

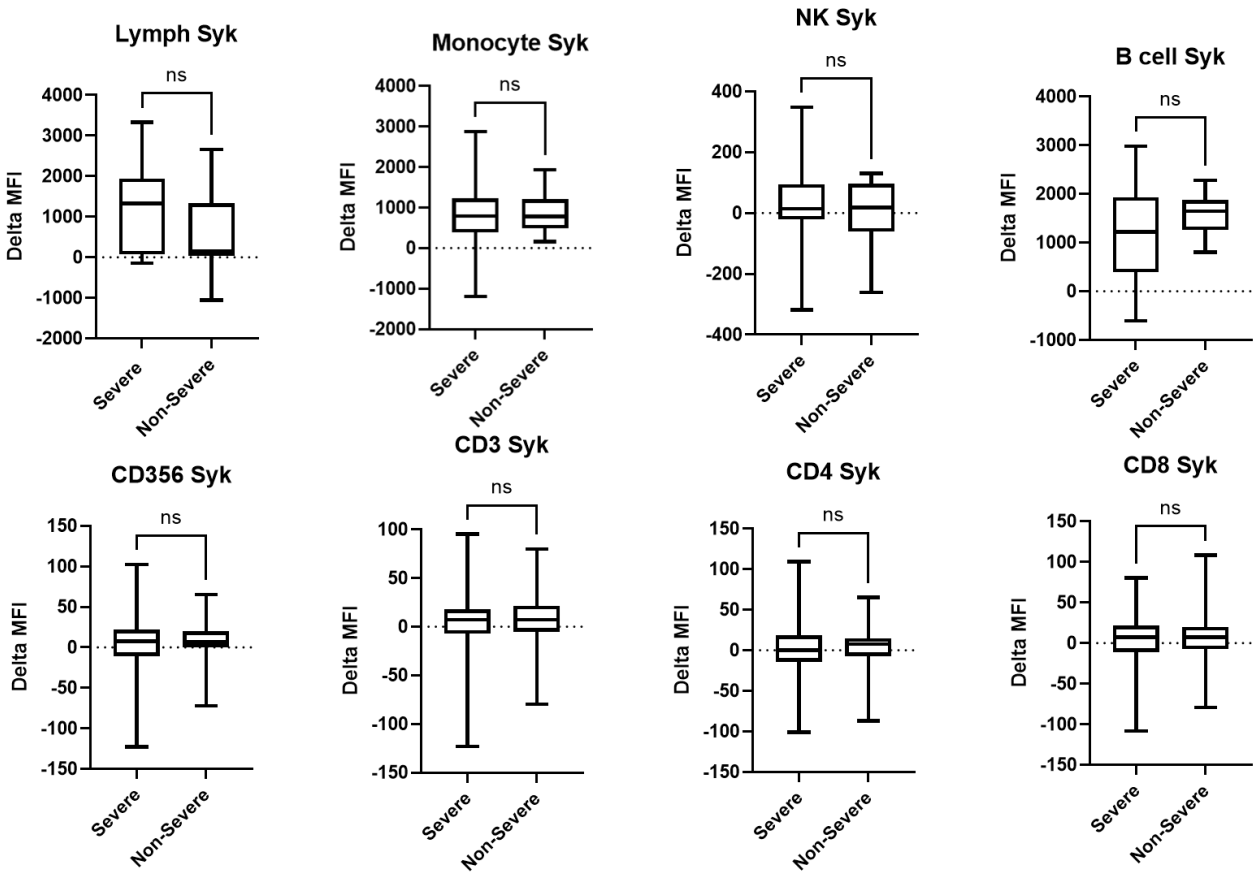
Appendix 5 Intracellular Immune Cell Signalling



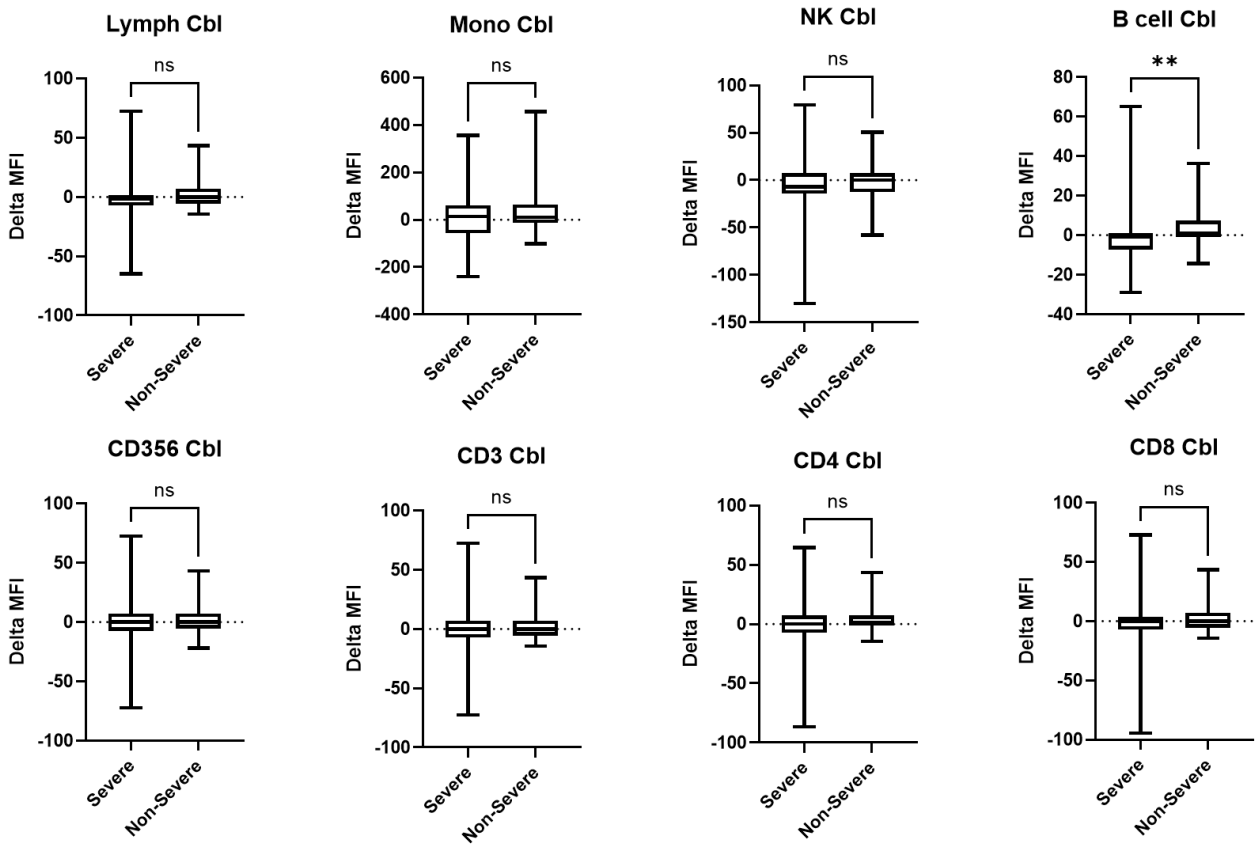
Increment in pSTAT 3 activity after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)



