

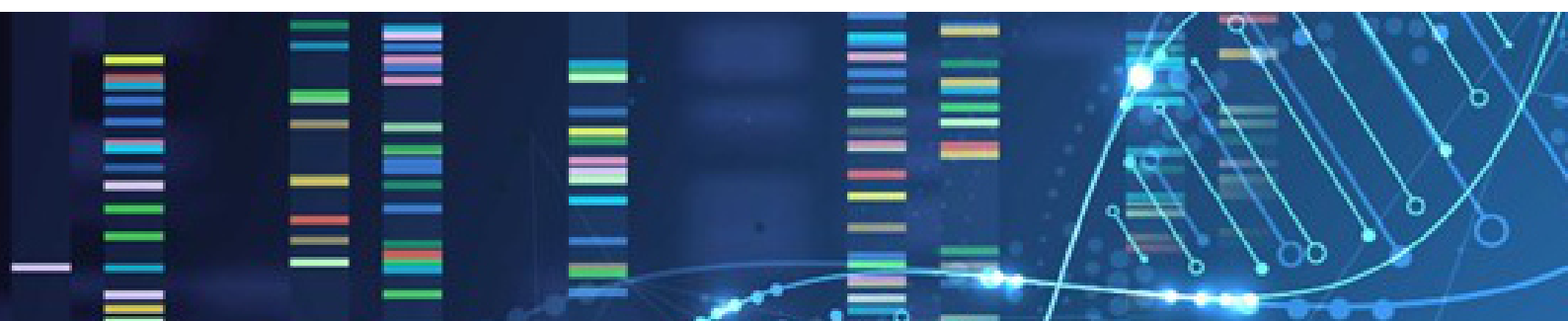
DOUTORAMENTO
MEDICINA

Integrating liquid biopsies into postoperative early-stage lung cancer

Joana Sena Freitas Espiga de Macedo Carona

D

2023



**Integrating liquid biopsies into
postoperative early-stage lung cancer**

Joana Sena Freitas Espiga de Macedo Carona

Orientação do Professor Doutor Venceslau Hespanhol
Coorientação do Professor Doutor José Carlos Machado

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Aos meus filhos Francisca e Alexandre.

Ao Alexandre.

Aos meus Pais.

Em memória dos meus Avós, Maria Cândida,
Maria Estrela e José Alberto.

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PREFÁCIO

Dada a minha formação profissional e académica no Hospital de São João e no Instituto Português de Oncologia do Porto, bem como a aprendizagem proporcionada pelos meus professores ao longo das diferentes fases da minha vida, acredito que passados 24 anos como médica, 16 anos como especialista em oncologia médica e 7 anos como consultora de oncologia médica, é minha obrigação e responsabilidade transmitir a minha experiência a toda a comunidade científica ao meu alcance.

A minha principal motivação sempre foi ajudar a tornar o cancro uma doença menos devastadora, ciente de que é a segunda maior causa de morte no mundo. Fruto da minha formação profissional e académica e do meu espírito cívico, acredito que o meu conhecimento, em Oncologia Médica, se não fosse difundido na comunidade científica seria um ato egoísta, não honrando o juramento de Hipócrates. Como médico é nossa obrigação integrar todos os recursos humanos e técnicos disponíveis, a atividade clínica multidisciplinar, a investigação clínica e básica e o ensino graduado e pós-graduado.

Por isso, primeiro como médica clínica e depois como oncologista clínica a minha preocupação foi dedicar o meu tempo, motivada pela minha prática clínica a investir nos meus conhecimentos na área das ciências básicas através de uma formação académica. Através do investimento nas duas principais autoestradas, prática clínica e ciências básicas, a minha ambição foi sempre servir da melhor forma os nossos doentes não só com conhecimento da prática clínica, mas sobretudo como é que o conhecimento das ciências básicas pode influenciar e mudar a nossa visão para realizarmos os nossos atos clínicos com a missão de melhor tratar e cuidar de pacientes oncológicos no dia a dia. Ao longo da minha experiência, simples curiosidades clínicas de “como?” e “porquê?” só podem ser respondidas na maioria das vezes por recurso às ciências básicas.

Durante os meus anos académicos, muitos artigos foram elaborados abrangendo um amplo espectro em diversas áreas médicas e oncológicas, com alguns artigos científicos publicados em revistas indexadas, mas com baixo fator de impacto, à data fruto da minha inexperiência no meio. A minha preocupação sempre foi divulgar o meu conhecimento científico junto da comunidade científica, através de comunicações orais, apresentações de pósteres em reuniões nacionais e internacionais e publicação de artigos científicos em revistas indexadas. É obrigação de todos os médicos difundir na comunidade científica, a sua experiência médica adquirida e superar todos os obstáculos com resiliência.

Como oncologista médica, tenho o privilégio de apoiar os pacientes tanto clinicamente como no desenvolvimento de novas abordagens de diagnóstico e tratamento. A minha grande motivação é promover e vislumbrar a atualização contínua de todos os parceiros científicos sob a orientação de três grandes pilares nos quais acredito: Visão, Missão e Ambição.

A Visão será sempre ir além do que se espera e com a Missão permanente de nos desenvolvermos, fazermos melhor e nos atualizarmos cientificamente; nunca esquecendo que esta Missão é cumprida por todos nós, que cuidamos diariamente dos nossos doentes. A nossa Ambição como médicos é preservar a qualidade científica, protegendo seus recursos humanos e realizando o Ato Médico Fundamental: tratar e cuidar de vidas humanas.

RESUMO

O cancro do pulmão é um problema de saúde pública mundial, com alta taxa de incidência e mortalidade. Além das políticas de combate ao tabagismo e das diretrizes de rastreamento, o cancro do pulmão é uma prioridade de saúde pública de nossa população.

O advento da genética molecular dos tumores trouxe consigo a possibilidade de modificar esta situação, quer através do refinamento do diagnóstico, quer da identificação de alvos terapêuticos específicos, quer sobretudo por eventualmente permitir o diagnóstico precoce da doença. Apesar de numerosos trabalhos terem já demonstrado a utilidade das técnicas da genética molecular ao longo do tempo e com o surgimento de técnicas inovadoras no cancro do pulmão, a sua implementação na rotina clínica em Portugal tem sido um desafio ao longo da última década.

Quando iniciei o meu primeiro trabalho em cancro do pulmão, no contexto do Mestrado em Medicina e Oncologia Molecular, as mutações do anti-oncogene p53 em amostras clínicas de pacientes foram analisadas por sequenciação. Na sequência desse trabalho, constatei com as limitações da integração do diagnóstico molecular na prática clínica por rotina.

Mais tarde, no âmbito dos trabalhos conducentes ao grau de Doutoramento, a investigação teve como base o uso da biópsia líquida baseada no ADN tumoral circulante (ADNtc) de células neoplásicas. Esta metodologia, mudou o paradigma da monitorização dos doentes oncológicos, minimizando a exposição a procedimentos invasivos, proporcionando um maior conhecimento sobre o perfil mutacional do tumor, e avaliando a resposta à cirurgia pulmonar. Monitorizar a sua expressão ao longo do tempo, avaliar a sua relação com os parâmetros clínico-patológicos e a sua associação com a progressão clínica e imagiológica, tornou-se fundamental na abordagem do doente oncológico. Recorrendo à técnica de sequenciação de nova geração, foi realizada a correspondência do perfil mutacional entre o tumor e o ADNtc aquando da alta cirúrgica e durante a última avaliação.

Com este estudo observacional prospetivo, realizado numa única instituição, foi incluída uma coorte de 34 pacientes com cancro de pulmão em estadios precoces. Foram usados testes de classificação sinalizada de pares pareados de Wilcoxon e modelos de regressão linear.

Verificou-se que, a maior concentração ADN tumoral circulante é libertada na veia pulmonar quando comparado com as amostras de sangue periférico colhidas antes da cirurgia ($p = 0,002$). O maior aumento foi observado na alta cirúrgica quando comparado com as duas primeiras colheitas ($p = <0,001$, $p = 0,001$ respetivamente). Na alta cirúrgica e na última observação, foi identificado significado estatístico quando comparado ao pré-operatório, veia pulmonar e à primeira observação após a alta ($p < 0,0001$, $p < 0,0001$, $p < 0,0001$ respetivamente). Não houve significado estatística ao comparar as biópsias líquidas obtidas no último seguimento com a expressão do ADNtc na alta cirúrgica ($p=0,851$).

A correlação entre a concentração de ADNtc nos cinco momentos de colheita e as quatro características clínico-patológicas mostrou que pacientes com menos de 70 anos tiveram redução significativa da concentração de ADNtc no momento pré-operatório e na alta cirúrgica ($\beta = -16.734$ [-27,707; -5,760]; $p=0.003$; $\beta = -21.785$ [-38,447; -5,123]; $p=0.010$) em oposição a um aumento da concentração de ADNtc na

veia pulmonar e no último seguimento ($\beta = 8,369$ [0,359; 16,378]; $p=0,041$; $\beta = 34,402$ [12,549; 56,254]; $p=0,002$) todos com nível de confiança de 95%.

O perfil mutacional do tumor foi avaliado, assim como a frequência alélica de 26 dos 34 pacientes. Cinco amostras foram excluídas devido a problemas técnicos e em 3 amostras foram identificadas translocações. Nos casos em que mutações acionáveis foram identificadas em biópsias de tecido, a sua expressão foi avaliada por NGS na alta cirúrgica, onde a expressão quantitativa do ADNtc foi maior, para o perfil mutacional. Nenhuma mutação somática foi identificada na alta cirúrgica dos 26 pacientes, a mutação esperada foi encontrada em 5 de 6 amostras de plasma de pacientes no momento pré-operatório e em 2 de 6 amostras de plasma de pacientes no momento da veia pulmonar. Dois dos seis pacientes com mutações acionáveis apresentaram progressão da doença.

De um total de 34 pacientes, sete pacientes apresentaram progressão imagiológica. Esses sete pacientes também apresentaram valores medianos superiores de ADNtc na última observação quando comparados aos valores de ADNtc na alta cirúrgica (recidiva molecular). Os valores absolutos de quantificação de ADNtc mostram que o tempo médio para recidiva molecular foi de 343,86 dias [94; 504] e o tempo médio de progressão imagiológica foi de 647 dias [150; 959]. Nestes sete pacientes, a recidiva do ADNtc é quantificável e a doença oncológica mensurável é posteriormente confirmada por tomografia computadorizada (TC).

A quantificação do ADN tumoral livre circulante e o perfil mutacional por NGS são ferramentas promissoras, permitindo através da genotipagem, a detecção de doença molecular residual pós-cirúrgica e o diagnóstico da recorrência, antecipando e complementando a recidiva clínica e radiológica. Destacar a tentativa de integrar a genética molecular na prática clínica e identificar as principais barreiras à sua implementação em pacientes com cancro do pulmão tem sido uma missão ambiciosa ainda a cumprir plenamente na rotina da prática clínica diária.

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PREFACE

Given my professional and academic training at the Hospital de São João and the Portuguese Institute of Oncology in Oporto, as well as the learning path provided by my teachers throughout the different stages of my life, I believe that after twenty-four years as a physician, sixteen years as a Medical Oncology Specialist and seven years as an Assistant Medical Oncology Consultant, it is my obligation and responsibility to share my humble medical experience to the whole scientific community.

My primary motivation has always been to help make cancer a less devastating disease, aware that it is the second leading cause of death, globally. As a result of my professional and academic education, training, and civic duty, I believe that my knowledge, in Medical Oncology, if not diffused in the scientific community would be a selfish act of not honouring my Hippocrates oath. As a physician, one must integrate the human and technical resources available, the multidisciplinary clinical activity, and clinical and basic research gained throughout my undergraduate and postgraduate education.

Therefore, first as a clinical physician and then as a clinical oncologist, my concern was to dedicate my time, motivated by my clinical practice, to investing in my knowledge in basic sciences through academic training. Through investment in the two main highways, clinical practice and basic sciences, my Ambition has always been to best serve our patients not only with knowledge of clinical practice but above all, how knowledge of basic sciences can influence and change our Vision to carry out our clinical acts with the Mission of better treating and caring for cancer patients daily. Throughout my experience, simple clinical considerations of how and why can only be answered most of the time, by resorting to basic sciences.

During my academic years, many written papers, ranged from a broad spectrum within diverse medical and oncological areas. Owing to my partial inexperience, many scientific papers were published in indexed journals, with a low impact factor. My concern was always to share my scientific “know-how” within the scientific community, through oral communications and poster presentations at national and international meetings, a most important means through scientific papers in indexed journals. Acquired medical experience is an obligation of all physicians to spread knowledge and overcome all obstacles with resilience.

As a Medical Oncologist, I have the privilege of supporting patients both clinically as part of my role and working to develop new diagnostic and treatment approaches. My great motivation is to promote and envision the continuous updating of all scientific partners under the guidance of three main pillars in which I believe in Vision, Mission and Ambition.

The Vision will always be to reach beyond the expected and with the ongoing Mission of developing, always doing better and scientifically updating ourselves; never neglecting that this Mission should be performed by those who care for the sick daily. Our Ambition should be to preserve scientific quality, protect all human resources and execute the Fundamental Medical Act: to treat and care for human lives.

SUMMARY

Lung cancer is a worldwide public health problem with an overwhelmingly high incidence and mortality rate. In addition to tobacco-control policies and screening guidelines, lung cancer is a priority in caring for our population healthcare.

The development of tumour molecular genetics carries the promise of altering this state of affairs, as it should lead to a more precise classification of tumours, identify specific molecular targets for therapy and, above all, allow the development of new methods for early diagnosis and identification of high-risk population for lung cancer. Regardless of the numerous studies demonstrating the usefulness of molecular genetic techniques throughout time with emerging innovative techniques in lung cancer, its routine clinical implementation in Portugal has been a challenge over the last decade.

When I started my first work in the context of the master's in molecular medicine and oncology in lung cancer, I analysed the anti-oncogene p53 mutations in clinical samples of patients diagnosed with lung cancer through sequentiation of PCR products. An attempt to identify the main practical limitations for integrating molecular genetics in routine clinical practice was also carried out.

Later, in the context of doctoral studies, the investigation was based on liquid biopsies of circulating tumour DNA (ctDNA) from plasma, which changed the paradigm in monitoring cancer patients, to minimize the exposure to invasive procedures, providing greater insight into the mutational profile of the tumour, and evaluating the response to lung cancer surgery. The aim was to monitor its expression across time, its relationship with clinicopathological parameters and its association with clinical and imageology progression. Next-generation sequencing technique, allowed the analysis of mutational profile correspondence between tumour and plasma ctDNA at surgical discharge and last follow-up.

This prospective observational study, conducted at a single institution, included a cohort of 34 patients with early-stage lung cancer. Wilcoxon-matched pairs signed-rank tests and linear regression models were used.

More ctDNA was shed directly from the tumour bed into the vein that drains blood directly from the tumour when compared to peripheral blood samples obtained before surgery (PV > PO; $p = 0.002$) and a greater increase was observed at surgical discharge when compared to the first two withdrawals (SD > PV and SD > PO; $p = <0.001$). At surgical discharge (SD), ctDNA concentration when compared with preoperative (PO), pulmonary vein (PV) and first follow-up (F1) and the differences were statistically significant ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$ respectively). No statistically significant differences were observed when comparing the last follow-up to surgical discharge ctDNA expression ($p = 0.851$).

The correlation between the ctDNA concentration at the five collection times and the four clinicopathological characteristics showed that patients younger than 70 years had a significant reduction in the ctDNA concentration in the preoperative period and at surgical discharge ($\beta = -16,734$ [- 27.707; -5.760]; $p=0.003$; $\beta = -21.785$ [-38.447; -5.123]; $p=0.010$) as opposed to an increase in the concentration of ctDNA in the pulmonary vein and in the last follow-up ($\beta = 8.369$ [0.359; 16.378]; $p=0.041$; $\beta = 34.402$ [12.549; 56.254]; $p=0.002$) all with a confidence level of 95%.

Of the 34 patients, only 26 patients had appropriate tumour samples for mutational profiling through the NGS technique. Five samples were not processed for technical issues and three tumour samples showed translocations.

In the cases where actionable mutations were identified in tissue biopsies, their expression was evaluated by NGS at surgical discharge, where quantitative ctDNA expression was greatest, for mutational profiling. No somatic mutations were found at the surgical discharge of the 26 patients. We found the expected mutation in five out of six patients at preoperative time point and in two out of six patients at pulmonary vein time point. During surveillance, two of the six patients with actionable mutations showed imaging progression.

Of the 34 patients, seven patients had imaging progression. These seven patients also had higher median ctDNA values at the last observation when compared to ctDNA values at surgical discharge (molecular recurrence). The absolute values of ctDNA quantification show that the mean time to molecular relapse was 343.86 days [94; 504] and the mean time to imaging progression was 647 days [150; 959]. In these seven patients, ctDNA recurrence is quantifiable and measurable oncologic disease later confirmed by computed tomography (CT).

The quantification of circulating tumour DNA and the mutational profile through NGS are promising tools, allowing genotyping, the detection of post-surgical residual molecular disease and the diagnosis of recurrence, anticipating and complementing clinical and radiological recurrence. Highlighting the attempt to integrate molecular genetics in routine clinical practice and identify the main practical barriers to its implementation in lung cancer patients has been an ambitious mission yet to be fully accomplish in clinical practice.

ABBREVIATIONS

ALK (anaplastic lymphoma kinase)
BRAF (b-raf proto-oncogene)
CT: (computed tomography)
cfDNA (cell-free circulating DNA)
ctDNA (circulating tumour DNA or cell-free circulating tumour DNA)
CTC (circulating tumour cells)
CTLA-4 (cytotoxic T lymphocyte protein 4)
DNA (deoxyribonucleic acid)
EGFR (epidermal growth factor receptor)
ERBB2 (erb-b2 receptor tyrosine kinase2)
EMA (european medicine agency)
FDA (food and drug administration)
IO (immunotherapy or immuno-oncology agents)
KRAS (KRAS proto-oncogene GTPase)
NGS (next-generation sequencing)
NTRK1/2/3 (neurotrophic tyrosine receptor kinase)
MAF (mutant allele frequency)
MET (mesenchymal-epithelial transition proto-oncogene)
MP (mutational profile)
MRD (minimal residual disease)
NRAS (NRAS proto-oncogene GTPase)
OS (overall survival)
PD-L1 (programmed death ligand 1)
PD1 (programmed cell death protein 1)
PCR (polymerase chain reaction)
PFS (progression free survival)
RET (rearranged during transfection)
RNA (ribonucleic acid)
ROS1 (ROS proto-oncogene 1)
TP53 (tumour protein p53)

PUBLICATIONS

This thesis includes results published in the three following articles:

Chapter 1

Genetic alterations in Lung Cancer: Assessing limitations in routine clinical use

Joana Espiga de Macedo, Ângela M. S. Costa, Inês A.M. Barbosa, Sandra Rebelo, Conceição Souto de Moura, Luís Teixeira da Costa, Venceslau Hespanhol

Rev Port Pneumol, Jan 2007, vol.13, no.1, p.9-34

[https://doi.org/10.1016/S2173-5115\(07\)70320-2](https://doi.org/10.1016/S2173-5115(07)70320-2)

Chapter 2

Implementation of a Pilot Study to Analyze Circulating Tumour DNA in Early-Stage Lung Cancer

Joana Espiga de Macedo, Tiago Taveira-Gomes, José Carlos Machado, Venceslau Hespanhol

Acta Médica Portuguesa: aceite para publicação a 4 de maio de 2023

Chapter 3

Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Radiological Diagnosis

Joana Espiga de Macedo

Br J cancer Res 2020; 3(1): 357 - 361.

[BJCR-143.pdf \(britishjournalofcancerresearch.com\)](#)

The following scientific information published as an opinion paper and three posters presented at a Lung Cancer Meeting, were also used in the elaboration of the discussion of the thesis although they are not fully included in its core.

ctDNA-What is it's Advantage in Clinical Practice in Lung Cancer at the Present Time

Joana Espiga de Macedo, Manuela Machado, Marta Diogo Pereira, Joana Reis, Francisco Viana Machado, Matilde Amaral, Conceição Souto Moura, Fátima Carneiro, João Maciel, Pedro Bastos, Venceslau Hespanhol

J Can Sci Res 2017, 3:S1

[ct DNA - What is it's Advantage in Clinical Practice in Lung Cancer at the Present Time \(longdom.org\)](#)

cfDNA Analysis Implementation in Early-Stage Lung Cancer

Joana Espiga de Macedo, J.C.M. Machado, V.M. Hespanhol

J Thorac Oncol; Volume 17, Issue 9, Supplement, September 2022, Page S576

<https://doi.org/10.1016/j.jtho.2022.07.1043>

Liquid biopsies in the setting of Early-Stage Lung Cancer for the Detection of Actionable Mutations

Joana Espiga de Macedo, J.C.M. Machado, V.M. Hespanhol

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<https://doi.org/10.1016/j.jtho.2022.07.1042>

Incorporating cfDNA Detection to CT Scan Assessment in Post-Surgical Lung Cancer Patients

Joana Espiga de Macedo, J.C.M. Machado, V.M. Hespanhol

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INTRODUCTION

History of cancer

The human body is a complex ecosystem that faces diverse selective pressures and changes and reacts to its environment. Cancer appears as a clone of cells within our tissues, with an extreme capacity for growth, capable of invading adjacent tissues and unrespecting frontiers. Cancer is a multicellular live organism. Acquired genetic instability throughout time induces the appearance of many “offspring-cells” from the primary clone with its genetic information (genotype) and different cells arise with their own “outlook” (phenotype). This leads not only to the diversity between cancer types but also within an individual tumour. These emerging new cells may or may not have a survival advantage. If they do not, they are destroyed by other cells. If they do have an advantage, they survive and proliferate and substitute other cells. The dwelling between survival and proliferation or cell death (apoptosis) is the dichotomy that leads to cancer cell growth, invasion and seeding of tumour colonies to different sites in the body (metastatic).

Cancer is a genetic disease. More than 95% is caused by environmental causes leading to somatic mutations as stated by Peter Nowell in 1976 ¹. A minor percentage of cancer is due to hereditary factors, namely germinative mutations. Cancer is a process of Darwinian selection, where the host and the whole ecosystem performs an extremely important and crucial part ¹.

Cancer occurs due to a stepwise accumulation of somatic mutations as a baseline. However, we all know that cell proliferation *per si* does not induce cancer. Its' progression is due to the interaction of host cell factors, such as inflammatory environment, growth factors, stromal activation and DNA-damaging promoter agents (oncogenes), with cancer cells ². Cancer cells need to overcome normal body cells through proliferation, evade the host immune system and eventually co-operate with local cells to obtain oxygen to survive through angiogenesis and finally, migrate and colonize other parts of the body. Altogether the effect and risk of carcinogenesis are empowered, leading to cancer as the peak of the iceberg.

The concept of immune surveillance must not be neglected. When a tumour cell is presented to the immune system, one of two pathways can be chosen. First, the immune system recognizes tumour neo-antigens as “foreign” and leads these tumour cells to elimination. This is the innate immune response. On the other hand, tumour cells may gain immune resistance and induce immune “Tolerance” and the immune system favours or become pro-tumourigenic. In other words: the tumour survives, and immune evasion occurs ². This process has been defined by many authors as immunoediting and travels through three main phases: elimination, equilibrium and escape ³.

The most important factors for cancer evaluation and treatment are Genomics, Morphology (the creation of shape), Histopathology (disease development; the effects that the tumour had on the patient) and Hypoxia/ Metabolism. No less important are also Immunological and Psychological factors in which epigenetics play a major role. Morphology has been the “gold standard” for histological diagnosis of cancer since Rudolph Virchow's statement in 1855, “*Omnis cellula e cellula*”, every cell stems from another cell. He defined that a Disease is not only characterized by clinical symptoms but also by typical anatomic changes, or pathological anatomy ⁴. It was only towards the end of the 20th century that Virchow's theory was taken seriously, stating that specific

cancers such as lung cancer are indeed strongly associated with long-term inflammation⁵.

As stated by Peter Nowell the time for a normal cell to change to a new type of tissue, “neoplastic state”, with a selective growth advantage resulting from genetic instability. Nevertheless, these “neoplastic cells” may be eliminated by metabolic disadvantages, immunological control or by the “guardian of the genome”, the tumour suppressor gene p53, which plays an important role in cell cycle regulation, stress response, DNA repair and promotion of programmed cell death (apoptosis). The presence of p53 gene mutations is also considered a poor prognostic factor^{6,7}. These neoplastic cells may stay in a dormant state for a long, causing no harm to their host, or if the ecosystem is favourable may jump into a proliferating state, escaping all controls.

Epidemiology

Worldwide, according to Globocan 2020 estimates that there were 19.3 million new cancer cases and almost 10.0 million deaths from cancer in 2020. The global cancer burden is expected to be 28.4 million cases in 2040, a 47% rise from 2020^{8,9}. It has been estimated that in 2020, there will be 247,270 new cases of lung cancer (130,340 male cases and 116,930 female cases)¹⁰. The leading cause of death from cancer disease worldwide is lung cancer with a survival rate of 19% at 5 years or more after diagnosis^{11,12}. With an estimated 2.2 million new cancer cases and 1.8 million deaths, lung cancer is the second most diagnosed cancer and the leading cause of cancer death in 2020, representing approximately one in 10 (11.4%) cancers diagnosed and one in 5 (18.0%) deaths. In men, lung cancer is the leading cause of cancer morbidity and mortality, whereas, in women, it is third in incidence, following breast and colorectal cancer, and second in mortality, after breast cancer^{8,9}.

Cancer’s rising prominence as a leading cause of death reflects the increase of ageing and population growth, marked declines in mortality rates of stroke and coronary heart disease¹³, as well as the socio-economic development of each country¹⁴.

Etiopathogenesis

Seeing that the overwhelming majority (85-90%) of these cases are associated with smoking, a sweeping change in smoking habits would, in theory be the measure to have the highest impact on these numbers¹⁵. Second-hand or passive smokers have also shown an increase of 26% in having lung cancer¹⁶. With the introduction of effective tobacco-control policies, namely the MPOWER package by the WHO¹⁷, and regulations and screening guidelines, the mortality from lung cancer is expected to decrease by 48% in males and 23% in females. Nevertheless, 140,730 deaths in 2020 are estimated to be due to lung cancer¹⁰. Having said this, given the difficulty in changing human conduct and given the fact that lung cancer is a disease which develops over a long period, it does not seem likely that a drastic reduction in incidence and mortality rate of lung cancer shall be achieved, through preventative measures only over the coming decades.

Tobacco is the main etiopathogenic factor for lung cancer development. Environmental exposures to toxic substances such as asbestos, heavy metals, radon gas, polycyclic aromatic hydrocarbons and family risk factors are also known⁶. Certain types of bronchopulmonary diseases are predisposing to lung cancer, in the sense of favouring and triggering neoplastic lesions. Such as, tuberculosis, chronic bronchitis, lung fibrosis and conditions that lead to cicatricial processes. The patients with chronic

obstructive pulmonary disease have twice the risk of developing lung cancer, like asbestos workers who developed interstitial fibrosis (asbestosis) ¹⁸⁻²⁰. Concerning nutritional factors that interfere in lung cancer genesis, some deserve particular attention as the deficit of antioxidant vitamins, namely the vitamins A and E ⁶. Previous history of epithelial neoplasia, particularly of the respiratory and upper digestive tract (head and neck, oesophagus and lungs), presents a higher risk of developing simultaneous second primary neoplasms or subsequent. Recent studies have shown that mice exposed to electronic cigarettes increase the susceptibility to growing lung cancer compared to those not exposed ²¹.

When we talk about lung cancer, we are referring only to carcinomas of the lung originating from the respiratory epithelium (bronchi, bronchioles and alveoli) ⁶. The histogenesis of lung cancer is not entirely understood. Metaplastic changes of the bronchial mucosa result from a response to chronic and repeated exposure to inhaled compounds, developing a reactive inflammatory response to cell injury. Basal mucosal cells initiate a proliferative response producing mucus-producing goblet cells. Metaplasia consists of the replacement of ciliated and goblet epithelium by stratified epidermoid. Due to cellular atypia and high-activity mitosis, dysplasia appears at the level of the mucosa. When the entire mucosa is involved, we have a carcinoma *in situ*. Basement membrane invasion and infiltration of malignant cells into the underlying stroma are the first signs of invasion by cancer. The evolution time of this process is speculative, but it may take to develop about 10 to 20 years ⁶.

