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A Microfluidic Chip for Size-Based Isolation, Detection and Characterization of Circulating Tumour Cells in Lung Cancer

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Abstract

Lung cancer has the highest mortality related to cancer disease, affecting millions of people every year worldwide. The high mortality rate is mainly related to late diagnosis, when there is already tumour evolution, metastasis formation and less chances of survival. Tumour heterogeneity influences the efficiency of the applied treatment, commonly leading to relapse. Traditional methods of diagnosis and treatment are scarce and largely ineffective since the clinical profile of each patient determines their response to a certain therapy. Over the past decades, efforts towards the development in the field of oncology have been strongly focused on personalised treatment.

Although tissue biopsy is still the golden standard and provides useful information, liquid biopsy has emerged as a non-invasive, repeatable technique that is believed to transform clinical practice, presenting greater reproducibility and sensitivity in the diagnosis and monitoring of cancer, that can constitute as a solution for the intrinsic molecular cancer heterogeneity. Incorporation of liquid biopsy in routine consultations, may allow early-stage cancer diagnosis, as well as better monitoring and treatment selection. However, further developments must be caried out, so that liquid biopsies may be suitable for clinical settings and complemented by imaging and tissue biopsy traditional approaches.

Liquid biopsy focuses on the detection of materials originated in the tumour, also named biomarkers. One of the most analysed biomarkers in liquid biopsies is circulating tumour cells (CTCs). These cells, originated from the primary lung cancer, detach from the original site and travel the body through the bloodstream, inducing new lesion formations in other organs. CTCs' role in the process of metastasis is extremely useful to better understand how tumour evolution occurs. For CTCs to able to reach the bloodstream and provoke secondary tumours, they suffer phenotypical alterations through a process called epithelial to mesenchymal transition (EMT), in which cells lose their epithelial features and acquire mesenchymal ones, conferring them more mobility and aggressive characteristics. There is an extend amount of information that can be obtained through CTC analysis, including RNA, DNA and proteins. Ultimately, this strategy can support in the assessment of tumour profiling, correlation between CTC count and cancer burden, choice of treatment, monitorization of treatment and detection of minimal residual disease after

therapy. Combining different biomarkers within the same study is considered as being a promising strategy to obtain the most complete tumour profile possible.

Although CTCs present high potential, their isolation is extremely challenging due to their low concentration in blood, when comparing to other haematological cells. To overpass this impasse, enrichment techniques have been developed. These can be label dependent, relying on the presence or absence of specific cell surface markers of CTCs, or label independent techniques, that focus on the physical characteristics of CTCs.

Microfluidics systems are based on cell sample processing, in which cells flow through arrays of a microscale geometric design, incorporated with constriction to dictate the movement of CTCs and capture them according to their physical characteristics, such as size. Microfluidic devices have been widely used in liquid biopsy, replacing traditional, more expensive and not so effective methods. These may function based on immune-affinity, physical separation or immunomagnetic strategies. Microfluidic systems can be used in CTCs isolation, allowing rapid and high sensitivity capture, as well as enhanced cell recovery.

The main goal of this project was to isolate, detect and characterize CTCs present in blood samples from lung patients, using a size-based PDMS microfluidic device. The successful isolation of CTCs using the microfluid chip was assessed through cell spiking assays, using a urinary transitional cell carcinoma cell line. Diluted plasma samples were processed through the microfluidic device using a syringe pump. CTCs were isolated within the system and phenotypical characterized by cell surface epithelial and mesenchymal markers. Characterized samples were observed under a fluorescence microscope, as CTCs counting per sample was carried out. The obtained results obtained were correlated with the clinical data of the patients, regarding type and TNM stage. Follow-up monitorization of patients was performed, analysing the variation of CTCs count between a first assessment at diagnoses and follow-up after treatment therapy. DNA and RNA were extracted from the samples that presented the highest numbers of CTCs, and nucleic acids concentration was assessed. A proteomics study was implemented to analyse the composition of proteins present in isolated CTCs, specifically the presence of PD-L1 positive expression in comparison to a sample with no expression of this protein. A combined study of cfDNA methylation from plasma and CTC counts was done, to further assess the complementary information obtained by the study of the two biomarkers.

The work developed within this work demonstrated the successful application of a size-based microfluidic system to isolate and quantify CTCs, as well as perform the extraction of nucleic acids for further molecular characterization of the tumour, preliminary proteomic profiling of CTCs and assess another circulating biomarker from the same sample, cell free DNA. Microfluidic chips display potential to assist in the diagnosis, stratification, and follow-up of lung cancer patients.