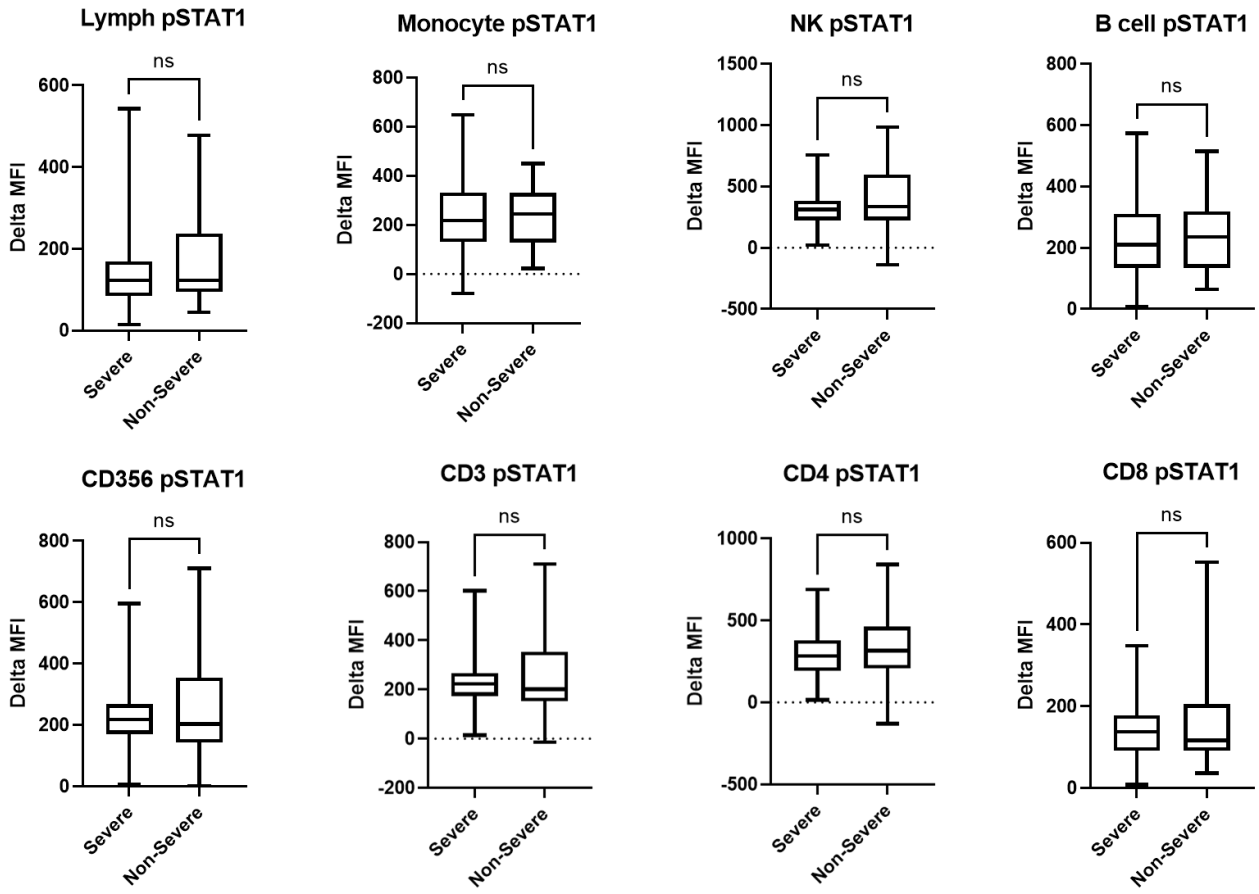
Increment in pSTAT 6 activity after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)



Increment in Syk activity after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)



Increment in Cbl activity after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)



Increment in pSTAT1 activity after stimulation in peripheral blood immune cell subpopulations at first presentation in severe and non-severe disease groups (defined by O2 requirement vs no O2 requirement)

