Protocol

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TRACERx

TRAcking non-small cell lung Cancer Evolution through therapy (Rx)

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Please note: This study protocol must not be applied to patients treated outside the TRACERx Study. UCL CTC can only ensure that approved study investigators are provided with amendments to the protocol.

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1. PROTOCOL SUMMARY

1.1. Summary of Study Design

Title:	TRAcking non-small cell lung Cancer Evolution through therapy (Rx)
Short Title/acronym:	TRACERx
Sponsor name & reference:	UCL - UCL/12/0279
Funder name:	Cancer Research UK Rosetrees Trust Academy for Medical Sciences UCL Biomedical Research Centre
Clinicaltrials.gov no:	NCT01888601
Design:	A prospective observational cohort study of patients with non-small cell lung cancer (NSCLC), in which translational research is the fundamental aspect of the study.
Primary objectives:	• Define the relationship between intratumour heterogeneity and clinical outcome following surgery and adjuvant therapy (including relationships between intratumour heterogeneity and clinical disease stage and histological subtypes of NSCLC). • Establish the impact of adjuvant platinum-containing regimens upon intratumour heterogeneity in relapsed disease.
Key secondary objectives:	• Development and validation of intratumour heterogeneity (ITH) ratio index (I_{TB}) as a prognostic and predictive biomarker in relation to DFS and OS.
Primary endpoints:	• Intratumour heterogeneity quantified by the ratio index I_{TB} • Disease-free survival • Overall survival
Target accrual:	842 patients, of which 270 are expected to have a first recurrence and agree to provide a biopsy of the site of local recurrence/metastases
Inclusion criteria:	• Written Informed consent • Patients ≥18 years of age, with early stage I-IIIA disease who are eligible for primary surgery • Histopathologically confirmed NSCLC, or a strong suspicion of cancer on lung imaging necessitating surgery (e.g. diagnosis determined from frozen section in theatre) • Primary surgery in keeping with NICE guidelines planned (see section 9.3)

	• Agreement to be followed up in a specialist centre • Performance status 0 or 1 • Suspected tumour at least 15mm in diameter on pre-operative imaging
Exclusion criteria:	• Any other current malignancy or malignancy diagnosed or relapsed within the past 5 years (other than non-melanomatous skin cancer, stage 0 melanoma *in situ*, and *in situ* cervical cancer) • Psychological condition that would preclude informed consent • Treatment with neo-adjuvant therapy for current lung malignancy deemed necessary • Adjuvant therapy other than platinum-based chemotherapy and/or radiotherapy • Known Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) or syphilis infection. • Sufficient tissue, i.e. a minimum of two tumour regions, is unlikely to be obtained for the study based on pre-operative imaging Patients found to have pre-invasive lesions rather than invasive cancer following surgery, such as adenocarcinoma *in situ* or minimally invasive lesions will be withdrawn. However, the surgical tissue already collected will be sent to the central laboratory, but these patients will not be followed-up in the study or required to provide any further blood samples. If these patients subsequently develop invasive cancer, the date of diagnosis and the tumour histology will be reported on the electronic data capture system.
Planned number of sites:	Hospital sites: approximately 15-20 Translational research laboratories: Translational Cancer Therapeutics Laboratory - London, Department of Cancer Studies and Molecular Medicine - Leicester, Department of Clinical Immunology – Birmingham, CEP GCLP laboratories - Manchester, Illumina, Boreal Genomics, Natera, Genentech, and Syncona
Treatment summary:	All recruited patients will be suitable for primary surgery, in accordance with NICE guidelines. Further treatments (e.g. chemotherapy) would be given according to standard of care. No treatments are specified as part of this observational study.
Collection of tissue samples:	Baseline: • From the primary tumour and normal tissue from the resected specimen, surplus to diagnostic and pathological requirements. After first confirmed recurrence:

	• Biopsy (after consent) of local-regional and/or metastatic sites Following progression, after the first recurrence: • Biopsy (after consent) of local-regional and/or metastatic sites
Collection of blood samples	<u>Baseline (before surgery)</u>: • 1 x 10mL for germ line DNA • 4 x 10mL for cfDNA • 2 x 10mL for cfDNA high sensitivity assay for approximately 100 patients (Nb. recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.) • 2 x 10mL CellSave tube, for circulating tumour cells (pulmonary blood); for approximately 200 patients • 1 x 10mL for immunology • Leicester only – additional 1 x 10mL cfDNA • Manchester only – additional 1 x 10mL CellSave tube. This may be omitted in patients who provide the 40mL immunology sample. <u>48 hours after surgery:</u> • 2 x 10mL for cfDNA high sensitivity assay for approximately 100 patients (Nb. recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.) <u>Follow-up after surgery:</u> 5 years of follow up in the adjuvant setting; every 3 months in years 1-2, then every 6 months in years 3-5. For patients who receive adjuvant chemotherapy, the first post-surgery sample should be collected just before cycle 1 treatment and the next sample should be collected 6 months from the day of surgery • 2 x 10mL for cfDNA • 2 x 10mL for cfDNA high sensitivity assay for approximately 100 patients (for up to 3 years of follow-up only) (Nb. recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.) • All patients at ONE follow-up visit only – 1 x 50mL for immunology • Up to 30 patients (identified following surgery) at ONE follow-up visit only (preferably pre-chemotherapy OR, if that is not possible, following recovery of the neutrophil, lymphocyte and monocyte counts post-chemotherapy) – 1 x 50mL for a proof of concept neo-antigen assay

	• Up to 30 patients (identified at baseline) at ONE follow-up visit only (preferably pre-chemotherapy, OR, if that is not possible, following recovery of the neutrophil, lymphocyte and monocyte counts post-chemotherapy) - additional 1 x 140mL for neo-antigen assay product development • Leicester only – additional 1 x 10mL cfDNA <u>At first confirmed recurrence</u>: • 4 x 10mL for cfDNA • 2 x 10mL for cfDNA high sensitivity assay for approximately 100 patients (only if first recurrence is within 3 years of surgery) (Nb. recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.) • 1 x 10mL CellSave tube (circulating tumour cells) • 1 x 10mL for immunology • Leicester only – additional 1 x 10mL cfDNA • Patients additionally participating in relevant clinical trials (eg. DARWIN trials) only – additional 1 x 40mL for immunology <u>Follow-up after first recurrence:</u> At first CT on treatment for first recurrence • 2 x 10mL for cfDNA • 1 x 10mL CellSave tube • Leicester only – additional 1 x 10mL cfDNA • Patients additionally participating in relevant clinical trials (eg. DARWIN trials) only – additional 1 x 40mL for immunology (post cycles 1, 2, 4 and 6 only) <u>At progression, after the first recurrence</u>: • 2 x 10mL for cfDNA • 1 x 10mL CellSave tube • Leicester only – additional 1 x 10mL cfDNA • Patients additionally participating in relevant clinical trials (eg. DARWIN trials) only – additional 1 x 40mL for immunology <u>Following progression</u>: At up to 4 more time points, for example at the first CT scan whilst on treatment and at subsequent progressions • 2 x 10mL for cfDNA Leicester only – additional 1 x 10mL cfDNA <u>Completion of all treatment</u>: • 4 x 10mL for cfDNA • 1 x 10mL CellSave tube Leicester only – additional 1 x 10mL cfDNA

Translational research:	• Multi-region tissue sampling (including DNA and RNA sequencing and immunological analyses) • Blood samples for germ line DNA • Blood samples for circulating free tumour DNA (cfDNA) • Blood samples for circulating tumour cells • Blood samples for immunology • Blood samples for development of a neo-antigen assay
Anticipated duration of recruitment:	3 years
Duration of patient follow up:	5 years
Definition of end of study:	When the last patient who is alive has undergone 5 years of follow up (if the study objectives can be addressed reliably before this time, end of study could be sooner).

2. INTRODUCTION

2.1. Background and justification

Lung cancer, the leading cause of cancer-related death, accounts for nearly 1.4 million deaths worldwide every year, with an annual incidence of over 41,000 in the UK (age standardised incidence of 48 per 100,000). Mortality from lung cancer accounts for over 452,000 deaths in China, 342,000 in Europe, 162,000 in the US and 35,000 in UK. Approximately 78% of cases are non-small cell lung cancer (NSCLC), representing 32,000 new cases per annum in the UK. Despite surgery and adjuvant chemotherapy, the overall 5-year survival remains 67% for stage IB, 49% for stage II and 39% for stage III NSCLCs [1]. Although advances have been made for maintenance therapy, the efficacy of standard platinum-based chemotherapeutic treatment of NSCLC has reached a plateau, particularly in the adjuvant setting, so a more radical approach is needed to develop novel therapeutic modalities. After 30 years of clinical and translational research in this disease, the median survival of patients with stage IIIB/IV NSCLC has improved from approximately 5 months to only 12 months [2] and 15 months with maintenance chemotherapy. Increasing evidence implicates intratumour heterogeneity as a central factor [3]. Understanding how cancer clonal heterogeneity impacts upon survival outcome and how cancer subclones compete, adapt and evolve through the disease course in relation to therapy is an area of unmet clinical and scientific need.

2.1.1. Current Evidence for Intratumour Heterogeneity in NSCLC

Intratumour heterogeneity is increasingly recognised as a major hurdle to achieve improvements in therapeutic outcome and biomarker validation [4,5]. Intratumour genetic diversity provides a substrate for tumour adaptation and evolution. However, the evolutionary genomic landscape of NSCLC and how it changes through the disease course has not been studied in detail. Washington University [3] has recently described subclonal populations of cells with distinct allelic variant frequencies in primary NSCLCs. However, the spatial distribution of such subclones and how they change over time is unknown.

Spatial Heterogeneity and Branched Evolution in Primary NSCLC
Phylogenetic tree analysis of multi-region exome sequence datasets from a single tumour allows discrimination of conserved early genetic mutations present at all sites of the primary tumour from later mutations present in part of the tumour and/or metastatic sites and potential relationships of such changes with ploidy shifts, chromosomal instability and mutational processes that may change during the course of tumour progression (Figure 1).

This analysis has confirmed spatial intratumour heterogeneity of NSCLC subclones. Preliminary multi-region sequencing analysis of seven patients demonstrates branched evolution and the presence of subclones with distinct subclonal driver events spatially separated within the same tumour, or between primary tumour and lymph node/regional metastatic sites. These results, similar to our experience in clear cell renal carcinoma, demonstrate that through multi-region analysis, the total number of driver events detectable in the whole tumour expands, with up to 10 extra somatic putative driver events detectable through multi-regional analysis, separated spatially within the same tumour or lymph node site. The data also illustrate the large differences in phylogenetic structures in these early stage, surgically operable, tumours.

The tumours have at least one candidate subclonal driver event, previously described in lung adenocarcinoma, present in one tumour region but not another. Analysis of patients L003 and L007 reveal as many or more spatially separated candidate subclonal driver events than truncal early founder events, illustrating the scale of intratumour heterogeneity present in some primary NSCLCs. When focal and recurrent copy number events in NSCLC are also included, the total number of heterogeneous

spatially separated driver events expands even further (omitted from Figure 1 for simplicity). L007 illustrates the scale of diversity present between 3 mediastinal and hilar lymph nodes (R2, R3 and R4), which continue to evolve private somatic events distinct from the primary disease and from neighbouring lymph nodes. The scale of intratumour heterogeneity witnessed in at least half of these small primary tumours appears to be more profound than that witnessed in the considerably larger metastatic clear cell carcinomas of the kidney subject to the same level of stringent analysis.

