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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 [β = -16 734 (-27 707; - 5760); $p = 0.003$; β = -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 [β = 8369 (0.359; 16 378); $p = 0.041$; β = 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-operatory 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-operatory 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 de 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 estádio inicial.

Métodos: Uma coorte de 47 pacientes com cancro de pulmão em estádio 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 momento 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 [β = -16 734 (-27 707; -5760); $p = 0,003$; β = -21 785 (-38 447; -5123); $p = 0,010$] em oposição a um aumento da concentração de ctDNA na veia pulmonar e no último seguimento [β = 8369 (0,359; 16 378); $p = 0,041$; β = 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 estádio inicial.

Palavras-chave: Detecção 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

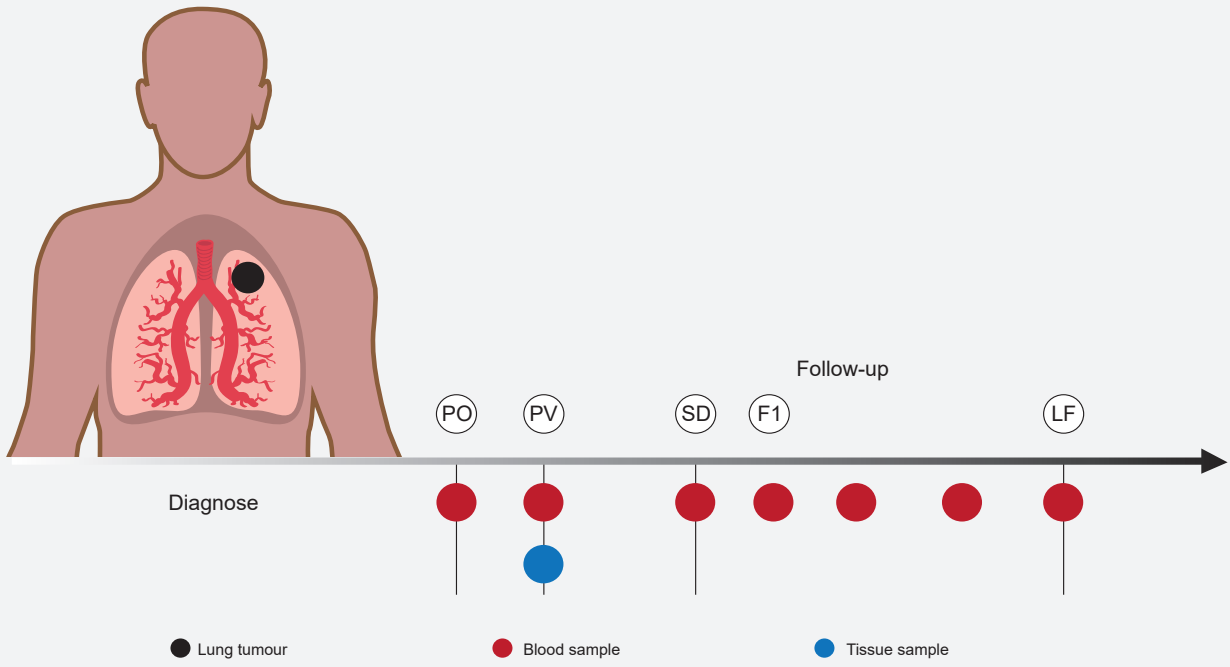

Figure 1 – Flowchart
PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

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 panelTM were used for tissue biopsies and Oncomine 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 Kruskall–Walli's test, a non-parametric version of the one-way ANOVA model, was performed between all-time points. Linear regression