Four main histological types of lung cancer considered are squamous cell carcinoma, adenocarcinoma, large cell lung carcinoma and small cell lung carcinoma, constituting 90-95% of all primitive lung tumours. The combined forms, the carcinomas adenosquamous and mixed small and large cell carcinomas, represent 2 to 4% of the total, being an argument in favour of the common origin of all types of bronchial carcinoma. Lung cancer embraces two broad categories: non-small cell lung cancer (NSCLC) corresponds to approximately 80% of all lung cancer cases, and small cell lung cancer accounts for 15% ^{22,23}.

The definitions for tumour, node, metastasis (TNM) and stage grouping are defined according to the eighth edition of the AJCC Cancer Staging Manual ²⁴.

History of liquid biopsies

Finding minimally invasive methods to access cancer has been the major goal of oncology research. Tissue biopsies have for long been the “gold standard” for histological diagnosis and immunohistochemical, genetic and molecular studies of the various neoplasms ²⁵. A single “snapshot” of the tumour has highlighted bias information, given the high level of spatial and temporal inpatient and intratumour heterogeneity ²⁶. Inpatient heterogeneity being the genomic landscape may differ between tumour sites within a patient. Intratumour heterogeneity being the genomic landscape within a single tumour manifestation may not be uniform. This was demonstrated by tissue biopsies taken from different parts of the primary tumour or metastases. Tissue biopsies may not fully represent the tumour genotype.

A unique procedure that could give us the whole genomic profile of the tumour in a non-invasive, harmless, and easily executed technique was by passed with a procedure known as liquid biopsies. According to two sources, the National Cancer Institute and ESMO Precision Medicine Glossary defined liquid biopsies as a test done on fluid biological samples (i.e. blood, cerebrospinal fluid, urine, saliva) that contain

different components (i.e. circulating tumour cells (CTCs), cell-free circulating DNA (cfDNA), circulating tumour DNA (ctDNA), extracellular vesicles (EVs) microRNA (miRNA), proteins) consisting in nucleic acids fragments derived from cancer cells and healthy tissues ^{27,28}.

cfDNA refers to both encapsulated and non-encapsulated cell-free DNA fragments found in different bodily fluids found in both healthy and diseased individuals. It is thought that it is generated through apoptosis, necrosis, and active secretion of EVs and CTCs. The fraction of cfDNA derived from tumour cells is known as circulating tumour DNA (ctDNA). All molecular alterations of cancer cells can also be detected in ctDNA ^{26,27}. It is also known that cfDNA levels are higher in a cancer patient when compared to a healthy subject. However, cfDNA isn't exclusive to cancer and can also be increased in pro-inflammatory diseases, autoimmune disorders, surgery, tissue injury as well as pregnancy and physical exercise. ctDNA is the DNA released by the primary or metastatic neoplastic cells, into the bloodstream and which can be isolated from peripheral blood with a simple blood test. In cancer patients, ctDNA represents a small proportion of total cfDNA, varying from <0.1% to 10%. ctDNA levels are related to the tumour burden, tumour location, vascularity and cellular turnover and tend to be higher in metastatic cancer and increase in disease progression (Table 1). The analysis of ctDNA from a blood sample will correspond to a "screenshot" of the primary and metastatic tumour; an overtime analysis will provide the whole "motion picture" of tumour evolution in a longitudinal time setting.

The first publication occurred in 1948 when circulating cell-free deoxyribonucleic acid (cfDNA) was first identified in the blood of healthy individuals by Mandel and Metals ²⁹. Leon *et al.* later described higher levels of circulating free DNA (cfDNA) in the serum of cancer patients compared to healthy individuals ³⁰. This led to the hypothesis that tumours shed DNA fragments in the bloodstream. Six years later, Shapiro demonstrated the correlation between cfDNA concentration between benign and malignant lung nodules ³¹. In 1989, Stroun *et al.* demonstrated that DNA found in the plasma of cancer patients originated from tumour cells due to their genomic instability ³². In the early '90s, in two studies specific KRAS proto-oncogene GTPase and NRAS proto-oncogene GTPase mutations of ctDNA were identified in pancreatic adenocarcinoma and acute myelogenous leukemia ^{33,34}.

The idea that liquid biopsy matches tissue biopsy has been around for several years now. In ASCO 2016, a study funded by Guardian Health Inc. obtained liquid biopsies from 15000 cancer patients (lung cancer 37%, breast cancer 14%, colorectal cancer 10% and other cancers 38%). From 386 of these patients, tumour biopsies were also available. When comparing tissue samples with circulating tumour DNA (ctDNA) by sequencing method, the results reported an overall accuracy of 87% (336/386 patients) ^{35,36}. In a 640 pan-cancer patient study, Bettegowda *et al.* established a correlation between quantification of cfDNA with stage and tumour burden in colon, breast and lung cancer. Significant differences in cfDNA concentrations between cancer types and a 100-fold higher cfDNA concentration in stage IV patients versus stage I ³⁷. In the NILE study ³⁸, which enrolled 307 patients, only 18% of patients had complete tissue genotyping for all eight guideline-recommended genomic biomarkers. Only one of the eight biomarkers was identified in 21.3% of patients using tissue *versus* 27.3% for ctDNA (n=282; p<0.0001 for non-inferiority). The clinical sensitivity of ctDNA relative to tissue, was 80% for the eight biomarkers recommended. The FDA approved EGFR, ALK, ROS1 and BRAF concordance of >98.2% and 100% positive predictive value for ctDNA vs tissue. Nevertheless, ctDNA median turnaround time was faster than tissue (9 vs 15 days;

p<0.0001). Using ctDNA in addition to tissue increased the detection of somatic mutations by 48%.

In the last few years, scientists have eagerly sought the identification of reliable biomarkers with a high capacity for innovation in predicting the course of cancer. The ideal biomarker should monitor the pacemaker of clonal evolution and be a surrogate indicator of future events, being it recurrence, progression, response, or resistance to ongoing therapy. The analytes used for liquid biopsies are plasma cfDNA, ctDNA, CTCs, exosomes and miRNAs. The complete integration of all circulating analytes into the bloodstream has been recently designated as the “tumour circulome”³⁹.

However, tumour cells release both mutant DNA and nonmutant DNA fragments into the circulation. The nonmutant fragments are more abundant than the mutant fragments. The tumour microenvironment and other somatic cells in the body release nonmutant DNA, which is of greater abundance than the tumour-derived fraction. ctDNA can now be distinguished from non-tumour-derived circulating DNA through genetic and epigenetic signatures retained from the primary tumour or through distinct fragmentation patterns.

Table 1: Summary of advantages and limitations of cfDNA and ctDNA

	Advantages	Limitations
cfDNA ^{37,40,41,42}	. Increased in cancer patients comparing to healthy individuals . Genetic and epigenetic alterations reflect those of the original tumour . Representation of the tumour heterogeneity and dynamics . Highly sensitive assays available (PCR, NGS) . Half-life (16min to 2,5hours) . fragments of ≈166p	. Markedly diluted compared to germline circulating DNA . Positively correlates with tumour size and staging . Increased in some benign or premalignant conditions . High costs
ctDNA ^{42,43}	. Highly studied . Clinical approved for targeted treatments . High specificity (no false positive results)	. Short half-life (less than 1 hour) . Fragments of 134 to 144bp . Low concentration (<1%) . Low sensitivity (possibility of false negative results)

The comprehensive genomic profiling through liquid and tissue builds the strength of each type of assay as shown in Table 2. They should be considered as complementary tools to track molecular residual disease throughout time.

Table 2: Genotyping through plasma NGS vs standard of care tissue samples

	Blood	Tissue
STRENGTHS	. Less invasive/less morbidity . Simpler to obtain/faster results . Less biased detection of genomic alterations vs single tissue biopsy site . Makes repeat biopsy more feasible . Could allow real time monitoring	. Standard of care . More confidence in negative results . Higher sensitivity for certain types of alterations
LIMITATIONS	. Not all patients have ctDNA . Negative results should be confirmed with tissue testing	. Invasive procedure with potential complications . Tumour heterogeneity may not be captured . Finite resource in many patients

Liquid biopsies can potentially be the key to obtaining a broader view of tumour heterogeneity, collecting sequential data reflecting the spectrum of the disease at different time points. It may help to identify early detection of progression, treatment failures at high-risk patients and mechanisms of resistance to distinct drugs. In clinical practice, we should tailor each treatment for each individual patient and a specific cancer type. An even more ambitious goal for liquid biopsies is early cancer detection in asymptomatic individuals, thereby improving early diagnosis and reducing cancer mortality rate ⁴⁴.

Technological evolution

One of the first challenges considered was how to discriminate the ctDNA from DNA free from normal cells. It is now difficult to remember that, for a long time, the biomarkers analysis was limited to 1-5 genes in the clinical routine. The main goal is to identify clinically relevant genetic alterations. Amongst the most frequent techniques used in liquid biopsy are digital polymerase chain reaction (dPCR) and next-generation sequencing (NGS).

The sensitivity of dPCR has improved over time, however, with the increase in the number of biomarkers and the need for them to be analysed simultaneously, several technical problems arise, for example, the amount of sample required for DNA extraction ²⁵. dPCR also requires prior knowledge of the mutation's status, for specific probes are needed which will limit its application. An advantage of dPCR is that it has been shown to be a cost-effective method ⁴³.

The new NGS, allows, in just 10ng of DNA, to determine the detection of mutations, translocations or variations of copies in many genes simultaneously. This NGS technique allows for saving material and obtaining a fast response with acceptable sensitivities and specificities⁴⁶, assuming an essential role for the major objective is the understanding of

the complexity of cancer through the analysis of the genome by sequencing of the exon or the entire tumour genome. The only two validated liquid biopsy tests by the FDA and EMA are the cobas® EGFR Mutation Test for NSCLC and the QIAGEN therascreen® EGFR RGQ PCR kit. They were also considered the only tests with clinical utility ^{47,48,49}.

In May 2017, the guidelines for NGS suggestions for liquid biopsies were validated by the College of American Pathologists, the Association of Molecular Pathology as well as the European Society of Pathology ^{50,51}. As a consequence of the many published studies and many others currently underway, there has recently been a need for standardized validation, and an OncoNetwork Global Consortium was created: CANCER-ID ⁵¹⁻⁵³. Also, with the collaboration of the International Liquid Biopsy Standardization Alliance (ILSA) ⁵⁴, the whole scientific community will use the same language and the same techniques to make translational research into a single language.

According to the Association for Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO), and College of American Pathologists (CAP) guidelines, gene alterations can be classified based on a joint consensus recommendation (JCR) of a 4-tiered system ⁴⁹. Globally, all observed variants should be reported respecting all the Human Genome Variation Society (HGVS) indications ⁵⁵. Considering the degree of complexity of genomic variants and their interpretation in the context of a subtype of cancer within an individual patient may reveal complex and challenging interpretations. Thus, the entity Molecular Tumour Boards (MTBs) have gained a major impact in multidisciplinary meetings where several health caregivers will discuss the best therapeutic treatment directed to specific cancer, with its own diversity, within an individual patient. The Memorial Sloan Kettering Cancer Center (MSK), and the European Society for Medical Oncology Scale for Clinical Actionability of Molecular Targets (ESCAT) have created guidelines or tools for modern oncologists in the era of implementing precision in the field of oncology. This is a fundamental tool for MTBs to perform a correct and critical interpretation of molecular results ^{56,57}.

With the increasing development of molecular medicine and expanding fields in translational cancer research, we may further change our clinical rational to a new era of precision cancer medicine. Consequently, we may improve the quality of life of our patients, with less toxicity, more cost-effective decisions and above all, improve response rate and survival.

Defining the whole genetic “picture” or genomic landscape of each patient will soon be considered a standard of care and will require all genetic information concerning each cancer, to offer precision medicine by customizing healthcare by molecular analyses ⁵⁸.

Translational application

The pharmacokinetics, or rather the better understanding of the cells biological activity associated with the evolution of the tumour and its genetic alterations, created the bridging between basic research and clinical investigation. Bridging knowledge means using basic concepts of cancer, such as genomic changes and gene or protein expression patterns, and their application in clinical practice. It is known as Translational Investigation, travelling from the biology of the tumour cell to clinical and therapeutic intervention, which is supposed to benefit a subset of patients.

Milholland *et al.* published in 2017 a study which showed that, on average, one mutation per cell division occurs ⁵⁹. However, another study published in the Cell in 2012,

in which the complete genome of a patient with breast cancer was sequenced, detected about 70,000 mutations, of which only five had an active role in carcinogenesis that is, all other mutations had no "driving force" to lead to tumour development ⁶⁰. These are the so-called "Passenger mutations" which do not cause interference in the carcinogenic process. More specifically, they are somatic mutations within either a coding or non-coding region of the genome that does not confer a selective growth advantage under a given set of selective pressures. In contrast, the five most mutated mutations are "Driver mutations" defined as genomic mutations that fall within a cancer gene regulatory region and change the cancer gene function or activity. These play a critical role in the tumour's objectives: cancer initiation, proliferation, angiogenesis, migration, metastasis and survival. It is estimated that on average cancer has four driver mutations, but it may vary according to cancer type ⁶¹. Cancer result from somatic mutation and clonal selection. There are two types of cancer genes: oncogenes that promote cellular progression and tumour suppressor genes that act to oppose the process that can drive cancer progression.

Traditionally, the anatomical, pathological and imaging tools allowed, in combination, an evaluation of each solid tumour and it's staging according to the clinical classification of Tumour, Nodule and Metastasis (TNM 8th edition) ²⁴. Until recently, most treatments in oncology are based on staging and classification. Adding information obtained through the development of tumour molecular genetics is essential to improve the therapy for each patient and each tumour. During the last two decades, we have watched its implications in altering this situation of tumour classification and identifying specific molecular targets for therapy. However, a limited number of mutations change the behaviour of the tumour, and the cellular complexity is much higher and depends on the characteristics of the host, the microenvironment, the immunological profile, and lifestyle factors.

Science is in constant turnover. There are no static genetic, immunological, or environmental "signatures" in an oncological patient. There is a dynamic interaction between each other and between them and an individual patient. Which one prevails will depend on biomarkers, performance status, co-morbidities and preference according to side effects and quality of life. Therefore, treatment discussion for each tumour in an individual patient has been more refined, having overall survival of our patients with the best quality of care as the priority ².

With the advances in genomics, transcriptomic and epigenetic research, cancer is now considered, a heterogeneous disease. It occurs because some pathways are blocked, and tumours find different "motorways" to reach the same end: Growth, Proliferation and Migration. Cancer is a dynamic disease, where green signs open and the motorway is free to travel (proliferation) as an alternative to red signs that have blocked this passage, leading to apoptosis. Cancer is a clonal disease, where predominate cells survive depending on which traffic light is on or off. It is up to physicians to investigate, namely by rebiopsying these tumours or using liquid biopsy for ctDNA ⁶², when we withstand progression or acquired resistance to a specific therapy, to determine the driving clone ¹⁴.

The reduction in lung cancer mortality rate depends on significant improvements in diagnostic methods and treatments efficacy. The study of tumour molecular genetics allows us reasonable expectations today of meeting those twin goals. One of these reasons is that tumours are evolving processes in which a phylogenetic system is achieved, as the spectrum of molecular alterations of tumours becomes better known, mapping the diseases behaviour more minutely than at present. Secondly, the rise to

specific therapeutic targets allows the well-thought-out development and application of highly effective treatments. Finally, pinpointing the alterations causing the first traces of oncogenesis could revolutionise the early diagnosis of cancers.

In the era of Precision Medicine, our goal is to eliminate the concept that “one size fits all” model of patient management based on average response to empirical treatment. Shifting treatment based on genotyping the tumour and detecting genomic alterations of solid tumours, namely microsatellite instability, tumour burden, among other biomarkers. All these joint efforts will lead to a broader knowledge of each tumour and translates into better treatment for each patient. Bridging the clinical gaps with basic science will allow Precision Oncology, to completely change the current paradigms of cancer management at the present times.

With Translational Investigation, patients will benefit from new investigational techniques and improve their quality of care and of life with tailored treatments to disease biology and predicted treatment response. Oncology is taking a 360° clockwise turn, favouring the era of Precision Medicine.

History of Lung Cancer Treatment

Lung cancer treatment is determined according to histology, morphology, stage, genetic profile and most importantly, the patient’s performance status and willingness to perform therapy. These choices may range from surgery, to radiotherapy, chemotherapy, molecularly targeted therapy, and immunotherapy, alone or in combination, and best supportive care.

Radical resection is the cornerstone for patients with early-stage lung cancer (stage I, II and IIIA N0-N1) who are medically fit corresponding to approximately 16%^{22,23}. In node-negative (N0) disease, 13 to 23% of cases, recurred after curative resection (R0); recurrence is higher in node-positive cases (N1). Approximately 30% of patients with locally advanced disease, stage IIIA-C (T3T4, N2-N3) are not candidates for surgery. Concurrent chemotherapy followed by immunotherapy is the standard of care^{24,63}. So far, targeted therapy has not proven an overall survival benefit in early-stage lung cancer patients.

Approximately 80% of recurrences occur in the first two years following resection in distant sites, with a 5-year survival of 30%⁶⁴⁻⁶⁶. Unfortunately, most NSCLCs, approximately 65%, present locally advanced or metastatic disease, and regional therapy is not an option. These numbers clearly show that at the treatment level, reducing the mortality rate depends on significant improvements in prevention, diagnostic methods, and efficacy of treatments.

In advanced lung cancer disease, targeted therapies have improved clinical outcomes in a significant way, improving not only quality of life but also progression-free survival and overall survival. According to genetic alterations, specific therapies were developed, as illustrated in Table 3. If no targetable mutations are identified, and no contraindication for PDL1 (programmed death ligand 1), PD1 (programmed cell death protein 1) receptor, CTLA-4 (cytotoxic T lymphocyte protein 4) inhibitors exist, immunotherapy or Immuno-oncology agents (IO) may be an option (Table 4) in monotherapy or combination as a first-line option in squamous and non-squamous stage IV lung cancer. Chemotherapy is always an optional therapy, as is best supportive care^{2, 67}.

Understanding the cornerstone of carcinogenesis permits us to comprehend how lung cancer behaves and responds to strategic oncological treatments.

Table 3: Molecular Targeted Therapies

Genetic Alteration (Driver Event)	%	Targeted Agents with Activity Against Driver Event in Lung Cancer
EGFR	10-30	Afatinib ⁶⁸ Erlotinib ⁶⁹ Dacomitinib ⁷⁰ Gefitinib ^{71,72} Osimertinib ⁷³
ALK	5	Alectinib ^{74,75} Brigatinib ⁷⁶ Ceritinib ⁷⁷ Crizotinib ^{78,74} Lorlatinib ⁷⁹
ROS1	1-2	Ceritinib ⁸⁰ Crizotinib ⁸¹ Entrectinib ⁸²
BRAF V600E	1-3	Dabrafenib/Trametinib ⁸³ Dabrafenib ⁸³
MET exon 14	2-4	Capmatinib ⁸⁴ Crizotinib ⁸⁵ Tepotinib ^{85,6}
NTRK1/2/3	0.2	Larotectinib ⁸⁷ Entrectinib ⁸⁸
RET	1-2	Sapercatinib ⁸⁹ Pralsetinib ⁹⁰ Cabozantinib ^{91,92} Vandetanib ⁹³
ERBB2 (HER2) *	-	Ado-trastuzumab emtansine ⁹⁴ Fam-trastuzumab deruxtecan-nxki ⁹⁵
High-level MET amplification *	-	Crizotinib ^{96,97} Capmatinib ⁹⁸
KRAS G12C	-	Sotorasib ⁹⁹ Adagrasib ¹⁰⁰

* Emerging biomarkers

Table 4: Immunotherapy or Immuno-oncology agents (IO)

	Immunotherapy or Immuno-oncology agents
anti-CTLA4	Ipilimumab ^{101,102} Tremelimumab ^{103,104}
anti-PD-L1	Avelumab ¹⁰⁵ Durvalumab ¹⁰⁴ Atezolizumab ^{106,107}
anti-PD1	Pembrolizumab ¹⁰⁸⁻¹¹⁰ Nivolumab ^{101,102}

AIM

With all this potential, and in the face of many published studies showing the usefulness of molecular genetic techniques as auxiliary diagnostic tools, it is somewhat surprising that the latter are not in common use in routine clinical practice in Portugal. This study is part of our plan to establish and implement in the medium-term protocols for transposing molecular genetic knowledge and techniques from bench to clinical practice.

Our first aim is to identify the main practical barriers to implementing molecular genetic techniques. To do that, we focussed on studying the anti-oncogene p53 mutations in clinical samples of patients diagnosed with lung cancer by sequencing. This choice was induced by p53 being the most common target of mutations in lung cancer and by the fact that there are indications of these mutations having prognostic value. In 2007 we used a p53 mutation screen in multiple clinical samples from a series of lung cancer patients to attempt to identify the main practical limitations in integrating molecular genetics in routine clinical practice.

Recently, cancer diagnosis and follow-up in solid tumours is revolutionizing by a new concept of questioning what is happening in the blood of cancer patients. This concept of liquid biopsies technology allows us to collect circulating cancer cells or DNA fragments from tumours shed into the bloodstream. We shall focus on circulating tumour DNA (ctDNA) as the most advanced clinical method used at the present time to screen for actionable mutations that are routinely used in advanced lung cancer both at the diagnostic and post-progression stages, their usefulness in surgically resectable lung cancer has not yet been addressed. Our aim is to know whether cancer cells or ctDNA remaining in the body after surgery will be important in predicting tumour recurrence. Although the number of cancer cells remaining after a “curative surgery” is small, if any, and the detection of minimal residual disease has been a technical challenge, recent advances through NGS are set to change this situation.

The second aim of this study was to integrate the routine use of ctDNA in lung cancer surgical patients. Evaluate its quantitative evolution of ctDNA across time and its correlation with clinicopathological variables at five specific collection time points.

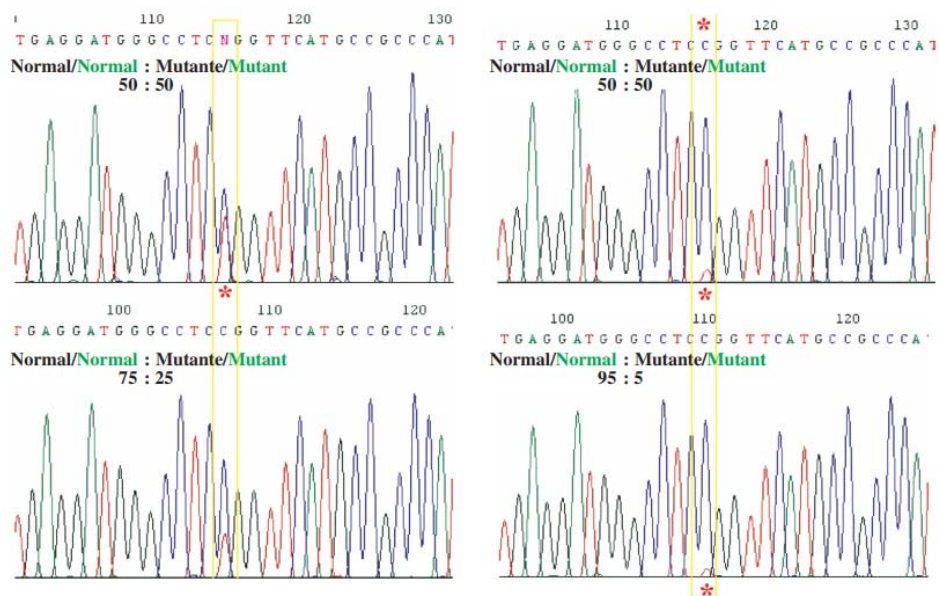
Thirdly, assessing whether cancer cells remain in the body after surgery will be important in predicting tumour recurrence through genotyping tumour and plasma samples for actionable mutations where ctDNA expression is greatest. To understand the usefulness of ctDNA expression as a guide to detect residual postsurgical ctDNA and disease progression.

Finally, evaluate the association between ctDNA concentration (molecular) and imaging (CT) progression.

MATERIAL & METHODS

In each chapter of lung cancer investigation, different molecular genetic techniques are used in a rationale of a timeline of technological evolution. The material and methods used are specified and described in the three papers of the core of the thesis.

Chapter 1 - Genetic alterations in Lung Cancer: Assessing limitations in routine clinical use.



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**Alterações genéticas no cancro do pulmão:
Avaliação das limitações ao seu uso na rotina clínica**

***Genetic alterations in lung cancer:
Assessing limitations in routine clinical use***

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Resumo

O cancro do pulmão é a causa mais frequente de mortalidade por cancro no mundo, sendo responsável por cerca de 1,1 milhões de mortes por ano. A sobrevivência média dos doentes é geralmente curta, por a doença se encontrar em estádios avançados na altura do diagnóstico, mas também devido à falta de eficácia dos tratamentos disponíveis. O advento da genética molecular dos tumores trouxe consigo a possibilidade de modificar esta situação, quer através do refinamento do diagnóstico, quer da identificação de alvos terapêuticos específicos, quer sobretudo por – pelo menos em teoria – permitir o diagnóstico precoce da doença. No entanto, e apesar de numerosos trabalhos terem já demonstrado a utilidade das técnicas da genética molecular no estudo do cancro do pulmão, o seu uso na rotina clínica em Portugal tem sido limitado.

Abstract

Lung cancer is the most frequent cause of cancer mortality worldwide, responsible for approximately 1.1 million deaths per year. Median survival is short, both as most tumours are diagnosed at an advanced stage and because of the limited efficacy of available treatments. The development of tumour molecular genetics carries the promise of altering this state of affairs, as it should lead to a more precise classification of tumours, identify specific molecular targets for therapy and, above all, allow the development of new methods for early diagnosis. Despite numerous studies demonstrating the usefulness of molecular genetic techniques in the study of lung cancer, its routine clinical use in Portugal has, however, been limited. In this study, we used a p53 mutation screen in multiple clinical samples from a series of lung cancer pa-

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No presente estudo, utilizou-se a pesquisa de mutações no anti-oncogene p53 em amostras clínicas de doentes com diagnóstico de cancro do pulmão como método para identificar as dificuldades práticas à integração da genética molecular na rotina clínica. Os resultados obtidos sugerem que o principal factor limitante a essa integração é a obtenção de amostras de ADN de qualidade, um problema que pode ser superado pela alteração das práticas correntes de recolha de amostras.

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Palavras-chave: Cancro, pulmão, clínica, genética molecular, mutação, p53.

tients to attempt to identify the main practical limitations to the integration of molecular genetics in routine clinical practice. Our results suggest that the main limiting factor is the availability of samples with good quality DNA; a problem that could be overcome by alterations in common sample collection and storage procedures.

Rev Port Pneumol 2007; XIII (1): 9-34

Key-words: Cancer, lung, clinical, molecular genetics, mutation, p53.

A sobrevivência média global destes doentes aos 5 anos é de cerca de 15%

Introdução

O cancro do pulmão é actualmente a neoplasia mais comum em todo o mundo, representando por si só cerca de 13% dos novos casos de cancro^{1,2}. É também a principal causa de morte por cancro, sendo responsável por aproximadamente um quinto dos óbitos, ou cerca de 1,1 milhões de mortes anuais³. Esta situação torna-se ainda mais preocupante se considerarmos que os avanços terapêuticos dos últimos 20 anos tiveram uma reduzida repercussão na sobrevivência dos doentes com cancro do pulmão. Actualmente, a sobrevivência média global destes doentes aos 5 anos é de cerca de 15%⁴. No entanto, e tal como para outras neoplasias, o prognóstico depende muito do estadiamento à data do diagnóstico: para os estádios precoces, submetidos a cirurgia (que representam apenas 15% dos casos), a sobrevivência aos 5 anos é de cerca de 80%, mas para a maioria dos casos que são diagnosticados em estádios avançados ela é muito inferior (por exemplo, 5% para um doente com carcinoma não de pequenas células em estágio IIIB ou 1%

Introduction

Lung cancer is currently the most common form of cancer the world over, in itself responsible for around 13% of new cancer cases^{1,2}. In parallel it is also the main cause of cancer death, responsible for approximately one fifth of all cancer deaths, or around 1.1 million deaths per year³. This state of play becomes even more worrying if we consider that the advances in treatment of the last 20 years have had a reduced impact on lung cancer patient survival. The current mean global 5-year survival rate of these patients is around 15%⁴. Just as with other forms of cancer, the prognosis depends greatly on the stage at the date of diagnosis. While for early stages undergoing surgery (only about 15% of cases), the 5-year survival rate is approximately 80%, for the greater part of cases, diagnosed in advanced stages, the rate is much lower; 5% for a non-small cell carcinoma patient in stage IIIB and 1% in stage

ALTERAÇÕES GENÉTICAS NO CANCRO DO PULMÃO: AVALIAÇÃO DAS LIMITAÇÕES AO SEU USO NA ROTINA CLÍNICA

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em estágio IV)⁵. Estes números demonstram claramente que, ao nível do tratamento, a redução da taxa de mortalidade depende de melhorias significativas, quer dos métodos de diagnóstico, quer da eficácia das terapias.