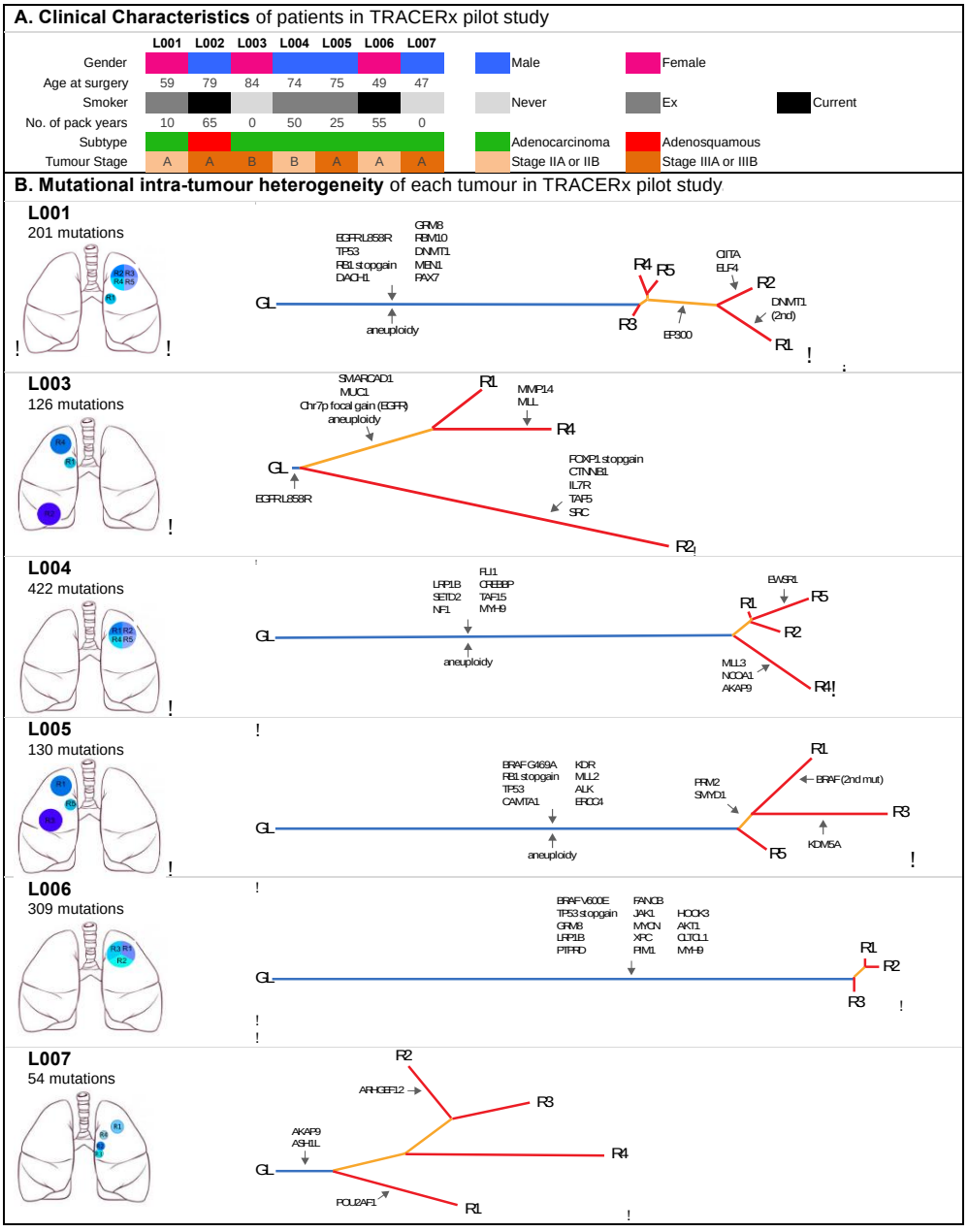


Figure 1: Mutational intratumour heterogeneity of primary NSCLC tumours.
A: Clinical characteristics of the 7 patients. **B:** Anatomical tumour regions are indicated in schematic diagrams of the thorax of each patient, and the mutational intratumour heterogeneity of each tumour is represented by phylogenetic trees. Potential driver mutations are indicated along the trunk and branches of the phylogenetic trees. Blue lines represent the ubiquitous mutations (trunk of the phylogenetic tree), yellow lines represent the shared mutations in a subset of regions (branches of the phylogenetic tree) and red lines represent the private mutations unique to a specific region (branches of the phylogenetic tree). The relative lengths of the lines are proportional to the number of mutations that are ubiquitous,

shared or private within each tree for each patient (not comparable between patients). The total number of mutations per patient is indicated.

The importance of intratumour heterogeneity is increasingly recognised as a driver of tumour progression, drug resistance and treatment failure in solid tumours [6-13]. The presence of subclonal driver events, exacerbated by underlying genomic instability mechanisms, may prove a significant challenge to biomarker development and drug target discovery efforts and contribute to drug resistance and poor survival outcome in human cancers [6,14-16]. Given evidence for the association of genetically distinct subclonal cell populations with distinct functional properties, it seems likely that the presence of such diversity, as measured by the number of subclonal somatic events might be associated with poor clinical outcome. Landau et al, 2013 provided evidence for the selection of subclones harbouring distinct driver events through therapy [17] in chronic lymphocytic leukaemia (CLL). They showed 8 patients with subclonal mutations present prior to therapy demonstrated shorter failure free survival times between treatments compared to 4 patients without subclonal events.

Unmet clinical need: The spatial separation of tumour subclones, the changing nature of the disease over time or the impact of such diversity upon disease outcome have not been addressed. TRACERx will address this area of unmet need to attempt to prospectively define thresholds of tumour heterogeneity for risk stratification, the impact of therapy upon genomic complexity and define the processes that drive lung cancer evolution over time.

2.1.2. Tumour Heterogeneity and Outcome and Impact of Platinum on Genome Complexity in NSCLC

It is unclear why adjuvant chemotherapy following surgery is effective for some patients with primary NSCLC but not others. An increasing body of evidence supports the association of patterns of intratumour heterogeneity, in multivariate analyses, with poor survival outcome in NSCLC and other solid tumours [18]. Chromosomal instability, a driver of intratumour heterogeneity is associated with cancer drug resistance and numerous studies have documented the association of chromosomal instability with poor outcome in NSCLC specifically [11,18-22]. The impact of intratumour heterogeneity on evolutionary fitness together with the documented relationships of heterogeneity with drug resistance supports the potential predictive nature of this candidate biomarker. Cytotoxic therapies have also been shown to influence the genomic landscapes of drug resistant disease [23,24]. These studies raise the concern that increased genomic complexity in cytotoxic refractory tumours may potentiate tumour adaptation later in the course of the disease. However, these studies are based on the analysis of small retrospective cohorts and the true relationship between intratumour heterogeneity and clinical outcome, and the impact of platinum cytotoxics upon the tumour genomic landscape is currently unknown.

Unmet clinical need: TRACERx will prospectively assess whether an intratumour heterogeneity index (I_{TB}) defines patient cohorts that do and do not derive benefit from adjuvant therapy. TRACERx will attempt to validate intratumour heterogeneity as an effective biomarker (prognostic or predictive) of disease free survival through a controlled (National Institute of Clinical Excellence guidelines) prospective longitudinal national cohort study. It will determine whether the index of intratumour heterogeneity provides additional information independent of standard factors such as tumour stage and histology. Defining how platinum therapy impacts upon tumour complexity at relapse and understanding how this relates to the genetic profile of the tumour prior to treatment in the adjuvant setting, may contribute to a better understanding of patient stratification for adjuvant therapy. TRACERx will therefore prospectively define the potential impact of platinum therapy upon the genomic architecture of tumours following disease relapse.

2.1.3. Longitudinal Studies to Define Drivers of Intratumour Heterogeneity and Drug Resistance

Increasingly, deep sequencing analyses are revealing the vast clonal heterogeneity present in solid tumours including NSCLC and the spatial and temporal dynamics of cancer subclones that emerge during the disease course and following acquisition of drug resistance [5,25]. Burrell et al, 2013 have shown that through the integration of complex cancer informatics datasets from multi-region tumour sequencing analysis or comparative genomic hybridisation (CGH) datasets with functional genomics follow-up of candidate somatic events, drivers of intratumour heterogeneity can be defined *in vivo*. They also found that one such mechanism driving tumour heterogeneity, DNA replication stress, may be targetable, suggesting that defining such processes in longitudinal solid tumour cohorts may have therapeutic relevance to attempt to limit tumour heterogeneity, adaptation and cancer evolution [26].

Unmet clinical need: Identification of drivers of cancer diversity and evolutionary fitness has the potential to offer new therapeutic opportunities to limit tumour adaptation. TRACERx will utilise genomic data derived from in depth multi-region sequencing analyses of NSCLCs over time to develop an improved understanding of the relationships of phenotypic and genetic intratumour heterogeneity with cancer evolution and identify further drivers of genomic instability. Laboratory work to define the functional relevance of these genomic datasets in NSCLC will be funded separately. Ultimately it is hoped that this will support the development of novel therapeutic approaches to limit relapse and improve outcomes in NSCLC.

2.1.4. Development of Minimally Invasive Methods to Study Tumour Evolution

Tumour heterogeneity has been shown to present profound problems from a tumour sampling perspective (10, 29), particularly in the metastatic setting with the emergence of metastases that may be composed of distinct tumour subclones (10, 18). The advent of technologies to detect circulating tumour cells (CTCs) and circulating free tumour DNA (cfDNA) has the potential to revolutionise and simplify the monitoring of tumour evolution over time. Diaz et al, 2012 and Shaw et al, 2012 have shown that cfDNA analysis is technically straightforward with limited cost and has demonstrable utility in disease monitoring [25,27]. Krebs et al, 2011 has also demonstrated that CTC number in NSCLC is an independent prognostic biomarker [28]. Analysis of single purified CTCs allows questions to be addressed regarding the co-existence of mutations within individual cells and therefore a more direct analysis of intercellular heterogeneity.

Unmet Need: The difficulties inherent in repeat tumour sampling emphasise the unmet need for the development of sensitive, minimally-invasive circulating biomarkers (CTCs and cfDNA) to attempt to resolve the extent of heterogeneity within one patient. Circulating biomarkers have the potential to monitor minimal residual disease (MRD), forecast early progression, document subclonal evolution through therapy and shed light on the genetic events permissive for tumour progression, metastases and intrinsic or acquired drug resistance [29]. Moreover, in patients with advanced metastatic disease where biopsies of multiple tumour sites are not only impractical, but also distressing for the patient, it will be important to formally determine how well CTCs and cfDNA analyses reflect tumour heterogeneity present in primary and recurrent disease and provide genetic information that may forecast the emergence of drug resistance. Therefore, in parallel with molecular analysis of spatially separated and sequential tumour biopsies, a unique TRACERx biobank will be developed of isolated CTCs and extracted cfDNA collected serially from TRACERx patients, with which to subsequently investigate their clinical utility.

2.1.5. Impact of Tumour Heterogeneity on Tumour Neo-antigenic repertoire and on Immunity

The discovery of novel targets to improve outcome is a key element in any comprehensive programme of lung cancer research. The impressive results seen with anti-PD1 in NSCLC demonstrates the likely pre-existence of immunity against tumour antigens expressed on cancer cells which can be harnessed for patient benefit [30]. The close association of tumour response to anti-PD-1 targeted therapies with tumoural expression of PD-L1 [30] demonstrates that assessment of the tumour immunobiological status, and the heterogeneity of such candidate immune biomarkers, may be critical in deciding whether an immunologically targeted therapy will be appropriate for a particular patient and which therapy to select. Furthermore, whilst emerging evidence suggests that intratumour heterogeneity may significantly limit the anti-tumour activity of targeted therapeutics [31], its overall effect on the anti-cancer immune response may prove tractable, since high levels of intratumoural mutational diversity may generate neo-antigens perceived by the immune system as non-self, thus providing relevant targets for immune-based therapies [31-33].

Unmet clinical need: TRACERx will provide the future resource to define how intratumour heterogeneity impacts upon cancer immunity throughout tumour evolution and therapy. Such studies will help to define how the clinical evaluation of intratumour heterogeneity will inform patient stratification and the development of combinatorial therapies incorporating conventional, targeted and immune-based therapeutics.

2.1.6. Clonal Dominance and Clinical Outcome

Until recently, the term actionable mutation was used to define the presence of a somatic mutation or copy number event in a single biopsy that might suggest a defined targeted therapeutic approach. However, emerging evidence for spatial intratumour heterogeneity in breast cancer [6], renal cell carcinoma [34], glioblastoma [35], pancreatic cancer [13,36] and medulloblastoma [8] have led to calls to consider the role of clonal dominance when defining actionable events. The TRACERx consortium has demonstrated evidence for similar spatial heterogeneity in NSCLC (Figure 1). Indeed in NSCLC, it has been suggested that subclonal EGFR somatic mutational heterogeneity may be an understudied mechanism of drug resistance [37].

Unmet clinical need: We aim to define early founder events, if they exist, that are suitable for clinical trial stratification and to prospectively assess the role of drug target intratumour heterogeneity in the early emergence of resistance and poor disease-free survival (DFS) outcomes. TRACERx will provide the platform to distinguish early founder events (present in the trunk of the tumour's evolutionary tree) from subclonal drivers. We will relate the clonal dominance of targetable events such as EGFR activating mutations to progression free survival (PFS) intervals for EGFR targeted therapies in the advanced disease setting. In so doing, we will attempt to define a new process for drug development, stratifying PFS outcomes based on clonal dominance of the targetable event in NSCLC. Clonal dominance of targetable lesions will be determined by deep sequencing paired primary and recurrent samples through the UCL-GCLP cancer gene panel.

3. STUDY DESIGN

Prospective observational cohort study in primary NSCLC. 842 patients with stage I-IIIA NSCLC will be recruited and followed up for 5 years.

3.1. Study Objectives

To study the NSCLC genomic landscape between primary and metastatic sites and the evolutionary dynamics of intratumour heterogeneity over time combined with detailed clinical, histopathological and cancer phenotypic annotation for each patient, in order to significantly improve the outcomes of NSCLC patients (e.g. reduce their chance of recurrence and improve survival).

3.1.1. Primary objectives:

- Define the relationship between intratumour heterogeneity and clinical outcome (disease-free survival and overall survival) following surgery and adjuvant therapy (including relationships between intratumour heterogeneity and clinical disease stage and histological subtypes of NSCLC).
- Establish the impact of adjuvant platinum-containing regimens upon intratumour heterogeneity in relapsed disease compared to primary resected tumour.

3.1.2. Secondary objectives:

- Development and validation of an intratumour heterogeneity ratio index I_{TB} as a prognostic or predictive biomarker in relation to its association with DFS and OS
- Infer a complete picture of NSCLC evolutionary dynamics: Define drivers of genomic instability, metastatic progression and drug resistance by identifying and tracking the dynamics of somatic mutational heterogeneity, and chromosomal structural and numerical instability present in the primary tumour and at metastatic sites. Individual tumour phylogenetic tree analysis will:
    a) Establish the order of somatic events in relation to genomic instability onset and metastatic progression
    b) Decipher genetic “bottlenecking” events following metastasis and drug therapy
    c) Establish dynamics of tumour evolution during the disease course from early to late stage NSCLC.
- Initiate a longitudinal minimally-invasive circulating tumour cells (CTC) and cfDNA biobank to develop analytical methods for the early detection and monitoring of tumour evolution over time.
- Develop a longitudinal tissue resource to serve as a platform to assess the relationship between genetic intratumour heterogeneity and the host immune response.
- Isolate monocytes and lymphocytes for the *in vitro* generation of neoantigen-reactive T cells to be tested for immunoreactivity to matched tumour samples
- Develop a repository of lung cancer cell lines, organoids and in vivo mouse models of patient-derived lung cancers that can be used as *in vitro* and *in vivo* models to study the aetiology of lung diseases, including lung cancer and predict response to therapeutics and resistance in lung cancer.
- Define relationships between intratumour heterogeneity and targeted/cytotoxic therapeutic outcome.