References

1. Unknown. Oxford English Dictionary - Stratification.Unknown.
2. Abdelnour C, Agosta F, Bozzali M, Fougère B, Iwata A, Nilforooshan R, et al. Perspectives and challenges in patient stratification in Alzheimer's disease. Alzheimers Res Ther. 2022;14(1):112.
3. Rosen RD SA. TNM Classification. National Library of Medicine 2023.
4. Brierley J. The evolving TNM cancer staging system: an essential component of cancer care. Cmaj. 2006;174(2):155-6.
5. Gospodarowicz MK, Miller D, Groome PA, Greene FL, Logan PA, Sobin LH. The process for continuous improvement of the TNM classification. Cancer. 2004;100(1):1-5.
6. Feng SH, Yang ST. The new 8th TNM staging system of lung cancer and its potential imaging interpretation pitfalls and limitations with CT image demonstrations. Diagn Interv Radiol. 2019;25(4):270-9.
7. Planchard D, Popat S, Kerr K, Novello S, Smit EF, Faivre-Finn C, et al. Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2018;29(Suppl 4):iv192-iv237.
8. Rami-Porta R, Asamura H, Travis WD, Rusch VW. Lung cancer - major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin. 2017;67(2):138-55.
9. Jin Y, Chen M, Yu X. Comparison of the 7(th) and proposed 8(th) editions of the AJCC/UICC TNM staging system for non-small cell lung cancer undergoing radical surgery. Sci Rep. 2016;6:33587.
10. Charalampidis C, Youroukou A, Lazaridis G, Baka S, Mpoukovinas I, Karavasilis V, et al. Pleura space anatomy. J Thorac Dis. 2015;7(Suppl 1):S27-32.
11. Rabinowitz JG, Cohen BA, Mendleson DS. The Pulmonary Ligament. Radiologic Clinics of North America. 1984;22(3):659-72.
12. Yalcin NG, Choong CKC, Eizenberg N. Anatomy and Pathophysiology of the Pleura and Pleural Space. Thoracic Surgery Clinics. 2013;23(1):1-10.
13. Miserocchi G. Physiology and pathophysiology of pleural fluid turnover. Eur Respir J. 1997;10(1):219-25.
14. Light RW, Macgregor MI, Luchsinger PC, Ball WC, Jr. Pleural effusions: the diagnostic separation of transudates and exudates. Ann Intern Med. 1972;77(4):507-13.
15. Sharma K, Fultariya L, Reddy Mallimala P, Shah K, Sharma V. Comparison of the Efficacy of Light's Criteria With Serum-Effusion Albumin Gradient and Pleural Effusion Glucose. Cureus. 2023;15(8):e43319.
16. Sahn SA. The pathophysiology of pleural effusions. Annu Rev Med. 1990;41:7-13.
17. Zocchi L. Physiology and pathophysiology of pleural fluid turnover. Eur Respir J. 2002;20(6):1545-58.
18. Ferreiro L, Toubes ME, San José ME, Suárez-Antelo J, Golpe A, Valdés L. Advances in pleural effusion diagnostics. Expert Rev Respir Med. 2020;14(1):51-66.
19. Psallidas I, Kalomenidis I, Porcel JM, Robinson BW, Stathopoulos GT. Malignant pleural effusion: from bench to bedside. Eur Respir Rev. 2016;25(140):189-98.

20. Sahn SA. Pleural diseases related to metastatic malignancies. Eur Respir J. 1997;10(8):1907-13.
21. Loveland P, Christie M, Hammerschlag G, Irving L, Steinfort D. Diagnostic yield of pleural fluid cytology in malignant effusions: an Australian tertiary centre experience. Intern Med J. 2018;48(11):1318-24.
22. Lim E, Baldwin D, Beckles M, Duffy J, Entwisle J, Faivre-Finn C, et al. Guidelines on the radical management of patients with lung cancer. Thorax. 2010;65 Suppl 3:iii1-27.
23. Tsim S, Paterson S, Cartwright D, Fong CJ, Alexander L, Kelly C, et al. Baseline predictors of negative and incomplete pleural cytology in patients with suspected pleural malignancy - Data supporting 'Direct to LAT' in selected groups. Lung Cancer. 2019;133:123-9.
24. Morgensztern D, Waqar S, Subramanian J, Trinkaus K, Govindan R. Prognostic impact of malignant pleural effusion at presentation in patients with metastatic non-small-cell lung cancer. J Thorac Oncol. 2012;7(10):1485-9.
25. Porcel JM, Gasol A, Bielsa S, Civit C, Light RW, Salud A. Clinical features and survival of lung cancer patients with pleural effusions. Respirology. 2015;20(4):654-9.
26. Hallifax RJ, Haris M, Corcoran JP, Leyakathalikhan S, Brown E, Srikantharaja D, et al. Role of CT in assessing pleural malignancy prior to thoracoscopy. Thorax. 2015;70(2):192-3.
27. Lung cancer: diagnosis and management. NICE.NICE guideline [NG122].
28. Porcel JM, Hernández P, Martínez-Alonso M, Bielsa S, Salud A. Accuracy of fluorodeoxyglucose-PET imaging for differentiating benign from malignant pleural effusions: a meta-analysis. Chest. 2015;147(2):502-12.
29. Arnold DT, De Fonseka D, Perry S, Morley A, Harvey JE, Medford A, et al. Investigating unilateral pleural effusions: the role of cytology. Eur Respir J. 2018;52(5).
30. Łochowski MP, Kozak J. Video-assisted thoracic surgery complications. Wideochir Inne Tech Maloinwazyjne. 2014;9(4):495-500.
31. Michaud G, Berkowitz DM, Ernst A. Pleuroscopy for diagnosis and therapy for pleural effusions. Chest. 2010;138(5):1242-6.
32. Bibby AC, Dorn P, Psallidas I, Porcel JM, Janssen J, Froudarakis M, et al. ERS/EACTS statement on the management of malignant pleural effusions. Eur Respir J. 2018;52(1).
33. Hooper C, Lee YC, Maskell N. Investigation of a unilateral pleural effusion in adults: British Thoracic Society Pleural Disease Guideline 2010. Thorax. 2010;65 Suppl 2:ii4-17.
34. Rahman NM, Ali NJ, Brown G, Chapman SJ, Davies RJ, Downer NJ, et al. Local anaesthetic thoracoscopy: British Thoracic Society Pleural Disease Guideline 2010. Thorax. 2010;65 Suppl 2:ii54-60.
35. Sundaralingam A, Bedawi EO, Harriss EK, Munavvar M, Rahman NM. The Frequency, Risk Factors, and Management of Complications From Pleural Procedures. Chest. 2022;161(5):1407-25.
36. Ryu JS, Ryu HJ, Lee SN, Memon A, Lee SK, Nam HS, et al. Prognostic impact of minimal pleural effusion in non-small-cell lung cancer. J Clin Oncol. 2014;32(9):960-7.