O estudo da genética molecular dos tumores permite-nos, hoje em dia, uma expectativa razoável de se poderem atingir esses dois objetivos, por várias razões. Em primeiro lugar, resulta do facto de os tumores serem processos evolutivos⁶ que as alterações genéticas determinantes para o seu desenvolvimento formam a base do melhor sistema de classificação possível para os tumores: um sistema filogenético. É claro que, dadas as limitações actuais dos nossos conhecimentos, um tal sistema ainda não é viável. Contudo, à medida que o espectro de alterações moleculares dos tumores for sendo mais bem conhecido, deverá ser possível usar esses dados para obter um diagnóstico mais preciso e prever com maior exactidão do que actualmente o comportamento da doença – incluindo a resposta às terapias. Segundo, as alterações genéticas presentes nos tumores originam directamente alvos terapêuticos específicos que podem permitir o desenvolvimento e aplicação racionais de terapias altamente eficazes – como demonstrado pelos exemplos do inibidor de bcr-abl (imatinib) para leucemia mieloide crónica e dos inibidores do EGFR (erlotinib e gefitinib) em casos específicos de cancro do pulmão. Finalmente, a natureza multietápica da evolução tumoral⁶⁻⁸ implica que a identificação das alterações responsáveis pelos passos iniciais da oncogénese pode revolucionar o diagnóstico precoce das neoplasias. Com todo este potencial, e os numerosos estudos já publicados demonstrando a utilidade das técnicas da genética molecular como métodos auxiliares de diagnóstico, é algo surpreendente que ainda não se tenha generalizado o

IV)⁵. These numbers clearly show that at treatment level, reducing the mortality rate depends on significant improvements both in diagnostic methods and in the efficacy of treatment.

The study of tumour molecular genetics allows us reasonable expectations today of meeting those twin goals. There are several reasons for this. One of these reasons is the fact that tumours are evolving processes⁶ in which the genetic alterations determining their development form the base of the best classification system possible for tumours; a phylogenetic system. Obviously, given the current limits to our knowledge, such a system is not yet viable but as the spectrum of molecular alterations of tumours becomes better known, it should be possible to use these data to obtain a more precise diagnosis and map the disease's behaviour – including response to treatment – more minutely than at present.

A second reason for hope is that the genetic alterations present in tumours give direct rise to specific therapeutic targets which could allow the well thought-out development and application of highly effective treatments. Examples of this are the bcr-abl inhibitor (Imatinib) for chronic myeloid leukaemia and the EGFR inhibitors (Erlotinib and Gefitinib) used in specific cases of lung cancer. Finally, the multistage nature of tumoral development⁶⁻⁸ means that pinpointing the alterations causing the first traces of oncogenesis could revolutionise the early diagnosis of cancers. With all this potential, and in the face of many published studies showing the usefulness of molecular genetic techniques as auxiliary diagnostic tools, it is somewhat surprising that the latter are not in general use in

A identificação das alterações responsáveis pelos passos iniciais da oncogénese pode revolucionar o diagnóstico precoce das neoplasias

seu uso na rotina clínica em Portugal. O estudo ora apresentado, inserido no nosso plano de, a médio prazo, estabelecer e implementar protocolos que permitam a transposição dos conhecimentos e das técnicas da genética molecular da bancada para a clínica, visou primariamente a identificação dos principais entraves de ordem prática a essa implementação. Para tanto, decidimos concentrar-nos na análise de mutações do anti-oncogene p53 em amostras clínicas de doentes com diagnóstico de cancro do pulmão, uma escolha motivada pelo facto de o p53 ser o alvo mais frequente de mutações em cancro do pulmão e de haver indícios de que essas mutações têm valor prognóstico.

Material e métodos

Seleção e avaliação clínica de doentes

Foram admitidos neste estudo doentes com cancro do pulmão, caracterizado cito e histologicamente, em seguimento na consulta de Pneumologia Oncológica do Hospital de São João, entre Maio de 2004 e Junho de 2005. Todos os doentes seleccionados assinaram consentimento informado. Em caso de suspeita de neoplasia do pulmão, foram obtidas história clínica pormenorizada e história de hábitos tabágicos (por consulta de processos clínicos ou entrevista). Seguidamente, foram efectuados exame físico e exames auxiliares de diagnóstico, incluindo exames laboratoriais, radiografia e TAC (tomografia axial computadorizada) torácicos e provas funcionais respiratórias. Perante evidência clínica e imagiológica sugestiva de cancro do pulmão, os doentes foram submetidos a exames de diagnóstico invasivos, como broncofibroscopia ou biópsia aspirativa transtorácica (BAT), consoante a localização do tumor. As broncofibroscopias, efectuadas com introdução transnasal de broncoscópio Olympus P30, P40 ou broncoveidos-

routine clinical practice in Portugal. This study is part of our plan to establish and implement in the medium-term protocols for transposing molecular genetic knowledge and techniques from bench to clinical practice. Our first aim is to identify the main practical barriers to implementing molecular genetic techniques. In order to do that, we focussed on analysing the anti-oncogene p53 mutations in clinical samples of patients diagnosed with lung cancer. This choice was occasioned by p53 being the most common target of mutations in lung cancer and by the fact that there are indications of these mutations having prognostic value.

Material and methods

The selection and clinical evaluation of patients

We studied lung cancer patients whose cancer had been characterised cytologically and histologically following oncological pulmonology consultations at the Hospital de São João between May 2004-June 2005. All patients selected gave their written informed consent to the study. A detailed clinical history and smoking habits history were obtained whenever lung cancer was suspected, by consulting case files or conducting interviews. The next step was a physical examination and auxiliary diagnostic exams. These included laboratory investigation, chest X-ray and CT (computerised tomography) scan and lung function tests. When clinical and imaging findings suggested lung cancer, patients underwent invasive diagnostic procedures such as bronchofibroscopy or transthoracic aspiration biopsy (TAB) depending on the location of the tumour. The bronchoscopies were performed with the transnasal introduction of the Olympus P30 or P40 bron-

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cópio, permitiram obter amostras das lesões suspeitas (lavado brônquico, escovagem brônquica, biópsia brônquica). Os procedimentos intrabronquiais foram realizados de acordo com a estratégia diagnóstica mais adequada para cada doente (lavagem brônquica com 10 ml de solução salina estéril, escovado brônquico e biópsia brônquica nos locais onde existiam sinais directos ou indirectos de envolvimento neoplásico). A BAT permitiu obter citologia aspirativa das lesões. O diagnóstico anatomopatológico foi efectuado de acordo com a classificação da OMS em vigor e o estadiamento clínico dos doentes foi feito de acordo com a Revisão do Sistema Internacional de Estadiamento do Cancro do Pulmão^{5,9}.

Obtenção de material para estudo molecular

Amostras de expectoração por emissão, obtidas antes da intervenção endoscópica, foram armazenadas em tubos “cónicos” de 50 ml numa solução de 2% carbowax/50% etanol (fixador de Saccomanno)^{10,11}. A fracção do lavado brônquico não utilizada para diagnóstico foi transferida para tubos “cónicos” de 15 ml, submetida a centrifugação durante 5 minutos a 10 000 rpm à temperatura ambiente, e após rejeição do sobrenadante armazenada a -80°C, em etanol a 95%^{12,13}. A extremidade da escova resultante da escovagem brônquica foi colocada em tubos tipo *eppendorf* de 1,5 ml com etanol a 70% e armazenada a 4°C¹⁴. Fracções das biópsias brônquicas da massa tumoral não utilizadas para fins de diagnóstico foram armazenadas a -80°C, sem fixação^{12,15}.

Isolamento e purificação de ADN das amostras clínicas

O ADN genómico de cada uma das amostras foi isolado e extraído com o *kit* de isolamento de DNA Puregene, da Gentra Systems. Em re-

choscope or bronchovideoscope, allowing collection of suspect lesion samples (bronchial lavage, bronchial brushing, bronchial biopsy). The intrabronchial proceedings were carried out in accordance with the most appropriate diagnostic strategy for each patient (bronchial lavage with 10ml of sterile saline solution and bronchial brushing and bronchial biopsy at the locales having direct or indirect signs of cancerous involvement). TAB was used to obtain aspiration cytology of the lesions. The prevailing WHO classifications were used to make an anatomic pathology diagnosis and patients' clinical staging was classified in accordance with the Revisions in the International Systems for Staging Lung Cancer^{5,9}.

Obtaining material for molecular study

Expectoration samples, taken before the endoscopy procedure was carried out, were stored in 50ml conical tubes in a 2% Carbowax/50% ethanol solution (Sacomanno Fixative)^{10,11}. The portion of the bronchial lavage not used in diagnosis was transferred to 15ml conical tubes, centrifuged for 5 minutes at 10 000 rpm at room temperature and the fluid which had risen to the surface was then discarded from it. The portion was then stored in 95% ethanol at -80°C^{12,13}. The tip of the brush used in the bronchial brushing was placed in 1.5ml Eppendorf-type tubes with 70% ethanol and stored at 4°C¹⁴. Portions of the tumoral mass resulting from the bronchial biopsies not used for diagnosis were stored at -80°C with no fixing^{12,15}.

Isolation and purification of the DNA of the clinical samples

The genomic DNA of each sample was isolated and extracted with the Gentra Systems Puregene DNA isolation kit. The samples

sumo, após centrifugação das amostras e rejeição dos sobrenadantes, adicionaram-se 30 µl de solução de *lise* e 0,15 µl de solução de proteinase K (20 mg/ml), por cada miligrama de amostra. O lisado foi incubado durante 4-5 horas a 65°C e, após digestão, centrifugação e arrefecimento a uma temperatura próxima da temperatura ambiente, procedeu-se à precipitação proteica. O material sobrenadante, contendo o ADN, foi retirado para um novo tubo contendo isopropanol a 100% e misturado suavemente até precipitação do ADN. Após centrifugação a 13 000-16 000g durante 3 minutos, o sobrenadante foi rejeitado e o precipitado lavado com 150 µl de etanol a 70%. Após remoção do excesso de etanol e secagem, o ADN foi reidratado num volume de água bidestilada autoclavada. As amostras foram submetidas a avaliação de qualidade por electroforese de uma aliquota em gel de agarose e armazenadas a -80°C até uso posterior.

Amplificação do ADN genómico

Dois fragmentos de ADN genómico, contendo os exões 5 a 9 do p53, foram amplificados por *polymerase chain reaction* (PCR), usando os pares de oligómeros indicados no Quadro I. As reacções de amplificação foram efectuadas num volume total de 25 µl, contendo 350ng de cada oligómero, 1,5mM de MgCl₂ (Fermentas), 1,25 unidades de Taq DNA polimerase, 0,25 mM de cada nucleótido (QBiogene) e 1 µl de ADN genómico, num tampão de reacção adequado, com (NH₄)₂SO₄ (Fermentas). O protocolo de amplificação incluiu um passo inicial de 3 mn a 94°C, seguido de 40 ciclos de 30s a 95°C, 1mn a 58°C e 1mn a 72°C, de um período de 3mn a 72°C e de arrefecimento a 4°C.

Purificação dos produtos de PCR

Os produtos de amplificação de ADN genómico foram separados em gel de agarose a 1%

were centrifuged and that rising to the surface discarded, then 30µl of lysis solution and 0.15µl of Proteinase K solution (20mg/ml) for each milligram of sample were added. This was incubated for 4-5 hours at 65°C and protein precipitation was made after digestion, centrifuging and cooling to near room temperature. DNA-bearing material rising to the surface was collected into a new tube containing 100% isopropyl alcohol and carefully shaken until DNA precipitation occurred. After centrifugation at 13 000-16 000g for 3 minutes, the material rising to the surface was discarded and the precipitation lavaged with 150µl of 70% ethanol. After removing the excess ethanol and drying, the DNA was rehydrated in twice distilled autoclaved water. The samples' quality was evaluated using Agarose gel electrophoresis and stored at -80°C for later use.

Amplification of genomic

DNA Two fragments of genomic DNA containing p53 exons 5-9 were amplified by polymerase chain reaction (PCR) using the oligomer pairs stated in Table I. The amplification reactions were made in a total volume of 25µl containing 350ng of each oligomer, 1.5mM of MgCl₂ (Fermentas), 1.25 units of Taq DNA polymerase, 0.25mM of each nucleotide (QBiogene) and 1µl of genomic DNA in a suitable reaction solution with (NH₄)₂SO₄ (Fermentas). The amplification protocol included an initial step of 3mn at 94°C, followed by 40 30sec cycles at 95°C, 1min at 58°C and 1min at 72°C, then 3min at 72°C and cooling at 4°C.

Purification of the PCR products

The products of genomic DNA amplification were separated in 1% Agarose gel and

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Quadro I – Oligómeros de ADN utilizados neste trabalho e suas aplicações

Designação	Localização	Cadeia	Sequência	Aplicação
LTC04-038	Intrão 4	Directa	AGC TCC TGA GGT GTA GAC G	Amplificação de exões 5 e 6
LTC04-040	Intrão 6	Complementar	CCC GGA GGG CCA CTG AC	Amplificação de exões 5 e 6
LTC04-041	Intrão 6	Directa	CCC TGC TTG CCA CAG GTC	Amplificação de exões 7 a 9 Apoiar amplificação específica de M09
LTC04-039	Intrão 9	Complementar	GAA ACT TTC CAC TTG ATA AGA G	Amplificação de exões 7 a 9 Apoiar amplificação específica de M09
LTC04-056	Intrão 4	Directa	GCT ACA CAT GTT TGT TTC TTT G	Sequenciação de exões 5 e 6
LTC04-055	Intrão 7	Complementar	TAG TAT GGA AGA AAT CGG TAA G	Sequenciação de exão 7
LTC04-042	Intrão 7	Directa	ATG GGA CAG GTA GGA CCT G	Sequenciação de exões 8 e 9
LTC05-151	Exão 7	Directa	GGG CGG CAT GAA CCG GAT	Amplificação específica de M09
LTC05-152	Exão 7	Complementar	TGA TGG TGA GGA TGG GCA	Amplificação específica de M09

Table I – DNA Oligomers used in this study and their applications

Designation	Location	Chain	Sequence	Application
LTC04-038	Intron 4	Direct	AGC TCC TGA GGT GTA GAC G	Amplification of exons 5 and 6
LTC04-040	Intron 6	Complementary	CCC GGA GGG CCA CTG AC	Amplification of exons 5 and 6
LTC04-041	Intron 6	Direct	CCC TGC TTG CCA CAG GTC	Amplification of exons 7-9 Specific support amplification of M09
LTC04-039	Intron 9	Complementary	GAA ACT TTC CAC TTG ATA AGA G	Amplification of exons 7-9 Specific support amplification of M09
LTC04-056	Intron 4	Direct	GCT ACA CAT GTT TGT TTC TTT G	Sequentionation of exons 5 and 6
LTC04-055	Intron 7	Complementary	TAG TAT GGA AGA AAT CGG TAA G	Sequentionation of exon 7
LTC04-042	Intron 7	Direct	ATG GGA CAG GTA GGA CCT G	Sequentionation of exons 8 and 9
LTC05-151	Exon 7	Direct	GGG CGG CAT GAA CCG GAT	Specific amplification of M09
LTC05-152	Exon 7	Complementary	TGA TGG TGA GGA TGG GCA	Specific amplification of M09

e os fragmentos de tamanho esperado isolados e purificados por filtração por coluna de Sephadex-G50Fine (Amersham) e precipitação com etanol. Os precipitados foram lavados uma vez com uma solução de 70% etanol e res-suspendidos em 20 µl de uma solução de loTE (3 mM Tris.HCl, pH 7,5 e 0,2 mM EDTA).

fragments of the desired size isolated and purified by filtration by Sephadex-G50Fine column (Amersham) and precipitation with ethanol. The precipitations were lavaged once with a 70% ethanol solution and resuspended in 20µl of loTE (3mM Tris.HCl, pH 7.5 and 0.2mM EDTA).

Pesquisa de mutações

A pesquisa de mutações foi efectuada por sequenciação dos produtos de PCR com o *kit* BigDye v3.1, da Applied BioSystems, usando 1µl de produto de PCR e 50ng do oligómero adequado (indicado no Quadro I) num volume total de 5 µl, com o seguinte protocolo: Incubação inicial a 96°C durante 1 minuto, seguida de 35 ciclos de 15s a 96°C, 15s a 54°C e 2mn a 60°C, de uma incubação final de 4mn a de 60°C e de arrefecimento a 4°C. Os produtos de reacção foram purificados por filtração por coluna de Sephadex-G50Fine, misturados com 10 µl de formamida (Applied BioSystems) e analisados num sequenciador ABI prism 3100. As sequências obtidas foram analisadas por inspecção visual dos electroforetogramas e com o programa DNASIS v5.0-Demo (Hitachi). Nos casos em que foram detectadas mutações, estas foram confirmadas por repetição integral dos procedimentos de amplificação e sequenciação.

Clonagem de sequências mutantes

A clonagem de produtos de PCR onde haviam sido identificadas mutações foi feita por “clonagem T:A”, usando o pTA4, um vector desenvolvido no laboratório para o efeito. Aproximadamente 30 ng de produto foram ligados a 10 ng de pTA4, previamente cortado com Eam1105I (Fermentas) e purificado por separação em gel e coluna de Sephadex. A reacção de ligação foi efectuada por incubação com 5 unidades de T4 DNA ligase (Fermentas), num tampão adequado (Fermentas), num volume de 20 ml, a 16°C, por um período de 12-16h. Os produtos de ligação foram purificados por extracção com fenol-clorofórmio e precipitação com etanol¹⁶. Após lavagem com etanol a 70%, os precipitados foram ressuspensos em 15 µl de uma solução

Research into mutations

Research into the mutations was done by sequentiation of PCR products with the BigDye v3.1 kit (Applied BioSystems), using 1µl of PCR product and 50ng of suitable oligomer (shown in Table I) in a total volume of 5µl, with the protocol as follows: initial incubation at 96°C for 1 minute followed by 35 15sec cycles at 96°C, 15sec at 54°C and 2min at 60°C with a final incubation of 4min at 60°C and cooling at 4°C. The reaction products were purified by filtration by Sephadex-G50Fine column, mixed with 10µl de formamide (Applied BioSystems) and analysed in ABI prism 3100 DNA sequencer. The sequences obtained were analysed by visual inspection of the electrophoretogram and using the DNASIS v5.0-Demo (Hitachi) program. Where mutations were detected, they were confirmed by repetition *in toto* of the amplification and sequentiation processes.

Cloning of the mutant sequences

Cloning of the PCR products wherein mutants were identified was achieved by T/A-based subcloning using TA4, a vector developed in the laboratory for that end. Approximately 30ng of the product were bound to 10ng of pTA4, previously cut with Eam1105I (Fermentas) and purified by separation in Sephadex gel and column. The ligation reaction was effected by incubation with 5 units of T4 DNA ligase (Fermentas) in a suitable solution (Fermentas) in a volume of 20ml at 16°C for a 12-16h period. The ligation products were purified by **extraction** with **phenol-chloroform** and precipitation with ethanol¹⁶. The precipitations were lavaged with a 70% ethanol solution and resuspended in 15µl of loTE. A third

de loTE. Um terço deste volume foi utilizado para transformar *E.coli* electrocompetentes (preparadas no laboratório) com um electroporador MicroPulser (Biorad). Após electroporação, as bactérias foram misturadas com 1 ml de LB (Pronadisa) contendo 100 mg/ml de ampicilina (Eurobio) e plaqueadas em LB-agar (Pronadisa) com igual concentração de ampicilina e incubadas a 37°C por 12-16h. Múltiplas colónias resultantes de cada transformação foram inoculadas em LB e incubadas a 37°C por 12-16h. O ADN plasmídico de cada uma foi isolado pelo método da *lise* alcalina¹⁶ e utilizado em reacções de sequenciação com o oligómero LTC04-055, para identificar os clones contendo sequências mutantes. Foram feitos *stocks* de um clone contendo cada uma destas e de um clone com a sequência normal: após centrifugação de uma fracção das respectivas culturas e rejeição do sobrenadante, as bactérias foram ressuspendidas numa mistura 3:1 de LB (contendo ampicilina) e glicerol esterilizado e armazenadas a -80°C até utilização posterior.

Amplificação de ADN plasmídico

Foram feitos riscados dos clones armazenados a -80°C em placas de LB com ampicilina. O ADN plasmídico correspondente ao fragmento de p53 foi amplificado com os respectivos oligómeros (LTC04-041 e LTC04-039) directamente a partir de uma colónia “repicada” para uma mistura contendo todos os reagentes necessários à amplificação, em quantidades proporcionais às indicadas para as amplificações de ADN genómico, mas num volume final de 10 ml. As reacções de PCR foram feitas com o mesmo perfil de temperaturas das amplificações de ADN genómico, mas com apenas 35 ciclos de temperatura.

of this volume was used to transform electrocompetent *E.coli* cells (prepared in the laboratory) with a MicroPulser electroporator (Bio-rad Laboratories). After electroporation, the bacteria were mixed with 1ml of LB (Pronadisa, Spain) containing 100mg/ml of ampicillin (Eurobio) and LB-agar plates (Pronadisa, Spain) with an equal concentration of ampicillin and incubated at 37°C for 12-16h. The multiple colonies resulting from each transformation were inoculated in LB and incubated at 37°C for 12-16h. The plasmidic DNA from each one was isolated by the alkaline lysis method¹⁶ and used in sequencing reactions with the oligomer LTC04-055 to identify the clones containing mutant sequences. Stocks were harvested of a clone containing each one of these and of a clone with a normal sequence. After centrifugation of a portion of the respective cultures and discarding that rising to the surface, the bacteria were resuspended in a 3:1 LB mixture (containing ampicillin) and sterilised glycerol and stored at -80°C for later use.

Amplification of the plasmidic DNA

The clones stored at -80°C were streaked onto LB plates with ampicillin. The plasmidic DNA corresponding to a fragment of p53 was amplified with the respective oligomers (LTC04-041 and LTC04-039) straight from a transplanted colony to a mixture containing all the reagents necessary for amplification, in quantities proportional to those indicated for genomic DNA amplifications, but in a final volume of 10ml. The PCR reactions were made with the same profile as the temperatures of the amplifications of genomic DNA but with only 35 cycles of temperature.

Amplificação específica de ADN contendo mutações

A amplificação específica de sequências mutantes foi feita num volume de reacção de 25 µl, a partir de cerca de 15 ng de produtos de PCR de DNA plasmídico, em condições semelhantes às acima descritas, com as seguintes alterações: a) A concentração de cada nucleótido foi apenas de 0,125 mM; b) Foram usados apenas 20 ciclos de temperatura; c) O protocolo de termociclagem foi “assimptótico”, isto, é incluiu temperaturas de hibridação decrescentes – 5 x 64°C, 5 x 62°C e 10 x 60°C.

Resultados

No período de selecção de doentes para este estudo (Maio de 2004 a Junho de 2005), foi possível recolher, para estudo molecular, um total de 29 amostras clínicas, provenientes de 15 doentes com cancro do pulmão. As amostras obtidas e os resultados das extracções de ADN a partir delas estão discriminados no Quadro II. Como pode constatar-se pela análise do quadro, em 6 casos não foi possível obter ADN a partir da amostra, e noutros 3 o ADN obtido não apresentou qualidade suficiente para permitir a amplificação dos fragmentos desejados, o que reduziu o total de amostras passíveis de análise molecular para 20 (4 biópsias brônquicas, 6 lavados brônquicos, 5 escovados brônquicos e 5 amostras de expectoração), provenientes de 12 doentes.

Para testar a possibilidade de análise molecular das amostras de ADN obtidas, procedemos a uma pesquisa de mutações no antioncogene p53, uma vez que este gene é o mais frequente alvo de mutações conhecido em cancro do pulmão e que há dados que indicam terem essas mutações valor prognóstico^{17,18,19}. A pesquisa de mutações cingiu-se aos exões 5 a 9 do p53, que codificam a zona central – funcionalmen-

Specific amplification of mutation-containing DNA

The specific amplification of mutant sequences was made in a reaction volume of 25µl, from around 15ng of PCR product of plasmidic DNA in conditions similar to those described above, with the following alterations: a) the concentration of each nucleotide was only of 0.125mM; b) Only 20 cycles of temperature were used; c) The thermocycling protocol was ‘asymptotic’ in that it included descending hybridization temperatures – 5 x 64°C, 5 x 62°C and 10 x 60°C.

Results

During the May 2004-June 2005 selection period of patients for this study it was possible to collect a total of 29 clinical samples from 15 lung cancer patients for molecular study. Table II details the samples obtained and the results of the DNA extraction from these. An analysis of the table shows that it was not possible to obtain DNA from the sample in 6 cases while in another 3 the DNA obtained was not of sufficient quality for an amplification of the desired fragments. This cut the total number of the samples suitable for molecular analysis to 20 (4 bronchial biopsies, 6 bronchial lavages, 5 bronchial brushings and 5 expectoration samples) which came from 12 patients.

We researched the mutations in the anti-oncogene p53 in order to test the possibility of a molecular analysis of the DNA samples obtained as this gene is the most common target of mutations known in lung cancer and data indicate these mutations have prognostic value^{17,18,19}. Research into mutations centres on exons 5-9 of p53. These codify the central zone of the corresponding protein. This zone is

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Quadro II – Amostras clínicas para diagnóstico molecular e resultados das extracções de ADN

Doente	Anatomia patológica	Amostra			
		BB	LB	EB	Ex
D 01	CPNPC	sADN	sQld		sADN
D 02	CPNPC			sADN	
D 03	Adenocarcinoma			3E	
D 04	Adenocarcinoma			4E	
D 05	Adenocarcinoma			5E	
D 06	Adenocarcinoma			sADN	
D 07	Adenocarcinoma		7L		7X
D 08	CPNPC		8L		
D 09	CPPC	9B		9E	9X
D 10	Adenocarcinoma		sADN	sQld	10X
D 11	CPNPC		11L	sQld	
D 12	CPNPC	sADN			12X
D 13	CPPC	13B	13L		13X
D 14	Carcinoma epidermóide	14B	14L		
D 15	Adenocarcinoma	15B	15L	15E	

Abreviaturas: **CPNPC** – Carcinoma do pulmão não de pequenas células; **CPPC** – Carcinoma do pulmão de pequenas células; **sADN** – Extração não forneceu ADN; **sQld** – ADN extraído não tinha qualidade para amplificação. Amostras designadas de acordo com o número do doente e o tipo (**BB** – biópsia brônquica; **LB** – lavado brônquico; **EB** – escovado brônquico; **Ex** – Expectoração).