- Through UCL-GCLP gene panel in a certified laboratory environment, TRACERx will define clonally dominant disease drivers (paired primary-metastatic site comparisons in at least 270 patients with relapsed disease) to address the role of clonal driver dominance in targeted therapeutic response, and to guide stratification of lung cancer treatment and future clinical study inclusion in collaboration with the CR-UK Stratified Medicines Phase II program.
- Develop analytical methods for determining morphological heterogeneity within separate tumour regions.
- Tissue samples obtained from lung resections, both tumour and normal, will be analysed using high-power microscopy, such as electron microscopy, in order to obtain information regarding cellular structures.

3.1.3. Additional research questions to be examined:

- How is intratumour heterogeneity manifested in tumour histology? Does heterogeneity in histological pattern predict molecular heterogeneity?
- Can morphologically distinct areas of tumour regions (guided by digital microscopy) be investigated, using techniques such as deep sequencing, to demonstrate a correlation between genetic and phenotypic heterogeneity?
- How is intratumour heterogeneity manifested in CTCs and cfDNA at resection (qualitatively and quantitatively)? Are truncal, actionable driver mutations detectable in blood and informative?
- How is intratumour heterogeneity manifested in CTCs and cfDNA at recurrence?
- Can CTCs be used to detect and track actionable mutations and therefore guide treatment stratification?
- Do CTCs and cfDNA detected following disease recurrence (stage IV) reflect an evolved or selected subclone(s) of the original tumour? Is there an informative pattern of branched mutations in CTCs and/or cfDNA that maps to tumour evolution in matched biopsies from metastatic site(s) or low frequency subclones detectable in the primary?
- How does the genetic landscape of CTCs and cfDNA detectable at diagnosis and recurrence reflect the somatic mutational landscape of the original primary and metastatic disease?
- Can cfDNA detect minimal residual disease following tumour resection and can the dynamics of cfDNA predict tumour recurrence?
- After therapy, are new subclones selected that emerge and are detectable in CTCs and cfDNA?
- Do CTCs and cfDNA present in patients with advanced metastatic disease represent further selection of subclones over time and do they provide information about drug resistance mechanisms and escape pathways?
- What is the overall impact of distinct drivers of intratumour heterogeneity (e.g. somatic hypermutation vs chromosomal instability) on immune infiltration and function throughout tumour progression and therapy?
- What proportion of tumour infiltrating lymphocytes recognise neo-antigens generated by intratumour heterogeneity and how does therapeutic intervention affect the frequency and activity of such cells?
- Can novel T cell receptor (TCR) phosphopeptides be identified in patients with NSCLC that may serve as a platform for the development of a future immunotherapeutic strategy to circumvent tumour heterogeneity?

- What are the most relevant immune-modulatory pathways controlling the function of tumour infiltrating lymphocytes and how does this correlate with different drivers of intratumour heterogeneity before and after therapy?
- As with a biopsy to determine molecularly targeted therapy selection so with a biopsy to determine immunotherapy choice – is the immunobiological analysis of a single biopsy representative of the rest of the tumour and is a biopsy of the primary in any way representative of synchronous or metachronous metastatic disease?
- What is the spatial and temporal concordance of any immunobiological variable?
- What are the molecular drivers of the immunobiological landscape? What drives a prominent or negligible immune response in any individual NSCLC?
- Is there any evidence for the presence of tumour-related genetic abnormalities in histologically normal lung tissue that represents the 'normal' boundaries of a tumour?
- Examine correlations between imaging findings of cancer heterogeneity with the genomics data.
- Can brain metastasis-initiating cells be identified in cells derived from resected NSCLC tumour samples, in the context of in vivo models?
- Does the multi-region ploidy status of NSCLC tumours, identified by various methods including image cytometry, correlate with genomic sequencing data and clinical outcome?
- Can tumour homogenates be used to decipher the clonal evolution of NSLCC tumours and to identify candidate clinically relevant tumour-specific aberrations?

3.2. Study Activation

UCL CTC will ensure that all study documentation has been reviewed and approved by all relevant bodies and that the following have been obtained prior to activating the study:

- Research Ethics Committee approval
- 'Adoption' into NIHR portfolio
- NHS permission
- Adequate funding for central coordination
- Confirmation of sponsorship, and adequate insurance provision

4. SELECTION OF SITES/SITE INVESTIGATORS

4.1. Site Selection

In this protocol study 'site' refers to the hospital where study-related activities are conducted.

Sites must be able to comply with:

- Clinical care, follow up schedules and all requirements of the study protocol
- Data collection requirements, including adherence to electronic data capture timelines as per section 11.3
- Sample collection, processing and storage requirements
- Monitoring requirements, as outline in the protocol (section 14 and study monitoring plan)

4.1.1. Selection of Principal Investigator and other investigators at sites

Sites must have an appropriate Principal Investigator (PI), i.e. a health care professional authorised by the site, to lead and coordinate the work of the study on behalf of the site. Other investigators at site wishing to participate in the study must be trained and approved by the PI. PIs may be medical doctors, surgeons or pathologists.

4.1.2. Training requirements for site staff

All site staff must be appropriately qualified by education, training and experience to perform the study related duties allocated to them, which must be recorded on the site delegation log.

CVs for all staff must be kept up-to-date, signed and dated and copies held in the Investigator Site File (ISF). An up-to-date, signed copy of the CV for the PI must be forwarded to UCL CTC upon request.

GCP training is required for all staff responsible for study activities. The frequency of repeat training may be dictated by the requirements of their employing institution, or 2 yearly where the institution has no policy, and more frequently when there have been updates to the legal or regulatory requirements for the conduct of clinical trials.

4.2. Site initiation and Activation

4.2.1. Site initiation

Before a site is activated, the UCL CTC study team will arrange a site initiation with the site. The PI, and site research team must attend this initiation visit. The site will be trained in the day-to-day management of the study and essential documentation required for the study will be checked.

Site initiation will be performed for each site by site visit/teleconference with site/investigator meetings

4.2.2. Required documentation

The following documentation must be submitted by the site to UCL CTC prior to a site being activated by UCL CTC study team:

- Study specific Site Registration Form (identifying relevant local staff)
- All relevant institutional approvals (e.g. local NHS permission)
- A completed site delegation log that is initialled and dated by the PI

- A copy of the PI's current CV that is signed and dated

In addition, the following agreements must be in place:

- a signed Clinical Trial Site Agreement (CTSA) between the Sponsor and the relevant institution (usually a NHS Trust)

4.2.3. Site activation letter

Once the UCL CTC study team has received all required documentation and the site has been initiated, a site activation letter will be issued to the PI, at which point the site may start to approach patients.

Once the site has been activated by UCL CTC, the PI is responsible for ensuring:

- adherence to the most recent version of the protocol and TSPs for tissue/blood collection, processing and storage/transport;
- all relevant site staff are trained in the protocol requirements;
- appropriate recruitment and medical care of patients in the study;
- timely completion of the electronic data capture forms (including assessment of adverse events);
- prompt notification and assessment of serious adverse reactions that are associated with the study procedures, for example, due to the biopsies following recurrence or progression.

5. INFORMED CONSENT

5.1. Study entry

Sites are responsible for assessing a patient's capacity to give informed consent.

All patients will be asked for consent prior to surgery. Sites must ensure that all patients have been given the current approved version of the patient information sheet, are fully informed about the study and have confirmed their willingness to take part in the study by signing the current approved consent form.

Sites must assess a patient's ability to understand verbal and written information in English and whether or not an interpreter would be required to ensure fully informed consent. If a patient requires an interpreter and none is available, the patient should not be considered for the study.

The PI, or, where delegated by the PI, other appropriately trained site staff, are required to provide a full explanation of the study prior to study entry. During these discussions, the current approved patient information sheet for the study should be discussed with the patient. Patients should be given adequate time to consider and discuss participation in the study. If appropriate patients can be consented on the same day. A follow-up phone call can be made to the patient after they have consented and prior to surgery if deemed necessary. Written informed consent on the current approved version of the consent form for the study must be obtained before any study-specific procedures are conducted. The discussion and consent process must be documented in the patient notes.

Site staff are responsible for:

- checking that the correct (current approved) versions of the patient information sheet and consent form are used;
- checking that information on the consent form is complete and legible;
- checking that the patient has completed/initialled all relevant sections and signed and dated the form;
- checking that an appropriate member of staff has countersigned and dated the consent forms to confirm that they provided information to the patient;
- checking that an appropriate member of staff has made dated entries in the patient's medical notes relating to the informed consent process (i.e. information given, consent signed etc.);
- giving the patient a copy of their signed consent form, patient information sheet, and patient contact card;
- following registration: adding the patient study number to all copies of the consent form, which should be filed in the patient's medical notes and investigator site file.

The right of the patient to refuse to participate in the study without giving reasons must be respected. All patients are free to withdraw at any time. Also refer to section 15 (Withdrawal of Patients).

5.2. Consent process for a pulmonary blood sample

Sites that are able to perform pulmonary blood sampling will approach patients (approximately 200 patients in total planned for Manchester) to provide a CTC blood sample during their surgery. A separate consent will be obtained for collection of this sample.

5.3. Consent process for patients with recurrent/metastatic disease

Patients who develop local-regional or metastatic recurrence/progression after surgery and during follow-up, should be considered for metastasectomy or a biopsy from the site of recurrence. A separate consent will be obtained to perform this, and more details about the procedure provided in a specific information sheet.

6. SELECTION OF PATIENTS

6.1. Pre-Registration Evaluation

Patients must give written informed consent **before** any study specific screening investigations may be carried out. Please refer to section 9 for details of assessments.

6.2. Screening Log

A screening log must be maintained by the site and kept in the Investigator Site File. This must record all patients identified with NSCLC and eligible for primary surgery and the reasons why they were not registered in the study if this is the case. The log must be sent to UCL CTC when requested, with patient identifiers removed prior to sending. This is expected to be done approximately every 6 months or the first 12 months of the study. A review will then be undertaken to determine if further screening logs are required.

6.3. Patient Eligibility

Queries in relation to the eligibility criteria should be addressed prior to registration. Patients are eligible for the study if all the inclusion criteria are met and none of the exclusion criteria apply.

6.3.1. Inclusion criteria

- Written Informed consent
- Patients ≥18 years of age, with early stage I-IIIA disease who are eligible for primary surgery
- Histopathologically confirmed NSCLC, or a strong suspicion of cancer on lung imaging necessitating surgery (e.g. diagnosis determined from frozen section in theatre)
- Primary surgery in keeping with NICE guidelines planned (see section 9.3)
- Agreement to be followed up in a specialist centre
- Performance status 0 or 1
- Suspected tumour at least 15mm in diameter on pre-operative imaging

6.3.2. Exclusion criteria

- Any other current malignancy or malignancy diagnosed or relapsed within the past 5 years (other than non-melanomatous skin cancer, stage 0 melanoma *in situ*, and *in situ* cervical cancer)
- Psychological condition that would preclude informed consent
- Treatment with neo-adjuvant therapy for current lung malignancy deemed necessary
- Adjuvant therapy other than platinum-based chemotherapy and/or radiotherapy
- Known Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) or syphilis infection.
- Sufficient tissue, i.e. a minimum of two tumour regions, is unlikely to be obtained for the study based on pre-operative imaging

6.3.3. Patient ineligibility following registration

Following registration, patients should be withdrawn if:

- There is insufficient tissue
- The patient is unable to comply with protocol requirements
- There is a change in histology from NSCLC following surgery, or NSCLC is not confirmed during or after surgery.
- Change in staging to IIIB/IV following surgery
- The operative criteria are not met (e.g. incomplete resection with macroscopic residual tumours (R2)); see section 9.3 for a list of accepted surgical procedures. Patients with microscopic residual tumours (R1) are eligible and should remain in the study
- Adjuvant therapy other than platinum-based chemotherapy and/or radiotherapy is administered

Patients found to have pre-invasive or minimally invasive adenocarcinoma (MIA) lesions following surgery will be withdrawn. However, the surgical tissue already collected will be sent to the central laboratory but these patients will not be followed-up in the study or required to provide any further blood samples. If these patients subsequently develop invasive cancer, the date of diagnosis and the tumour histology will be reported on the electronic data capture system. For the purposes of the study, the following are considered as pre-invasive lesions:

- Squamous dysplasia
- Squamous cell carcinoma in situ (CIS)
- Atypical adenomatous hyperplasia (AAH)
- Adenocarcinoma *in situ* (AIS)

If any of these occur, patients will be replaced to ensure the target sample size is met. Samples collected up until the point of study withdrawal will be biobanked centrally for future ethically approved research unless the patient withdraws consent for the provision of these samples:

- If a patient is post-operatively diagnosed with a lung cancer sub-type other than NSCLC, a pre-invasive lung lesion, or minimally invasive adenocarcinoma, and subsequently withdrawn as per the criteria above, samples that have already been collected should be sent to the central laboratory for biobanking.

- If a patient is post-operatively found to not have a diagnosis of cancer, or is diagnosed with lung metastases from a previous cancer other than lung, and subsequently withdrawn from the study, the fresh tissue for immunological analyses and the 10ml immunology blood sample for UCL do not need to be sent to the central laboratory. However, any other samples that have already been collected may still be of interest. Please call the TRACERx study coordinator at UCL CTC to discuss.

Please refer to section 15 for withdrawals.

7. REGISTRATION PROCEDURES

7.1. Registration

Patient registration will be performed via a remote electronic data capture system hosted by UCL CTC. Following pre-registration evaluations (as detailed in section 6.1), confirmation of eligibility and consent of a patient at a site, the registration should be completed on the remote data capture system (**https://rde.ctc.ucl.ac.uk/**).