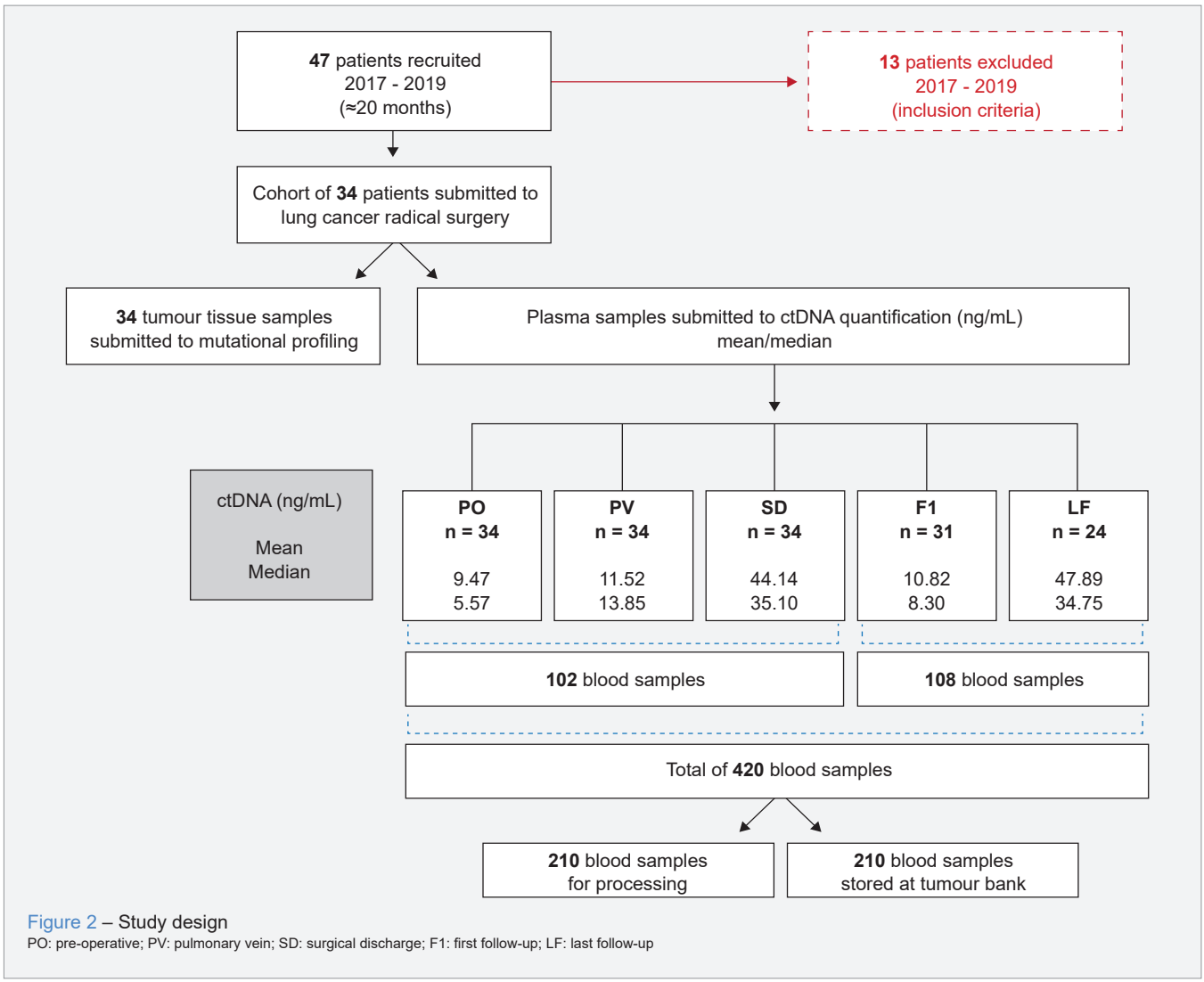

Figure 2 – Study design
PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up

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%).

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)

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 Kruskall–Walli's test results were inferior to 0.001.

The correlation between ctDNA concentration according to five-time points (dependent variables) and the four clinicopathological 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 [β= -16 734 (-27 707; -5 760); $p = 0.003$; β = -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 [β = 8369 (0.359; 16 378); $p = 0.041$; β = 34 402 (12 549; 56 254); $p = 0.002$, with a confidence level of 95%]. The joint model value is β = -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.