Table II – Clinical samples for molecular diagnosis and results of the DNA extractions

Patient	Anatomic pathology	Sample			
		BB	BL	BBr	Ex
D 01	NSCLC	sDNA	sQld		sDNA
D 02	NSCLC			sDNA	
D 03	Adenocarcinoma			3Br	
D 04	Adenocarcinoma			4Br	
D 05	Adenocarcinoma			5Br	
D 06	Adenocarcinoma			sDNA	
D 07	Adenocarcinoma		7L		7Ex
D 08	NSCLC		8L		
D 09	SCLC	9B		9Br	9Ex
D 10	Adenocarcinoma		sDNA	sQld	10Ex
D 11	NSCLC		11L	sQld	
D 12	NSCLC	sDNA			12Ex
D 13	SCLC	13B	13L		13Ex
D 14	Epidermoid carcinoma	14B	14L		
D 15	Adenocarcinoma	15B	15L	15Br	

Abbreviations: **NSCLC** – Non-small cell lung cancer; **SCLC** – Small cell lung cancer; **sDNA** – Extraction did not supply DNA; **sQld** – DNA extracted was not of suitable quality for amplification. Samples designated in accordance with the number and type of patient (**BB** – bronchial biopsy; **BL** – bronchial lavage; **BBr** – bronchial brushing; **Ex** – Expectoration).

te mais importante – da correspondente proteína e onde se concentram 80% das mutações no p53²⁰. Foram testados diferentes protocolos de amplificação, por PCR, das sequências correspondentes aos cinco exões indicados, tendo-se finalmente optado por proceder a uma amplificação em dois segmentos, o primeiro contendo os exões 5 e 6 e o segundo os exões 7 a 9, de acordo com as condições indicadas (material e método). Seguidamente, compararam-se diferentes métodos de purificação dos produtos de PCR, tendo-se optado pela combinação electroforese em gel de agarose/filtração por coluna de Sephadex/precipitação com etanol, por proporcionar os melhores resultados na sequenciação dos produtos.

Os protocolos de amplificação, purificação e sequenciação estabelecidos foram então utilizados na análise dos ADN extraídos das amostras clínicas, tendo-se obtido os resultados indicados na Fig. 1 e nos Quadros III e IV – onde, para comparação, se incluem igualmente os resultados da avaliação anatomopatológica das amostras. Como pode verificar-se, foram detectadas mutações no p53 em 6 amostras de 4 doentes diferentes: 4 biópsias brônquicas, 1 lavado brônquico e 1 escovado brônquico. Em todos estes casos, a sequenciação detectou simultaneamente a sequência normal e uma sequência mutante, o que pode dever-se, quer ao facto de apenas um alelo do p53 estar afectado nos tumores, quer à presença de uma fracção importante de células não neoplásicas nas amostras. Esta segunda explicação parece mais provável, já que, nos casos em que as mutações foram detectadas em duas amostras do mesmo doente, as sequências normal e mutante estavam presentes em diferentes proporções. Estes resultados confirmam a possibilidade de realização de estudos de genética molecular em amostras clínicas de rotina de cancro de pulmão.

functionally the most important, and the one in which 80% of the p53 mutations concentrate²⁰. Different PCR amplification protocols were tested of the sequences corresponding to the five exons in question. The protocol finally selected was an amplification in two segments. The first segment contained the exons 5 and 6 and the second exons 7-9, in accordance with that described in the Material and Methods section above. Next different purification methods of the PCR products were compared and the one selected was an agarose gel electrophoresis/filtration by Sephadex column/precipitation with ethanol combination to obtain the best results in sequentiation of the products.

The established amplification, purification and sequentiation protocols were then used in the analysis of the DNA extracted from the clinical samples. The results gleaned are given in Fig. 1 and in Tables III and IV, which also include the findings of the anatomic pathology examination for purposes of comparison. It can be seen that p53 mutations were detected in 6 samples, from 4 different patients; 4 bronchial biopsies, 1 bronchial lavage and 1 bronchial brushing. In every case, the sequentiation detected the normal sequence and the mutant sequence simultaneously. This could be due to either the fact that only one p53 allele is affected by the tumours, or the presence of a significant portion of non-cancerous cells in the samples. This second explanation seems the more likely seeing as in the cases in which mutations were detected in two samples from the same patient, the normal and mutant sequences were present in different proportions. These results confirm the possibility of carrying out molecular genetic studies in routine clinical lung cancer samples.

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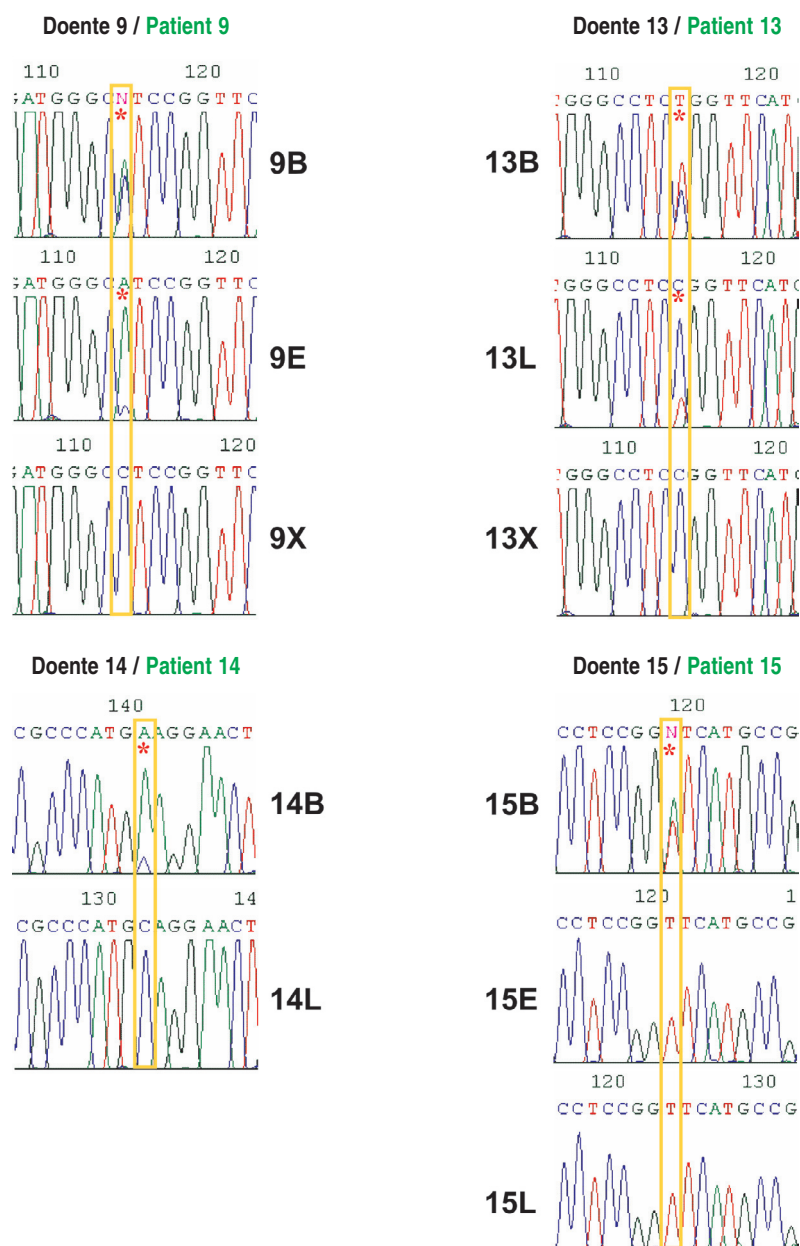


Fig . 1 – Identificação de mutações em amostras clínicas de cancro do pulmão. Pormenores dos electroforetogramas correspondentes à sequenciação do exão 7 de diversas amostras com o oligómero LTC04-055, mostrando a presença (*), ou não, de mutações. Amostras designadas de acordo com o número do doente e o tipo (**B** – biópsia brônquica; **L** – lavado brônquico; **E** – escovado brônquico; **X** – Expectoração).

Fig. 1 – Identification of the mutations in clinical lung cancer samples. Details of the electrophoretograms corresponding to sequentiation of exon 7 of various samples with the oligomer LTC04-055, showing the presence (*), or not, of mutations. Samples designated in accordance with patient number and type (**B** – Bronchial biopsy; **L** – Bronchial lavage; **E** – Bronchial brushing; **X** – Expectoration).

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Quadro III – Resultados das análises anatomopatológica e molecular

Doente	Amostra e análise							
	BB		LB		EB		Ex	
	AP	Mol	AP	Mol	AP	Mol	AP	Mol
D 01	CPNPC		nAval		nAval		nAval	
D 02			NEG		CPNPC			
D 03			NEG		NEG	NEG		
D 04			NEG		NEG	NEG		
D 05			NEG		NEG	NEG		
D 06								
D 07			AdeCa	NEG			nAval	NEG
D 08			nAval	NEG				
D 09	CPPC	M09			CPPC	M09	nAval	NEG
D 10					AdeCa		NEG	NEG
D 11	CPNPC		CPNPC	NEG				
D 12	CPNPC						NEG	NEG
D 13	CPPC	M13	nAval	M13			nAval	NEG
D 14	CaEpi	M14	nAval	NEG				
D 15	AdeCa	M15	nAval	NEG	NEG	NEG		

Abreviaturas: **BB** – Biópsia brônquica; **LB** – Lavado brônquico; **EB** – Escovado brônquico; **Ex** – Expectoração; **AP** – Anatomopatológica; **Mol** – Molecular; **CPNPC** – Carcinoma do pulmão não de pequenas células; **CPPC** – Carcinoma do pulmão de pequenas células; **CaEpi** – Carcinoma epidermóide; **nAval** – Amostra não avaliada; **NEG** – Resultado negativo; **M** – Mutação detectada (indexada com o número do doente); **AdeCa** – Adenocarcinoma.

Table III – Anatomic pathology and molecular analysis results

Patient	Sample and Analysis							
	BB		BL		BBr		Ex	
	AP	Mol	AP	Mol	AP	Mol	AP	Mol
D 01	NSCLC		nEval		nEval		nEval	
D 02			NEG		NSCLC			
D 03			NEG		NEG	NEG		
D 04			NEG		NEG	NEG		
D 05			NEG		NEG	NEG		
D 06								
D 07			AdeCa	NEG			nEval	NEG
D 08			nEval	NEG				
D 09	SCLC	M09			SCLC	M09	nEval	NEG
D 10					AdeCa		NEG	NEG
D 11	NSCLC		NSCLC	NEG				
D 12	NSCLC						NEG	NEG
D 13	SCLC	M13	nEval	M13			nEval	NEG
D 14	EpiCa	M14	nEval	NEG				
D 15	AdeCa	M15	nEval	NEG	NEG	NEG		

Abbreviations: **BB** – Bronchial biopsy; **BL** – Bronchial lavage; **BBr** – Bronchial brushing; **Ex** – Expectoration; **AP** – Anatomic pathology; **Mol** – Molecular; **NSCLC** – Non-small cell lung cancer; **SCLC** – Small cell lung cancer; **EpiCa** – Epidermoid Carcinoma; **nEval** – sample not evaluated; **NEG** – Negative result; **M** – Mutation detected (indexed with the patient number); **AdeCa** – Adenocarcinoma.

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Quadro IV – Características das mutações identificadas

Mutação	BB	LB	EB	Ex	Exão	Bases	Aminoácido	Códdão
M09	+	ND	+	–	7	G:C → T:A	Arg → Met	249
M13	+	+	ND	–	7	G:C → A:T	Arg → Gln	242
M14	+	–	ND	ND	7	G:C → T:A	Cys → Phe	248
M15	+	–	–	ND	7	A:T → T:A	Asn → Ile	247

Abreviaturas: **BB** – Biópsia brônquica; **LB** – Lavado brônquico; **EB** – Escovado brônquico; **Ex** – Expectoração; **M** – Mutação detectada (indexada com o número do doente) **ND** – Amostra não disponível; abreviaturas dos aminoácidos de acordo com as convenções

Table IV – Characteristics of the mutations identified

Mutation	BB	BL	BBr	Ex	Exon	Bases	Amino-acid	Codon
M09	+	ND	+	–	7	G:C → T:A	Arg → Met	249
M13	+	+	ND	–	7	G:C → A:T	Arg → Gln	242
M14	+	–	ND	ND	7	G:C → T:A	Cys → Phe	248
M15	+	–	–	ND	7	A:T → T:A	Asn → Ile	247

Abbreviations: **BB** – Bronchial biopsy; **BL** – Bronchial lavage; **BBr** – Bronchial brushing; **Ex** – Expectoration; **M** – Mutation detected (indexed with the patient number) **ND** – No sample available; amino-acids abbreviated in accordance with the conventions

Por outro lado, como indicado nos Quadros III e IV, embora em dois casos as mutações tenham sido detectadas em duas amostras diferentes, em todos os casos com mutações pelo menos uma amostra proveniente do mesmo doente deu um resultado negativo na análise molecular. Em teoria, estes resultados poderiam ser devidos, quer à ausência de células tumorais nas respectivas amostras, quer a uma baixa sensibilidade do método de pesquisa de mutações utilizado. Para tentar distinguir entre estas duas possibilidades, começámos por avaliar a percentagem de ADN mutante que poderia ser detectada com segurança. Em primeiro lugar, clonaram-se, num vector adequado, os fragmentos de ADN contendo cada uma das sequências mutantes e também a sequência normal, gerando-se assim fontes de “sequências puras” dos diferentes tipos. O ADN plasmídico foi depois utilizado para obter produtos de PCR (amplificados com os mesmos oligómeros

On the other hand, Tables III and IV show that although in two cases the mutations were detected in two different samples, in every case with mutations at least one sample coming from the same patient gave a negative result in molecular analysis. In theory, these results may be attributed to either the absence of cancerous cells in the respective samples or to the low sensitivity of the method used to investigate the mutations. In order to distinguish between these two possibilities, we began by assessing the percentage of mutant DNA which can safely be detected. Firstly we cloned in a suitable vector the DNA fragments containing each of the mutant sequences and also a normal sequence, thus creating sources of ‘pure sequences’ of the different types. The plasmidic DNA was then used to obtain PCR products (amplified with the same oligomers used for the genomic DNA) with the various sequences.

usados para o ADN genómico) com as várias sequências. A sequenciação de diferentes misturas de produto normal e mutante – Fig. 2 – sugere que percentagens de ADN tumoral inferiores a 10% poderiam originar falsos negativos na análise molecular. Para melhorar esta sensibilidade, decidimos desenhar oligómeros capazes de discriminar entre as sequências normal e mutantes, de forma a tentar amplificar estas especificamente. A Fig. 3 exemplifica o tipo de resultado obtido: para a mutação M09, foi possível obter condições de amplificação capazes de detectar especificamente a sequência mutante numa mistura contendo mais de 99% de ADN normal.

The sequentiation of the different mixtures of the normal and mutant product – Fig. 2 – suggests that percentages of tumoral DNA lower than 10% can give rise to false negatives in molecular analysis. To raise this sensitivity, we decided to design oligomers capable of distinguishing between the normal and mutant sequences so as to specifically try to amplify these. Fig. 3 shows the type of result obtained; for the MO9 mutation, it was possible to obtain amplification conditions capable of specifically detecting the mutant sequence in a mixture containing over 99% of normal DNA.

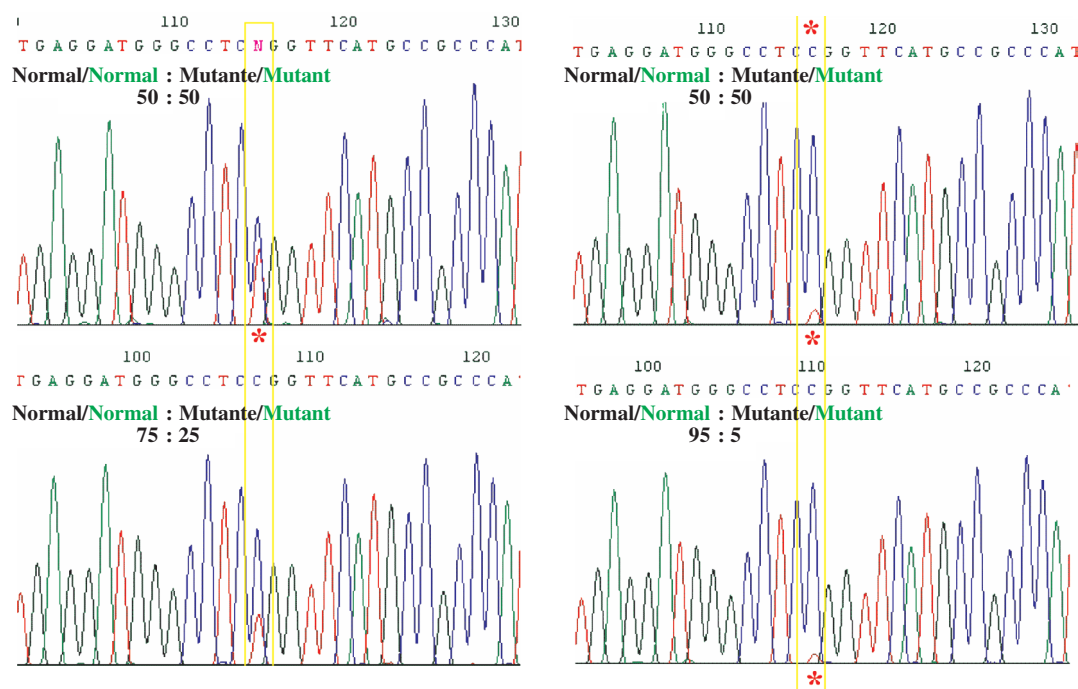


Fig. 2 – Avaliação da sensibilidade do método de detecção de mutações. Pormenores dos electroforetogramas correspondentes à sequenciação do exão 7 de diferentes misturas de produtos de PCR com sequências normal e mutante (M13). Para percentagens de ADN normal superiores a 90%, a identificação da mutação é pouco fiável.

Fig. 2 – Evaluation of the sensitivity of the mutation detection method. Details of the electrophoretograms corresponding to sequentiation of exon 7 of different mixtures of the PCR products with normal and mutant sequences (M13). Identification of mutation is not very reliable for percentages of normal DNA above 90%.

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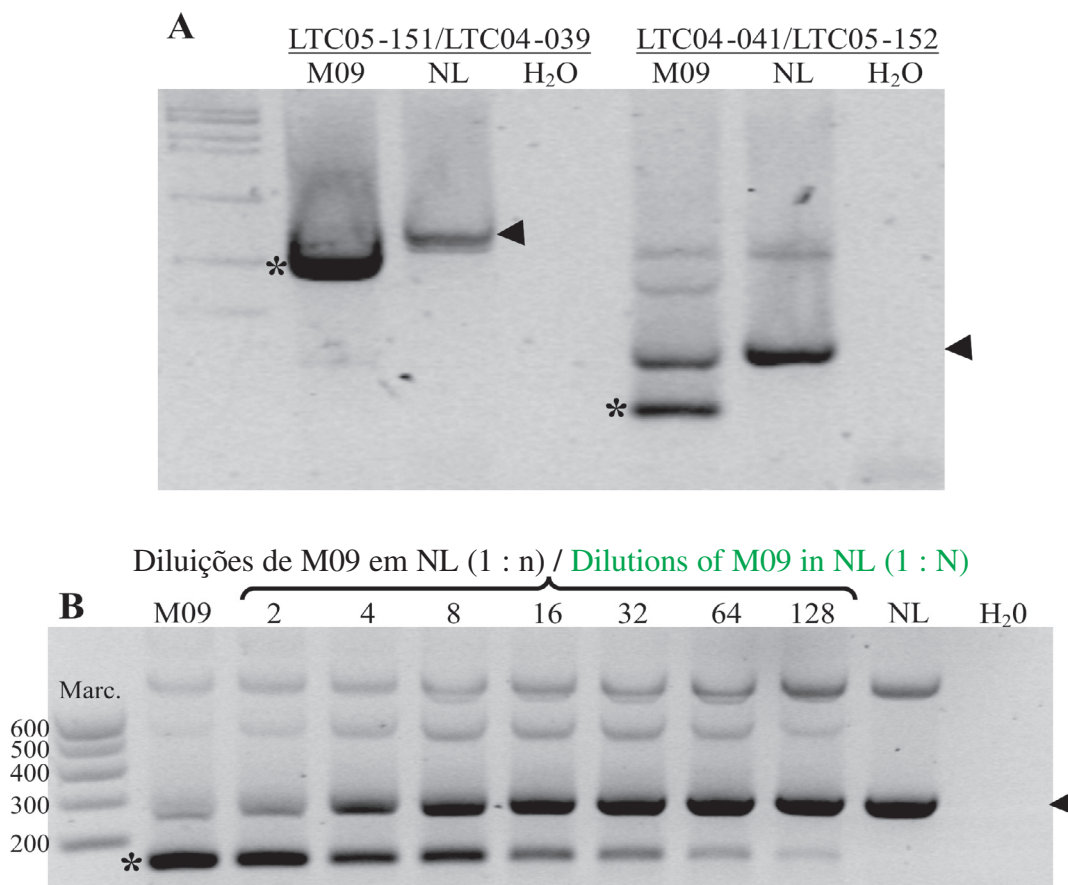


Fig. 3 – Detecção de mutações específicas em amostras contendo pequenas fracções de ADN mutante. **A** – Teste de pares de oligómeros para amplificação específica de ADN mutante. As bandas de tamanhos correspondentes aos produtos de amplificação específicos da sequência mutante do p53 estão assinaladas com um asterisco. As reacções de PCR originam ainda outros produtos (pontas de seta). **B** – Amplificação específica da sequência mutante, a partir de diluições sucessivas de ADN mutante (M09) em ADN normal (NL), usando o par de oligos LTC04-041/LTC05-152 (155pb). A sequência mutante (*) é detectável até uma diluição de 1:128. É visível como, paralelamente à redução de produto específico, se dá um aumento na quantidade de produto inespecífico (ponta de seta).

Fig. 3 – Detection of specific mutations in samples containing small fractions of mutant DNA. **A** – Oligomer pairs test for specific amplification of mutant DNA. The bands with sizes corresponding to the specific amplification products of the mutant p53 sequence are marked with an asterisk. The PCR reactions give rise to yet more products (arrowheads). **B** – Specific amplification of mutant sequence, from the successive dilutions of mutant DNA (M09) in normal DNA (NL), using oligo pair LTC04-041/LTC05-152 (155pb). The mutant sequence (*) is detectable up to a dilution of 1:128. It is visible when, in parallel with reduction of specific product, the quantity of nonspecific product is increased (arrowhead).

Discussão e conclusões

O cancro do pulmão é um grave problema de saúde pública que afecta cerca de um milhão e duzentas mil e mata um milhão e cem mil pes-

Discussion and conclusions

Lung cancer is a serious public health problem affecting around one million, two hundred thousand people and killing one million,

soas por ano a nível mundial²¹. Uma vez que, na sua esmagadora maioria (85-90%), estes casos estão associados ao consumo do tabaco, a alteração radical dos hábitos tabágicos seria, em abstracto, a medida com maior impacto nestes números²². No entanto, dada a dificuldade em alterar os comportamentos humanos, e ainda o facto de o cancro do pulmão ser uma doença que se desenvolve durante um longo período de tempo, não parece provável conseguir, através apenas de medidas preventivas, uma redução drástica na incidência e mortalidade por cancro de pulmão nas próximas décadas. Do lado da terapia, constata-se – aliás como para outros tipos de tumores – que a ablação cirúrgica representa de longe a melhor opção, indicando que é fundamental melhorar, por um lado, o diagnóstico e, por outro, os tratamentos não cirúrgicos. Em teoria, o progresso da genética molecular dos tumores deve abrir novas possibilidades em ambos os domínios, já que permitirá: 1) Uma classificação mais precisa dos tumores, com as consequentes vantagens ao nível do prognóstico e da escolha das abordagens terapêuticas; 2) A identificação de alvos terapêuticos, contribuindo assim para o desenvolvimento e aplicação de novos fármacos mais específicos e eficazes; 3) A identificação de grupos de indivíduos com risco acrescido de desenvolvimento da doença; 4) A detecção precoce – potencialmente por métodos não invasivos – dos tumores; 5) O estabelecimento de métodos mais sensíveis de acompanhamento de doentes para avaliação da evolução da doença, incluindo detecção “precoce” de recidivas; 6) Melhorar a sensibilidade na avaliação de amostras clínicas, como margens cirúrgicas ou biópsias ganglionares, com as consequentes melhorias no estadiamento e tratamento da doença. Embora algumas destas aplicações não sejam ainda possíveis, em particular no cancro

one hundred thousand people per year worldwide²¹. Seeing as the overwhelming majority (85-90%) of these cases is associated to smoking, a sweeping change in smoking habits would in theory be the measure to have the greatest impact on these numbers²². This said, given the difficulty in changing human behaviour, and also given the fact that lung cancer is a disease which develops over a long period of time, it does not seem likely that a drastic reduction in the incidence and mortality rate of lung cancer will be achieved through preventative measures only over the coming decades. In terms of treatment, it is felt – just as it is for other types of tumours – that surgical excision is by far the best option, meaning that it is fundamental to improve both the diagnostic process and the non-surgical treatments. In theory, progress in the molecular genetics of tumours should open up new possibilities in both domains, as it allows: 1) A more precise classification of tumours, which has advantages for prognosis and choice of treatment option; 2) The identification of therapeutic targets, adding to the development and application of new, more specific and efficacious pharmaceutical products; 3) The identification of groups of individuals with a higher risk of developing the disease; 4) The early detection of tumours, potentially through non-invasive methods; 5) The establishing of more sensitive methods of monitoring patients to evaluate progression of the disease, including early detection of reoccurrences; 6) Improved sensitivity in the evaluation of clinical samples, such as surgical margins or ganglia biopsies, which brings better prospects for the disease’s staging and treatment. While some of these applications are not yet possible, particularly in lung cancer, which is less understood at a molecular

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do pulmão, por ser menos compreendido a nível molecular do que outros tumores (como o cancro do cólon e recto), parece-nos que estão já reunidas as condições técnicas para a sua introdução na rotina clínica.

O presente trabalho, inserido no nosso plano de levar a cabo a transposição de técnicas do laboratório de investigação para a prática clínica, teve como objectivo primário identificar as principais dificuldades de ordem prática a essa implementação. Neste sentido, os resultados apresentados no Quadro II são muito esclarecedores. Em primeiro lugar, o número de doentes dos quais foi possível recolher pelo menos uma amostra clínica para análise molecular durante um período de 13 meses foi de apenas 15, ou pouco mais de um por mês, o que é muito inferior ao número de casos diagnosticados no mesmo período. Segundo, em cerca de 20% dos casos não foi possível purificar ADN das amostras e noutros 10% o ADN obtido não era de suficiente qualidade para a análise, o que reduziu o número de doentes efectivamente incluídos no estudo a doze – menos de um por mês. Terceiro, mesmo considerando apenas estes doze doentes, o número total de amostras foi pequeno: 20, de um máximo possível de 48, na maioria dos casos estando apenas uma amostra de cada doente disponível para análise molecular. Todos estes dados mostram que o principal obstáculo à introdução da genética molecular na rotina clínica por nós encontrado foi a dificuldade de obtenção de amostras, em particular com ADN de qualidade. Para tentar resolver este problema, é necessário compreender as suas causas. Nalguns casos, a ausência de amostra será devida às particularidades do exame de diagnóstico: se, por exemplo, não for observada uma área suspeita durante a bron-

level than other tumours (such as cancer of the colon and cancer of the rectum), we feel the applications already meet the technical conditions that will allow them to be introduced into routine clinical practice.

Our study is part of our plan to transpose laboratory investigation techniques into clinical practice. As such, its main aim is to identify the main practical barriers to this implementation. Accordingly, the results shown in Table II bring a great deal to bear here. Firstly, during a 13 month period the number of patients from whom it was possible to collect at least one clinical sample for molecular analysis was only 15. This translates as just over one per month, which is markedly lower than the number of cases diagnosed in the same time period. Secondly, it was not possible to purify the DNA in the samples in approximately 20% of the cases and in a further 10% the DNA obtained was not of sufficient quality to analyse. This cut down the number of patients effectively included in the study to twelve – less than one per month. Thirdly, even considering only these twelve patients, the total number of samples was small; 20 from a possible maximum of 48 and in the majority of cases, only one sample from each patient was available for molecular analysis. All these data evidence that the main barrier we found to the introduction of molecular genetics into clinical routine was the difficulty in obtaining samples, in particular those with quality DNA. It is necessary to understand the causes of this problem to try to solve it. In some cases, the lack of a sample is a result of the characteristics of the diagnostic exam. For example, if a suspect area is not seen during the bronchoscopy, a biopsy is not carried out, or if a patient experiences problems during the ex-

cofibroscopia, não é feita uma biópsia; ou, se o doente tiver problemas durante o exame, este pode ter que ser interrompido antes de se obterem todas as amostras pretendidas. No entanto, este tipo de situação dificilmente pode explicar o baixo número de doentes incluídos no estudo. A explicação mais plausível é que o reduzido número de amostras se deve à não incorporação da colheita na rotina clínica – o que pode igualmente explicar a significativa percentagem de amostras que não fornecem ADN de qualidade (ou o não fornecem de todo); estas resultam provavelmente da secundarização das amostras para análise molecular em relação às usadas para diagnóstico anatomopatológico. Evidentemente, esta secundarização – que tem a sua expressão mais evidente na utilização das amostras inicialmente destinadas a análise molecular para o diagnóstico anatomopatológico, em caso de necessidade – é plenamente justificada do ponto de vista ético, já que é no melhor interesse do doente. Contudo, ela dá origem a uma situação de difícil resolução: a análise molecular é secundarizada porque não é parte do *standard* da rotina (se o fosse não se utilizariam para outros fins amostras a ela destinadas) e essa secundarização é o principal obstáculo à sua introdução na rotina. A resolução do problema deverá passar por uma sensibilização dos clínicos para a utilidade das análises moleculares; deste modo, será possível aumentar gradualmente o número de amostras recolhidas e, consequentemente, estabelecer e otimizar protocolos que consolidem a genética molecular como meio auxiliar de diagnóstico.