A study number will be assigned for the patient once the registration process has been completed and must be recorded by the site on the master subject log. If there are any difficulties, sites should contact the UCL CTC:

Telephone number:	+44 (0)20 7679 9880
UCL CTC Office hours:	09:00 to 17:00 Monday to Friday

Once a patient has been registered onto the study:

- Patients must be provided with the following:
  - A copy of their signed consent form and patient information sheet
  - A patient contact card. Site on-call contact details for 24 hour medical care must be added to this card and patients advised to carry this with them at all times while participating in the study
- An email with the patient's study number should be sent to UCL CTC (**ctc.tracerx@ucl.ac.uk**) to confirm registration of a patient**.** UCL CTC will add the patient to the TRACERx tracker to enable the tracking of study samples.

8. TREATMENT

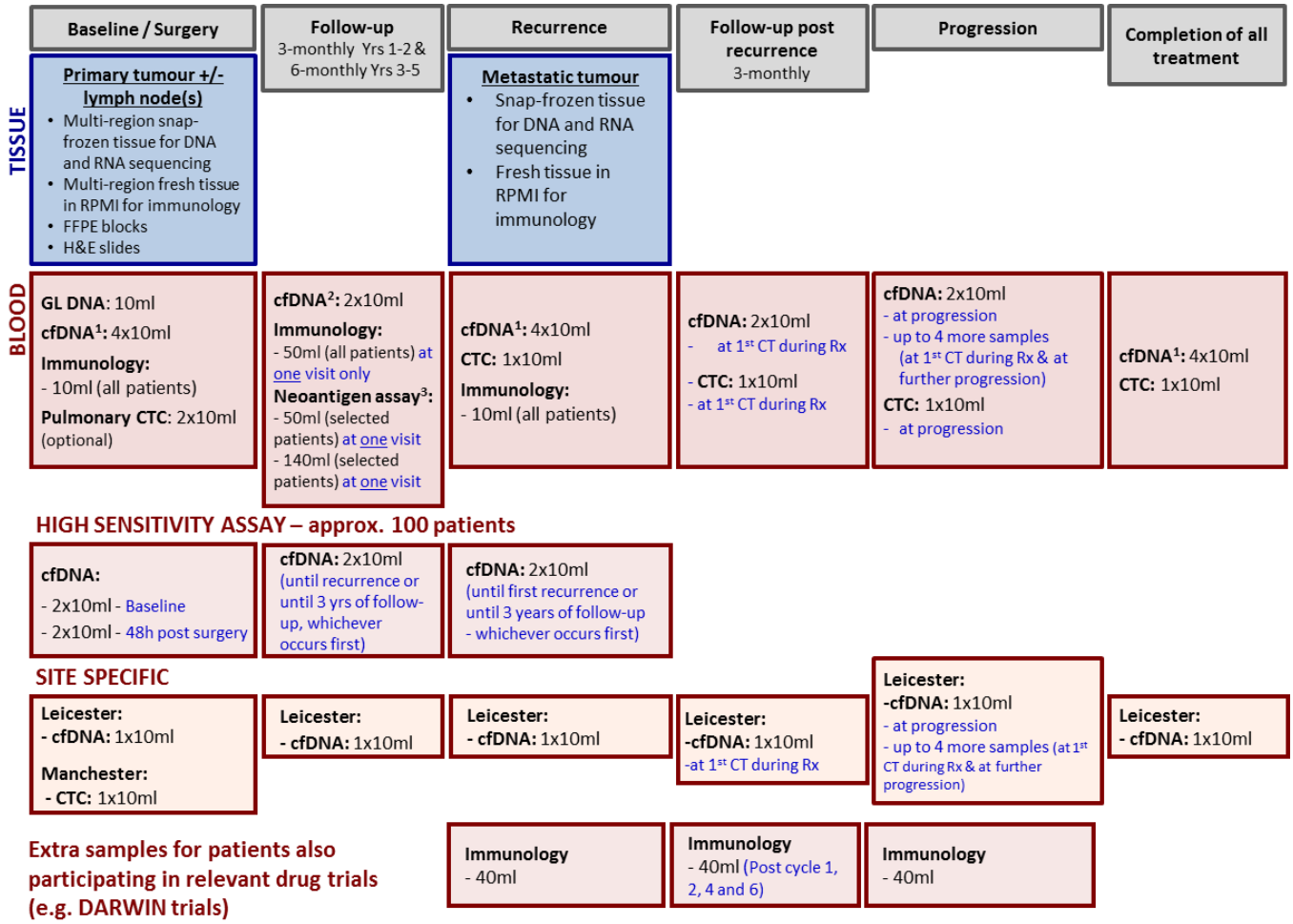
All eligible patients will be amenable to primary surgery in keeping with NICE guidelines. Further treatment will be according to standard of care determined by NICE guidelines.

9. ASSESSMENTS AND COLLECTION OF BIOLOGICAL SPECIMENS

Each patient is expected to be followed up for a maximum of 60 months. All efforts should be made by the Site to contact the patient's GP to assess their condition, if a patient fails to attend a clinic or cannot be followed up at each respective site.

A set of Trial Specific Procedures will be used by centres to obtain and process biological samples.

Below is a summary of what biological specimens would be collected and when. See also Appendix 3



Abbreviations: GL DNA: germ line DNA, cfDNA: circulating free tumour DNA, CTC: circulating tumour cell, Rx: treatment.

[1]: Samples to be collected separately: 20ml to be sent to UCL and 20ml to be sent to Leicester

[2] If patient is having adjuvant chemotherapy, the first sample is to be collected pre-cycle 1 instead of at 3 months, and second sample to be collected at 6 months after surgery

[3] Sites will be informed which patients should be asked for either of the neo-antigen assay blood samples. If the patient is receiving adjuvant chemotherapy then the sample should be taken pre-cycle 1 wherever possible.

9.1. Baseline Assessments

The following assessments are required prior to patient registration, in addition to collecting data on demographics (such as age and sex):

- Medical history, and specified clinical characteristics
- Information about smoking exposure and other patient characteristics, using the baseline questionnaire to be completed by the patient. Sites should send the original questionnaire into the UCL CTC and keep a copy in their site file
- ECOG performance status
- Smoking status
- Details from routine PET-CT or CT scan (chest, abdomen (and pelvis if clinically indicated)) to be recorded on study CRF.

9.2. Blood sampling before surgery

Baseline blood sample collection should take place preferably within the 1 - 2 weeks prior to surgery if possible.

- Blood sample for germ line DNA – 10mL
- Blood samples for cfDNA – 4 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)
- Blood sample for CTC – 1x CellSave tube (minimum 10mL, Manchester only).
- Pulmonary blood sample for CTC – 2 x CellSave tube (minimum 10mL) – but only for ~200 patients from sites who are able to collect pulmonary blood.
- Blood sample for immunology – 10mL in all patients

High Sensitivity Assay

In an initial cohort of approximately 100 patients an additional 2 x 10mL of blood will be collected at each cfDNA sampling time point until first recurrence or until 3 years of follow-up (whichever occurs first). An additional sample will also be obtained at 48h post-surgery.

The cfDNA will be analysed for common mutations using a highly sensitive, multiplexed assay to detect and quantify variants including point mutations and insertion/deletions in genes such as EGFR, KRAS and PIK3CA.

Update: recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.

- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture

9.3. Tissue sampling during surgery

Patients will undergo surgery according to NICE guidelines. The following assessments/sampling will be completed:

- Date of surgery and surgical details:
    - lobectomy (either open or thoracoscopic)
    - lung parenchymal-sparing operations (segmentectomy or wedge resection) if a complete resection can be achieved

  - extensive surgery (bronchoangioplastic surgery, bi-lobectomy, pneumonectomy) if necessary to obtain clear margins, hilar and mediastinal lymph node sampling or en bloc resection

- Tissue (primary tumour and associated lymph node, if applicable, and normal tissue from the resected specimen) surplus to diagnostic and pathological requirements will be used for:
    - Multi-region sampling for subsequent analyses including DNA and RNA sequencing of frozen tissue
    - Multi-region sampling for immunological analyses on fresh tissue
    - Production of formalin-fixed, paraffin embedded tissue blocks
    - Production of histological slides from tumours
  - For large tumours – multi-region sampling for above analyses in addition to immunological analyses on frozen tissue and production of region-specific formalin-fixed paraffin embedded tissue blocks. Where possible, cell line derivation and establishment of xenograft models from fresh tissue.
  - For smaller tumours – tissue will be collected with priority given to sequencing analyses
  - For pre-invasive lesions detected after surgery, tissue may be sampled and used for research purposes provided diagnostic requirements are met. These patients will be withdrawn from the study and so not be followed-up for the study or required to provide any further study blood samples. However, the date lesions progress to invasive cancer will be reported on the electronic data capture system.

(Refer to the TSPs for tissue/blood collection, processing and storage/transport for further guidelines).Formalin-fixed, paraffin embedded tissue blocks will be kept for the duration of the study, unless requested to be returned for diagnostic purposes. At the end of the study any remaining tissue will be stored in a biobank for future ethically approved research.

H&E slides may be requested from sites for central review. These slides will be scanned and then returned to site.

9.4. Assessments during adjuvant chemotherapy (if given)

For each cycle of adjuvant chemotherapy the following details should be collected:

- Details of adjuvant chemotherapy
- ECOG performance status
- Smoking status
- Adverse Reactions - assessed only in relation to adjuvant chemotherapy (grades 3-5 only) and those associated with study procedures – e.g. venepuncture

9.5. Assessments and blood sampling after surgery, during 5 years follow up

After surgery/completion of adjuvant chemotherapy (if given), patients will be followed up according to national guidelines, which is every 3 months for the first 2 years, then every 6 months in years 3-5. They are expected to receive standard of care, i.e. platinum-based adjuvant therapy according to NICE guidelines. The electronic data capture forms should be completed to document the following:

- ECOG performance status
- Smoking status
- Chest x-ray
- Details from PET-CT or CT if done
- Adverse Reactions - assessed only in relation to adjuvant chemotherapy (grades 3-5 only) and those associated with study procedures – e.g. venepuncture

In addition, the following would be requested:

- Blood sample for cfDNA – 2 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)
- Blood sample for immunology at ONE follow-up visit only – 1 x 50mL
- Blood sample for a proof of concept neo-antigen assay at ONE follow-up visit only – 1 x 50mL (up to 30 patients, who will be identified following surgery). This should preferably be taken pre-chemotherapy where applicable OR, if that is not possible, following recovery of the neutrophil, lymphocyte and monocyte counts post-chemotherapy.
- Blood sample for neo-antigen assay product development at ONE follow-up visit only – 1 x 140mL (up to 30 patients, who will be identified at baseline). This should preferably be taken pre-chemotherapy where applicable OR, if that is not possible, following recovery of the neutrophil, lymphocyte and monocyte counts post-chemotherapy

- For patients who do not receive adjuvant chemotherapy, every 3 months from the day of surgery for years 1-2 and every 6 months in years 3 -5 until first recurrence.
- For patients who receive adjuvant chemotherapy, the first post-surgery sample should be collected just before cycle 1 treatment and the next sample should be collected 6 months from the day of surgery. Sample collection should then be every 3 months for years 1-2 following surgery and every 6 months in years 3-5 until first recurrence.

High Sensitivity Assay

In an initial cohort of approximately 100 patients an additional 2 x 10mL of blood will be collected at each cfDNA sampling time point until first recurrence or until 3 years of follow-up (whichever occurs first). The cfDNA will be analysed for common mutations using a highly sensitive, multiplexed assay to detect and quantify variants including point mutations and insertion/deletions in genes such as EGFR, KRAS and PIK3CA.

Update: recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.

9.6. First recurrence

Patients who have a recurrence (based on chest X-ray, CT scan or other clinical diagnosis) will be treated as per standard of care. The following will be requested at the time of recurrence:

- Blood sample for CTC – 1x CellSave tube (minimum 10mL)
- Blood samples for cfDNA – 4 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)
- Blood sample for immunology – 10mL in all patients,

The following should also be recorded:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done – RECIST 1.1
- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture and biopsies

High Sensitivity Assay
In an initial cohort of approximately 100 patients an additional 2 x 10mL of blood will be collected for cfDNA only if first recurrence is within 3 years of surgery.
The cfDNA will be analysed for common mutations using a highly sensitive, multiplexed assay to detect and quantify variants including point mutations and insertion/deletions in genes such as EGFR, KRAS and PIK3CA.

Update: recruitment to this cohort is now complete, but collection of samples at the subsequent time points will continue for existing patients.

Patients additionally participating in relevant clinical trials (eg. DARWIN trials) only
In patients who also consent to relevant clinical trials (eg. DARWIN trials) an additional 1 x 40mL of blood will be collected for immunology at the time of first recurrence.

During treatment for first recurrence
The following will be requested at the time of the first CT scan, on treatment for first recurrence:

- Blood sample for CTC – 1 x CellSave tube (minimum 10mL)
- Blood samples for cfDNA – 2 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)

The following should also be recorded:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done
- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture

Further follow-up will be at the end-of-treatment CT scan (RECIST 1.1) and 3 monthly thereafter. If the patient does not receive treatment at the time of first recurrence, follow-up will be every 3 months.

The following will be collected:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done

- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture

Patients additionally participating in relevant clinical trials only
In patients who also consent to relevant clinical trials (eg. DARWIN trials) an additional 1 x 40mL of blood will be collected for immunology at the end of treatment cycle 1, 2, 4 and 6.

9.7. Progression, following recurrence

At the first evidence of documented progression based on chest X-ray, CT scan (RECIST 1.1) or clinical progression, patients will be asked to provide the following:

- Blood sample for CTC – 1 x CellSave tube
- Blood sample for cfDNA – 2 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)

The following should also be recorded:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done
- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture and biopsies

Patients participating in relevant clinical trials (eg. DARWIN trials) only
In patients who consent to relevant clinical trials (e.g. DARWIN trials) an additional 1 x 40mL of blood will be collected for immunology at the time of progression.