37. Cantó A, Ferrer G, Romagosa V, Moya J, Bernat R. Lung cancer and pleural effusion. Clinical significance and study of pleural metastatic locations. Chest. 1985;87(5):649-52.
38. Roberts JR, Blum MG, Arildsen R, Drinkwater DC, Jr., Christian KR, Powers TA, et al. Prospective comparison of radiologic, thoracoscopic, and pathologic staging in patients with early non-small cell lung cancer. Ann Thorac Surg. 1999;68(4):1154-8.
39. Excellence NIfHaC. Lung cancer: diagnosis and management [NG122] [Internet]: NICE; 2019 [updated 08 March 2024. 2019:[
40. Cancer Research UK - Lung Cancer Statistics. 2024.
41. Navani N, Baldwin DR, Edwards JG, Evison M, McDonald F, Nicholson AG, et al. Lung Cancer in the United Kingdom. J Thorac Oncol. 2022;17(2):186-93.
42. Christensen HM, Huniche L. Patient perspectives and experience on the diagnostic pathway of lung cancer: A qualitative study. SAGE Open Med. 2020;8:2050312120918996.
43. Bradley JD, Hu C, Komaki RR, Masters GA, Blumenschein GR, Schild SE, et al. Long-Term Results of NRG Oncology RTOG 0617: Standard- Versus High-Dose Chemoradiotherapy With or Without Cetuximab for Unresectable Stage III Non-Small-Cell Lung Cancer. J Clin Oncol. 2020;38(7):706-14.
44. Peters S, Weder W, Dafni U, Kerr KM, Bubendorf L, Meldgaard P, et al. Lungscape: resected non-small-cell lung cancer outcome by clinical and pathological parameters. J Thorac Oncol. 2014;9(11):1675-84.
45. Postmus PE, Kerr KM, Oudkerk M, Senan S, Waller DA, Vansteenkiste J, et al. Early and locally advanced non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2017;28(suppl_4):iv1-iv21.
46. Ball D, Mai GT, Vinod S, Babington S, Ruben J, Kron T, et al. Stereotactic ablative radiotherapy versus standard radiotherapy in stage 1 non-small-cell lung cancer (TROG 09.02 CHISEL): a phase 3, open-label, randomised controlled trial. Lancet Oncol. 2019;20(4):494-503.
47. Chang JY, Lin SH, Dong W, Liao Z, Gandhi SJ, Gay CM, et al. Stereotactic ablative radiotherapy with or without immunotherapy for early-stage or isolated lung parenchymal recurrent node-negative non-small-cell lung cancer: an open-label, randomised, phase 2 trial. Lancet. 2023;402(10405):871-81.
48. Seo YS, Kim HJ, Wu HG, Choi SM, Park S. Lobectomy versus stereotactic ablative radiotherapy for medically operable patients with stage IA non-small cell lung cancer: A virtual randomized phase III trial stratified by age. Thorac Cancer. 2019;10(6):1489-99.
49. Zhao Y, Feng HM, Tian J, Li B, Wang C, Ge L, et al. Stereotactic body radiation therapy versus more fractionated radical radiotherapy for adults with stage I/II non-small cell lung cancer: a systematic review and network meta-analysis: Cochrane Database Syst Rev. 2022 Apr 8;2022(4):CD014744. doi: 10.1002/14651858.CD014744. eCollection 2022.
50. Petrella F, Rizzo S, Attili I, Passaro A, Zilli T, Martucci F, et al. Stage III Non-Small-Cell Lung Cancer: An Overview of Treatment Options. Curr Oncol. 2023;30(3):3160-75.
51. Cancer TAJCo. AJCC Cancer Staging Manual.8th Edition.
52. Excellence NIfHaC. Pembrolizumab with carboplatin and paclitaxel for untreated metastatic squamous non-small-cell lung cancer [Guideline]. NICE; 2022 [updated 9 Feb 2022.