Resumo

O cancro do pulmão apresenta a maior mortalidade relacionada com doenças cancerígenas, afetando milhões de pessoas em todo o mundo. A alta taxa de mortalidade está relacionada principalmente com o diagnóstico tardio, quando normalmente já houve evolução do tumor, inclusive formação de metástases e menor chance de recuperação. A heterogeneidade do tumor influencia a eficiência do tratamento aplicado, comumente levando à reincidência do tumor após terapia. Os métodos tradicionais de diagnóstico e tratamento são escassos e pouco eficazes, uma vez que o perfil clínico de cada paciente determina sua resposta a uma determinada terapia. Nas últimas décadas, os desenvolvimentos no campo da oncologia têm sido notáveis e têm-se concentrado maioritariamente no tratamento personalizado a cada paciente.

Embora a biópsia de tecido ainda seja a técnica mais usada e forneça informações úteis no diagnóstico do tumor, a biópsia líquida surgiu como uma técnica não invasiva e repetível, que se acredita poder transformar a prática clínica, apresentando maior reprodutibilidade e sensibilidade no diagnóstico e acompanhamento do cancro, o que pode constituir uma solução para a heterogeneidade molecular intrínseca do cancro. A futura incorporação da biópsia líquida nas consultas de rotina, pode permitir o diagnóstico do cancro ainda em estádio inicial, bem como melhor acompanhamento e seleção do tratamento. No entanto, ainda são necessários vários estudos de modo a perceber como as biópsias líquidas se podem tornar adequadas para uma situação clínica ou hospitalar, ainda assim, complementadas por abordagens tradicionais como a biópsia de tecido e os exames de imagem.

A biópsia líquida traduz-se na deteção de materiais originados no tumor, também chamados de biomarcadores. Um dos biomarcadores mais analisados em biópsias líquidas são as células tumorais circulantes (CTCs). Essas células, originadas no tumor primário de pulmão, desprendem-se do local de origem e viajam pelo corpo através da corrente sanguínea, induzindo a formação de novas lesões em outros órgãos. O papel das CTCs no processo de metástase é extremamente útil para entender melhor como ocorre a evolução do tumor. Para as CTCs poderem entrar na corrente sanguínea e conseguir formar tumores secundários, estas sofrem alterações fenotípicas por meio de um processo denominado transição epitelial para mesenquimal (EMT), no qual as células perdem as suas características epiteliais e adquirem características mesenquimais, conferindo-

lhes maior mobilidade e maior agressividade. Há uma grande quantidade de informação que pode ser obtida por meio das CTCs, incluindo a análise de RNA, DNA e proteínas. Em última análise, esta estratégia permite a avaliação do perfil do tumor, correlação entre a contagem de CTCs e a evolução do tumor, auxiliar na escolha do tratamento, monitorização do tratamento aplicado e deteção de doença residual mínima após a terapia. A combinação de diferentes biomarcadores num mesmo estudo é considerada uma estratégia promissora para se obter um perfil tumoral mais completo.

Apesar do alto potencial na utilização das CTCs, o seu isolamento é extremamente difícil devido à sua baixa concentração no sangue, quando comparada a de outras células hematológicas. As denominadas técnicas de enriquecimento destas células foram desenvolvidas para contornar esse mesmo problema. Estas técnicas podem ser dependentes da presença ou ausência de marcadores específicos de superfície celular das CTCs, ou podem ser técnicas independentes de marcação, concentrando-se nas características físicas das CTCs.

Nos sistemas de microfluídica baseados no processamento de amostras com células, estas fluem por sequências geométricas delineadas em microescala, de forma a controlar o movimento dos CTCs e capturá-los de acordo com suas características físicas, como o tamanho. Dispositivos microfluídicos têm sido amplamente utilizados em biópsias líquidas, substituindo métodos tradicionais, mais caros e não tão eficazes. A tecnologia de microfluídica pode funcionar com base na afinidade imunológica, separação física ou estratégias imunomagnéticas. Estes sistemas, usados no isolamento de CTCs, permitem uma captura rápida e de alta sensibilidade, bem como uma recuperação celular aprimorada.