Os resultados por nós obtidos deverão, esperamos, contribuir para este processo de sensibilização. De facto, mesmo com o protocolo relativamente simples utilizado, foi possível

amination, the examination could be halted before the desired samples are obtained. While these types of troublesome situations may perhaps go a little way towards explaining the low number of patients included in the study, the most plausible explanation is that the reduced number of samples is due to sample collection not being part of clinical routine. This could equally explain the significant percentage of samples which did not yield quality DNA (or any at all); these probably came from secundarisation of the samples for molecular analysis in relation to the samples used for anatomic pathology diagnosis. It is evident that this secundarisation, most strongly expressed when samples originally destined for molecular analysis are used for anatomic pathology diagnosis, should this need arise, is fully justified from an ethical viewpoint in that it is in the patient's best interest. However, it gives rise to a situation that is difficult to resolve; the 'Cinderella' status of molecular analysis is a result of this form of analysis playing no part in standard routine practice. If it did, samples scheduled for molecular analysis would not be used for other purposes. This secundarisation is the biggest barrier to introducing molecular analysis into routine practice. Solving the problem must involve sensitising clinics to the usefulness of molecular analysis. This would make it possible to gradually increase the number of samples collected and consequently establish and optimise protocols which firm up the role of molecular genetics as an auxiliary diagnostic tool.

The results we obtained will, we hope, serve as a contribution to this sensitisation process. On this head, even with the relatively simple protocol used, detecting mutations in the sam-

detectar mutações nas amostras de um terço dos doentes (4 em 12). Se, por outro lado, se excluirmos os dois casos em que as únicas amostras disponíveis eram de expectoração – para as quais, por razões técnicas (vide abaixo), é provável que o método utilizado não tenha sensibilidade suficiente – e se tiver em conta que a percentagem de mutação do p53 no cancro do pulmão ronda os 60%, verifica-se que a *aplicação prática* da análise molecular foi muito bem sucedida, com uma eficiência próxima do máximo teórico²³.

Uma análise mais pormenorizada dos resultados apresentados nos Quadros III e IV dá uma ideia mais precisa do que já foi conseguido e do que é importante melhorar na metodologia de análise molecular utilizada. Do lado positivo, constata-se que se detectaram mutações em todas (4/4) as amostras de biópsias; que a fracção de sucesso diagnóstico exclusivamente por análise molecular (4/10, excluindo as expectorações) é comparável às verificadas para análise anatomopatológica de escovados brônquicos (3/7) ou lavados brônquicos (2/6); que em nenhum caso há evidência de se ter falhado a detecção de uma mutação numa amostra em que ela estava presente – já que as duas amostras de escovados brônquicos negativas na análise molecular e positivas na análise anatomopatológica podem corresponder a tumores sem mutações no p53. Por outro lado, estas duas amostras efectivamente indicam a principal limitação da metodologia empregue (e, em certa medida, da análise molecular disponível para o cancro do pulmão): sensibilidade. Quando se consideram apenas as amostras disponíveis tanto para análise anatomopatológica como molecular, esta identificou 4 dos 6 casos diagnosticados por aquela. Se é verdade que a ausência de mutações

ples of a third of the patients (4 out of 12) was possible. Moreover, if we exclude the two cases in which the only samples available were sputum samples (for which it is probable that the method used for technical reasons did not have the required degree of sensitivity, see below) and bear in mind that the percentage of p53 mutation in lung cancer is around 60%, it can be seen that the practical application of molecular analysis was very successful, showing an efficacy close to that of its theoretical maximum²³.

The more detailed analysis of the results which is given in Tables III and IV conveys a more precise idea of that which was achieved and that which it is important to improve in the molecular analysis methodology used. On a plus note, if mutations were detected in all (4/4) of the biopsy samples, then the success rate for diagnosis exclusively by molecular analysis (4/10, with the sputum samples excluded) is comparable to that seen in the anatomic pathology analysis of bronchial brushings (3/7) or bronchial lavages (2/6). Further, no case bore evidence of a failed detection of a mutation in a sample in which it proved to be present, if we consider that the two bronchial brushing samples which were negative in the molecular analysis and positive in the anatomic pathology analysis could correspond to tumours without p53 mutations. Conversely, these two samples effectively indicate the main limitation of the methodology employed; sensitivity, a limitation to some degree of the molecular analysis available for lung cancer. When we consider only the samples available for both anatomic pathology analysis and molecular analysis, the latter's sensitivity allowed identification in 4/6 cases,

A identificação de alterações moleculares em genes específicos permite, mais do que o diagnóstico, uma classificação mais completa dos tumores

no p53 pode explicar os restantes dois casos, isso implica que a pesquisa de mutações somente no p53 – pelo menos tal como empregue aqui – é menos sensível do que a análise anatomopatológica. Isto, por sua vez, pode parecer sugerir que não há vantagem na utilização presente das técnicas moleculares em cancro do pulmão. Para esclarecer este ponto, devemos, em primeiro lugar, salientar que o p53 foi escolhido apenas como um exemplo, para testar a possibilidade de introdução da genética molecular na rotina clínica. Existem múltiplas alterações genéticas nos tumores do pulmão, e espera-se que a lista de alvos genéticos a testar venha a crescer rapidamente, em particular em resultado de buscas em larga escala⁸. Para além disso, e como foi referido, a identificação de alterações moleculares em genes específicos permite, mais do que o diagnóstico, uma classificação mais completa dos tumores – o que tem implicações no prognóstico e nas decisões terapêuticas. Mesmo no caso do p53, há evidência nesse sentido, embora aparentemente só para tumores em estádios mais precoces, mas no caso do EGFR (e outros deverão seguir-se-lhe) as coisas são muito mais claras, com excelentes respostas à terapia nos casos de tumores com mutações específicas na zona do gene que codifica o domínio catalítico da proteína^{18,24,25}.

Há, no entanto, um segundo problema de sensibilidade que não pode ser resolvido com o estudo de mais alvos moleculares. Como já referido, as discrepâncias observadas nos resultados da análise molecular de diferentes amostras dos mesmos doentes (Quadros III e IV) podem ser devidos a uma baixa sensibilidade do método de pesquisa de mutações utilizado – que estimamos poder produzir falsos negativos para amostras com

as compared with the former's 6/6. If it is correct that the absence of p53 mutations can explain the remaining two cases, this implies that the investigation of p53 mutations only – at least as used here – is less sensitive than anatomic pathology analysis. This in its turn could suggest that there is no advantage in the current use of molecular techniques in lung cancer. To clarify this point, we should firstly underline that the p53 was only chosen as an example, to test the possibility of introducing molecular genetics into clinical routine. There are multiple genetic alterations in lung tumours and it is expected that the list of genetic targets to test will grow rapidly, in particular as a result of large-scale searches⁸. Beyond this, and as mentioned above, the identification of molecular alterations in specific genes allows more than diagnosis; it allows a more complete classification of tumours, which has implications for prognosis and treatment options. While there is evidence of this even in the case of p53, although seemingly only for tumours at early stages, things are much clearer in the case of EGFR (to be followed by others), with excellent responses to treatment in the cases of tumours with specific mutations in the zone of the gene which codifies the catalytic domain of protein^{18,24,25}. There is also a second problem of sensitivity, one which cannot be resolved by the study of more molecular targets. As already stated, the discrepancies seen in the results of the molecular analysis of different samples from the same patients (Tables III and IV) may be due to the low sensitivity of the method of research into the mutations which was used. We estimate this could yield false negatives in

percentagens de ADN mutante inferiores a 10%. Do mesmo modo, não podemos excluir que os dois casos com discrepâncias entre as análises molecular e anatomopatológica sejam devidos a esta limitação de sensibilidade. Finalmente, afigura-se-nos muito provável que as amostras de expectoração contenham uma fracção relativamente pequena de ADN tumoral – e portanto que este possa não ter sido detectado, ainda que contivesse mutações no p53. Isto significa que o protocolo por nós adoptado é adequado para amostras ricas em ADN tumoral – como em geral se espera que sejam as biópsias –, mas a sua sensibilidade pode tornar-se limitante à medida que a fracção de ADN tumoral vai diminuindo. Esta limitação de sensibilidade pode ser superada através de diferentes abordagens – consoante a aplicação pretendida. Uma delas, por nós adoptada neste estudo, consiste em empregar métodos de detectar mutantes *específicos*. Estes métodos, já anteriormente usados em cancro do pulmão²⁶, são actualmente capazes, graças a melhoramentos significativos nos últimos anos, de detectar uma molécula mutante entre dez mil normais^{27,28}. São adequados para aplicações em que a mutação em causa foi previamente identificada, como análises de margens cirúrgicas e gânglios linfáticos, ou detecção e quantificação de doença residual – e, consequentemente, de detecção precoce de recidiva –, inclusivamente a partir de amostras não invasivas^{26,29-31}. São menos apropriados para aplicações como a detecção precoce de doença, ou a identificação de subgrupos de risco, a não ser nos raros tumores frequentemente associados a mutações muito específicas³². Para a maior parte dos tumores, como o cancro do pulmão, existem três opções principais: 1) A modifi-

samples with mutant DNA percentages lower than 10%. In the same way, we cannot exclude that the two cases with discrepancies between the molecular and anatomic pathology analysis may be due to this limit in sensitivity. Finally, we deem it very likely that the sputum samples contain a relatively small amount of tumoral DNA, which may not have been detected even if it contained p53 mutations. This means that while the protocol we adopted is suitable for samples rich in tumoral DNA – as biopsies are generally expected to be – its sensitivity could become limiting as the tumoral DNA fraction decreases. This limitation to the sensitivity could be overcome through different approaches used in line with the application desired. One of them, which we adopted in this study, consists of using methods of detecting specific mutants. These methods, already used in lung cancer²⁶, are currently capable of detecting one mutant molecule among ten thousand normal ones, thanks to significant improvements in the last few years^{27,28}. They are suitable for applications in which the mutation in cause has been previously identified, such as analysis of the surgical margins and lymphatic ganglions, or the detection and quantification of the residual disease – and consequently of early detection of reoccurrence – solely from the non-invasive samples^{26,29-31}. They are less suitable for applications such as the early detection of disease, or the identification of risk sub-groups, unless in the rare tumours commonly associated to very specific mutations³². There are three main options for the greater part of tumours, such as lung cancer: 1) The mod-

cação do procedimento de identificação de mutações por nós utilizado por inclusão de um passo de fraccionamento dos produtos amplificados (por exemplo por clonagem), seguida de análise de muitas fracções³³ – uma opção difícil de transpor para a rotina clínica, dada o seu elevado custo em mão-de-obra e recursos materiais. 2) O desenvolvimento de métodos de detecção de mutações baseados em testes de função proteica, em microrganismos ou células de mamíferos – uma abordagem ainda muito experimental que pretendemos explorar a médio prazo. 3) A optimização de métodos de detecção simultânea de múltiplas mutações específicas – que, conjugada com dados estatísticos sobre a ocorrência de mutações²³, deverá permitir a detecção precoce de um número crescente de tumores. Como decorre dos resultados aqui apresentados, os nossos esforços deverão concentrar-se, no futuro próximo, no estabelecimento deste tipo de metodologias e sua aplicação no refinamento do diagnóstico, no seguimento de doentes e na implementação de programas de rastreio de cancro do pulmão com base molecular.

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ification of the mutation identification procedure we used, to include a step of fractioning of the amplification products (by cloning, for example), followed by analysis of the fractions³³ – a option which is difficult to transpose to routine clinical practice given its high costs in terms of manpower and material resources. 2) The development of mutation detection methods based on protein function tests in micro-organisms or mammalian cells – a still very experimental approach which we intend to explore in the medium-term. 3) The refinement of simultaneous detection methods of specific multiple mutations which, taken in conjunction with statistical data on the occurrence of mutations²³ should allow the early detection of an increasing number of tumours. As the results we present show, our efforts should focus in the near future on the establishing of these types of methodologies and their use in refining diagnosis, monitoring patients and the implementation of molecular-based lung cancer tracking programmes.

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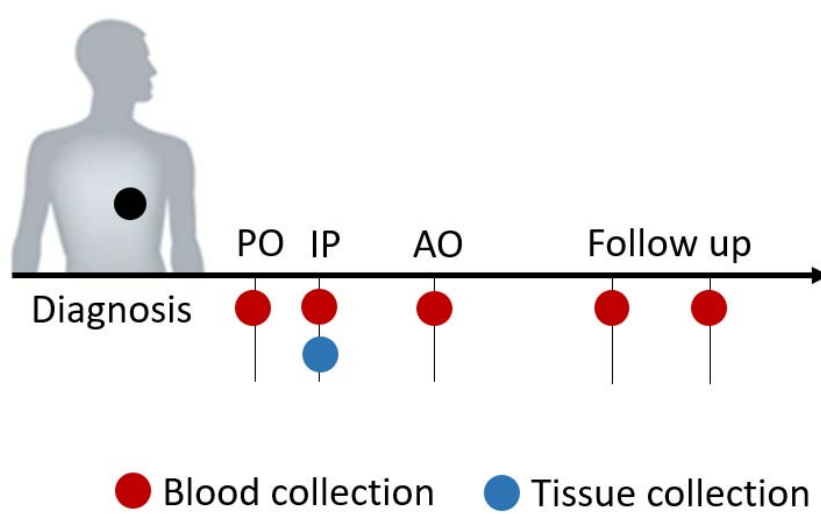
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Chapter 2 - Implementation of a Pilot Study to Analyze Circulating Tumour DNA in Early-Stage Lung Cancer



PO – Pre-operative
IP – Intra-operative
AO – After operation

Implementation of a Pilot Study to Analyze Circulating Tumor DNA in Early-Stage Lung Cancer

Implementação de um Estudo Piloto para Análise de ADN Tumoral Circulante no Cancro do Pulmão em Estádio Inicial

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ABSTRACT

Introduction: Liquid biopsies based on plasma circulating tumour deoxyribonucleic acid (ctDNA) have shown promise in monitoring lung cancer evolution. The expression of ctDNA across time, its relationship with clinicopathological parameters and its association with lung cancer progression through imaging allow us to weigh how useful ctDNA could be in monitoring surgically resectable lung cancer. The aim of this study was to assess the impact of ctDNA analysis implementation in early-stage lung cancer.

Methods: A cohort of 47 patients was sequentially recruited. Only 34 patients with early-stage lung cancer were included. All patients had a tissue specimen and five blood samples drawn: at the preoperative stage, from the pulmonary vein, at surgical discharge, at the first follow-up and at the last follow-up. All blood samples were evaluated for ctDNA expression.

Results: On average, the maximum yield of ctDNA was obtained in liquid biopsies at the surgical discharge of patients when compared with PO, PV, and F1 ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$ respectively). No statistically significant differences were found when comparing the last follow-up to surgical discharge ctDNA expression ($p = 0.851$). The correlation between ctDNA concentration according to five-time points and the four clinicopathological characteristics showed that patients younger than 70 years had a statistically significant reduction of the concentration of ctDNA at the preoperative and surgical discharge time point [$\beta = -16\,734$ (-27 707; -5760); $p = 0.003$; $\beta = -21\,785$ (-38 447; -5123); $p = 0.010$], as opposed to an increase of the concentration of ctDNA at the pulmonary vein and last follow-up time points [$\beta = 8369$ (0.359; 16 378); $p = 0.041$; $\beta = 34\,402$ (12 549; 56 254); $p = 0.002$] all with a confidence level of 95%. In the cases where actionable mutations were identified in tissue biopsies, the expected mutation was found in five out of six patients plasma samples at the pre-operative time point and in two out of six patients plasma samples at the pulmonary vein time point. Two out of six patients with actionable mutations had disease progression.

Conclusion: The results of this pilot study suggest that the maximum yield of ctDNA is obtained at the surgical discharge of the patients and that the pre-operative timepoint is the one offering the highest sensitivity for the detection of actionable mutations in ctDNA in early-stage lung cancer.

Keywords: Circulating Tumor DNA; Early Detection of Cancer/methods; High-Throughput Nucleotide Sequencing; Lung Neoplasms; Mutation; Neoplasm Staging

RESUMO

Introdução: As biópsias líquidas baseadas no ácido desoxirribonucleico tumoral circulante (ctADN) no plasma têm-se mostrado promissoras na monitorização da evolução do cancro do pulmão. A expressão do ctADN ao longo do tempo, sua relação com parâmetros clínico-patológicos e sua associação com a progressão do cancro do pulmão através da imagem, permitem-nos avaliar o quanto útil o ctADN pode ser na monitorização do cancro do pulmão ressecável cirurgicamente. O objetivo deste estudo foi avaliar o impacto da implementação da análise de ctADN no cancro do pulmão em estágio inicial.

Métodos: Uma coorte de 47 pacientes com cancro de pulmão em estágio inicial foi recrutada sequencialmente. Apenas 34 pacientes foram incluídos. Todos os pacientes colheram uma amostra de tecido e cinco amostras de sangue: no pré-operatório, da veia pulmonar, na alta cirúrgica, no primeiro seguimento e no último seguimento. Todas as amostras de sangue foram avaliadas quanto à expressão de ctADN.

Resultados: Em média, o rendimento máximo de ctADN foi obtido em biópsias líquidas obtidas na alta cirúrgica dos pacientes quando comparado com as colheitas nos momentos pré-operatório, da veia pulmonar, e no primeiro seguimento ($p < 0,0001$, $p < 0,0001$, $p < 0,0001$, respetivamente). Não houve significado estatístico ao comparar as biópsias líquidas obtidas no último seguimento com a expressão do ctADN na alta cirúrgica ($p = 0,851$). A correlação entre a concentração de ctADN nos cinco momentos de colheita e as quatro características clínico-patológicas mostrou que pacientes com menos de 70 anos tiveram redução significativa da concentração de ctADN no momento pré-operatório e na alta cirúrgica [$\beta = -16\,734$ (-27 707; -5760); $p = 0,003$; $\beta = -21\,785$ (-38 447; -5123); $p = 0,010$] em oposição a um aumento da concentração de ctDNA na veia pulmonar e no último seguimento [$\beta = 8369$ (0,359; 16 378); $p = 0,041$; $\beta = 34\,402$ (12 549; 56 254); $p = 0,002$] todos com nível de confiança de 95%. Nos casos em que foram identificadas mutações acionáveis em biópsias de tecido, a mutação esperada foi encontrada em cinco de seis amostras de plasma de pacientes no momento pré-operatório e em duas de seis amostras de plasma de pacientes no momento da veia pulmonar. Dois dos seis pacientes com mutações acionáveis apresentaram progressão da doença.

Conclusão: Os resultados deste estudo piloto sugerem que o rendimento máximo do ctDNA é obtido na alta cirúrgica dos pacientes e que o momento pré-operatório é o que oferece a maior sensibilidade para a deteção de mutações acionáveis no ctDNA no cancro do pulmão em estágio inicial.

Palavras-chave: Deteção Precoce de Cancro/métodos; DNA Tumoral Circulante; Estadiamento de Neoplasias; Mutação; Neoplasias do Pulmão; Sequenciamento de Nucleotídeos em Larga Escala

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INTRODUCTION

Lung cancer (LC) is the deadliest tumor worldwide, with nearly 1.8 million deaths in 2020.¹⁻³ The best subgroup of patients with a chance of surgery with curative intent are those at an early stage (I - IIIA) corresponding to 20% to 25% of the cases of non-small cell lung cancer (NSCLC) diagnosed each year even though radical resection has curative intent and is the cornerstone for patients with early-stage LC, tumor relapse occurs in about 30% to 70% of patients. Adjuvant chemotherapy can reduce the risk of disease recurrence by 16% and increase five-year overall survival by 5.4% when compared with placebo.⁴⁻⁷

Early detection of recurrence is associated with better outcomes. Screening with low-dose computed tomography (CT) has been shown to reduce LC mortality in high-risk population, but its implementation is low due to socio-economical limitations.⁸ Until now, no biomarkers with high specificity and sensitivity could identify patients at high-risk of recurrence, and TNM staging and performance status are the only tools that clinicians can rely on.

In 1948, circulating cell-free tumor deoxyribonucleic acid (cfDNA) was first identified in human blood by Mandel *et al.*⁹ The discovery of cfDNA in blood samples is what has been defined as a form of 'liquid biopsy'. The cfDNA released from cancer patients is referred to as circulating tumor DNA (ctDNA) and is only a portion of all cfDNA. The percentage of ctDNA in overall cfDNA of patients with cancer can range from 0.1% to over 10%. An extremely sensitive technique must be used to detect mutations or other changes present in ctDNA at low variant allele frequencies (AF) of 0.003% or lower.¹⁰

A cancer biomarker is a molecular component that can give us advantages in facing cancer. It should provide prognostic and predictive information, detecting the disease while measurable, within a high-risk population, while it is not yet clinically apparent.¹¹ Despite all this potential and many published studies showing the usefulness of molecular genetic techniques as auxiliary diagnostic tools, the latter are not being used in routine clinical practice in Portugal for early-stage lung cancer.¹² This study is part of our plan to establish and implement protocols for transposing molecular genetic knowledge and techniques from the bench to clinical practice in the medium-term.

The primary aim of this study was to integrate the routine use of ctDNA in lung cancer surgical patients, and to evaluate the quantitative evolution of ctDNA across time and its correlation with clinicopathological variables at five specific data collection time points.

Even though liquid biopsies are a useful strategy to screen for actionable mutations that are routinely used in advanced lung cancer both at the diagnostic and post-progression stages, their usefulness in surgically resectable lung cancer has not yet been addressed. Our second aim was to assess whether cancer cells remaining in the body after surgery will be important in predicting tumor recurrence by assessing the importance of genotyping tumor and plasma samples for actionable mutations where ctDNA is most expressed. Finally, we aimed to assess if ctDNA is a critical tool in the postsurgical management of lung cancer patients with actionable mutations, as well as its usefulness as a guide to detect residual postsurgical ctDNA and disease progression.

METHODS

Study cohort

A prospective, single-center, observational study was conducted by the Centro Hospitalar Universitário de São João (CHUSJ), EPE. The study protocol was approved by the Ethics Committee of Centro Hospital Universitário de São João, EPE on May 11, 2017 (approval number: CES01). The methods were conducted under the precepts of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

All patients had to sign an informed consent form to be included in the study. Eligible patients were either women or men, aged over 18 years old, and had pathological confirmation of early-stage non-small cell lung cancer (as per the criteria of the American Joint Committee on Cancer, 8th edition, criteria). Forty-seven patients were initially recruited from our center between the May 29, 2017 and January 28, 2019. Thirteen patients were excluded as they either did not meet the inclusion criteria or because data for subsequent analysis was not available. This study cohort included 34 patients. The study design was distributed between the Cardiothoracic and the Pulmonology departments. Patients were referred to the Cardiothoracic Department after full diagnosis and staging procedures and accepted for surgical treatment in multidisciplinary thoracic diseases oncologic group meetings. Thirty-four patients with NSCLC underwent radical surgery at the Department of Cardiothoracic Surgery. Information related to clinical and radiological evolution, as well as information related to treatments they had undergone during the disease, and their response, was collected throughout time (Fig. 1).

Sample collection

Tumor samples (TS) were collected during surgical resection. Five blood samples were taken from each of the patients:

at the preoperative peripheral stage (PO), from the pulmonary vein (PV), at surgical discharge (SD), at first follow-up (F1), and last follow-up (LF). Additional samples drawn during follow-up at other intermediate points were also accepted, according to the flowchart (Fig. 1). A total of 34 samples of tumor tissue and 34 peripheral blood samples were collected at the preoperative time point, 34 blood samples were collected during the intraoperative act from the corresponding pulmonary vein, and 34 peripheral blood samples were obtained at surgical discharge. During the surgical period, 102 blood samples were collected, and during follow-up, a total of 108 samples were withdrawn. This total 34 tissue samples and 210 viable blood samples that were sent for processing to the I3S Institute. Another 210 blood samples were stored in the tumor bank of the Hospital of São João in the Department of Pathology, for later use, if necessary for further evaluations. The final number of samples analyzed was 210 (Fig. 2).

Laboratory procedures

The tumor sample was collected immediately after surgical resection. Histological specimens were fixed with formalin (formalin-fixed paraffin-embedded tissue, FFPE). After pathological and immunohistochemical evaluation, samples were used for DNA extraction using the QIAamp DNA Mini Kit. DNA was quantified with NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) or Qubit® 2.0 Fluorometer (Invitrogen, Waltham, MA, USA).

Blood samples collected into EDTA tubes were processed within one to four hours after withdrawal, were centrifuged at 1600 g/min for 10 minutes, and peripheral blood lymphocytes were separated and stored at -80°C until use. The plasma was collected by centrifuging the supernatant from the blood samples again at 18 000 g/min for five minutes and was stored at -80°C until further use.¹³⁻¹⁷ Ion Ampliseq Colon and Lung panel™ were used for tissue biopsies and OncoPrint lung circulating free tumor DNA assay for circulating free tumor DNA (cfDNA) samples. All amplified products will be used to prepare libraries and sequenced using the ion PGMTM or S5TM system. The QuantStudio 3D Digital PCR system TM will be used to confirm selected results.

Statistical analysis

The sample size was limited by the availability of specimens for subsequent analysis following a sequential standard molecular diagnostic approach. Most of the analysis was descriptive. Categorical data were described as absolute (n) and relative frequencies. Medians, interquartile ranges (IQR), and minimum and maximum values were determined for continuous variables. Non-parametric Wilcoxon-matched pairs signed-rank tests were used to infer the difference in the quantity of ctDNA (ng/mL) present in the blood of lung cancer patients at different and between different data collection time points (PO, PV, SD, F1 and LF). Significance values were adjusted using the Bonferroni correction for several tests. A Kruskal-Wallis test, a non-parametric version of the one-way ANOVA model, was performed between all-time points. Linear regression models were developed to estimate the effect of the four categorical variables (age, sex, smoking status, tumor size) in ctDNA concentration during each specific data collection time point. Additionally, we created a linear regression model with the same equation and repeated measurements to consider all time points in the same analysis. In order to account for the potential effect of outliers in all models, we used a robust estimator of the effects instead of a model-based estimator.

Molecular relapse ctDNA progression was defined as the quantity of ctDNA (ng/mL) present in the plasma samples at the last follow-up greater than at surgical discharge. Imaging features of relapse were defined according to the comparative assessment of the last CT evaluation and the CT performed after surgery.

RESULTS

Clinicopathological characteristics

Over 20 months, 47 patients were assessed for eligibility in this study, starting on May 29, 2017 until January 28, 2019. Thirteen patients were excluded according to inclusion criteria (Fig. 2). This cohort of patients included 34 patients, 15 of the female sex (44.12%) and 19 of the male sex (55.88%), with a median age at diagnosis of 64.5 years, with an interval between 50 and 79 years of age. The mean follow-up time was 699.88 days, and the median follow-up time was 809.50 days (range: 3 to 1337 days).