53. Imyanitov EN, Iyevleva AG, Levchenko EV. Molecular testing and targeted therapy for non-small cell lung cancer: Current status and perspectives. Crit Rev Oncol Hematol. 2021;157:103194.
54. Cardoso F, Senkus E, Costa A, Papadopoulos E, Aapro M, André F, et al. 4th ESO-ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC 4)†. Ann Oncol. 2018;29(8):1634-57.
55. Jiang L, Su X, Zhang T, Yin X, Zhang M, Fu H, et al. PD-L1 expression and its relationship with oncogenic drivers in non-small cell lung cancer (NSCLC). Oncotarget. 2017;8(16):26845-57.
56. Luo SY, Lam DCL. Oncogenic driver mutations in lung cancer. Translational Respiratory Medicine. 2013;1(1):6.
57. Colombo N, Sessa C, du Bois A, Ledermann J, McCluggage WG, McNeish I, et al. ESMO-ESGO consensus conference recommendations on ovarian cancer: pathology and molecular biology, early and advanced stages, borderline tumours and recurrent disease†. Ann Oncol. 2019;30(5):672-705.
58. Holmes M, Mahar A, Lum T, Kao S, Cooper WA. Real-world programmed death-ligand 1 prevalence rates in non-small cell lung cancer: correlation with clinicopathological features and tumour mutation status. J Clin Pathol. 2021;74(2):123-8.
59. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res. 2020;10(3):727-42.
60. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. N Engl J Med. 2016;375(19):1823-33.
61. Association AL. EGFR and Lung Cancer [Webpage]. 2021 [updated 28/10/2021; cited 2022 2022]. Available from: https://www.lung.org/lung-health-diseases/lung-disease-lookup/lung-cancer/symptoms-diagnosis/biomarker-testing/egfr#:~:text=EGFR%2Dpositive%20lung%20cancer%20represents,minimal%20to%20no%20smoking%20history.
62. Carbonnaux M, Souquet PJ, Meert AP, Scherpereel A, Peters M, Couraud S. Inequalities in lung cancer: a world of EGFR. Eur Respir J. 2016;47(5):1502-9.
63. Jiao Q, Bi L, Ren Y, Song S, Wang Q, Wang YS. Advances in studies of tyrosine kinase inhibitors and their acquired resistance. Mol Cancer. 2018;17(1):36.
64. Fukuoka M, Wu YL, Thongprasert S, Sunpaweravong P, Leong SS, Sriuranpong V, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol. 2011;29(21):2866-74.
65. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012;13(3):239-46.
66. Huang H. Anaplastic Lymphoma Kinase (ALK) Receptor Tyrosine Kinase: A Catalytic Receptor with Many Faces. Int J Mol Sci. 2018;19(11).
67. Association AL. ALK and Lung Cancer [Webpage]. 2021 [updated 28/10/2021; cited 2022 16/9/2022]. Available from: https://www.lung.org/lung-health-diseases/lung-disease-lookup/lung-cancer/symptoms-diagnosis/biomarker-testing/alk-lung-cancer.

68. Collazo-Lorduy A, Jiménez B, Castro-Henriques M, Remon J. Chapter 2 - ALK rearranged lung cancer: TKI treatment and outcome. In: Friboulet L, editor. Therapeutic Strategies to Overcome ALK Resistance in Cancer. 13: Academic Press; 2021. p. 31-53.
69. D'Angelo A, Sobhani N, Chapman R, Bagby S, Bortoletti C, Traversini M, et al. Focus on ROS1-Positive Non-Small Cell Lung Cancer (NSCLC): Crizotinib, Resistance Mechanisms and the Newer Generation of Targeted Therapies. Cancers (Basel). 2020;12(11).
70. Shen L, Lu S. Crizotinib versus pemetrexed-based chemotherapy in patients with advanced ROS1-rearranged non-small cell lung cancer. Journal of Clinical Oncology. 2019;37(15_suppl):9101-.
71. Brufsky AM, Dickler MN. Estrogen Receptor-Positive Breast Cancer: Exploiting Signaling Pathways Implicated in Endocrine Resistance. Oncologist. 2018;23(5):528-39.
72. Farrar MC JT. Tamoxifen. StatPearls Publishing; 2023.
73. Excellence NIfHaC. Advanced Breast Cancer: Diagnosis and Treatment: NICE; 2009 [updated 16 August 2017. Available from: https://www.nice.org.uk/guidance/cg81/chapter/recommendations#systemic-disease-modifying-therapy.
74. Bhatnagar AS. The discovery and mechanism of action of letrozole. Breast Cancer Res Treat. 2007;105 Suppl 1(Suppl 1):7-17.
75. Giordano SH, Temin S, Kirshner JJ, Chandarlapaty S, Crews JR, Davidson NE, et al. Systemic therapy for patients with advanced human epidermal growth factor receptor 2-positive breast cancer: American Society of Clinical Oncology clinical practice guideline. J Clin Oncol. 2014;32(19):2078-99.
76. Albanell J, Codony J, Rovira A, Mellado B, Gascón P. Mechanism of action of anti-HER2 monoclonal antibodies: scientific update on trastuzumab and 2C4. Adv Exp Med Biol. 2003;532:253-68.
77. G Curigliano LC-B, A Gennari, N Harbeck, C Criscitiello and D Trapani. ESMO Metastatic Breast Cancer Living Guidelines. Ann Oncol. 2021;v1.1(32(12)):1475 - 95.
78. Sabaawy HE, Ryan BM, Khiabanian H, Pine SR. JAK/STAT of all trades: linking inflammation with cancer development, tumor progression and therapy resistance. Carcinogenesis. 2021;42(12):1411-9.
79. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol. 2020;20(11):651-68.
80. Thomas SJ, Snowden JA, Zeidler MP, Danson SJ. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. Br J Cancer. 2015;113(3):365-71.
81. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395(10223):497-506.
82. Yesudhas D, Srivastava A, Gromiha MM. COVID-19 outbreak: history, mechanism, transmission, structural studies and therapeutics. Infection. 2021;49(2):199-213.
83. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor Recognition by the Novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. J Virol. 2020;94(7).
84. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol. 2015;1282:1-23.