Blood samples for cfDNA (2 x 10mL for all sites, plus 1 x 10mL for Leicester only) should be requested up to 4 more times: at the first CT scan on treatment for progression after first recurrence, at subsequent progressions and at the first CT scan on treatment for these progressions if treatment administered.

Further follow-up will be at the end-of-treatment CT scan (RECIST 1.1) and 3 monthly thereafter. If the patient does not receive treatment at the time of progression, follow-up will be every 3 months.

The following will be collected:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done
- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture and biopsies

9.8. Blood sampling after end of treatment

On completion of all treatment the following samples should be obtained:

- Blood sample for CTC – 1 x CellSave tube
- Blood sample for cfDNA – 4 x 10mL
- Blood samples for cfDNA – 1 x 10mL (Leicester only)

The following should also be recorded:

- ECOG performance status
- Smoking status
- Details from PET-CT/CT/CXR if done (RECIST 1.1)
- Adverse Reactions - assessed only in relation to those associated with study procedures – e.g. venepuncture

9.9. Biopsies

During follow up, all patients should be approached to provide biopsies of the sites of metastases and/or local-regional recurrence. This would be done at the time of the first recurrence, and then again, if the cancer progresses afterwards (before further lines of treatment are given). Consent will be obtained from patients before each biopsy.

The TRACERx consortium includes only centres that perform repeat tumour sampling in the metastatic setting as part of routine clinical practice for some patients. These centres have appropriate interventional radiology suites to perform biopsy procedures, experienced thoracic physicians highly competent in endobronchial ultrasound-guided (EBUS) tumour sampling and interventional radiologists competent in imaging-directed tumour sampling procedures. Audits performed at UCLH thoracic oncology unit and nationally within the United Kingdom confirm that biopsy of sites of recurrence in the chest is a relatively safe procedure with reversible haemoptysis occurring in less than 1-3% and pneumothorax in 2-5% of patients [38].

Patients who undergo further biopsies will also be offered the UCL-GCLP gene panel, which may provide additional opportunities for therapeutic studies in the metastatic setting. This information could be given to and discussed with patients via their clinician, who will consider the appropriateness of treatment clinical trials, e.g. one of the DARWIN trials. Any eligibility criteria for subsequent clinical trials are not assessed as part of TRACERx; there would be study-specific protocols, Patient Information Sheets and Consent Forms. Some biopsies may therefore be consented and carried out as part of these clinical trials.

9.10. Imaging scans

For each patient, a copy of the imaging PET-CT/CT scan should be obtained at each of the following times, where such a scan has been performed:

- Baseline
- First recurrence
- All points of progression, following the first recurrence

All centres should send the PET-CT/CT scans for centralised review within 3 months of the scan date via PACS links. Please contact the TRACERx study coordinator if transfer via PACS is not possible.

Scans must be anonymised and labelled with the patient's TRACERx number and timepoint (e.g. A_LTX000_baseline) in the file name.

The main purpose is to examine the association between the imaging data and the genomics data: correlating imaging data from different time points with the biological results (i.e. sequencing, CTC, cfDNA, or immunology). A CT scan of the tumour can be converted to data points that completely

describes all observable morphological features, and these can be computationally compared to traditional data points, such as mutations, copy number aberrations, gene expression values, or ITH measurements. Depending on the findings, images may also be requested for patients who have undergone other imaging modalities e.g. MRI as part of their standard care.

Transfer **anonymised** images with TRACERx study numbers via PACS to:

UCLH PACS
Junaid Chawdhury
Imaging IT Systems Administrator
020 344 73657
Admin-UCH.PACS@uclh.nhs.uk

Tumour measurements according to RECIST 1.1 will be collected using the electronic data capture system.

10. BIOLOGICAL SAMPLES

Please refer to the Trial Specific Procedures on processing biological samples for further guidelines.

10.1. Sending of samples

Samples should be sent to the following contacts below (please see 'TSP3: blood sample overview'):

- Tissue samples in RPMI Medium
- Frozen samples (UCL Laboratory will determine if sufficient tissue is available for immunology analyses and production of additional FFPE blocks)
- FFPE block(s) (from baseline surgery) plus anonymised pathology report (please add TRACERx study number)
- Blood sample for germ line DNA
- Blood sample for immunology
- H&E slides (include return address) – Nb. the CTC will request slides for specific patients

  TRACERx
  Dr Raju Veeriah
  Translational Cancer Therapeutics Laboratory
  UCL Cancer Institute
  Paul O'Gorman Building
  72 Huntley Street
  London, WC1E 6BT

- Blood sample for neo-antigen assay

  TRACERx
  Dr Raju Veeriah
  Translational Cancer Therapeutics Laboratory
  UCL Cancer Institute
  Paul O'Gorman Building
  72 Huntley Street
  London, WC1E 6BT

- Plasma samples for cfDNA

  UCLH – 2x sample at surgery, 2x sample from first recurrence, 2x sample from the end of all treatment, and all samples to be tested using the highly sensitive, multiplexed assay (approx. 100 patients initially)

  TRACERx
  Dr Raju Veeriah
  Translational Cancer Therapeutics Laboratory
  UCL Cancer Institute
  Paul O'Gorman Building
  72 Huntley Street
  London, WC1E 6BT

  Leicester – all other plasma samples cfDNA samples
  Jacqui Shaw
  TRACERx

Department of Cancer Studies and Molecular Medicine
University of Leicester
Level 3 Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester, LE2 7LX

- Blood samples for CTC

  PACCAR
  Dr Jackie Pierce
  TRACERx
  CEP GCLP laboratories
  Cancer Research UK Manchester Institute
  University of Manchester
  Wilmslow Road
  Withington
  Manchester, M20 4BX

11. DATA MANAGEMENT AND DATA HANDLING GUIDELINES

Data will be collected from sites using an electronic data capture system.

Source data are contained in source documents and must be accurately transcribed on to the electronic data capture system. Examples of source documents are hospital records which include laboratory and other clinical reports etc. Source data must not be entered directly into the electronic data capture system.

Where copies of supporting source documentation (e.g. autopsy reports, pathology reports, CT scan images etc.) are being submitted to UCL CTC, the patient's study number must be clearly indicated on all material and any patient identifiers removed/blacked out prior to sending to maintain confidentiality.

11.1. Data Entry

All entries made to the electronic data capture system must be by staff who are listed on the site staff delegation log and authorised by the PI to perform this duty. The PI is responsible for the accuracy of all data reported in the electronic data capture system.

11.2. Missing Data

To avoid the need for unnecessary data queries, data entered must be checked at site to ensure there are no blank fields. When data are unavailable, the status of the field should be changed to 'Not Available' (only use if every effort has been made to obtain the data).

11.3. Timelines for Data Entry

Data entry to the electronic data capture system must be completed as soon as possible after patient visit and within 1 month of the patient being seen.

Sites who persistently fail to enter data within the required timelines may be suspended from recruiting further patients into the study by UCL CTC and subjected to a 'for cause' monitoring visit. See section 14.3 ('For Cause' On-Site Monitoring) for details.

11.4. Data Queries

Data entered into the electronic data capture system will be checked for completeness, accuracy and consistency, including checks for missing or unusual values. Queries and guidance for resolution will be sent to the data contact at site.

12. SAFETY REPORTING

12.1. Definitions

Adverse Reaction (AR)

All untoward and unintended events causally related to a 'Study Procedure'; where a causal relationship between a 'Study Procedure' and an event is at least a reasonable possibility, i.e. the relationship cannot be ruled out.

Serious Adverse Reaction (SAR)

An adverse reaction that:

- Results in death
- Is life threatening (the term "life-threatening" refers to an event in which the patient was at risk of death at the time of the event. It does not refer to an event that hypothetically might have caused death if it were more severe)
- Requires in-patient hospitalisation or prolongs existing hospitalisation
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly or birth defect
- Is otherwise medically significant (e.g. important medical events that may not be immediately life-threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention to prevent one of the other outcomes listed above)

Related & Unexpected SARs

A serious adverse reaction, the nature or severity of which **is not consistent** with the applicable Study Procedure.

Study Procedure means the biopsy procedure and/or blood sampling procedure for the purposes of obtaining research samples within the study.

12.2. Reporting Procedures

Severity

Severity of each event must be determined by using the Common Terminology Criteria for Adverse Events (CTCAE) v4.0 as a guideline, wherever possible. The criteria are available online at:

http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf

In those cases where the CTCAE criteria do not apply, severity should be coded according to the following criteria:

1 = Mild (awareness of sign or symptom, but easily tolerated)
2 = Moderate (discomfort enough to cause interference with normal daily activities)
3 = Severe (inability to perform normal daily activities)
4 = Life threatening (immediate risk of death from the reaction as it occurred)
5 = Fatal (the event resulted in death)

For the purposes of the study, only grades 3-5 events associated with adjuvant chemotherapy should be recorded on the case report forms, and events of all grades associated with Study Procedures

Causality

The PI, or other delegated site investigator, must perform an evaluation of causality for each event.

UCL CTC will consider events evaluated as possibly, probably or definitely related to be adverse reactions.

- **Possibly**

  There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after the study procedure). However, the influence of other factors may have contributed to the event (e.g. the patient's clinical condition, other concomitant treatments).

- **Probably**

  There is evidence to suggest a causal relationship and the influence of other factors is unlikely.

- **Definitely**

  There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.

As this is an observation cohort study where patients follow their normal clinical pathways, and the study introduces two procedural interventions (blood sampling and biopsies), the PI, or other delegated site investigator should not report to UCL CTC events where there is no causality to the study procedure or adjuvant chemotherapy.

12.2.1. Serious Adverse Reactions (SARs)

All SARs that occur between the between the start of the first study procedure and 30 days post the last study procedure must be submitted to UCL CTC by fax within **24 hours** of observing or learning of the event, using the study specific SAR Report. All sections on the SAR Report must be completed. If the event is **not being reported within 24 hours** to UCL CTC, the circumstances that led to this must be detailed in the SAR Report to avoid unnecessary queries.

SAR Follow-Up Reports

All SARs must be followed-up until resolution and until there are no further queries. The PI, or other delegated site investigator, must provide follow-up SAR Reports if the SAR had not resolved at the time the initial report was submitted.

SAR Processing at UCL CTC

On receipt of the SAR Report, UCL CTC will check for legibility, completeness, accuracy and consistency. Expectedness will be evaluated, to determine whether or not the case qualifies for expedited reporting, using the list of expected adverse reactions in protocol appendix 2.

The CI, or their delegate (e.g. a clinical member of the TRACERx consortium), may be contacted to review the SAR and to perform an evaluation of causality on behalf of UCL CTC. In addition, if UCL CTC has considered expectedness difficult to determine, the CI, or their delegate, will be consulted for their opinion at this time.

12.3. Related and unexpected SARs

If the event is evaluated as a related and unexpected SAR, UCL CTC will submit a report to the REC within 15 calendar days. Where there are conflicting evaluations of causal relationship by the site and UCL CTC/CI, both opinions will be reported.

UCL CTC will inform all PIs of any related and unexpected SARs that occur on the study. PIs will receive expedited related and unexpected SAR reports that must be processed according to local requirements.

12.4. Safety Monitoring

UCL CTC will provide safety information to the TRACERx consortium, and the IDMC on a periodic basis for review.

Study safety data will be monitored to identify:

- new adverse reactions to the study procedures

Should UCL CTC identify or suspect any issues concerning patient safety at any point throughout the study, the CI or TRACERx consortium will be consulted for their opinion.

13. INCIDENT REPORTING

13.1. Incident Reporting

Organisations must notify UCL CTC of all deviations from the protocol or GCP immediately. UCL CTC may require a report on the incident(s) and a form will be provided if the organisation does not have an appropriate document (e.g. Trust Incident Form).

If site staff are unsure whether a certain occurrence constitutes a deviation from the protocol or GCP, the UCL CTC study team can be contacted immediately to discuss.

14. STUDY MONITORING AND OVERSIGHT

Participating sites and PIs must agree to allow study-related on-site monitoring, Sponsor audits and regulatory inspections by providing direct access to source data/documents as required. Patients are informed of this in the patient information sheet and are asked to consent to their medical notes being reviewed by appropriate individuals on the consent form.

UCL CTC will determine the appropriate level and nature of monitoring required for the study. Risk will be assessed on an ongoing basis and adjustments made accordingly.

14.1. Central Monitoring

Sites will be requested to submit screening logs and staff delegation logs to UCL CTC at the frequency detailed in the study monitoring plan or on request and these will be checked for consistency and completeness. Also refer to sections 4.2.2 (Required documentation) and 6.2 (Screening Log).

Ensuring patient eligibility is the responsibility of the PI or other delegated Investigator(s). Checks of the criteria listed on the registration form will be undertaken by an appropriately trained UCL CTC staff member prior to registration. Also refer to section 7.1 (Registration).

Details relating to the informed consent process will be collected on the registration form and are subject to review by CTC as part of patient eligibility.

Sites will be requested to conduct quality control checks of documentation held within the Investigator Site File at the frequency detailed in the study monitoring plan. Checklists detailing the current version/date of version controlled documents will be provided for this purpose.

Data received at UCL CTC will be subject to review in accordance with section 11.4 (Data Queries).

Where central monitoring of data and/or documentation submitted by sites indicates that a patient may have been placed at risk, the matter will be raised urgently with site staff and escalated as appropriate (refer to section 13 (Incident Reporting) and 14.3 ('For Cause' On-Site Monitoring) for further details).