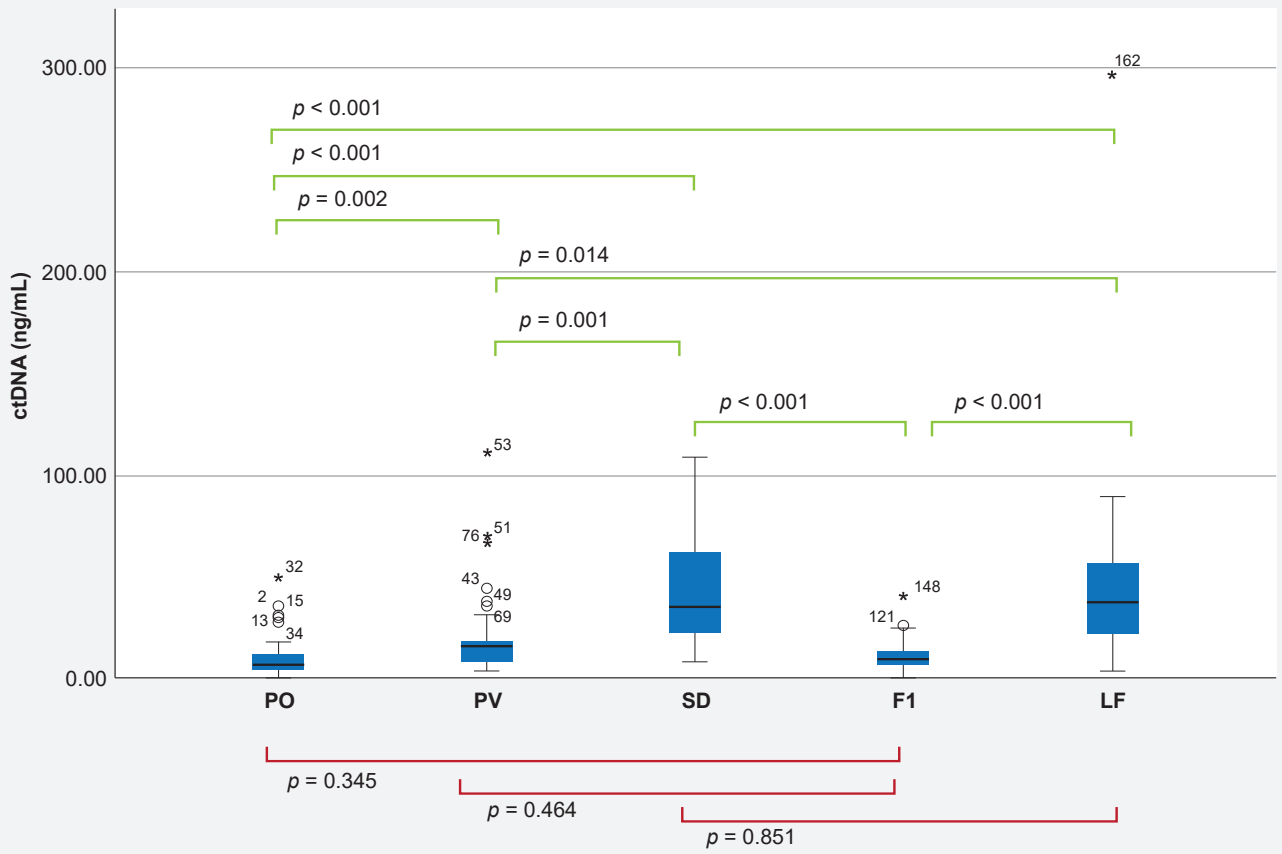

Figure 3 – ctDNA concentration at five time points (p adjusted value)
PO: pre-operative; PV: pulmonary vein; SD: surgical discharge; F1: first follow-up; LF: last follow-up
Green lines: statistical significance. Red lines: not statistically significant.

Table 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 ≤ 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

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 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

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 *EGFR* p.Asp770delinsGlyTyr	0.1 9.8 0	Negative 44.0 0	Negative 48.9 5	Negative 57.1 499	**Positive** **828**
#14	55.8 *EGFR* 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 *BRAF* p.Val600Glu	Negative 6.4 0	Negative 10.8 0	Negative 31.1 5	Negative 5.6 29	Negative
#28	9.7 *EGFR* p.Leu858Arg	**0.1** 4.2 0	Negative 4.2 0	Negative 12.2 4	Negative 62.13 312	Negative
#29	8.1 *EGFR* p.Leu747_Ala750delinsPro	**0.1** 3.6 0	**0.1** 10.2 0	Negative 14.9 5	Negative 10.9 22	Negative
#34	30.6 *EGFR* p.Leu747_Ala750delinsPro	**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

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 Chaudhuri[22] 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 patients[4], 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 nonsignificant 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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