85. WHO. WHO Director-General's opening remarks at the media briefing on COVID-19 – 11 March 2020. 2020.
86. Organisation WH. WHO COVID-19 Dashboard. 2024.
87. Virology: Coronaviruses: Nature. 1968;220(5168):650. doi: 10.1038/220650b0.
88. Bhat EA, Khan J, Sajjad N, Ali A, Aldakeel FM, Mateen A, et al. SARS-CoV-2: Insight in genome structure, pathogenesis and viral receptor binding analysis - An updated review. Int Immunopharmacol. 2021;95:107493.
89. Chilamakuri R, Agarwal S. COVID-19: Characteristics and Therapeutics. Cells. 2021;10(2).
90. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. N Engl J Med. 2020;382(13):1199-207.
91. Prevention CfDCa. Symptoms of COVID-19 [Web page]. CDC; 2022 [updated 11/08/2022.
92. Knight SR, Ho A, Pius R, Buchan I, Carson G, Drake TM, et al. Risk stratification of patients admitted to hospital with covid-19 using the ISARIC WHO Clinical Characterisation Protocol: development and validation of the 4C Mortality Score. BMJ. 2020;370:m3339.
93. Loo J, Spittle DA, Newnham M. COVID-19, immunothrombosis and venous thromboembolism: biological mechanisms. Thorax. 2021;76(4):412-20.
94. Khalid MF, Selvam K, Jeffry AJN, Salmi MF, Najib MA, Norhayati MN, et al. Performance of Rapid Antigen Tests for COVID-19 Diagnosis: A Systematic Review and Meta-Analysis. Diagnostics (Basel). 2022;12(1).
95. Bachman J. Chapter Two - Reverse-Transcription PCR (RT-PCR). In: Lorsch J, editor. Methods in Enzymology. 530: Academic Press; 2013. p. 67-74.
96. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. Jama. 2020;323(13):1239-42.
97. Guan WJ, Liang WH, Zhao Y, Liang HR, Chen ZS, Li YM, et al. Comorbidity and its impact on 1590 patients with COVID-19 in China: a nationwide analysis. Eur Respir J. 2020;55(5).
98. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, et al. Estimates of the severity of coronavirus disease 2019: a model-based analysis. Lancet Infect Dis. 2020;20(6):669-77.
99. Docherty AB, Harrison EM, Green CA, Hardwick HE, Pius R, Norman L, et al. Features of 20 133 UK patients in hospital with covid-19 using the ISARIC WHO Clinical Characterisation Protocol: prospective observational cohort study. Bmj. 2020;369:m1985.
100. Hariri L, Hardin CC. Covid-19, Angiogenesis, and ARDS Endotypes. N Engl J Med. 2020;383(2):182-3.
101. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, et al. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. Jama. 2020;323(16):1574-81.
102. Clift AK, Coupland CAC, Keogh RH, Diaz-Ordaz K, Williamson E, Harrison EM, et al. Living risk prediction algorithm (QCOVID) for risk of hospital admission and mortality from coronavirus 19 in adults: national derivation and validation cohort study. Bmj. 2020;371:m3731.

103. Williamson EJ, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, et al. Factors associated with COVID-19-related death using OpenSAFELY. Nature. 2020;584(7821):430-6.
104. Gupta RK, Harrison EM, Ho A, Docherty AB, Knight SR, van Smeden M, et al. Development and validation of the ISARIC 4C Deterioration model for adults hospitalised with COVID-19: a prospective cohort study. Lancet Respir Med. 2021;9(4):349-59.
105. Ranard BL, Megjhani M, Terilli K, Doyle K, Claassen J, Pinsky MR, et al. Identification of Endotypes of Hospitalized COVID-19 Patients. Front Med (Lausanne). 2021;8:770343.
106. Collins. Phenotype. Collins English Dictionary.
107. Coperchini F, Chiovato L, Croce L, Magri F, Rotondi M. The cytokine storm in COVID-19: An overview of the involvement of the chemokine/chemokine-receptor system. Cytokine Growth Factor Rev. 2020;53:25-32.
108. Balkhi MY. Mechanistic understanding of innate and adaptive immune responses in SARS-CoV-2 infection. Mol Immunol. 2021;135:268-75.
109. Jarczak D, Nierhaus A. Cytokine Storm-Definition, Causes, and Implications. Int J Mol Sci. 2022;23(19).
110. Ghazavi A, Ganji A, Keshavarzian N, Rabiemajd S, Mosayebi G. Cytokine profile and disease severity in patients with COVID-19. Cytokine. 2021;137:155323.
111. Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. Emerg Microbes Infect. 2020;9(1):1123-30.
112. Diamond MS, Kanneganti T-D. Innate immunity: the first line of defense against SARS-CoV-2. Nature Immunology. 2022;23(2):165-76.
113. Moss P. The T cell immune response against SARS-CoV-2. Nature Immunology. 2022;23(2):186-93.
114. Lee JS, Shin E-C. The type I interferon response in COVID-19: implications for treatment. Nature Reviews Immunology. 2020;20(10):585-6.
115. Li J, Pavlov I, Ehrmann S. High-Flow Oxygen vs Conventional Oxygen and Invasive Mechanical Ventilation and Clinical Recovery in Patients With Severe COVID-19. JAMA. 2022;327(11):1092-.
116. Perkins GD, Ji C, Connolly BA, Couper K, Lall R, Baillie JK, et al. Effect of Noninvasive Respiratory Strategies on Intubation or Mortality Among Patients With Acute Hypoxemic Respiratory Failure and COVID-19: The RECOVERY-RS Randomized Clinical Trial. JAMA. 2022;327(6):546-58.
117. Horby P, Lim WS, Emberson JR, Mafham M, Bell JL, Linsell L, et al. Dexamethasone in Hospitalized Patients with Covid-19. N Engl J Med. 2021;384(8):693-704.
118. Tocilizumab in patients admitted to hospital with COVID-19 (RECOVERY): a randomised, controlled, open-label, platform trial. Lancet. 2021;397(10285):1637-45.
119. Thiel V, Weber F. Interferon and cytokine responses to SARS-coronavirus infection. Cytokine Growth Factor Rev. 2008;19(2):121-32.
120. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, et al. Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. Science. 2020;369(6504):718-24.