Concerning smoking habits, 13 were non-smokers (38.23%), and 21 were former and active smokers (61.77%). According to the clinical stage of the tumor in question, we observed that cT1N0M0 was the predominant stage with twenty-one patients included (55.2%), from a cohort of 34 patients. Surgically, one patient underwent left pneumectomy, three bilobectomy, and thirty patients with lobectomy and mediastinal lymph node emptying in all cases. According to the pathological stage of the 32 naive patients, 20 patients presented with stage I (62.5%), 11 patients with stage II patients (34.37%), and one patient with stage III (3.13%). Two patients underwent neoadjuvant therapy followed by surgery, one staged as

ypT3N0M0R1 and the second one as ypT0N2M0R0. Twenty-seven patients had a diagnosis of adenocarcinoma (79.41%), six had a diagnosis of squamous cell carcinoma (17.65%), and one patient was diagnosed with large cell neuroendocrine carcinoma and adenocarcinoma (mixed pattern) (2.94 %). Concerning the prognostic factors, the following were considered: venous (V), lymphatic (L), perineural invasion (PN), invasion of the visceral pleura (VP) and residual tumor resection margin (R). The 34 patients presented the following results: ten patients with no prognostic factors (29.41%), five patients with one factor (14.71%), nine patients with two factors (26.47%), and ten patients with three prognostic factors (29.41%).

Of the 34 patients included in the study, ten patients stayed under surveillance (29.41%), seventeen patients underwent adjuvant chemotherapy (50.00%) and seven patients were lost in the follow-up (20.59%). Until the last evaluation, four patients died (11.76%) and the remaining are alive. Only one of the patients that died was lost during follow-up. All clinical features can be analyzed in Table 1.

Quantification of ctDNA plasma samples over time

Quantitative differences in ctDNA concentrations obtained from plasma were noted between the preoperative stage (PO), extracted from the pulmonary vein (PV), at surgical discharge (SD), at first follow-up (F1), and last follow-up (LF). The average volume of plasma obtained at surgical discharge was 3.85 mL. The absolute values of ctDNA of the 34 patients obtained overtime and CT last evaluation too [Appendix 1 Table A.1 (Appendix 1: <https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/19487/15190>)]. The mean and median molecular follow-up time were 413.8 and 417.0 days (range: 94 to 678 days) respectively. The mean and median imaging follow-up was 764.48 and 834.0 days (range: 150 to 1337 days) respectively.

More ctDNA was shed directly from the tumor bed into the vein that drains blood directly from the tumor when compared to peripheral blood samples obtained before surgery ($PV > PO$; $p = 0.002$) and a greater increase was observed at surgical discharge when compared to the first two withdrawals ($SD > PV$ and $SD > PO$; $p = <0.001$).

At the first follow-up (F1) or baseline assessment, lower ctDNA concentrations were identified when compared to surgical discharge. At surgical discharge (SD), ctDNA concentration when compared with PO, PV, and F1 and the differences were statistically significant ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$ respectively).

At the last follow-up (LF) or during longitudinal monitoring, ctDNA expression increased when compared with PO, PV, and F1 ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$ respectively) (Fig. 3: green lines). No statistically significant differences were observed when comparing the last follow-up to surgical discharge ctDNA expression ($p = 0.851$) and also between the first follow-up and PV or PO (Fig. 3: red lines). Significance values were adjusted using the Bonferroni correction for several tests performed with similar results [Appendix 1 Table A.2 (Appendix 1: <https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/19487/15190>)]. The Kruskal–Walli's test results were inferior to 0.001.

The correlation between ctDNA concentration according to five-time points (dependent variables) and the four clinico-pathological characteristics (categorical variables): sex, age, smoking status, and tumor size was analyzed. The number of patients included at each collection point and the mean time of blood withdrawal was 34 patients at PO, PV and SD, 31 at F1 and 24 at LF. The mean time of blood withdrawal was 0.0, 0.0, 5.5, 33.65, and 413.8 days respectively.

Patients younger than 70 years had a significant reduction of the concentration of ctDNA at the preoperative and surgical discharge time point [$\beta = -16\,734$ (-27 707; -5 760); $p = 0.003$; $\beta = -21\,785$ (-38 447; -5123); $p = 0.010$] with a confidence level of 95%. As opposed to an increase of the concentration of ctDNA at the pulmonary vein and last follow-up time points [$\beta = 8369$ (0.359; 16 378); $p = 0.041$; $\beta = 34\,402$ (12 549; 56 254); $p = 0.002$, with a confidence level of 95%]. The joint model value is $\beta = -9235$ (-15 352; -3118); $p = 0.004$ with a confidence level of 95%. The evaluation of ctDNA according to sex, smoking status, and tumor size at all time points was not statistically significant across all collection points as shown in Table 2.

Tumor mutational profile

Of the 34 patients, only 29 patients had appropriate tumor samples for mutational profiling through the NGS technique. Several mutations and different associations were identified. The allelic frequency was evaluated. Five samples were not processed for technical issues [Appendix Table A.3 (Appendix 1: <https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/19487/15190>)].

Within each tumor, the mutational profile in each tumor sample was determined by the allele frequency. The most predominant mutations were *TP53* and *KRAS* with 30.8% and 34.6%, followed by *EGFR* with 19.2% mutations. Other mutations such as *MET*, *BRAF*, *PIK3CA* and *MPL* were also identified. Three tumor samples showed translocations (EML4-ALK.E13A20).

Correlation of mutational profile of ctDNA from blood samples with tumor samples

Of the 34 patients, only 26 had appropriate ctDNA from blood samples at surgical discharge, where quantitative ctDNA expression was greatest, for mutational profiling. By NGS, no somatic mutations were identified at surgical discharge from the 26 patients.

Because of these results, only blood samples where actionable mutations were detected in tumor tissue were subjected to sequencing analysis at the pre-operative stage, from the pulmonary vein, at surgical discharge and last follow-up. These were cross-checked with the tumor mutational profile. The allelic frequency was evaluated for each one.

We performed NGS of liquid biopsies in cases where actionable mutations in *EGFR* and *BRAF* were identified in tissue biopsies. We found the expected mutation in five out of six patients at the pre-operative time point and in two out of six at the pulmonary vein time point. The mutation allele fraction detected was always very low in the range of 0.1% to 0.2%. During surveillance, two out of six patients showed imaging progression on days 828 and 724 as shown in Table 3.

DISCUSSION

The initial findings of this study led us to understand the differences in expression of ctDNA concentration over time, at specific time points, which is particularly relevant during the first week post-surgery.

According to the literature, the volume of plasma extracted is important so that a successful amount of ctDNA can be extracted. According to Crowley *et al*, the most frequently used protocols to obtain ctDNA required approximately 1 mL of plasma (3 mL of blood).¹⁸ In the studies by Veldore and Messaoudi a mean average of approximately 10 mL of plasma volume was required to increase analytical sensitivity.^{17,19} However, no standard collection volume has been established. At surgical discharge, our samples had a mean of 3.85 mL of plasma like in the study by Gale *et al*.²⁰

Regarding ctDNA concentration at surgical discharge, higher concentrations of ctDNA were observed due to surgery where more ctDNA was shed resulting from post-surgical trauma as observed in the study by Abbosh.²¹ The mean time for blood withdrawal was 5.5 days (range: 4 - 12 days) in our study when compared to the study by Abbosh where plasma samples were collected two to five days after surgery.

At the first follow-up (F1) or a landmark time point, a lower concentration of ctDNA was detected when compared to SD and LF (Fig. 3), at a mean of 33.65 days (range: 17 to 109 days). At this point, however, the results could not be affected by surgical trauma. This was also observed in the study by Chaudhuri²² where blood was collected within four months of treatment completion, and one month after surgery according to the studies by Zang and Li.^{23,24} At F1, a smaller interquartile amplitude was observed as well as at the PO and PV data collection points as confirmed in Fig. 3. The first follow-up is probably the best time to consider ctDNA post-surgery samples, as a prognostic biomarker of early-stage lung cancer.

Nevertheless, the patients that showed an increase of ctDNA at the last follow-up or during longitudinal monitoring at a mean of 413.8 days (range: 94 to 678 days) [Appendix 1, Table A.1 (Appendix 1: <https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/19487/15190>)], were those that eventually corresponded to the concept of molecular progression, or as a negative predictive biomarker of response to curative surgery. These results reflect those observed in the study by Chaudhuri, where samples were collected every 60 to 180 days after curative-intent treatment.²² During the last follow-up, these patients did not suffer surgical trauma or other traumatic interventions which could induce the release of ctDNA into circulation. The best probable timing to consider withdrawing blood samples for ctDNA quantification post-surgery is during intermediate time points until the last follow-up, where seven patients showed molecular relapse and CT progression [Appendix 1, Table A.1 (Appendix 1: <https://www.actamedicaportuguesa.com/revista/index.php/amp/article/view/19487/15190>)]. The ctDNA concentration at LF may be considered a predictive biomarker of tumor recurrence after curative surgical treatment. When comparing F1 with PV and PO, no statistically significant differences were found, neither between LF and SD; probably due to the expression of ctDNA, at each time point (Fig. 3; red lines). In between these, statistically significant differences were found as shown in Fig. 3 (green lines).

Considering the different time points, a greater tendency of expressing ctDNA with statistical significance was found in patients older than 70 years, as expected due to biological factors such as DNA damage over time and shortening telomeres, or in other words, physiological aging.^{25,26}

As described in the literature, approximately 53% of lung cancer cases occur in individuals between 55 to 74 years old and 37% occur in individuals over 75 years old, with the median age at diagnosis being 70 years old.²⁶ This justifies the age cut-off considered in this study. Our cohort of patients had a median age at diagnosis of 64.5 years, with an interval between 50 and 79 years of age. The increase of ctDNA concentration favoring patients younger than 70 years from the pulmonary vein time-point can be physiologically justified considering the tumoral drainage area. Although the median age of these patients at pulmonary vein time-point (n = 34) and at the last follow-up (n = 24) was, respectively, 64.5 years and

62.5 years, this could eventually support the increase of ctDNA concentration favoring patients younger than 70 years.

Moreover, the mutational profile analysis was performed through the NGS technique in tissue and plasma samples at surgical discharge where quantitative expression of ctDNA was greatest. Mutated allelic frequency (MAF) is the best way to evaluate ctDNA over time. MAF is important when facing two oncogenic driver mutations, mutations that are responsible for initiation of cancer, helping to define the predominant one, namely which one is leading to progression. Regarding the study by Veldoure *et al*, a concordance with an accuracy of 96.97% was observed between the mutational profile of tissue and plasma samples through allele-specific real-time PCR and NGS techniques.⁴

Although surgery offers the best chance of cure from early-stage non-small cell lung cancer, which corresponds to approximately 16% of all lung cancer patients⁴, many patients still suffer from recurrent disease which is thought to be due to the presence of minimal or molecular residual disease. Today's standard of care as postoperative adjuvant treatment for completely resected stage I-III NSCLC is platinum doublet chemotherapy which results in a 5% increase in 5-year survival.²⁷ On the other hand, 95% of patients are either those whose disease cannot be cured by the adjuvant treatment or those who are cured by surgery alone and thus do not require adjuvant therapy. Considering resected epidermal growth factor receptor (EGFR) mutated NSCLC patients', a recent meta-analysis has shown improved disease-free survival and a non-significant improvement in overall survival, in patients who received adjuvant EGFR inhibitors after curative surgery compared to those who received chemotherapy or no further treatment.²⁸ As well as other recent studies, it was demonstrated that after chemoradiotherapy, patients with detectable ctDNA had better outcomes when treated with immune checkpoint inhibitors than those with undetectable ctDNA following chemoradiotherapy regardless of further immunotherapy.²⁹ The importance of these observations highlights the importance of clinicians escalating or de-escalating therapy according to ctDNA expression after curative treatment.

In our pilot study, mutational profile concordance was not observed at the SD time point and was only observed at the PO and PV time points (with the PO time-point being the one offering the highest sensitivity). This may be justified by the extremely low mutated allelic frequency detected and by its proximity to the sensitivity threshold detection level of NGS.

Despite the limited number of cases, the results observed showed that, even in situations of early-stage lung cancer there is ctDNA identifiable by NGS from the liquid biopsy. The clinical relevance of this observation is still unclear. However, these results should strongly encourage research using non-invasive methods to identify the risk of recurrent tumors after surgery with curative intent, select the optimal adjuvant treatment for each lung cancer patient, minimize toxicity, and improve quality of life and survival. Our study supports the idea that MP concordance may predict the risk of recurrence in resected tumors as later confirmed in CT scan progression in two out of the six patients, and as also shown in recent studies. ctDNA longitudinal analysis appears to be a pioneering model to dynamically predict recurrence in a setting characterized by economic and tissue availability constraints.^{21,22,27}

Several limitations of our study should be acknowledged, namely that a cohort of 34 patients with early-stage LC underwent curative surgical treatment and no control arm was contemplated. A validation study will be necessary to confirm these preliminary results. The viability of the analysis was compromised due to the sample size (210 plasma samples and 24 out of 34 patients with plasma samples at LF), which may justify the low amount of statistically significant results obtained over time. The need for rapid processing creates logistic challenges and the potential for preanalytical variability caused by fluctuations in ctDNA concentration and purity due to differences in processing times. Consequently, samples were excluded from a rigid teamwork scheme under high observation. This study is part of our plan to establish and implement protocols for transposing molecular genetic knowledge and techniques from bench to clinical practice in the medium-term.

CONCLUSION

Our results suggest that the quantitative expression of ctDNA is greatest at surgical discharge and at last the follow-up time point; its' decline at the first follow-up time point is likely due to the elimination of ctDNA post-surgical injury, and this is probably the best timing to consider the value of ctDNA as a prognostic biomarker of early-stage LC.

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AUTHOR CONTRIBUTIONS

JEM: Study design, data collection and analysis, writing of the manuscript.
TTG, JCM, VH: Supervision and critical review.

PROTECTION OF HUMANS AND ANIMALS

The authors declare that the procedures were followed according to the regulations established by the Clinical Research and Ethics Committee and to the Helsinki Declaration of the World Medical Association updated in 2013.

DATA CONFIDENTIALITY

The authors declare having followed the protocols in use at their working center regarding patients' data publication.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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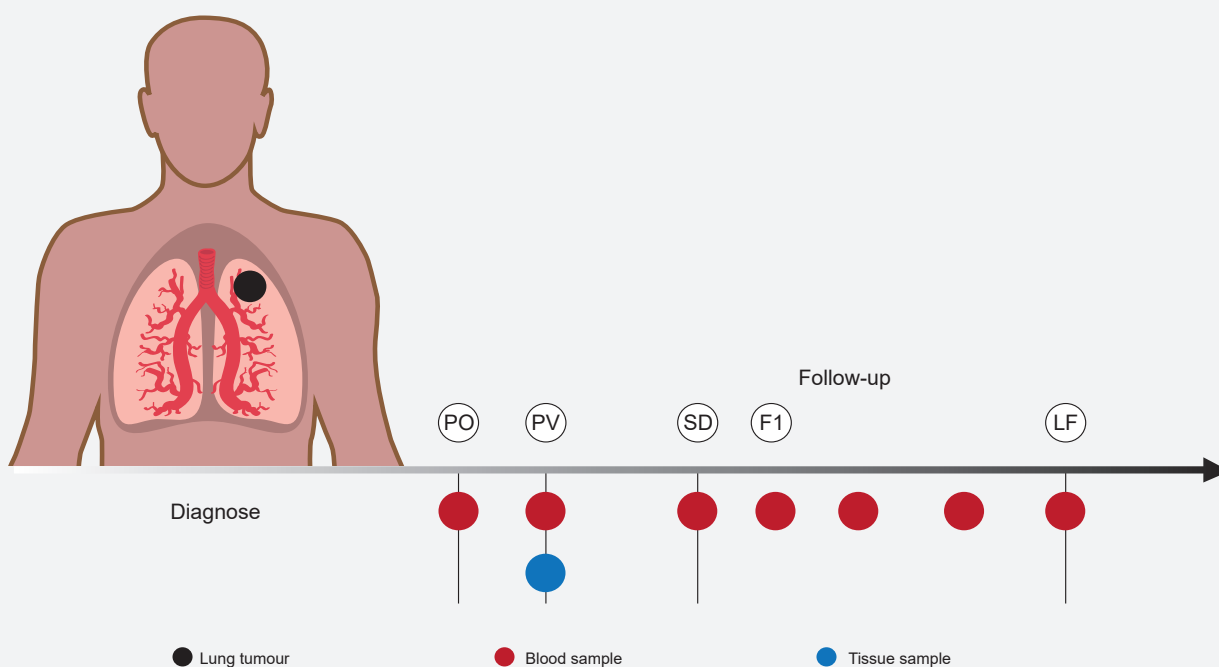
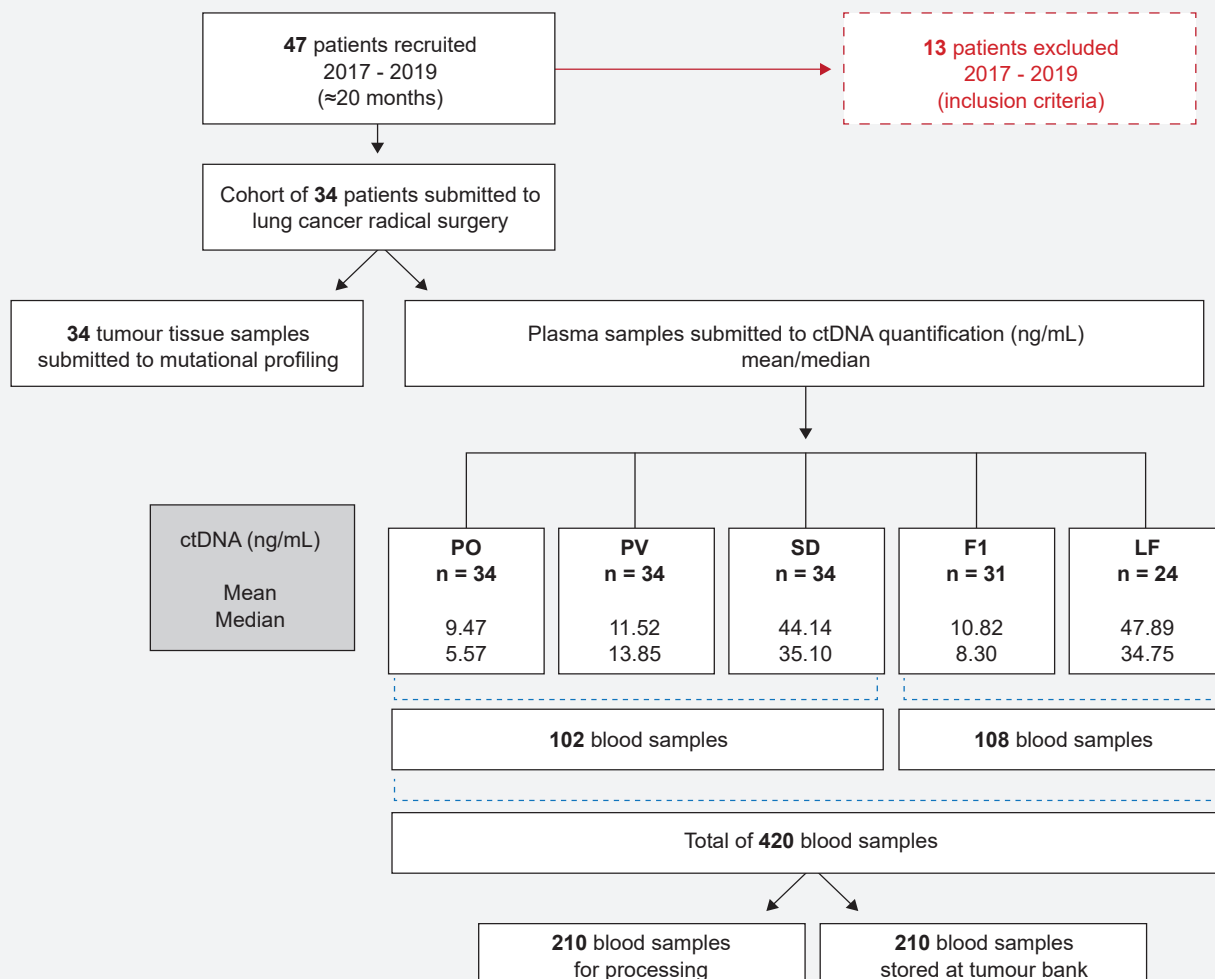


Figure 1 – Flowchart

PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

**Figure 2 – Study design**

PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

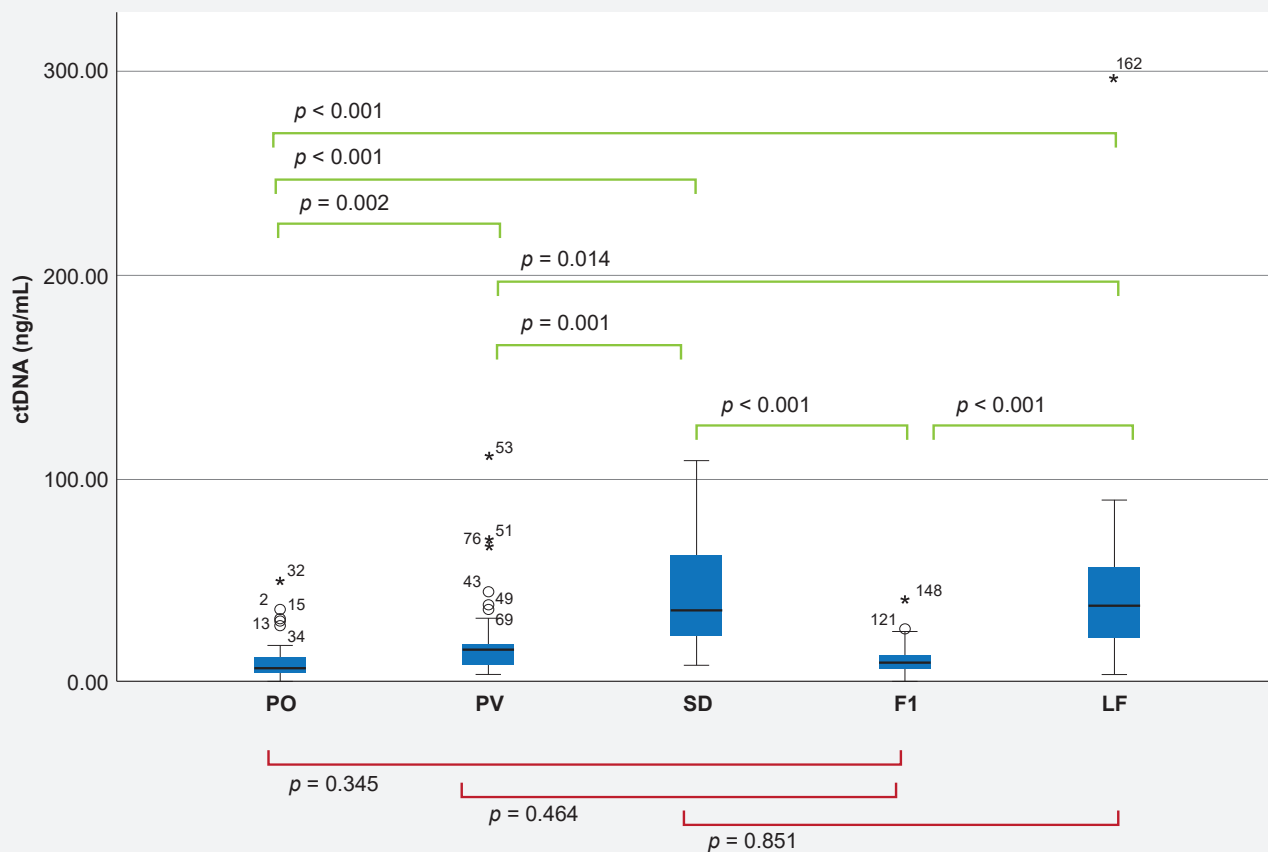


Figure 3 – ctDNA concentration at five time points (p adjusted value)

PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

Green lines: statistical significance. Red lines: not statistically significant.

Table 1 – Patients' clinical features

Variable	n = 34 (%)
Sex	
Female	15 (44.12)
Male	19 (55.88)
Age (years)	
Median	64.5
Range	50 to 79
Smoking status	
Non-smoker	13 (38.23)
Former & active smoker	21 (61.77)
Type of Surgery & mediastinal lymph node emptying	
Pneumectomy	1 (2.94)
Bilobectomy	3 (8.82)
Lobectomy	30 (88.24)
Histology	
Adenocarcinoma	27 (79.41)
Squamous carcinoma	6 (17.65)
Large cell neuroendocrine carcinoma & adenocarcinoma	1 (2.94)
Disease Stage (n = 32)	
I	20 (62.50)
II	11 (33.37)
III	1 (3.13)
ypTNM (n = 2)	
ypT3N0M0R1	1
ypT0N2M0R0	1
Number of Prognostic Factors	
0	10 (29.41)
1	5 (14.71)
2	9 (26.47)
3	10 (29.41)
Type of prognostic factors	
V1	16 (47.05)
L1	16 (47.05)
PN1	3 (8.82)
PV1	16 (47.05)
R1 (microscopic margins)	2 (5.88)
Post-surgical outcome	
Surveillance	10 (29.41)
Adjuvant chemotherapy	17 (50.00)
Lost in follow-up	7 (20.59)
Outcome	
Alive	30 (88.24)
Died	4 (11.76)
Follow-up (days)	
Mean time	699.88 (3 - 1337)
Median time	809.50 (3 - 1337)

Table 2 – Statistical analysis of ctDNA evolution according to five time points and clinicopathological features

	PO	PV	SD	F1	LF	Joint model
Age						
< 70 years vs > 70 years	β -16.734 IC: -27.707; -5.760 $p = 0.003$	β 8.369 IC: 0.359; 6.378 $p = 0.041$	β -21.785 IC: -38.447; -5.123 $p = 0.010$	β -4.920 IC: -12.404; 2.564 $p = 0.198$	β 34.402 IC: 12.549; 56.254 $p = 0.002$	β -9.235 IC: -15.352; -3.118 $p = 0.004$
Sex						
Female vs Male	β 1.323 IC: -7.087; 9.732 $p = 0.758$	β -7.579 IC: -17.839; 2.681 $p = 0.148$	β 2.485 IC: -22.065; 27.035 $p = 0.843$	β 0.212 IC: -3.951; 4.376 $p = 0.920$	β -15.852 IC: -46.416; 14.712 $p = 0.309$	β 1.316 IC: -3.827; 6.458 $p = 0.609$
Smoking status						
Non-smoker vs Active & former smoker	β -5.228 IC: -13.771; 3.314 $p = 0.230$	β 8.468 IC: -3.043; 19.978 $p = 0.149$	β 17.137 IC: -7.049; 41.322 $p = 0.165$	β -3.320 IC: -6.968; 0.329 $p = 0.075$	β -7.628 IC: -26.455; 11.200 $p = 0.427$	β -4.480 IC: -9.767; 0.807 $p = 0.095$
Tumour size						
T1 $\leq$ 3cm vs T2 > 3cm	β -0.388 IC: -7.174; 6.399 $p = 0.911$	β 7.443 IC: -1.007; 15.893 $p = 0.084$	β -18.505 IC: -42.897; 5.887 $p = 0.137$	β 1.520 IC: -3.739; 6.779 $p = 0.571$	β -5.313 IC: -52.750; 42.124 $p = 0.826$	β 0.738 IC: -4.760; 6.236 $p = 0.788$

PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

Table 3 – Tumor and ctDNA samples with actionable mutation (n = 6)

Patient #	Tumour sample AF (%) Mutated gene AA change	NGS PO AF (%) ctDNA (ng/mL) days	NGS PV AF (%) ctDNA (ng/mL) days	NGS SD AF (%) ctDNA (ng/mL) days	NGS LF AF (%) ctDNA (ng/mL) days	Imageology progression CT scan days
#6	27.0 <i>EGFR</i> p.Asp770delinsGlyTyr	0.1 9.8 0	Negative 44.0 0	Negative 48.9 5	Negative 57.1 499	Positive 828
#14	55.8 <i>EGFR</i> p.Leu747_Pro753delinsSer	0.2 2.3 0	0.1 7.0 0	Negative 16.6 7	Negative 17.8 447	Positive 724
#21	20.0 <i>BRAF</i> p.Val600Glu	Negative 6.4 0	Negative 10.8 0	Negative 31.1 5	Negative 5.6 29	Negative
#28	9.7 <i>EGFR</i> p.Leu858Arg	0.1 4.2 0	Negative 4.2 0	Negative 12.2 4	Negative 62.13 312	Negative
#29	8.1 <i>EGFR</i> p.Leu747_Alal750delinsPro	0.1 3.6 0	0.1 10.2 0	Negative 14.9 5	Negative 10.9 22	Negative
#34	30.6 <i>EGFR</i> p.Leu747_Alal750delinsPro	0.1 49.2 0	Negative 16.3 0	Negative 108.0 8	Negative 11.9 109	Negative

PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; ctDNA: circulating tumour DNA; AA: amino acid; AF: allele frequency; CT: computed tomography

Appendix. Tables

Table A.1 The absolute values of ctDNA of the 34 patients and CT evaluation.