O objetivo principal deste projeto foi isolar, detetar e caracterizar CTCs presentes em amostras de plasma de pacientes diagnosticados com cancro do pulmão, usando um dispositivo de PDMS, pelo qual a estratégia de isolamento das CTCs é realizada por tamanho. A eficiência do chip de microfluídica no isolamento das CTCs foi testada usando uma linha celular de carcinoma de células transicionais urinárias. As amostras de plasma diluídas foram processadas através do dispositivo de microfluídica usando uma bomba de seringa. As CTCs foram isoladas dentro do sistema e caracterizadas fenotipicamente por marcadores de superfície epiteliais e mesenquimais. As amostras caracterizadas foram observadas no microscópio de fluorescência e realizou-se a contagem de CTCs por amostra. Os resultados obtidos foram correlacionados com os dados clínicos dos pacientes, nomeadamente o tipo e o estádio TNM do cancro do pulmão. Foi realizada uma monitoração de seguimento dos pacientes, na qual se analisou variação da concentração de CTCs entre a primeira amostra recolhida no diagnostico e uma segunda amostra de acompanhamento, após tratamento médico. O DNA e RNA foram extraídos das amostras que apresentaram maior contagem de CTCs, e a concentração de ácidos nucleicos foi avaliada. O estudo da proteómica foi implementado para analisar a composição de proteínas em CTCs isoladas, especificamente as diferenças na análise de uma amostra com expressão positiva de PD-L1 em comparação a uma outra sem expressão desta proteína. Por fim, foi realizado um estudo combinado en-

tre a metilação de cfDNA presente na amostra de plasma e contagens de CTC, para avaliar com maior detalhe as informações complementares que podem ser obtidas pelo estudo dos dois biomarcadores.

O trabalho desenvolvido neste projeto demonstrou sucesso na aplicação de um sistema de microfluídica para o isolamento por tamanho e para a quantificação de CTCs, assim como para realizar a extração de ácidos nucleicos para posterior caracterização molecular do tumor, para a obtenção de um perfil de proteómica preliminar das CTCs e para avaliar outro biomarcador circulante proveniente da mesma amostra, o cfDNA. Os chips de microfluídica exibem potencial no auxílio de diagnóstico, estratificação e acompanhamento de pacientes com cancro de pulmão.

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Abbreviations

AdC	Adenocarcinoma
ALDH1	Aldehyde Dehydrogenase 1
ALK	Anaplastic Lymphoma Kinase
ASCO	American Society of Clinical Oncology
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B
BSA	Bovine Serum Albumin
CD45	Cluster Of Differentiation 45
cfDNA	Circulating Cell Free DNA
CK	Cytokeratin
CSCs	Cancer Stem Cells
CT	Computerized Tomography
CTC	Circulating Tumour Cell
ctDNA	Circulating Tumour DNA
CTLA-4	Cytotoxic Lymphocyte-Associated Protein 4
CTM	Circulating Tumour Microemboli
DAPI	4',6-Diamidino-2-Phenylindole
dsDNA	Double-Stranded DNA
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMA	European Medicines Agency
EMT	Epithelial To Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
EV	Extracellular Vesicle
FDA	Food And Drug Administration
ICI	Immune Checkpoint Inhibitors
ISET	Isolation By Size Of Tumour Cells
LC	Lung Cancer
LCC	Large Cell Carcinomas
LC-MS	Liquid Chromatography—Mass Spectrometry

LDCT	Low-Dose Computed Tomography
lncRNA	Long Non-Coding RNA
MET	Mesenchymal To Epithelial Transition
miRNA	microRNA
MRD	Minimal Residual Disease
MRI	Magnetic Resonance Imaging
NaCl	Sodium Chloride
NSCLC	Non-Small Cell Lung Cancer
ORA	Over-Representation Analysis
OS	Overall Survival
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
PD-L1	Programmed Death-Ligand 1
PDMS	Polydimethylsiloxane
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PFS	Progression-Free Survival
RB1	Retinoblastoma 1 Gene
RNA	Ribonucleic Acid
ROS1	Proto-Oncogene Tyrosine-Protein Kinase ROS
RT	Room Temperature
SAA	Serum Amyloid A Proteins
SCC	Squamous Cell Lung Cancer
SCLC	Small-Cell Lung Cancer
Si	Silicon
TEP	Tumour Educated Platelets
TKI	Tyrosine Kinase Inhibitor
TNM	Classification Of Malignant Tumours
Vim	Vimentin
WBC	White Blood Cell

Chapter 1

Introduction

The context in which the project was developed follows, as well as the main objectives and contributions. The structure in which the various themes are displayed is also presented.