14.2. On-Site Monitoring

On-site monitoring may be conducted, and details of monitoring activities will be included in the study monitoring plan which will be provided to Sites (note: this may be updated from time to time during the study).

Sites will be sent a letter in advance confirming when a monitoring visit is being planned. The letter will include a list of the documents to be reviewed, interviews that will be conducted, planned inspections of the facilities, who will be performing the visit and when the visit is likely to occur.

Monitoring Follow-up

Following a monitoring visit, the Study Monitor/Study Coordinator will provide a follow-up email to the site, which will summarise the documents reviewed and a statement of findings, deviations, deficiencies, conclusions, actions taken and/or actions required. The PI at each site will be responsible for ensuring that monitoring findings are addressed in a timely manner, and by the deadline specified.

14.3. 'For Cause' On-Site Monitoring

On-site monitoring visits may be scheduled where there is evidence or suspicion of non-compliance at a site with important aspect(s) of the study protocol/GCP requirements. Sites will be sent a letter in advance outlining the reason(s) for the visit. The letter will include a list of the documents that are to be reviewed, interviews that will be conducted, planned inspections of the facilities, who will be performing the visit and when the visit is likely to occur.

UCL CTC will assess whether it is appropriate for the site to continue participation in the study. Refer to section 13 (Incident Reporting

14.4. Oversight Committees

14.4.1. TRACERx Consortium

The consortium will include the Chief Investigator, clinicians and experts from relevant specialties and TRACERx study staff from UCL CTC. The TRACERx consortium will be responsible for overseeing the study. The group will meet regularly and will send updates to PIs (via newsletters or at Investigator meetings) and to the NCRI Lung Clinical Studies Group.

The TRACERx consortium will review substantial amendments to the protocol prior to submission to the REC. Review may be undertaken by a smaller sub-set of the group depending on the nature of the amendment. All PIs will be kept informed of substantial amendments through their nominated responsible individuals.

14.4.2. Independent Data Monitoring Committee (IDMC)

The role of the IDMC is to provide independent advice on data and safety aspects of the study. Meetings of the Committee will be held approximately every 12 months to review interim analyses or as necessary to address any issues.

The first review will be conducted 12 months after recruitment to assess the biopsy success rate. If this is <20% of the number of observed recurrence found at the time the IDMC will review the data again in 6 months and determine if this part of the study should continue.

A further review will be undertaken after the first 150 patients have been followed up for 12 months, to examine:

- Accrual rates
- Recurrence rates
- Repeat biopsies rate
- Safety data
- The reliability of intratumour heterogeneity indices
- Efficacy results relevant to the sample size assumptions

If the recruitment rate is considerably lower than 24 patients/month for the first 26 months after the initiation phase, further centres will be approached. A further review will be undertaken 6 months after the additional sites are activated. If accrual remains low 12 months after the additional sites are activated the IDMC may recommend early closure.

All members of the IDMC will sign an IDMC charter.

14.4.3. Role of UCL CTC

UCL CTC will be responsible for the day to day coordination and management of the study and will act as custodian of the data generated in the study (on behalf of UCL) UCL CTC is responsible for all duties relating to safety reporting which are conducted in accordance with section 12 (Safety Reporting).

15. WITHDRAWAL OF PATIENTS

Please also refer to section 6.3.3. In consenting to the study, patients are consenting to study, assessments, follow-up and data collection. If possible, we aim to replace patients who withdraw early (this can only be done during recruitment).

15.1. Discontinuation from Study Follow-up

A patient may be withdrawn from the study whenever continued participation is no longer in the patient's best interests, but the reasons for doing so must be recorded. Reasons for discontinuing may include:

- Intercurrent illness which prevents further follow-up
- Patient choice
- Any alterations in the patient's condition which justifies the discontinuation of follow-up in the investigator's opinion

In these cases patients remain within the study for the purposes of follow-up and data analysis.

If a patient expresses their wish to withdraw from the study, sites should explain the importance of remaining on study follow-up and for allowing existing collected data to be used. If the patient gives a reason for their withdrawal, this should be recorded.

Samples collected up until the point of study withdrawal will be biobanked for future ethically approved research unless the patient withdraws consent for the provision of these samples.

15.2. Future Data Collection

If a patient explicitly states they do not wish to contribute further data to the study their decision must be respected, with the exception of safety data, and recorded on the electronic data capture system. In this event details should be recorded in the patient's hospital records, no further data must be completed and entered to the electronic data capture system and no further data other than safety data submitted to UCL CTC.

15.3. Losses to Follow-Up

If a patient moves from the area, every effort should be made for the patient to be followed up at another participating study site and for this new site to take over the responsibility for the patient, or for follow-up via GP. Details of participating study sites can be obtained from the UCL CTC study team who must be informed of the transfer of care and follow up arrangements.

If a patient is lost to follow-up at a site every effort should be made to contact the patient's GP to obtain information on the patient's status.

16. STUDY CLOSURE

16.1. End of Study

For regulatory purposes the end of the study is expected to be when the last patient who is alive has undergone 5 years of follow up, at which point the 'declaration of end of study' form will be submitted to ethical committees, as required. If the study objectives can be addressed reliably before this time, end of study could be sooner.

Following this, UCL CTC will advise sites on the procedure for closing the study at the site.

16.2. Archiving of Study Documentation

At the end of the study, UCL CTC will archive securely all centrally held study related documentation for a minimum of 5 years. Arrangements for confidential destruction will then be made. It is the responsibility of PIs to ensure data and all essential documents relating to the study held at site are retained for a minimum of 5 years after the end of the study, in accordance with national legislation and for the maximum period of time permitted by the site.

Essential documents are those which enable both the conduct of the study and the quality of the data produced to be evaluated and show whether the site complied with the principles of GCP and all applicable regulatory requirements.

UCL CTC will notify sites when study documentation held at sites may be archived. All archived documents must continue to be available for inspection by appropriate authorities upon request.

16.3. Early Discontinuation of Study

The study may be stopped before completion on the recommendation of the IDMC (see section 14.3.2). Sites will be informed in writing by UCL CTC of reasons for early closure and the actions to be taken with regards the follow up of patients.

16.4. Withdrawal from Study Participation by a Site

Should a site choose to close to recruitment the PI must inform UCL CTC in writing. Follow up as per protocol must continue for all patients recruited into the study at that site and other responsibilities continue as per CTSA.

17. STATISTICS

17.1. Sample Size Calculation

17.1.1. Sample size for examining the relationship between intratumour heterogeneity and clinical outcome

The sample size is based on demonstrating a relationship between tumours with divergent intratumour heterogeneity index values and clinical outcome. Patients will be split evenly into those with a low and high intratumour heterogeneity index value (and other splits will be considered). Assuming a median Disease Free Survival (DFS) of 30 months and a hazard ratio (HR) of 0.77, with a 2-sided 5% significance level, 90% power, accrual period of 3 years and 5 years follow-up after the end of accrual, the sample size required is almost 400 per group (total of 800 patients). Assuming a 5% dropout rate, a total of 842 patients (421 per group) are required. This sample size is also large enough to detect a 10% improvement in a 5 year OS rate from 46% in the high Intratumour Heterogeneity Index (I_{TB}) to 56% in the low Intratumour Heterogeneity Index group (HR=0.75), with 80% power and a 2 sided type I error set at 5% (logrank test). A high/low I_{TB} value will be defined as values above/below the 50th percentile (median I_{TB}).

We have a target DFS effect of a 23% reduction in risk (hazard ratio 0.77), which means that our study is powered for an effect at least this large, including a 30% difference (which has been the target for progression-free survival in trials of advanced NSCLC, in relation to expected effects on OS).

17.1.2. Sample size to establish the impact of adjuvant platinum-containing regimens upon intratumour heterogeneity in relapsed disease

From a meta-analysis of 4 large randomised clinical trials of adjuvant chemotherapy following surgery [39], the 5 year disease-free survival (DFS) rate was 38% for those not receiving chemotherapy, and 42% for those who do. In TRACERx, where patients are followed up for 5 years, this is equivalent to about 500 events (recurrences or deaths) among 842 patients. Most of these events are expected to be a recurrence (as a first event), we assume that 60% accept to provide a second biopsy (and are able to undergo the biopsy), and 10% failure rate of tumour sampling at metastatic sites, leaving 270 patients with paired samples.

With a sample size of 270 patients, we will be able to detect a moderate standardised difference of ≥0.35 between the platinum therapy and ‘no therapy’ groups, using the change in intratumour heterogeneity ITB index, with 80% power and two-sided 5% test of statistical significance. At least 50% of patients are expected to have adjuvant chemotherapy after surgery, so of those patients who relapse, and in whom we expect to have a second biopsy sample, there could be about 130 who have had adjuvant platinum therapy and about 140 who have not (given the higher event rate in the no chemotherapy group). If 70% of patients have adjuvant chemotherapy, there could be about 185 who have had adjuvant platinum therapy and about 85 who have not, and this has 80% power to detect a standardised difference of ≥0.37.

17.1.3. IDMC review of sample size during the study

As with any research study, the estimates of sample size are dependent on the parameters used in the assumptions. We have used published evidence to justify the sample size, and have been conservative when considering the number of patients who have a recurrence and will agree to a second biopsy (required for one of the two primary objectives). We will therefore request the IDMC to review the sample size assumptions at their data reviews using observed data, and examine analyses of the biomarkers

and how well they predict DFS and OS. The actual results on the relationship between the biomarkers, DFS and OS will be kept confidential within the IDMC.

17.2. Statistical analyses

17.2.1. Assessment of intra-tumour heterogeneity through multi-region exome sequencing

We will employ an Intratumour Heterogeneity 'ratio index' I_{TB} to assess diversity with a single measure: for each tumour we will consider the proportion of ubiquitous mutations (those detected in all primary regions and lymphatic nodes) to the mean number of non-ubiquitious mutations (those detected in at least one but not all primary regions/lymphatic nodes sites) detected in each region/node i.e.

$$I_{TB}= \frac{\text{mean(\# of non−ubiquitious mutations per region or lymph node)}}{\text{number of ubiquitious mutations}}$$

For each region of each patient, the number of non-ubiquitous mutations present in that region will be determined. The mean number of non-ubiquitous mutations will be the sum across the regions divided by the number of regions. We note here that I_{TB} is then a specific measure of the more general concept of ITH.

For example, if a patient has 4 biopsies from a primary tumour and a lymph node site: regions 1, 2, 3 and 4 and 20 ubiquitous mutations were detected (i.e. were present in all 4 regions); and if the regions had 7, 10, 12 and 11 non ubiquitous respectively (i.e. a mean = (7+10+12+11)/4 =10), then the ITB for this patient would be 10 / 20 = 0.5.

17.2.2. Define the relationship between intratumour heterogeneity and clinical outcome

The primary outcome is disease-free survival (DFS; defined from the date of surgery to the date of recurrence/metastatic disease or death from any cause, whichever occurs first). The relationship between the intratumour heterogeneity ratio index I_{TB} and DFS will be assessed by performing multivariate Cox proportional hazards analyses including established covariates such as age, tumour stage, performance status, histology, sex, presence/absence microscopic residual tumours (R1), adjuvant therapy status (i.e. platinum therapy vs. no adjuvant therapy) and smoking status. The I_{TB} will be analysed as a dichotomised variable using various classifications in this analysis. The above analysis will be repeated for OS.

17.2.3. Validation of I_{TB} as a biomarker for DFS and OS

In order to determine the utility of I_{TB} as a potential prognostic or predictive biomarker of DFS or OS, analyses will be conducted to determine if certain thresholds of I_{TB} indices are effective at predicting DFS or OS.

The analyses will involve dividing the data into two groups: the training set and validation set, using published methods [40]. The training set will consist of the first two-thirds of DFS (or OS) events. The predictive model will be determined from the training set and will have DFS (or OS) as the response and the I_{TB} scores (as either continuous values or categorized into quantile groups – e.g. 25%, 50%, 75% ITB) as the independent variable, as well as the established covariates listed above. A Cox multivariate regression analysis using backward selection (and 10% statistical significance) will be applied to the training set, to produce the group of variables which together are the best at predicting DFS, and ITB index would be included among these if it is an independent factor. The regression

coefficients will then be used as scores to be applied to the validation dataset, consisting of the last third of DFS events, and used to predict 1-year DFS and OS rates. Sensitivity (or detection rate) would be defined as the proportion of patients who have an event at one year, who are classified (from the model) as 'positive' using the set of factors, while false-positive rate is the proportion who do not have an event at one year and are also classified as 'positive'. The precise definition of 'positive' will come from the modelling of the training dataset. If the sensitivities are at least 60%, then the standard errors would be ≤5%, yielding acceptably narrow 95% confidence intervals. We will also examine 2- and 3-year DFS and OS rates.

The observed survival times (distinguished from survival rates) from the validation set of patients will also then be compared to those predicted from the model. Criteria such as ROC analysis and simpler descriptive analysis, such as the predicted value being within +5%, +10% etc. of the observed will also be used. In all analyses, models with and without adjustment of prognostic factors will be used.

17.2.3.1. Subsidiary analyses

The above analyses will be performed with further measures of ITH to determine the relationship with DFS and OS. Inclusion of alternative indices as considered by Merlo et al [41] and the consortium [42], will inform the optimum heterogeneity index for clinical stratification. These will be:

Hill Family of Indices: Specifically, we will consider the family of diversity measures first introduced by Hill [43], which take into account sub-clonal structure within the tumour in addition to the mutational spectra considered by the ratio index. This family is defined in the Appendix 5. These indices are able to assess intra-region heterogeneity as well as inter-region heterogeneity and could therefore be used to examine pairwise differences between primary regions, lymph nodes and metastatic sites.

The Clone Index: The Hill index includes, as a special case, what we refer to as the Clone index, the total number of sub-clones detected in the tumour.