Patient Number	PO ctDNA (ng/ml) days n=34	PV ctDNA (ng/ml) days n=34	SD ctDNA (ng/ml) days n=34	F1 ctDNA (ng/ml) days n=31	F2 ctDNA (ng/ml) days	F3 ctDNA (ng/ml) days	F4 ctDNA (ng/ml) days	F5 ctDNA (ng/ml) days	F6 ctDNA (ng/ml) days	LF ctDNA (ng/ml) days n=24	CT Progression days n=31
2	2.69 0	9.53 0	39.41 5	12.64 24	4.91 121	5.86 380	6.4 471	4.2 513		44.9 641	Negative 1337
3	31.1 0	18.5 0	28.3 5	19.8 17						13.2 396	Positive 585
4	3.09 0	13.08 0	21.84 6	2.95 35	5.79 160	2.6 208	1.4 273			45.02 338	Positive 152
5	5.44 0	4.7 0	62.3 6	6.9 34	6.0 390					26.9 593	Negative 1049
6	9.8 0	44.0 0	48.9 5	14.9 28	1.8 113	5.9 134	0.828 145	0.7 273	10.3 372	57.1 499	Positive 828
7	1.36 0	3.39 0	23.78 7	7.46 28	2.70 202	2.63 356	6.2 421			20.0 504	Positive 884
8	6.4 0	9.0 0	88.4 5	25.3 28	8.2 266					9.1 3 80	Negative 308
9	1.66 0	7.26 0	17.40 5	2.09 29	3.55 56	4.65 105	4.12 189			44.9 338	Negative 1090
10	8.8 0	17.9 0	35.0 5	18.5 25	6.0 201	4.6 390	7.5 481			25.6 537	Negative 1013
11	11.8 0	15.2 0	40.4 5	6.4 29	0.9 197	4.5 231	9.0 379			296.0 540	Negative 534
12	7.7 0	35.5 0	88.5 5	12.2 28	5.9 401	8.6 531				22.6 678	Negative 812
14	2.3 0	7.0 0	16.6 7	3.8 28	1.3 160	4.6 216	6.9 384			17.8 447	Positive 724
15	34.9 0	69.8 0	34.1 5	11.5 25							Negative 246
16	4.9 0	13.6 0	24.4 5	6.6 32	2.4 298	10.0 373				15.75 617	Negative 1154
18	8.8 0	17.6 0	46.0 12	13.40 31	6.6 158	10.3 221	4.7 324	6.0 422		56.0 541	Negative 1134
19	13.5 0	17.5 0	90.0 6	12.9 29	10.9 147	10.4 238	10.2 343			88.67 434	Negative 1049
20	3.5 0	7.9 0	9.5 5	6.2 25	6.0 184					27.45 435	Negative 1243
21	6.4 0	10.8 0	31.1 5	5.6 29							Negative 984
22	7.3 0	21.4 0	50.7 8	19.8 31	16.6 95					28.34 184	Negative 333
23	4.6 0	3.7 0	42.0 4	5.6 33	3.8 148	15.1 265				47.76 361	Positive 959
24	5.3 0	15.3 0	25.1 6	12.9 28	15.1 127	11.0 169				71.33 400	Negative 1117
25	0 0	6.9 0	29.9 4	8.3 24	41.10 94	68.80 184				58.29 327	Positive 150
27	3.6 0	14.1 0	71.2 5	8.3 35	0.8 97	0.151 167				3.4 300	Negative 969
28	4.2 0	4.2 0	12.2 4	5.5 50	8.86 129					62.13 312	Negative 995
29	3.6 0	10.2 0	14.9 5	10.9 22							Negative 243
30	6.6 0	16.0 0	78.2 5	0.2 31							Negative 263
31	3.8 0	7.5 0	35.2 7	10.2 21							Negative 303
32	5.3 0	26.0 0	10.6 0	5.2 25	0.9 184					55.84 198	Negative 890

Patient Number	PO ctDNA (ng/ml) days n=34	PV ctDNA (ng/ml) days n=34	SD ctDNA (ng/ml) days n=34	F1 ctDNA (ng/ml) days n=31	F2 ctDNA (ng/ml) days	F3 ctDNA (ng/ml) days	F4 ctDNA (ng/ml) days	F5 ctDNA (ng/ml) days	F6 ctDNA (ng/ml) days	LF ctDNA (ng/ml) days n=24	CT Progression days n=31
34	49.2 0	16.3 0	108.0 8	11.9 109							Positive 710
35	5.7 0	11.0 0	22.0 3								No CT
36	29.2 0	30.7 0	53.0 3	39.7 65							Negative 807
37	9.2 0	17.97 0	56.5 5	7.7 65	4.6 99					28.40 164	Positive 834
39	2.6 0	6.0 0	7.7 8								No CT
40	17.7 0	66 0	137.78 5								No CT
										Molecular Follow-up	Imagiology Follow-up
Mean time (days) (min; max)										413.8 (94; 678)	764.48 (150;1337)
Median time (days) (min; max)										417.0 (94; 678)	834.0 (150;1337)

Legend: **PO:** Pre-Operative; **PV:** Pulmonary Vein; **SD:** Surgical Discharge; **F1:** First Follow-up; **LF:** Last Follow-up; **ctDNA:** circulating tumour DNA; **CT:** computed tomography

Table A.2 Pairwise method analysis according to ctDNA concentration and the five time points. Significance values were adjusted using the Bonferroni correction for several tests.

	p value	p adjusted value
PO vs LF	<0.001	<0.001
PO vs F1	0.345	1.000
PO vs SD	<0.001	<0.001
PO vs PV	0.002	0.021
PV vs LF	0.001	0.014
F1 vs PV	0.046	0.464
PV vs SD	<0.001	0.001
F1 vs SD	<0.001	<0.001
LF vs SD	0.851	1.000
F1 vs LF	<0.001	<0.001

Legend: **PO:** Pre-Operative; **PV:** Pulmonary Vein; **SD:** Surgical Discharge; **F1:** First Follow-up; **LF:** Last Follow-up

Table A.3 Tumour mutational profile

Sample Nº	Histology	Mutated Gene	AA change	AF (%)
2T1	Adenocarcinoma	KRAS	p.Gly12Asp	24,0
3T1	Squamous cells	TP53	p.Ala159Ser	23,0
4T1	Squamous cells	TP53	p.Arg110Leu	52,0
5T1	Adenocarcinoma	KRAS	p.Gly12Ala	9,0
6T1	Adenocarcinoma	EGFR	p.Asp770delinsGlyTyr	27,0
7T1	Adenocarcinoma	TP53	p.Met160Ile	42,0
8T1	Squamous cells	FBXW7	p.Arg505Gly	48,0
		TP53	p.Val157Phe	53,0
9T1	Adenocarcinoma	KRAS	p.Gly12Asp	34,0
10T1	Large cell neuroendocrine	MET	p.Asn375Ser	49,0
11T1	Squamous cells	TP53	p.Arg213Leu	61,5
12T1	Adenocarcinoma	Translocation	EML4-ALK.E13A20	NA
14T1	Adenocarcinoma	EGFR	p.Leu747_Pro753delinsSer	55,8
		TP53	p.Glu171fs	46,9
		TP53	p.Glu171Gly	46,9
15T1	Adenocarcinoma	KDR	p.Gln472His	12,7
		KRAS	p.Gly12Cys	19,0
		TP53	p.Ala347Thr	4,7
		TP53	p.Gly245Cys	5,5
16T1	Adenocarcinoma	KRAS	p.Gly12Asp	13,4
		SMARCB1	p.Thr72Lys	7,3
18T1	Squamous cells	NRAS	p.Gln61Lys	4,3
		TP53	p.Ala159Val	8,7
19T1	Adenocarcinoma	Translocation	EML4-ALK.E13A20	NA
20T1	Adenocarcinoma	PIK3CA	p.Asn1044Lys	46,8
		TP53	p.Val157Phe	35,4
21T1	Adenocarcinoma	PIK3CA	p.Glu542Lys	3,4
		BRAF	p.Val600Glu	20,0
22T1	Adenocarcinoma	KRAS	p.Gly12Asp	34,6
23T1	Adenocarcinoma	No tissue		
24T1	Adenocarcinoma	Bad quality DNA for sequencing		
25T1	Adenocarcinoma	Bad quality DNA for sequencing		

27T1	Adenocarcinoma	RET	p.Cys634Tyr	3,0
		MPL	p.Ser505Asn	1,9
		MPL	p.Ala519Thr	3,4
28T1	Adenocarcinoma	CTNNB1	p.Ser37Phe	1,2
		CTNNB1	p.Ala43Val	1,5
		KIT	p.Glu561Lys	1,2
		APC	p.Arg1114Ter	1,4
		APC	p.Glu1374Lys	1,3
		EGFR	p.Leu858Arg	9,7
		SMO	p.Arg199Trp	1,6
		BRAF	p.Gly464Glu	1,4
		PTEN	p.Gly165Glu	1,5
		SMAD4	p.Gln245Ter	1,3
		SRC	p.Gln531Ter	3,9
		SMARCB1	p.Arg158Ter	1,2
29T1	Adenocarcinoma	EGFR	p.Leu747_Ala750delinsPro	8,1
		EGFR	p.Ala750Pro	7,5
30T1	Adenocarcinoma	KRAS	p.Gly12Cys	12,0
		TP53	p.Arg337Cys	4,8
31T1	Adenocarcinoma	NO MUTATION DETECTED		
32T1	Adenocarcinoma	Translocation	EML4-ALK.E13A20	NA
34T1	Adenocarcinoma	EGFR	p.Leu747_Ala750delinsPro	30,6
		EGFR	p.Ala750Pro	27,8
35T1	Adenocarcinoma	NO MUTATION DETECTED		
36T1	Adenocarcinoma	PIK3CA	p.Arg108His	1,2
		TP53	p.Thr81Ile	3,9
37T1	Adenocarcinoma	KRAS	p.Gly12Asp	15,3
		TP53	p.Trp91Ter	1,2
		TP53	p.Ala84Val	1,2
39T1	Adenocarcinoma	KRAS	p.Gly12Cys	18,6
		TP53	p.Gln136Glu	12,6
40T1	Squamous cells	TP53	p.Ile162fs	11,3

Chapter 3 - Five Case Reports of Surgical Lung Cancer Patients
Who Presented Molecular Progression before Radiological
Diagnosis

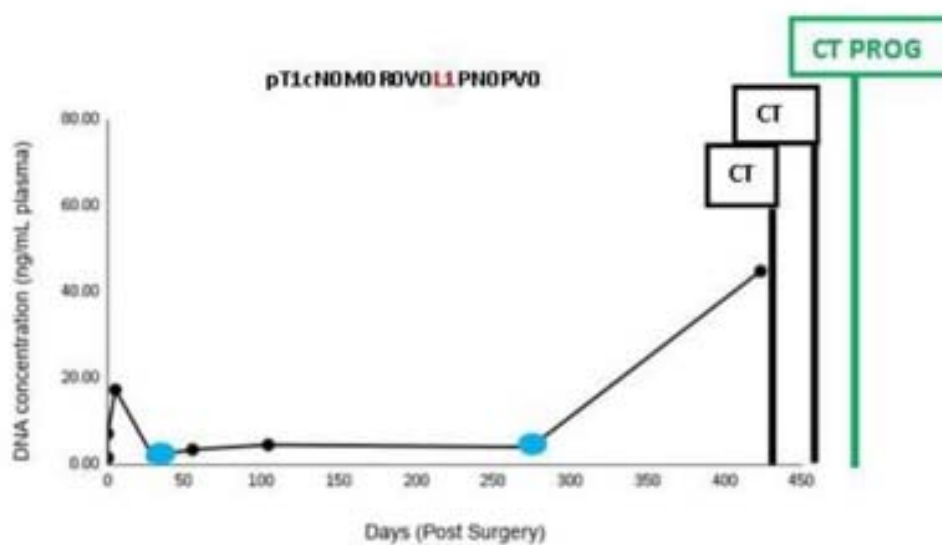


Figure 5.Fourth case report progression of the disease

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Case series

Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis

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Introduction

With the dynamic development of molecular medicine, we can aspire to personalize medicine. Our aim is not only survival and response rates, but also best quality of life, lowest toxicity and more cost-effective choices for each individual patient. The use of liquid biopsies, processed by next generation sequencing (NGS), has clinical utility and validity in the metastatic scenario. Evaluating its importance in early stage lung cancer is a challenging issue. We report five different cases submitted to lobectomy and lymph node dissection, where circulating free tumor DNA (cfDNA) plays an important role in the diagnosis of residual disease, progression or relapse. Blood samples analyzed were obtained before surgery (Pre-Operatory), from pulmonary vein (Intra-Operatory), before discharge (Post-Operatory) and during follow-up (F-UP) as can be observed in the workflow plan (Figure 1).

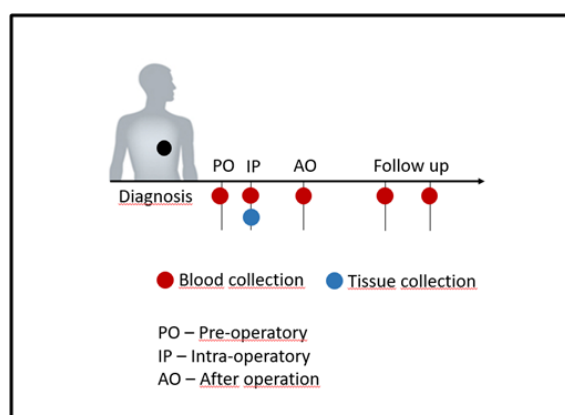


Figure 1. Workflow plan

First Case Report

A 69-year-old women, non-smoker, with

history of pulmonary tuberculosis was referenced with a thoracic computerized tomography (CT) scan with an opacity on the medium lobe. A bronchoscopy revealed an adenocarcinoma. A Positron Emission Tomography-CT (PET-CT) scan was negative. She was staged as cT1aN0M0. She was submitted to surgery and was staged as pT1aN0V0L0PN0PV0M0R0–IA1. The patient stayed in surveillance.

Tissue tumor DNA (tDNA) revealed an EGFR mutation with an allele frequency (AF) of 27% and cfDNA concentration was 9.8ng/ml before surgery. cfDNA from the pulmonary vein was 44.0ng/ml. On the fourth day post-surgery, plasma cfDNA concentration was 48.9ng/ml. Twenty-three days later, at her first appointment, plasma cfDNA had decreased to 14.9ng/ml. Twelve months after discharge plasma cfDNA had decreased to 10.3ng/ml.

Sixteen months after the first follow-up appointment, the value of cfDNA increased to 57.1ng/ml. CT scan performed four months later showed no progression. Only the CT scan 11 months after cfDNA increase or 27 months after the first follow-up appointment showed progression of the disease as can be observed on the Figure 2 (Table 1).

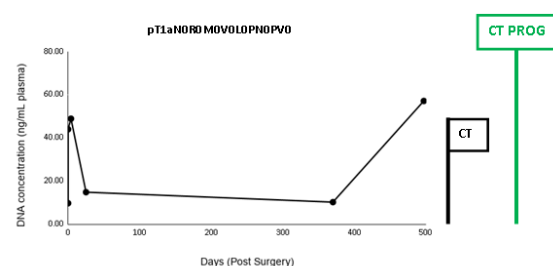


Figure 2. First case report Progression of the disease

Second Case Report

A 51-year-old man, smoker (30UPY), was referenced with a thoracic CT scan showing a nodule in the inferior right lobe with 41x34mm. Bronchoalveolar washing showed a squamous lung carcinoma. A PET-CT showed the same lesion with a SUVmax of 4.6. He was staged as cT2bN0M0. He was submitted to surgery and was staged as pT2aN0V0L0P-N0PV0M0R0 – IB. The patient stayed in surveillance.

tDNA revealed an TP53 mutation with an AF of 61.5% and plasma cfDNA concentration was 11.8ng/ml before surgery. cfDNA from the pulmonary vein was 15.2ng/ml. On the fourth day post-surgery, plasma cfDNA concentration was 40.4ng/ml. Twenty-four days later, plasma cfDNA had decreased to 6.4ng/ml. One year after discharge plasma cfDNA had 9.0ng/ml. The CT scan at that time was negative for disease progression. Seventeen months after the first follow-up appointment cfDNA increased to 296ng/ml. The patient died five months after the last plasma withdrawal from disease progression (Figure 3, Table 1).

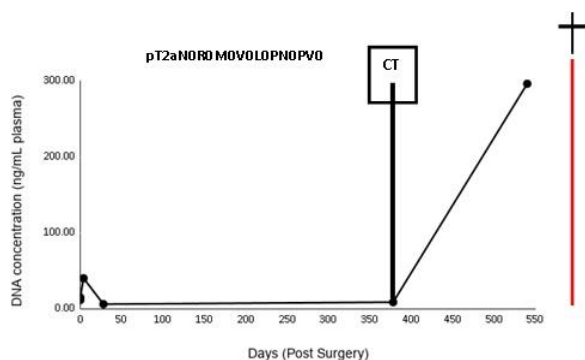


Figure 3.Second case report disease progression

Third Case Report

A 52-year-old man, smoker (36UPY), was referenced with a thoracic CT scan which showed a nodule in the medium lobe with 30x22mm. A lung biopsy revealed a squamous lung carcinoma. A PET-CT showed the same lesion with a SUVmax of 5.7. He was staged as a cT1cN0M0.

tDNA revealed a TP53 mutation with an AF of 52% and plasma cfDNA concentration was 3.09ng/ml before surgery. He was submitted to surgery and cfDNA concentration from the pulmonary vein was 13.08 ng/ml. On the fifth day post-surgery cfDNA concentration was 21.84ng/ml. Histology revealed a squamous carcinoma with invasion of the visceral pleura, pT2aN1M0V0L0PN0PV1R0, stage IIIIB. One month after surgery, plasma cfDNA concentration was 2.95ng/ml. Adjuvant chemotherapy was performed. Six months after first follow-up appointment CT scan

showed progression and 11 months after, plasma cfDNA concentration showed an increase of 45.02ng/ml, as well as a gradual increase during chemotherapy (Figure 4, Table 1). The patient died one month later.

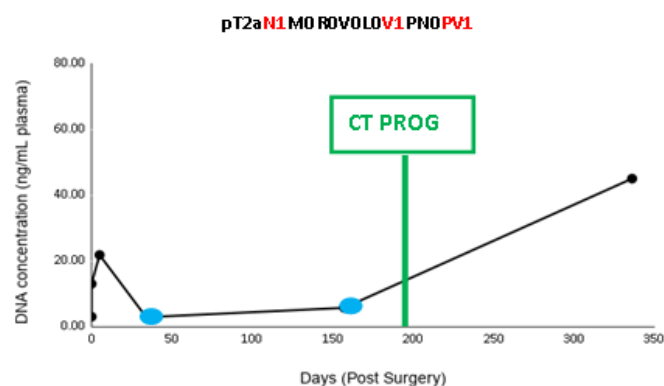


Figure 4.Third case report progression of the disease

Fourth Case Report

A 68-year-old man, smoker (100UPY), was referenced with a thoracic X-Ray with a suspicious image on the medium lobe. A CT scan showed a 16mm nodule on the medium lobe. The PET-CT showed the same lesion. A lung biopsy revealed an adenocarcinoma. He was staged as cT1bN0M0. He was submitted to surgery and was staged as pT1cN0M0R0V0L1P N0PV0 – IA. The patient performed adjuvant chemotherapy.

tDNA revealed an KRAS mutation with an AF of 34.0% and plasma cfDNA concentration was 1.66ng/ml before surgery. cfDNA from the pulmonary vein was 7.26ng/ml. On the fourth day post-surgery, plasma cfDNA concentration was 17.4ng/ml. Twenty-four days later, plasma cfDNA had decreased to 2.09ng/ml. One month after first follow-up appointment plasma cfDNA had 3.55ng/ml.

Ten months after first follow-up appointment the value of cfDNA increase to 44.9ng/ml. The CT scan performed twenty-three months after the first follow-up appointment showed progression of the disease as can be observed in the figure 5 (Table 1).

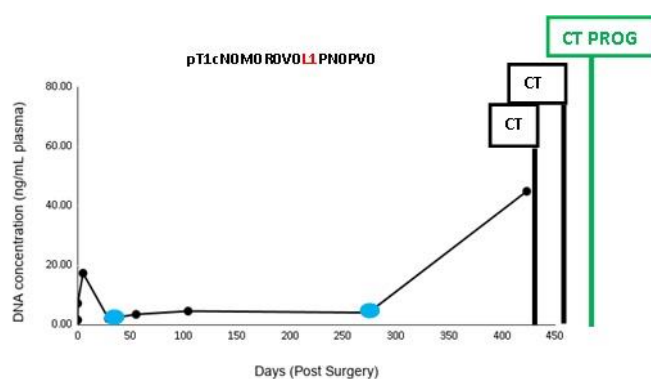


Figure 5.Fourth case report progression of the disease

Table 1. Timeline of cfDNAs and CT scans of Five Case Reports.

	H	pTNM	Pre-Op	Intra-Op	Post-Op	F-UP	F-UP	F-UP	F-UP	F-UP	CT scanProg.
1	A	pT1aN0M0R0 V0L0PN0PV0	13.07.17 9.8	13.07.17 44.0	17.07.17 48.9	09.08.17 14.9	19.07.18 10.3	23.11.18 57.1			18.10.19
2	S	pT2aN0M0R0 V0L0PN0PV0	12.10.17 11.8	12.10.17 15.2	16.10.17 40.4	9.11.17 6.4	25.10.18 9.0	04.04.19 296.0			October 2018 - normal
3	S	pT2N1M0R0 V1L0PN0PV1	06.07.17 3.09	06.07.17 13.08	11.07.17 21.84	9.8.17 2.95	12.12.17 5.79	08.06.18 45.02			16.01.18
4	A	pT1cN0M0R0 V0L1PN0PV0	28.09.17 1.66	28.09.17 7.26	02.10.17 17.40	26.10.17 2.09	22.11.17 3.55	10.01.18 4.65	04.04.18 4.12	31.08.18 44.9	09.09.19
5	A	pT2aN1M0R1 V1L1PN0PV0	05.03.18 0.00	05.03.18 6.9	08.03.18 29.9	28.03.18 8.3	06.06.18 41.10	04.09.18 68.80	25.01.19 58.29		31.08.19

H: Histology; A: Adenocarcinoma; S: Squamous carcinoma; Pre-Op: Pre-Operation; Intra-Op: Intra-Operative (pulmonary vein); Post-Op: Post-Operative; F-UP: follow-up; **BLUE**: chemotherapy; **Green**: cfDNA increase; Prog: Progression

Table 2. Timeline to Molecular and Radiological Progression after first follow-up appointment.

	Histology	cTNM	PET-CT (Suv-max)	pTNM	MDT	Time to MP (months)	Time to IP (months)
1	Adenocarcinoma	cT1aN0M0	-	pT1aN0M0R0V0L0PN0PV0	S	16	27
2	Squamous Carcinoma	cT2bN0M0	4.6	pT2aN0M0R0V0L0PN0PV0	S	17	-
3	Squamous Carcinoma	cT1cN0M0	5.7	pT2N1M0R0V1L0PN0PV1	C	11	6
4	Adenocarcinoma	cT1bN0M0	+	pT1cN0M0R0V0L1PN0PV0	C	10	23
5	Adenocarcinoma	cT2N1M0	5.4	pT2aN1M0R1V1L1PN0PV0	C	2	5

MDT: Multi-Disciplinary Team; Time to MP (months): Time to Molecular Progression; Time to IP (months): Time to Radiological Progression; S: surveillance; C: chemotherapy.

She was staged as cT2N1M0. She was surgery and the disease was staged as pT2aN1V1L1PN0PV0M0R1, with surgical intersection of an adenopathy.

tdNA revealed no mutation due to bad quality. cfDNA concentration was 0.0ng/ml before surgery. cfDNA from the pulmonary vein was 6.9ng/ml. She was discharged on the third day post-surgery, with a plasma cfDNA concentration of 29.9ng/ml. Twenty days after surgery, plasma cfDNA concentration a decreased (8.3ng/ml) (Figure 5). Adjuvant chemotherapy was performed.

Two months after first follow-up appointment and during chemotherapy cfDNA concentration was 41.10ng/ml and increased progressively during the next six months. CT scan showed progression at this time and tissue biopsy revealed an adenocarcinoma, TTF1+; tissue NGS revealed an epidermal growth factor receptor (EGFR) mutation on exon 19, p(Glu746_Ala750del) (AF39%) (Table 1). cfDNA before therapy was 68.80ng/ml. The patient started EGFR tyrosine kinase inhibitor (green dot in Figure 6). The patient maintains therapy with a clinical, imagiological stable disease and a gradual decrease cfDNA concentration.

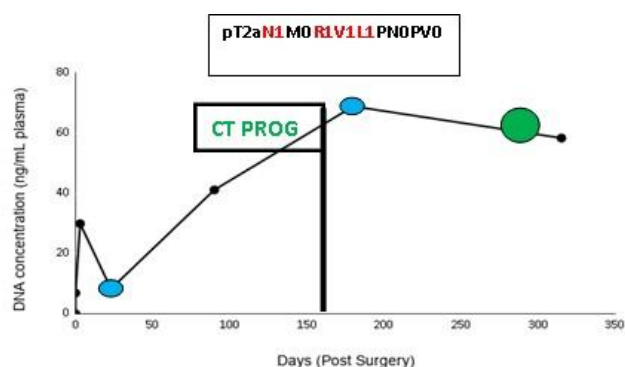


Figure 6. Fifth case report progression of the disease

Discussion

We report two cases who stayed in surveillance according to pathological staging (first and second case reports). Liquid biopsies showed to be crucial, to evaluate residual disease, stratify risk of relapse and identify the patients that showed molecular progression (increase in cfDNA) prior to imagiological diagnosis (Table 1 and 2). In the second case the patient unfortunately died before imagiological confirmation of disease progression. In both cases, once cfDNA increase was detected, 16 and 17 months after first follow-up appointment respectively, imagiological imaging should have been performed as soon as possible, in order to initiate appropriate treatment. In the last three cases adjuvant chemotherapy was performed. In the third and fourth case reports, the time from first follow-

up appointment until cfDNA progression was ten months. Being the fifth case an R1 resection, two months was the time to identify molecular progression in the same timing.

In all cases the difference of time to progression considering molecular and imagiological progression, observed in Table 1 and 2, show significant disparities in time (months). This difference is greatest in the patients who stayed in surveillance due to a less aggressive disease. While in the third and fourth cases the difference in time was smaller and in the fifth case even more (Table 1).

Conclusions

Lung Cancer is one leading cause of death worldwide, and patients would greatly benefit from an early diagnosis. Tissue biopsy is still considered the gold standard for diagnosis and there is a main potential to identify biomarkers. However, it is still an invasive and inadequate technique to diagnose tumor heterogeneity [1]. Through liquid biopsies real-time information on the molecular profile of the tumor can be obtained. cfDNA is the only procedure which identifies with high precision tumor biomarkers in order to target the disease with high precision.

A few active studies are ongoing with the aim of exploring the feasibility and utility of liquid biopsy in early stage NSCLC [2]. Results are yet to be awaited. Recent papers are favoring a molecular profile besides the classical TNM staging system may be used to better classify early lung cancer patients submitted to surgery. TNMB classification might be nearer than expected and with clinical implications soon [3,4]. In other words, liquid biopsies taken after surgery have shown to be a strong promising tool, biomarker, to identify the patients who will most benefit from adjuvant treatment or who are most likely to relapse when cfDNA is positive after surgery in a phenotypical negative local disease [5-7].

It seems that molecular profiling by using liquid biopsies may help us understand the aggressiveness of each tumor at a particular moment in time, besides the classical T (tumor size) and N (lymph nodal involvement) and the presence of other negative prognostic factors such as a positive involvement of lymphovascular, perineural, visceral pleura and margins resection [8]. An up or downstage of these characteristics can be evaluated by incorporating high or low molecular risk factors or genomic features such as an increase of cfDNA [9]. In other words, cfDNA could supplement the re-staging approaches after a curative surgery in lung cancer [10]. This is the main observation that can be withdrawn from these five case reports. Further trials need to be performed to change standard

of care of surveillance and diagnosis in lung cancer patients submitted to a curative surgery, with higher precision and efficiency.