121. Wang N, Zhan Y, Zhu L, Hou Z, Liu F, Song P, et al. Retrospective Multicenter Cohort Study Shows Early Interferon Therapy Is Associated with Favorable Clinical Responses in COVID-19 Patients. Cell Host Microbe. 2020;28(3):455-64.e2.
122. Maguire J, Khan I, McMenemin R, O'Rourke N, McNee S, Kelly V, et al. SOCCAR: A randomised phase II trial comparing sequential versus concurrent chemotherapy and radical hypofractionated radiotherapy in patients with inoperable stage III Non-Small Cell Lung Cancer and good performance status. Eur J Cancer. 2014;50(17):2939-49.
123. Semrau S, Klautke G, Fietkau R. Baseline cardiopulmonary function as an independent prognostic factor for survival of inoperable non-small-cell lung cancer after concurrent chemoradiotherapy: a single-center analysis of 161 cases. Int J Radiat Oncol Biol Phys. 2011;79(1):96-104.
124. Baracos VE, Reiman T, Mourtzakis M, Gioulbasanis I, Antoun S. Body composition in patients with non-small cell lung cancer: a contemporary view of cancer cachexia with the use of computed tomography image analysis. Am J Clin Nutr. 2010;91(4):1133s-7s.
125. Martin G, Tsim S, MacLay J, Stewart C, Blyth K. P6 Significance of minimal pleural effusion in non-small cell lung cancer. Thorax. 2016;71(Suppl 3):A86-A.
126. Tsim S, Stobo DB, Alexander L, Kelly C, Blyth KG. The diagnostic performance of routinely acquired and reported computed tomography imaging in patients presenting with suspected pleural malignancy. Lung Cancer. 2017;103:38-43.
127. Lim E, Clough R, Goldstraw P, Edmonds L, Aokage K, Yoshida J, et al. Impact of positive pleural lavage cytology on survival in patients having lung resection for non-small-cell lung cancer: An international individual patient data meta-analysis. J Thorac Cardiovasc Surg. 2010;139(6):1441-6.
128. Lim E, Ali A, Theodorou P, Nicholson AG, Ladas G, Goldstraw P. Intraoperative pleural lavage cytology is an independent prognostic indicator for staging non-small cell lung cancer. J Thorac Cardiovasc Surg. 2004;127(4):1113-8.
129. Marchetti G, Valsecchi A, Indellicati D, Arondi S, Trigiani M, Pinelli V. Ultrasound-guided medical thoracoscopy in the absence of pleural effusion. Chest. 2015;147(4):1008-12.
130. Loddenkemper R, Lee P, Noppen M, Mathur P. Medical Thoracoscopy/Pleuroscopy: step by step. Breathe. 2011;8:156-67.
131. McDonald CM, Pierre C, de Perrot M, Darling G, Cypel M, Pierre A, et al. Efficacy and Cost of Awake Thoracoscopy and Video-Assisted Thoracoscopic Surgery in the Undiagnosed Pleural Effusion. Ann Thorac Surg. 2018;106(2):361-7.
132. Arya R, Antonisamy B, Kumar S. Sample size estimation in prevalence studies. Indian J Pediatr. 2012;79(11):1482-8.
133. Roberts ME, Rahman NM, Maskell NA, Bibby AC, Blyth KG, Corcoran JP, et al. British Thoracic Society Guideline for pleural disease. Thorax. 2023;78(Suppl 3):s1-s42.
134. Clive AO, Kahan BC, Hooper CE, Bhatnagar R, Morley AJ, Zahan-Evans N, et al. Predicting survival in malignant pleural effusion: development and validation of the LENT prognostic score. Thorax. 2014;69(12):1098-104.
135. Physicians RCo. Spotlight Report on Molecular Testing in Advanced Lung Cancer. 2020 January 2020.
136. Mercer RM, Varatharajah R, Shepherd G, Lu Q, Castro-Añón O, McCracken DJ, et al. Critical analysis of the utility of initial pleural aspiration in the diagnosis and