Chromosomal Instability Index: heterogeneity is not simply manifested at the somatic mutational level, but also at the chromosomal level, in the form of structural and numerical chromosomal instability. We shall consider an index used by ourselves to define the relationship between chromosomal numerical heterogeneity (defined by modal centromeric deviation; MCD) and outcome [42]. To derive an MCD score based on centromeric signals, the number of centromeres in 50 nuclei will be counted for chromosome 2 and 15 in each tumour region. The mean (chromosome 15 and 2) percentage deviating from the modal centromere number will be utilised to define the MCD. To avoid false classification of CIN due to sectioning artefacts and to control for bimodality in diploid tumours, all centromere counts equal to one will be removed for the derivation of the CIN score. The MCD score will be compared to a measure of clonal heterogeneity, the Shannon Diversity Index (a special case of the Hill family of indices) H, which will be estimated for chromosome 15 and 2 using the formula:

$$H = -\sum_i p_i \ln(p_i)$$

where p_i is the frequency of centromere signal, i.

17.2.3.2. Additional endpoints in relation to I_{TB}

A separate analysis where the DFS time will be modelled against the I_{TB} as a continuous measure (on a scale from 0 to ∞) will be carried out to confirm a general relationship between survival and I_{TB}. This analysis will be done using non-parametric methods (such as Quantile and other regression models which take into account censoring). These models will explore the relationship between the survival

times and the I_{TB}. Because the I_{TB} is likely to be a heavy tailed distribution (i.e. some very extreme values), the mean survival time is unlikely to provide accurate estimates for varying I_{TB} levels. This analysis will help to identify a suitable model for predicting survival times from I_{TB} at the patient level. It is expected that as I_{TB} increases, both the DFS rate and DFS time will decrease.

The relationship between the proportion of patients with DFS and I_{TB} will be explored using survival techniques. The dependent variable will be DFS and the independent variable will be I_{TB}. The median (and other quantiles) proportion of patients alive for given I_{TB} values will be determined and a Kaplan Meier plot will be generated. A further plot will be generated so that varying categories of survival time (e.g. 0-2 months, >2-4 months, >4 months which might correspond to poor, average and good survival) can be displayed. These graphs will help to identify the relationship between I_{TB}, OS and DFS such as: 'what is the median I_{TB} associated with patients who have <2 months DFS?'

We will explore additional endpoints in relation to I_{TB}:

I) The relationship between time from recurrence to death and I_{TB} will be explored to investigate the role of ITH in the rate of death, post progression (i.e. do patients with greater ITH die quicker, once they have progressed)

II) The relationship between I_{TB} and tumour response in terms of both the RECIST (v1.1) criteria and the absolute and percentage change in tumour lesion measurements. Graphical displays (e.g. Waterfall plots) will be generated to explore these relationships.

For all the above analysis, estimates of effects (e.g. Hazard ratios or median times) will be presented with appropriate confidence intervals and p-values, where appropriate.

The analysis will be conducted for all 842 patients. Additional exploratory analyses will be carried out for specific subgroups (such as histological subtypes, disease stages and smoking status). Analyses will be based on determining the relationship between intratumour heterogeneity at baseline and survival (among all patients recruited to the study), as well as the changes between the initial and relapse biopsies (for those who have paired samples, following a relapse).

17.2.4. Establish the impact of adjuvant platinum-containing regimens upon intratumour heterogeneity in relapsed disease

In the group of patients for which we have paired primary and metastatic biopsies, we will recalculate the I_{TB} including the metastatic biopsy and determine the change from the initial value (i.e. excluding metastasis) as determined for objective 1A.

For example suppose we have, as before, a primary tumour with four regions harbouring 7, 10, 12 and 11 non-ubiquitous mutations and 20 ubiquitous mutations (I_{TB}=0.5) and suppose further that this patient undergoes a subsequent metastasis biopsy which reveals 5 non-ubiquitous mutations and for which two of the mutations previously classed as ubiquitous are not detected. This means that we now have 18 ubiquitous mutations, and each of the four initial regions now contain an extra two non-ubiquitous mutations (since they are not present in the metastatic site), so we can calculate the revised index for this tumour as: mean non-ubiquitous mutations = ((7+2) + (10+2) + (12+2) + (11+2) + 5) / 5 = 10.6, I_{TB} = 10.6/18 = 0.59. Thus, consideration of the metastatic site increases the ITB by 0.09 for this hypothetical patient.

Since the Hill indices can also assess intra-regional heterogeneity, we will additionally calculate Hill indices for each region, lymphatic node and metastasis and their pairwise differences.

The within patient differences for each of the ITH indices will be assessed using generalized linear models (GLM) with the primary endpoint as in 1A; important covariates including tumour stage will be included in the model. This analysis will provide information on:

I) How intra patient changes in ITH indices relate to other prognostic factors assessed through GLM methods
II) How survival is related to within patient changes in ITH and other prognostic factors determined with Cox regression methods.

In addition, the predictive value of each of the indices in terms of patient survival and adjuvant treatment status (adjuvant chemotherapy vs. non-adjuvant) will be determined through Cox regression modelling, including terms for an interaction between ITH and treatment status. The interaction term will provide a preliminary assessment of whether survival is dependent on a combination of ITH and treatment (adjuvant/non adjuvant) status. Analyses similar to those in 15.2.3 could also be performed, with OS as the endpoint to be predicted according to adjuvant treatment status and ITH (and change in ITH).

17.3. Assessment of study feasibility and early stopping

An assessment will be carried out after the first 150 patients have been followed up for 12 months, to examine the feasibility and safety of repeat biopsies (including biopsies from metastatic sites and the acceptance rate of patients), then every 12 months thereafter (see section 14.3.2)

If the recruitment rate is considerably lower than 24 patients/month for the first 26 months after the initiation phase, the study is unlikely to recruit 842 patients in 3 years, and if permitted by the IDMC, the consortium will approach at least 2 other ECMCs to request participation. If this does occur, there will be another data review at 6 months, then 12, and if accrual is similarly low, the IDMC will recommend early closure.

Throughout the study, we will also closely monitor the repeat biopsy acceptance rate, as a proportion of those who have a confirmed recurrence and data reported back to local study investigators every 6 months (i.e. for all patients and per centre). If it is found that many patients are declining this biopsy, the study investigators will determine how this can be improved, with involvement of the patient representative and whether additional recruiting sites should be enrolled.

The disease-free survival rate at 1 year should be approximately 70%, so 45 out of 150 patients (30%) are expected to have suffered disease recurrence or have died by 26 months after recruitment starts. We need at least 60% of patients to agree to the second biopsy to achieve 270 paired biopsies. Therefore a success rate of 20% will be considered too low at this early point in the study. If there are <10 repeat biopsies among the 150 patients (bearing in mind the number of recurrences), the IDMC will re-assess this data 6 months later, and if the rate is still as low as this, make recommendations on whether the study should be modified (recruit more sites) and consider how the study should continue. It should be noted that the repeat biopsies are only required for one of the two primary objectives.

18. ETHICAL APPROVALS

In conducting the study, the Sponsor, UCL CTC and sites shall comply with all laws and statutes, as amended from time to time, applicable to the performance of clinical studies including, but not limited to:

- the principles of ICH Harmonised Tripartite Guideline for Good Clinical Practice
- Human Rights Act 1998
- Data Protection Act 1998
- Freedom of Information Act 2000
- Human Tissue Act 2004
- Mental Capacity Act 2005
- the Research Governance Framework for Health and Social Care, issued by the UK Department of Health (Second Edition 2005) or the Scottish Health Department Research Governance Framework for Health and Community Care (Second Edition 2006)

18.1. Ethical Approval

The study will be conducted in accordance with the World Medical Association Declaration of Helsinki entitled 'Ethical Principles for Medical Research Involving Human Subjects' (1996 version) and in accordance with the terms and conditions of the ethical approval given to the study.

The study has received a favourable opinion from the NRES Committee London – Camden & Islington Research Ethics Committee. UCL CTC will submit Annual Progress Reports to the REC, which will commence one year from the date of ethical approval for the study.

18.2. Site Approvals

Evidence of approval from the Trust R&D for a study site must be provided to UCL CTC. Sites will only be activated when all necessary local approvals for the study have been obtained.

18.3. Protocol Amendments

UCL CTC will be responsible for gaining ethical approval, for amendments made to the protocol and other study-related documents. Once approved, UCL CTC will ensure that all amended documents are distributed to sites and CLRNs as appropriate.

Site staff will be responsible for acknowledging receipt of documents and for implementing all amendments.

18.4. Patient Confidentiality & Data Protection

Patient identifiable data, including, date of birth and NHS number will be required for the registration process and will be provided to UCL CTC. UCL CTC will preserve patient confidentiality and will not disclose or reproduce any information by which patients could be identified. Data will be stored in a secure manner and UCL CTC studies are registered in accordance with the Data Protection Act 1998 with the Data Protection Officer at UCL.

19. SPONSORSHIP AND INDEMNITY

19.1. Sponsor Details

Sponsor Name:	University College London
Address:	Joint Research Office Gower Street London WC1E 6BT
Contact:	Director of Research Support
Tel:	020 3447 9995/2178 (unit admin)
Fax:	020 3447 9937

19.2. Indemnity

University College London holds insurance against claims from participants for injury caused by their participation in this study. Participants may be able to claim compensation if they can prove that UCL has been negligent. However, if this study is being carried out in a hospital, the hospital continues to have a duty of care to the participant of the study. University College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. This applies whether the hospital is an NHS Trust or otherwise.

Hospitals selected to participate in this clinical study shall provide clinical negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary shall be provided to University College London, upon request.

20. FUNDING

Cancer Research UK and Rosetrees Foundation are funding the study, with additional funds from the Academy for Medical Sciences and UCL Biomedical Research Centre. Sites will receive payments from the Sponsor towards costs incurred; payments will be detailed in the CTSA signed between the Sponsor and each participating NHS trust.

21. PUBLICATION POLICY

The TRACERx consortium will have oversight of publications that arise directly from TRACERx. All collaborators who have actively contributed to the study will be named authors on all main study papers: from sites (including the site Principal Investigator, surgeons, pathologists, research nurse), the laboratories (including the scientists), and anyone else who has had a significant input into the conduct, analysis and interpretation of the study. There would either be a list of authors, or if this is too long they would be collectively called the 'TRACERx Consortium', with all names listed at the end of the paper in alphabetical order (and indicating their role in the study). Specialist papers focussing on a particular aspect may not require all collaborators to be authors. Sites and laboratories may not publish trial results prior to first publication by the named authors and need written consent from the TRACERx consortium to do so. The Chief Investigator will make the final decision on authorship.

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APPENDIX 1: ABBREVIATIONS

AAH	Atypical Adenomatous Hyperplasia
AIS	Adenocarcinoma *In Situ*
AR	Adverse Reaction
cfDNA	Circulating free tumour DNA
CI	Chief Investigator
CIS	Squamous Cell Carcinoma In Situ
CLRN	Comprehensive Local Research Network
CLL	Chronic Lymphocytic Leukaemia
CT	Computerised Tomography
CTCs	Circulating Tumour Cells
CTCAE	Common Terminology Criteria for Adverse Events
CTSA	Clinical Study Site Agreement
DNA	Deoxyribose Nucleic Acid
DFS	Disease Free Survival
ECOG	Eastern Cooperative Oncology Group
FFPE	Formalin Fixed Paraffin Embedded
GCP	Good Clinical Practice
H&E	Haematoxylin and Eosin stain
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IDMC	Independent Data Monitoring Committee
ITH	Intratumour Heterogeneity
NCRI	National Cancer Research Institute
NICE	National Institute of Clinical Excellence
NRES	National Research Ethics Service
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PET/CT	Positron Emission Tomography / Computerised Tomography
PFS	Progression Free Survival
PI	Principal Investigator
REC	Research Ethics Committee
RECIST	Response Evaluation Criteria in Solid Tumours
SAR	Serious Adverse Reaction
SUSAR	Suspected Unexpected Serious Adverse Reaction
TSP	Trial Specific Procedure
UCL CTC	CR UK and UCL Cancer Trials Centre

APPENDIX 2: EXPECTED ADVERSE REACTIONS

ARs Expected for Study Procedures (biopsy and blood sampling for study purposes)

The following ARs are commonly associated with the study procedures and will be considered 'expected':

Blood sampling

- Minor bruising
- Minor temporary discomfort

Biopsy procedures

1. Liver biopsy
    - Bruising
    - Bleeding
    - Pain
    - Infection
    - Allergic reaction to anaesthetic
    - Hypotension
    - Puncture of the gallbladder or lung, colon and kidney

2. Lung biopsy
    - Bruising
    - Minor bleeding
    - Significant bleeding
    - Pain
    - Flu-like symptoms
    - Sore nose
    - Sore throat
    - Infection
    - Allergic reaction to anaesthetic
    - Pneumothorax – self-limiting
    - Pneumothorax – requiring drainage
    - Hypoxia

3. Lymph node biopsy
    - Bruising
    - Bleeding
    - Pain
    - Infection
    - Pneumothorax
    - Allergic reaction to anaesthetic

4. Skin biopsy
    - Bruising
    - Bleeding
    - Pain
    - Infection
    - Allergic reaction to anaesthetic

5. Bone marrow biopsy
    - Bruising
    - Bleeding
    - Pain
    - Infection
    - Allergic reaction to anaesthetic
    - Swelling