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Update of results of “Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis”.

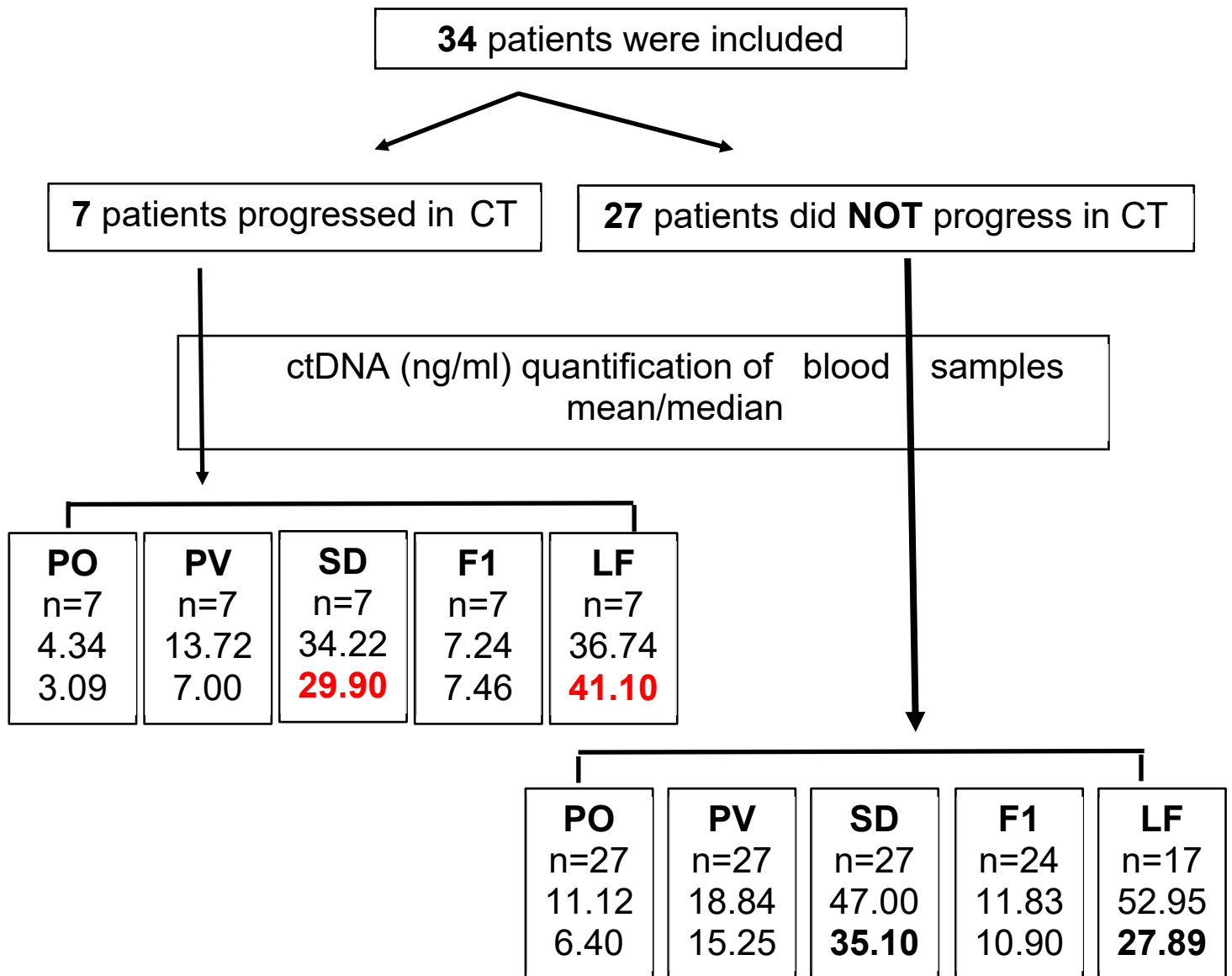
From a total of 34 patients, seven patients showed imagiology progression. Twenty-seven patients did not. These seven patients also presented higher median ctDNA values at the last follow-up compared to ctDNA values at surgical discharge (molecular relapse), in opposition to the 27 patients, shown in Figure 1 and Table 5. As also observed, tumours greater than 3cm, with more than one poor prognostic factor (PPF) and with adenocarcinoma were the clinicopathological features favouring progression (Table 6)¹¹¹.

The seven patients with ctDNA relapse and imagiology progression as shown in Table 7. The absolute values of ctDNA at last follow-up, can be compared with the other four-time points, but also the difference in days from molecular relapse to imagiology progression¹¹¹.

A linear regression analysis was performed considering the five-time points and crosschecked with molecular relapse (ctDNA) and imagiology progression (CT). Twenty-four patients were analysed and divided into two groups: group 1 included seven patients with progression of ctDNA and CT; group 2 with seventeen patients with ctDNA progression but without CT progression. An adjusted and raw analysis was performed, as observed in Table 8. No statistically significant results were found (Figure 2).

Once again, the absolute values of ctDNA quantification observed in Table 7 show that the mean time to molecular relapse was 343.86 days [94; 504] and the mean time for CT progression was 647 days [150; 959]. Before imagiology progression, molecular evidence shows metabolic cancer activity with a gradual increase of ctDNA values, to values superior to those quantified at SD time point as shown in Table 7. In these seven patients ctDNA relapse is quantified and mensurable oncological disease is later in time confirmed by CT scan.

Figure 1. Evaluation of CT progression (n=34)



Legend: PO: Pre-Operative; PV: Pulmonary Vein; SD: Surgical Discharge; LF Last Follow-up;

NGS: next generation sequencing; ctDNA: circulating tumour DNA.

Table 5. ctDNA expression at five time-points according to imagiology progression

CT scan	ctDNA (median)				
	PO	PV	SD	F1	LF
PROGRESSION n=7	3.09	7.00	29.90	7.46	41.10
NO PROGRESSION n=27	6.40	15.25	35.10	10.90	27.89

Legend: **PO:** Pre-Operative; **PV:** Pulmonary Vein; **SD:** Surgical Discharge; **LF:** Last Follow-up. **ctDNA:** circulating tumour DNA

Table 6. Patients' clinical features according to progression.

CT scan	T1≤3cm/T2>3cm	PPF 0/>1	Histology S/A
PROGRESSION n=7	2/5	1/6	1/6
NO PROGRESSION n=27	11/16	9/18	5/22

Legend: **PO:** PPF: poor prognostic factors; **S:** squamous carcinoma; **A:** adenocarcinoma

Table 7: Molecular (ctDNA) and imagiology (CT) progression (n=7)

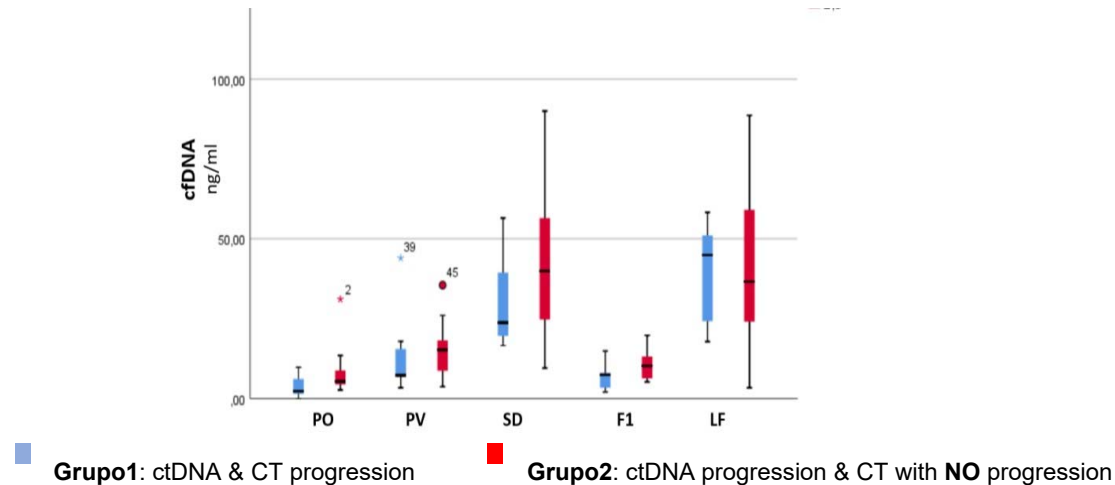
Patient Number	Tumour sample MUTATED GENE AF (%) AA change AF (%)	PO ctDNA (ng/ml) days	PV ctDNA (ng/ml) days	SD ctDNA (ng/ml) days	F1 ctDNA (ng/ml) days	F2 ctDNA (ng/ml) days	F3 ctDNA (ng/ml) days	F4 ctDNA (ng/ml) days	F5 ctDNA (ng/ml) days	F6 ctDNA (ng/ml) days	LF ctDNA (ng/ml) days	Molecular Relapse ctDNA (ng/ml) days	Imagiology Progression CT days
4 (3)	TP53 p.Arg110Leu 52,0	3.09 0	13.08 0	21.84 6	2.95 35	5.79 160	2.6 208	1.4 273			45.02 338	45.0 2 338	Positive 152
6 (1)	EGFR p.Asp770delinsGlyTyr 27,0	9.8 0	44.0 0	48.9 5	14.9 28	1.8 113	5.9 134	0.828 145	0.7 273	10.3 372	57.1 499	57. 1 499	Positive 828
7	TP53 p.Met160Ile 42,0	1.36 0	3.39 0	23.78 7	7.46 28	2.70 202	2.63 356	6.2 421			20.0 504	20. 0 504	Positive 884
14	EGFR p.Leu747_Pro753delins Ser 55,8	2.3 0	7.0 0	16.6 7	3.8 28	1.3 160	4.6 216	6.9 384			17.8 447	17. 8 447	Positive 724
23	No tissue	4.6 0	3.7 0	42.0 4	5.6 33	3.8 148	15.1 265				47.76 361	47.7 6 361	Positive 959
25 (5)	Bad quality DNA for sequencing	0 0	6.9 0	29.9 4	8.3 24	41.10 94	68.80 184				58.29 327	41. 10 94	Positive 150
37	KRAS p.Gly12Asp 15,3	9.2 0	17.97 0	56.5 5	7.7 65	4.6 99					28.40 164	28.4 0 164	Positive 834
												Molecular Relapse	Imagiology Progression
Mean time (days) (min; max)												343.86 (94; 504)	647.29 (150; 959)
Median time (days) (min; max)												361.0 (94; 504)	828.0 (150; 959)

Legend: PO: Pre-Operative; PV: Pulmonary Vein; SD: Surgical Discharge; F1: First Follow-up; LF: Last Follow-up; ctDNA: circulating tumour DNA; CT: computed tomography; (n°): corresponds to the number in the Table 1 of the publication “Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis”

Table 8. Statistical analysis of Group 1&2 at the five time points (n=24)

	PO		PV		SD		F1		LF	
	β raw	β adjusted	β raw	β adjusted	β raw	β adjusted	β raw	β adjusted	β raw	β adjusted
Group1 n=7	-4.117	-3.770	-0.936	-0.257	-11.523	-10.164	-4.060	-3.659	-16.580	-21.494
IC 95%	-9.347 to 1.113	-8.225 to 0.684	-9.667 to 7.796	-8.641 to 8.128	-31.029 to 7.983	-25.007 to 5.239	-8.297 to 0.177	-7.743 to 0.425	-66.102 to 32.943	-67.559 to 24.591
Group2 n=17	0	0	0	0	0	0	0	0	0	0
p value	0.123	0.097	0.834	0.952	0.247	0.196	0.060	0.079	0.512	0.361

Figure 2. ctDNA cluster boxplot at five time points according to M&I progression. (β raw & β adjusted).



Legend: PO: Pre-Operative; PV: Pulmonary Vein; SD: Surgical Discharge; F1: First Follow-up; LF: Last Follow-up; **ctDNA:** circulating tumour DNA. **Grupo1:** ctDNA & CT progression; **Grupo2:** ctDNA progression & CT with **NO** progression

. Confirmation of actionable mutations in tumour and blood samples of two case reports with molecular and imagiology progression.

From a total of seven patients with molecular (ctDNA) and imagiology (CT) progression ¹¹¹, only two cases were evaluated for a mutational profile from blood samples at four collection points (Table 9). The results were cross-checked with the tumour mutational profile. The concordance between the mutational profile of the tumour and ctDNA samples at PO, PV, SD and LF time-points for these two patients were positive for EGFR mutations only, two at the PO (patient 6 and 14) and one at the PV time point (patient 14).

Table 9: Molecular and CT progression (2 cases)

z	Tissue Samples MUTATED GENE AA change AF (%)	PO ctDNA (ng/ml) days	NGS PO AA change AF (%)	PV ctDNA (ng/ml) days	NGS PV AA change AF (%)	SD ctDNA (ng/ml) days	NGS SD AA change AF (%)	F1 ctDNA (ng/ml) days	LF ctDNA (ng/ml) days	NGS LF AA change AF (%)	Molecular Relapse ctDNA (ng/ml) days	Imagiology Progression CT days
6 (1)	EGFR p.Asp770delinsGlyTyr 27.0	9.8 0	EGFR p.Asp770delinsGlyTyr 0.1%	44.0 0	NEGATIVE	48.9 5	NEGATIVE	14.9 28	57.1 499	NEGATIVE	57.1 499	Positive 828
14	EGFR p.Leu747_Pro753delinsSer 55.8	2.3 0	EGFR p.Leu747_Pro753delinsSer 0.2%	7.0 0	EGFR p.Leu747_Pro753delinsSer 0.1%	16.6 7	NEGATIVE	3.8 28	17.8 447	NEGATIVE	17.8 447	Positive 724

Legend:

PO: Pre-Operative; **PV:** Pulmonary Vein; **SD:** Surgical Discharge; **F1:** First Follow-up; **LF:** Last Follow-up; **ctDNA:** circulating tumour DNA;

CT: computed tomography.

(n°): corresponds to the number in the Table 1 of the publication “Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis

RESULTS

From the three academic studies, Chapters 1, 2 and 3, the results obtained are the following:

In the first academic study in 2007, chapter 1, **“Genetic alterations in lung cancer: Assessing limitations in routine clinical use.”** we used a p53 mutation screen in multiple clinical samples from a series of lung cancer patients to attempt to identify the main practical limitations to the integration of molecular genetics in routine clinical practice. Our results suggest that the main limiting factor is the availability of samples, with good DNA quality. This problem could be overtaken, by improving the procedure for sample collection and storage.

The results of chapter 2 **“Implementation of a Pilot Study to Analyze Circulating Tumour DNA in Early-Stage Lung Cancer”** suggest that the quantitative expression of ctDNA is highest at surgical discharge and at last the follow-up time point; its’ decline at the first follow-up time point is likely due to the elimination of ctDNA post-surgical injury, and this is probably the best timing to consider the value of ctDNA as a prognostic biomarker of early-stage lung cancer.

From the third chapter, **“Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis”**, this observational study based on ctDNA results obtained on a longitudinal timeline allows us to state the following: Molecular profiling by using liquid biopsies may help us understand the aggressiveness of each tumour at a particular moment in time, besides the classical T (tumour size) and N (lymph nodal involvement) and the presence of other negative prognostic factors such as a positive involvement of lymphovascular, perineural, visceral pleura and margins resection. An up or downstage of these characteristics may be assessed by incorporating high or low molecular risk factors or genomic features such as increased ctDNA values. In other words, ctDNA could supplement the re-staging approaches after curative surgery for lung cancer. Liquid biopsies taken after surgery have shown to be a strong and promising tool, a biomarker, to identify the patients who will most benefit from adjuvant treatment or who are most likely to relapse when ctDNA is positive after surgery in a phenotypical negative local disease.

According to the update of Chapter 3, before imagiology progression, molecular evidence shows metabolic cancer activity with a gradual increase of ctDNA values, to values superior to those quantified at the SD time point. In these seven patients, ctDNA relapse is quantified and mensurable oncological disease is later in time confirmed by CT scan.

DISCUSSION

As previously mentioned, lung cancer is a serious public health problem and surgery still offers the best chance of cure for early-stage lung cancer patients, who are medically fit and can tolerate surgery, meaning that it is fundamental to improve both the early diagnostic process and the non-surgical treatments. In theory, progress in the molecular genetics of tumours should open new possibilities in both domains: early diagnosis and non-surgical treatments.

Concerning early diagnosis, the innovation of molecular techniques throughout time, has identified groups of individuals with a higher risk of developing the disease and early detection of tumours, potentially through non-invasive methods. Along with my academic investigation, the attempt to integrate molecular genetics in routine clinical practice and identify the main practical barriers to its implementation in lung cancer patients has been a constant challenge.

Even with thorough protocol elaboration, protocol information sessions, sensitising health professionals to the usefulness of molecular analysis, distribution of protocol flyers and e-mails alerting all involved health professionals; inclusively phone calls to recruited patients reminding them when sample collections took place, failures still occurred. Specifically due to patients experiencing problems during the examination, which had to be halted before the desired samples were obtained, the quality and quantity of samples obtained, time-interval from collection to preparation of samples and storage procedures were also a concern. The fact that the sample analysis plays no part in standard routine practice was the biggest barrier to introducing molecular analysis into routine practice in both projects. While these types of troublesome situations may perhaps go a little way towards explaining the low number of samples which did not yield good quality of DNA, included in both academic studies.

In our first academic project, we aimed at investigating anti-oncogene p53 mutations in clinical samples (bronchial lavage, bronchial brushing, bronchial biopsy) from a series of lung cancer patients through sequencing. This choice was occasioned by p53 being the most common target of mutations in lung cancer and by the fact that there are indications of these mutations having prognostic value. It should also be underlined that the p53 was only chosen as an example, to test the possibility of introducing molecular genetics into clinical routine.

The analysis of ctDNA in curative surgical lung cancer patients showed to be accomplished in the second academic project. With innovating molecular techniques such as next-generation techniques, it was proved to be possible to access the expression of ctDNA across time, and the impact of ctDNA analysis implementation in early-stage lung cancer. Results showed that the maximum yield of ctDNA is obtained at the surgical discharge of the patients and that the pre-operative timepoint is the one offering the highest sensitivity for the detection of actionable mutations in ctDNA in early-stage lung cancer ^{112,113}.

In this study, the most predominant mutations in each tumour sample were TP53 and KRAS with 30.8% and 34.6%, followed by EGFR with 19.2% mutations. Other mutations such as MET, BRAF, PIK3CA and MPL were also identified. Three tumour samples showed translocations (EML4-ALK.E13A20). Only EGFR and BRAF actionable mutations were evaluated. Our aim was also to look at the blood and detect if EGFR and BRAF actionable mutations which were still present in the body after a surgical curative

procedure. In this study, five out of six patients with EGFR + BRAF mutations were detected in the pre-operative blood samples and two patients in the pulmonary vein blood samples. In two case reports ctDNA increased on longitudinal bases disease progression was later confirmed in CT scan and EGFR actionable mutations were identified in both pre-operative blood samples and in one pulmonary vein blood sample (Table 17).

A recent study (LUCID- Lung Cancer Circulating Tumour DNA Study) assessing ctDNA in blood samples in early NSCLC patients submitted to surgery¹¹⁴, showed potential to identify patients who may benefit from further therapeutic intervention. In this study, ctDNA was taken from 88 patients with early-stage NSCLC before and after surgical treatment on a longitudinal basis as we performed in our pilot study. The authors concluded that ctDNA detected before and after surgery had shorter overall survival and recurrence-free survival.

Also, in a recent prospective study (LUMPCure study), which enrolled 261 patients with stages I to III NSCLC who underwent definitive surgery, the prognostic value of ctDNA-based on minimal residual disease detection in patients with NSCLC after radical resection was highlighted. Namely, to define the potentially cured population in localized NSCLC as those with longitudinal undetectable minimal residual disease. A subgroup analysis suggested a predictive value of minimal residual disease for adjuvant therapy and showed the nonessentiality of adjuvant therapy in this group of patients¹¹⁵.

For long has adjuvant (post-operative) chemotherapy been shown to improve survival in early-stage lung cancer patients as shown in a meta-analysis of 4584 patients (LACE Collaborative Group) with an overall 5-year survival of 5.4%^{116, 117}. The benefit was greater in patients with good performance status and with stage II and III disease. A recent randomised trial (IFCT 0002 trial) has shown no difference in survival in performing preoperative chemotherapy (induction or neoadjuvant chemotherapy) as opposed to postoperative chemotherapy¹¹⁸. So, pre-operative and post-operative chemotherapy is recommended for stage IB to III NSCLC¹¹⁷. The SWOG 9900 trial showed no difference in resection rate according to performing or not preoperative chemotherapy¹¹⁹. The NATCH trial also showed that with surgery alone to preoperative or postoperative chemotherapy, survival was equivalent in the three arms¹²⁰.

Evidence also exists in the neoadjuvant setting according to the immune state of the tumour, expression of PD-1/PDL-1. For tumours greater than 4cm, node-positive, no contraindication for immunotherapy, excluding oncogenic drivers such as EGFR mutations ALK rearrangements and who meet respectability criteria on surgical initial evaluation neoadjuvant chemotherapy and immunotherapy is recommended^{121,122,123}. As well as immunotherapy and chemoradiotherapy followed by surgery in patients with resectable and borderline resectable lung cancer¹²⁴. In the adjuvant setting, immunotherapy is also being contemplated after surgery and adjuvant chemotherapy¹²⁵. As previously mentioned, it is also known that patients with detectable ctDNA after chemoradiotherapy may benefit from consolidation immunotherapy in locally advanced non-small-cell lung cancer than those with undetectable ctDNA¹²⁶.

Patient 6 (nº1 in the paper “Five Case Reports of Surgical Lung Cancer Patients Who Presented Molecular Progression before Imagiological Diagnosis”) staged IA stayed in surveillance. An EGFR mutation was detected in the tissue sample. Molecular progression was detected at day 499 (16th month) after surgery and CT recurrence at day 829 (27th month).

Considering today's evidence based on the ADAURA trial, a phase 3 randomized trial, in the adjuvant setting of resected EGFR-mutated NSCLC, adjuvant EGFR TKIs

significantly improved disease-free survival and non-significantly improved overall survival ^{127, 128} and could have been performed in patient 6 and 14. On the other hand, the mutational profile of ctDNA could have detected molecular recurrence prior to the CT scan and an EGFR TK could have been initiated earlier.

Patient 25 (n°5 of the paper) was an R1 resection and adjuvant chemotherapy was performed. On the 94th day (3 months) after surgery, ctDNA recurrence occurred and a CT scan showed progression at the 150th day (5 months). In patient 37, KRAS mutation (p.Gly12Asp) was identified in the tissue sample. Molecular progression was detected on day 164th after surgery and on a CT scan on the 834th day.

In our study, nine patients had KRAS mutations, and four (44.4%) of them had G12C mutations. KRAS is a protein that is involved in cell signalling pathways, including those that are associated with cell growth and differentiation. KRAS mutations are detected in 23% of patients with NSCLC, with the G12C mutation being the most common. They are associated with poor prognosis, likely due to poor responses to most systemic therapies. Also, immune efficacy has been observed in those patients who have KRAS mutations. Although it has long been deemed "undruggable", with the development of drugs specifically binding the KRAS-G12C mutant protein, clinical trials that directly inhibit oncogenic RAS, the covalent KRAS-G12C inhibitors sotorasib and adagrasib, have recently made promising improvements to treat patients with advanced NSCLC carrying KRAS-G12C mutations ^{99,100}. In the adjuvant setting, this indication has not yet been considered.

So hopefully, considering the studies mentioned above, ctDNA may be contemplated, in future, a prognostic biomarker of according to the levels of ctDNA expression during follow-up, and a predictive biomarker of targetable treatments in the adjuvant setting of post-surgical lung cancer patients in clinical routine practice. Overall, our study adds to the accumulating evidence that supports the utility of ctDNA testing for the detection of minimal residual disease and recurrence prior to imaging progression. This may be used as a sensitive tool for identifying patients at high risk of relapse. It may also identify those that will benefit from additional adjuvant therapy from those at lower risk of relapse, for whom it may be possible to consider less intensive treatment.

Not neglecting the value of imaging in confirming no evidence of disease, as a clinician, it seems more comfortable if no evidence of blood ctDNA is also demonstrated, certifying "ctDNA clearance" during follow-up. Acknowledging, that in cases of ctDNA positivity and no evidence of disease in imaging during follow-up, as an oncologist, patients are until today considered in a state of high risk of recurrence and surveillance must be narrowed. Oncologists can only treat imagiologically evidence of cancer. Treating cancer blood recurrence through "ctDNA relapse" is a vision if proven worthy yet to be accomplished.

As the results show, our efforts should focus in the near future on establishing these types of methodologies and their use in refining diagnosis, monitoring patients and implementing molecular-based lung cancer tracking programmes.

CONCLUSION

Our concern is to define the added value of these studies to the clinical standard of care and ultimately lead to practice-changing protocol.

In summary, our results suggest that at surgical discharge and at the last follow-up, the quantitative expression ctDNA is greatest; its' decline at the first follow-up is likely due to the elimination of ctDNA post-surgical aggression and this is probably the best timing to consider ctDNA value as a prognostic biomarker of early-stage lung cancer. Nevertheless, the increase of ctDNA at the last follow-up after a decline at the first follow-up may be due to molecular progression and may be considered a predictive biomarker of recurrence after surgical curative treatment if no other cellular aggression has occurred. Besides the preoperative peripheral stage time point being one of the time points where older patients express more ctDNA, it is also the one offering the highest sensitivity for mutational profile concordance between the tumour and ctDNA samples. Not less relevant is the fact that 2 out of 6 patients where the correspondence of mutational profile was confirmed at the preoperative peripheral stage and pulmonary vein time-points, showed molecular and imaging progression.

The surrogate endpoint of an assay is to determine the "clearance of ctDNA" which depends on understanding ctDNA kinetics. Detection of minimal residual disease or the definition of ctDNA threshold depends on a retrospective analysis of a cohort of patients, to be validated and considered as a response. The clinical practice supports the new concept of "ctDNA clearance" correlated with disease-free survival and the concept of "ctDNA relapse prognostication" or detection of minimal residual disease posing opportunities to increase cancer cure in high-risk populations.

FUTURE PERSPECTIVES

For a long time, Physicians have dealt with a binominal ethical dispute between treating the Disease or the Patient. The secret of the care of the Patient is in caring for the Patient. Medicine wasn't born to "kill" death. Physician's aim is not to stop death, but rather treat and care for the living. Diseases *per se* do not exist, but diseased patients do¹²⁹⁻¹³². Liquid biopsies have revolutionized the era of translational research with direct benefits in the quality of care of our patients and with high public interest.

The possibility of identifying neoplastic ctDNA is a silent inoffensive tool, that acts as a cancer spy and can trigger appropriate reactions even before the disease (the enemy) or the doctors (the fighters), could have a minimal idea of its manifestation, besides all experience and scientific knowledge of time to progression or acquired resistance to target therapies¹³³.

A good spy (ctDNA) is the one which triggers the alarm discretely but with high sensitivity and specificity, to produce a good defence and an unexpected combat. It should be performed at the appropriate time (fit patient) permitting to use of the best armament (target therapies). This is what all clinicians, molecular scientists and patients should wish for: Excellent precision cancer medicine in a silent mode (minimal side effects) to preserve and recover benign territory before further one is damaged or lost⁵⁸.

The turnaround timer is quicker on liquid biopsies than on tissue biopsy. Liquid biopsies may be useful in early diagnosis, real-time monitoring of disease and estimating the risk of relapse (prognostic information) to define therapy selection, therapy resistance (predictive information) and secondary therapy selection¹³⁴⁻¹³⁶.

As the results we present show, our efforts should focus on establishing these types of methodologies and their use in refining diagnosis, monitoring patients for minimal residual disease and implementing molecular-based lung cancer tracking programmes¹³⁷.

Science progression has to be awaited to answer the question "how?" but it gets very confusing when you ask "why?"¹³⁸. As well as to understand "what" will be its consequences and "where" and "when" should these therapies act. Certainly, being the winner overall survival, the best combination in the correct sequence and in the right timing to these questions will predict the best outcome in caring for and treating our patients in a translational new era of Medicine.

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