management of suspected malignant pleural effusion. BMJ Open Respir Res. 2020;7(1).
137. Lou SK, Grenier S, Care M, McCuaig J, Stockley TL, Clarke B, et al. Validation of BRCA testing on cytologic samples of high-grade serous carcinoma. Cancer Cytopathol. 2021;129(11):907-13.
138. Varatharajah R, Shepherd G, Mercer R, Thanayanandan A, Lu Q, Tsikrika M, et al. Cytology positive pleural aspirations: sufficient to guide treatment?2019. OA490 p.
139. Navarra A MA, Barlow A et al. Detection of EGFR mutation status on pleural fluid samples in patient with lung adenocarcinoma. European Respiratory Journal. 2022;60.
140. Davies RS, Smith C, Edwards G, Butler R, Parry D, Lester JF. Impact of Cytological Sampling on EGFR Mutation Testing in Stage III-IV Lung Adenocarcinoma. Lung Cancer Int. 2017;2017:9614938.
141. Wu SG, Liu YN, Yu CJ, Yang JC, Shih JY. Driver mutations of young lung adenocarcinoma patients with malignant pleural effusion. Genes Chromosomes Cancer. 2018;57(10):513-21.
142. Cui W, Milner-Watts C, McVeigh TP, Minchom A, Bholse J, Davidson M, et al. A pilot of Blood-First diagnostic cell free DNA (cfDNA) next generation sequencing (NGS) in patients with suspected advanced lung cancer. Lung Cancer. 2022;165:34-42.
143. García-Pardo M, Czarnecka-Kujawa K, Law JH, Salvarrey AM, Fernandes R, Fan ZJ, et al. Association of Circulating Tumor DNA Testing Before Tissue Diagnosis With Time to Treatment Among Patients With Suspected Advanced Lung Cancer: The ACCELERATE Nonrandomized Clinical Trial. JAMA Netw Open. 2023;6(7):e2325332.
144. Porcel JM, Murata P, Porcel L, Bielsa S, Pardina M, Salud A. Prevalence, clinical characteristics, and outcome of pleural effusions in ovarian cancer. Pleura Peritoneum. 2021;6(2):75-81.
145. Nicoś M, Krawczyk P, Jarosz B, Sawicki M, Szumiłło J, Trojanowski T, et al. Analysis of KRAS and BRAF genes mutation in the central nervous system metastases of non-small cell lung cancer. Clin Exp Med. 2016;16(2):169-76.
146. Lindeman NI, Cagle PT, Aisner DL, Arcila ME, Beasley MB, Bernicker EH, et al. Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors: Guideline From the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology. Arch Pathol Lab Med. 2018;142(3):321-46.
147. Organisation WH. Coronavirus disease (COVID-19) pandemic: WHO; 2024 [Available from: https://www.who.int/europe/emergencies/situations/covid-19.
148. Osuchowski MF, Winkler MS, Skirecki T, Cajander S, Shankar-Hari M, Lachmann G, et al. The COVID-19 puzzle: deciphering pathophysiology and phenotypes of a new disease entity. Lancet Respir Med. 2021;9(6):622-42.
149. Gibson PG, Qin L, Puah SH. COVID-19 acute respiratory distress syndrome (ARDS): clinical features and differences from typical pre-COVID-19 ARDS. Med J Aust. 2020;213(2):54-6.e1.
150. Jhuti D, Rawat A, Guo CM, Wilson LA, Mills EJ, Forrest JI. Interferon Treatments for SARS-CoV-2: Challenges and Opportunities. Infect Dis Ther. 2022;11(3):953-72.

151. Ozger HS, Karakus R, Kuscu EN, Bagriacik UE, Oruklu N, Yaman M, et al. Serial measurement of cytokines strongly predict COVID-19 outcome. PLoS One. 2021;16(12):e0260623.
152. Xue C, Yao Q, Gu X, Shi Q, Yuan X, Chu Q, et al. Evolving cognition of the JAK-STAT signaling pathway: autoimmune disorders and cancer. Signal Transduct Target Ther. 2023;8(1):204.
153. Collotta D, Franchina MP, Carlucci V, Collino M. Recent advances in JAK inhibitors for the treatment of metabolic syndrome. Front Pharmacol. 2023;14:1245535.
154. Bitar M, Boldt A, Freitag M-T, Gruhn B, Köhl U, Sack U. Evaluating STAT5 Phosphorylation as a Mean to Assess T Cell Proliferation. Frontiers in Immunology. 2019;10.
155. Rincon-Arevalo H, Aue A, Ritter J, Szelinski F, Khadzhynov D, Zickler D, et al. Altered increase in STAT1 expression and phosphorylation in severe COVID-19. Eur J Immunol. 2022;52(1):138-48.
156. Pavey H, Kulkarni S, Wood A, Ben-Shlomo Y, Sever P, McEniery C, et al. Primary hypertension, anti-hypertensive medications and the risk of severe COVID-19 in UK Biobank. PLoS One. 2022;17(11):e0276781.
157. Dodington DW, Desai HR, Woo M. JAK/STAT - Emerging Players in Metabolism. Trends Endocrinol Metab. 2018;29(1):55-65.
158. Gusev E, Sarapultsev A, Solomatina L, Chereshnev V. SARS-CoV-2-Specific Immune Response and the Pathogenesis of COVID-19. Int J Mol Sci. 2022;23(3).
159. Song JW, Zhang C, Fan X, Meng FP, Xu Z, Xia P, et al. Immunological and inflammatory profiles in mild and severe cases of COVID-19. Nat Commun. 2020;11(1):3410.
160. Puzyrenko A, Felix JC, Ledeboer NA, Sun Y, Rui H, Sheinin Y. Cytotoxic CD8-positive T-lymphocyte infiltration in the lungs as a histological pattern of SARS-CoV-2 pneumonitis. Pathology. 2022;54(4):404-8.
161. Schaller T, Hirschbühl K, Burkhardt K, Braun G, Trepel M, Märkl B, et al. Postmortem Examination of Patients With COVID-19. Jama. 2020;323(24):2518-20.
162. Selvaraj V, Finn A, Lal A, Khan MS, Dapaah-Afriyie K, Carino GP. Baricitinib in hospitalised patients with COVID-19: A meta-analysis of randomised controlled trials. EClinicalMedicine. 2022;49:101489.