APPENDIX 3: SUMMARY OF ASSESSMENTS

		Baseline/ Pre-surgery	During surgery	Adjuvant chemo	Follow-up (no recurrence) 3-monthly (Yr1-2) 6 month (Yr 3-5)	First recurrence	Trt for first recurrence (at time of first CT scan)	End of treatment (EoT) and follow-up following recurrence (3 monthly until progression)	Progression	Trt for progression (at time of first CT scan)	End of treatment (EoT) and Follow-up following progression (3 monthly)	End of all Treatment
Medical history & clinical characteristics		X										
Patient questionnaire		X										
ECOG PS & smoking status		X		X	X	X	X	X	X	X	X	X
PET-CT/CT scan		X (CD)			X*	X* (CD) RECIST 1.1	X*	X* RECIST 1.1 - EoT only	X*(CD) RECIST 1.1	X*	X* RECIST 1.1 - EoT only	X*
			*as per standard practice (not mandated for TRACERx) but details required for CRF									
Surgical details			X									
CXR					X*	X*	X*	X*	X*	X*	X*	X*
			*as per standard practice (not mandated for TRACERx) but details required for CRF									
AR Check		X		X	X	X	X	X	X	X	X	X
Blood samples	Blood for germ line DNA (10mL)	X										
	Blood for cfDNA (20mL) - Plus an additional 20mL for high sensitivity assay for approx. 100 patients at baseline, 48 hours after surgery, and each time point until first recurrence or until 3 years of follow-up (whichever comes first) – recruitment to this cohort now complete. Follow up samples to continue for existing patients. * **Leicester patients only** – additional 1 x 10mL to be taken at all time points	X (x2)		X Sample to be collected pre-cycle 1 only	X	X (x2)	X		X (and up to 4 times at subsequent progression)			X (x2)
	Blood for CTC (1 x CellSave tube) ** **Manchester patients only** – additional 1 x CellSave tube to be taken at baseline	X (Manchester** only)				X (all sites)	X (all sites)		X (all sites)			X (all sites)
	Pulmonary CTC blood sample 2x CellSave tube **(additional consent form)**		X									
	Blood for immunology (10mL)	X				X						
	Blood for immunology (50mL)				X 1 visit only							
	Blood for neo-antigen assay (50ml or 140mL) – **selected patients only**			X 1 visit only pre-chemo if possible								
	Blood for immunology (40mL) - **patients in relevant clinical trials only (eg. DARWIN trials)**					X	X Post cycle 1, 2, 4 and 6 only		X			
Tissue	Multi-region snap-frozen tissue for DNA and RNA sequencing		X			X			X			
	FFPE block(s)		X									
	H&E slides (will be returned)		X									
	Fresh tissue in RPMI fresh medium		X			X			X			

APPENDIX 4: PROTOCOL VERSION HISTORY

Protocol:		**Amendments:**		
Version no.	**Date**	**Study Amendment no.**	**Protocol Section (no./title)**	**Summary of main changes from previous version.**
2.0	27/11/2013	N/A	N/A	First approved version for the study
3.0	03/07/2014	002	N/A	Update to TRACERx consortium list
			N/A	Amendment of the site Velindre to Cardiff & Vale
			3.1.2 Secondary objectives	Amendment of the secondary objective: Develop a repository of lung cancer cell lines, organoids and in vivo mouse models of patient-derived lung cancers that can be used as *in vitro* and *in vivo* models to study the aetiology of lung diseases, including lung cancer and predict response to therapeutics and resistance in lung cancer.
			3.1.2 Secondary objectives	Addition of the secondary objectives: -Develop analytical methods for determining morphological heterogeneity within separate tumour regions. -Tissue samples obtained from lung resections, both tumour and normal, will be analysed using high-power microscopy, such as electron microscopy, in order to obtain information regarding cellular structures.
			3.1.3 Additional research questions to be examined	Addition of the research question: Can CTCs be used to detect and track actionable mutations and therefore guide treatment stratification?
			6.3.2 Exclusion Criteria	Addition to exclusion criteria that patients who require neo-adjuvant chemotherapy will be ineligible
			6.3.2 Exclusion Criteria	Addition to exclusion criteria that if sufficient tissue is unlikely to be obtained for the study based on pre-operative imaging the patient will be ineligible
			6.3 Patient Eligibility 9.3 Tissue sampling during surgery	Eligibility criteria and wording in section 9.3 amended to clarify patients with in-situ lesions will not be followed-up for the study or required to provide study blood samples. The date the lesions become invasive will be captured on the CRF.

			6.3.3 Patient ineligibility following registration	Criteria for patients that may be withdrawn following registration updated to include any changes in cancer staging outside of IIIb or IV. Wording updated to clarify samples collected up until withdrawal will be biobanked.
			7.1 Registration	Addition that following registration, an email with the patient's study number should be sent to UCL CTC to confirm registration and the UCL CTC will then add the patient to the tracker.
			9 Assessments and collection of biological specimens	Additional details added for obtaining blood in ~100 patients initially for testing of cfDNA using a highly sensitive, multiplexed assay. Bloods will be obtained at the cfDNA sampling timepoints with an additional sample taken 48 hours post surgery. Samples will be taken until recurrence or until 3 years of follow-up (whichever comes first).
			9 Assessments and collection of biological specimens	Additional 10mL blood sample for cfDNA analyses to be taken at all cfDNA time points from Leicester patients only
			9 Assessments and collection of biological specimens	Additional 10 mL blood sample for circulating tumour cell analyses to be taken at baseline from 30 UCLH patients only, and then at first recurrence, at first CT on treatment for first recurrence, at progression and at completion of all treatment for those of the 30 patient cohort whose disease recurs.
			9 Assessments and collection of biological specimens	Additional 10 mL blood sample for circulating tumour cell analyses to be taken from 10 UCLH patients with EGFR, ALK, ROS, RET, KRAS or PD-L1 mutation, at first recurrence, at first CT on treatment for first recurrence, at progression and at completion of all treatment.
			9.1 Baseline assessments	Clarification that the original of the baseline questionnaire completed by the patient should be sent to the UCL CTC and a copy should be filed in the site file.
			9.2 Blood sampling before surgery	Addition that baseline blood sample collection should take place preferably within the 1 - 2 weeks prior to surgery if possible.
			9.2 Blood sampling before surgery	Additional 10 mL blood sample for circulating tumour cell analyses from Manchester patients at baseline only. This may be omitted in patients who provide the 40mL immunology sample.

			9.3 Tissue sampling during surgery	Addition that normal tissue will also be collected from the resected specimen as well as cancerous tissue
			9.3 Tissue sampling during surgery	Addition that histological slides may be made from tumour tissue, and where possible, fresh tissue from larger tumours will be used for cell line derivation and establishment of xenograft models.
			9.5 Assessments and blood sampling after surgery, during 5 years follow up	Timing of first cfDNA blood sample after surgery clarified for patients who receive adjuvant chemotherapy and those that do not.
			9.8 Blood sampling after end of treatment 10.1 Sending of samples	Additional 2 x 10ml blood for cfDNA analysis to be taken at the end of treatment to be sent to UCL Cancer Institute.
			12 Safety Reporting	Clarification that Adverse Reactions grades 3-5 related to adjuvant chemotherapy will be recorded as well as Adverse Reactions related to study procedures.
			14.3.1 TRACERx Consortium	Addition that review of substantial amendments may be undertaken by a smaller sub-set of the TRACERx consortium depending on the nature of the amendment
			15.1 Discontinuation from study follow-up	Clarification that samples collected until withdrawal will be biobanked unless the patient withdraws consent for the provision of these samples.
			Appendix 3 Summary of Assessments	Table updated to correct previous errors and to match proposed changes in the protocol.
4.0	22/12/2014	003	N/A	Update to TRACERx consortium list
			3.1.3 Additional research questions to be examined	Addition of the research question: Is there any evidence for the presence of tumour-related genetic abnormalities in histologically normal lung tissue that represents the 'normal' boundaries of a tumour?
			6.3 Patient Eligibility	Amendments so that patients who undergo post-operative radiotherapy are now eligible
			6.3.2 Exclusion Criteria	Addition of syphilis infection to the exclusion criteria

			6.3.3 Patient ineligibility following registration	Clarification that patients with microscopic residual tumours (R1) are eligible
			6.3.3 Patient ineligibility following registration	Clarification that patients with *in situ* lesions will be withdrawn, If these patients subsequently develop invasive cancer, the date of diagnosis and the tumour histology will be recorded on the EDCS
			6.3.3 Patient ineligibility following registration	Addition of details of which samples should be sent to the central laboratories if patients are withdrawn following surgery
5.0	28/07/2015	004	N/A	Addition that the days the TRACERx Study Coordinator can be contacted excludes Bank Holidays
			N/A	Update to TRACERx consortium list
			N/A	Update to 'Planned number of sites'
			4.1.1 Selection of Principal Investigator and other investigators at sites	Addition that Principal Investigators may be surgeons or pathologists as well as medical doctors
			6.3.2 Exclusion Criteria	Addition that stage 0 melanoma *in situ* is an exception to the exclusion criteria
			6.3.2 Exclusion Criteria	Clarification that treatment with neo-adjuvant therapy for current lung malignancy would exclude patients from the study
			6.3.2 Exclusion Criteria	Clarification that adjuvant radiotherapy does not exclude patients from the study
			6.3.2 Exclusion Criteria	Clarification that patients should be excluded if it is unlikely that a minimum of two tumour regions will be obtained for the study
			6.3.3 Patient ineligibility following registration	Clarification that adjuvant radiotherapy does not exclude patients from the study
			9.3 Tissue sampling during surgery	Clarification that patients with *in situ* lesions will be withdrawn

			9.10 Imaging scans	Clarification of the details of sending in imaging scans
			11.2 Missing data	Clarification on how to record on the electronic data capture system that data is unobtainable
			14.2 On-site monitoring	Addition of on-site monitoring section
			20 Funding	Addition that sites will receive payments from the sponsor
6.0			N/A	Update to TRACERx consortium list
			1.1 Summary of study design	Clarification that patients with pre-invasive lesions should be withdrawn
			1.1 Summary of study design	Addition of extra blood samples (50mL immunology, 50mL for a proof of concept neo-antigen assay, 140mL for neo-antigen assay development, and 40mL immunology for patients participating in relevant clinical trials). Update that the high sensitivity cfDNA cohort has closed to recruitment, but collection of samples at the subsequent time points will continue for existing patients.
			1.1 Summary of study design	Removal of phosphopeptidome blood sample for tumours >7cm Removal of additional 20ml cfDNA samples for the EGFR/BRAF cohort Removal of additional 10ml CTC samples for two specified cohorts of ULCH patients
			1.1 Summary of study design	Addition of inclusion criteria of minimum tumour size (15mm) on pre-operative imaging
			1.1 Summary of study design	Addition of new lab: Syncona
			3.1.2 Secondary objectives	Addition of extra secondary objective (isolation of cells for *in vitro* generation of neo-antigen reactive T cells)
			3.1.3 Additional research questions to be examined	Addition of three research questions
			6.3.1 Inclusion criteria	Addition of inclusion criteria of minimum tumour size (15mm) on pre-operative imaging

			6.3.3 Patient ineligibility following registration	Clarification that all pre-invasive lesions make a patient ineligible
			9. Assessments and collection of biological specimens	Overview updated to remove phosphopeptidome blood samples, additional 20ml EGFR/BRAF cohort cfDNA samples and additional 10ml CTC samples for two specified cohorts of ULCH patients. Update to include extra blood samples (50mL immunology, 50mL for a proof of concept neo-antigen assay, 140mL for neo-antigen assay development, and 40mL immunology for patients participating in relevant clinical trials).
			9.3 Tissue sampling during surgery	Clarification that all patients pre-invasive lesions will be withdrawn
			9.3 Tissue sampling during surgery	Addition of request of site H&E slides for central pathology review
			9.5 Assessments and blood sampling after surgery, during 5 years follow up	Addition of extra blood samples (50mL immunology, 50mL for a proof of concept neo-antigen assay, 140mL for neo-antigen assay development, and 40mL immunology for patients participating in relevant clinical trials). Removal of 20ml cfDNA blood samples for patients with EGFR/BRAF mutations. Update that the high sensitivity cfDNA cohort has closed to recruitment, but collection of samples at the subsequent time points will continue for existing patients.
			9.6 First recurrence	Removal of phosphopeptidome blood sample for tumours >7cm Removal of additional 20ml cfDNA samples for the EGFR/BRAF cohort Removal of additional 10ml CTC samples for two specified cohorts of ULCH patients Removal of 20ml cfDNA blood samples for patients with EGFR/BRAF mutations. Update that the high sensitivity cfDNA cohort has closed to recruitment, but collection of samples at the subsequent time points will continue for existing patients.

			9.6 First recurrence	Addition of 40mL immunology blood sample for patients participating in relevant clinical trials
			9.7 Progression, following recurrence	Removal of additional 20ml cfDNA samples for the EGFR/BRAF cohort Removal of additional 10ml CTC samples for two specified cohorts of ULCH patients
			9.7 Progression, following recurrence	Addition of 40mL immunology blood sample for patients participating in relevant clinical trials
			9.8 Blood sampling after end of treatment	Removal of additional 20ml cfDNA samples for the EGFR/BRAF cohort Removal of additional 10ml CTC samples for two specified cohorts of ULCH patients
			9.10 Imaging scans	Update to clarify that CT/PET-scans should be sent to UCLH via PACS rather than on CD, wherever possible.
			Appendix 1: Abbreviations	Addition of acronyms
			Appendix 3: Summary of assessments	Assessments table updated to **remove** phosphopeptidome blood samples, the extra 20ml cfDNA samples for patients with EGFR/BRAF mutation, and the additional 10ml CTC samples for two specified cohorts of ULCH patients. Assessments table updated to **add** extra blood samples (50mL immunology, 50mL for a proof of concept neo-antigen assay, 140mL for neo-antigen assay development and 40mL for patients participating in relevant clinical trials). Update that the high sensitivity cfDNA cohort has closed to recruitment, but collection of samples at the subsequent time points will continue for existing patients.