1.1 - Context and motivation

Lung cancer is the cancer with the highest rate of mortality worldwide [1]. In 2020, lung cancer was the leading cause of approximately 1.8 million deaths, with over 2.2 million new diagnoses [2]. High mortality is intrinsically related to late diagnosis. At a more advanced stage of the disease, metastases are more likely to form, in which the tumour develops beyond the primary site. This phenomenon usually translates into a poorer prognosis, less chances of treatment success and, consequently, a decrease in survival rate [3]. There are two main types of lung cancer. Non-small cell lung cancer (NSCLC), which presents approximately 85% of lung cancers cases, and small cell lung cancer (SCLC), that accounts for 10% to 15% [3, 4].

Besides the typical imaging techniques for cancer screening, the most common method and still most practiced in study of oncological diseases is tissue biopsy, where a portion of tumour tissue is removed from the patient for further cytologic analysis. However, this method is quite invasive, as it may have consequences for the patient and cannot be performed on a regular basis. This prevents continuous monitoring of the tumour's evolution. Thus, liquid biopsy emerged, a non-invasive method that allows continuous analysis of tumour biomarkers from a small sample of blood, urine or saliva. This strategy has shown improvements for patient's follow-up and disease characterization, and it may revolutionize cancer screening and monitoring in the near future. The diagnosis, prognosis and monitoring of lung cancer, including response to certain therapy or relapse of the disease, may become more accessible. When analysing specific biomarkers, such as circulating tumour cells (CTCs), circulating free DNA (cfDNA) or exomes, it is possible to analyse patient's individual clinical information e personalize treatment according to the characteristics of a specific tumour [5]. For instance, immunotherapy came as valuable strategy for cancer handling, where a specific therapy is applied according to proteins expressed in a certain tumour [6].

Circulating tumour cells (CTCs) are one the most used biomarkers in liquid biopsy. These are spread from the primary tumour to the bloodstream. These cells present high heterogeneity and can provide valuable information, such as DNA/RNA analysis and proteins expression. Furthermore, a correlation between the enumeration of CTCs present in the bloodstream and the aggressiveness of the tumour or efficiency of a treatment has been shown. However, tumour cells are extremely rare, especially compared to the remaining blood cells, which makes their isolation so challenging. For this reason, there is a great need to develop highly sensitive mechanisms for CTC capture and enrichment [7].

Microfluidics devices have improved liquid biopsies, in terms of circulating tumour biomarkers isolation and analysis. This approach not only requires smaller amounts of reagents, produces less waste, but also allows precise control and manipulation of the studying fluids which confers higher sensitivity and less cell loss. Further developments and incorporation with other types of technologies may achieve high sensibility and specificity of these systems, requirements for a qualified CTCs analysis, as well as for more efficient diagnosis, prognosis and monitoring of lung cancer patients [8].

1.2 – Objectives and contributions

Lung cancer is the cancer related disease with the highest mortality globally. Since the low survival rate is related to the late diagnosis of the disease and limitations regarding the disease follow-up, it is urgent to develop new approaches. In recent times, there has been increasing interest in liquid biopsy, including CTCs. These cells play a pivotal role in the formation of metastases and their concentration has been proved to be related to the aggressiveness of the cancer. Furthermore, there is the possibility of adapting the therapy to the patient's clinical case by the information that is retrieved from tumour cells, including DNA, RNA, proteins, metabolites. However, due to the rarity of CTCs in the bloodstream, their isolation and analysis may become difficult.

This highlights the importance of developing this study, which aims to isolate and analyse CTCs, through a non-invasive approach, which relies on a polydimethylsiloxane (PDMS) size-based microfluidic system. Once captured, CTCs were characterized with epithelial and mesenchymal surface markers and observed under a fluorescence microscope. CTCs were identified and correlated to the characteristics of the patients, such as histopathological type, stage and treatment status. When possible, DNA/RNA were extracted from the isolated cells and CTCs were also analysed for their proteomics profile. For a selection of samples, paired analysis with DNA methylation from cfDNA extracted from plasma was performed. The main goal of this project is to contribute to the improvement of early diagnosis and more effective methods in the study of lung cancer heterogeneity, providing a non-invasive tool for tumour screening, characterization and monitoring.

The following study was carried out within the scope of the master’s thesis project in Biomedical Engineering at FEUP, throughout the 2020/2021 academic year. The work was carried out at the Institute of Research and Innovation in Health (i3S) of the University of Porto, in the Biocomposites group.

The work developed in this dissertation has resulted in an e-poster communication, which received a distinction at IJUP2021 within the topic of Engineering.

The results obtained from this study are to be included in a research article, currently under preparation.

1.3 – Structure

The dissertation report has a total of 5 chapters. The structure is described next. Chapter 2 contains the literature review, where a gather of theoretical information, regarding the themes involved in the project, is presented. This theoretical introduction includes research regarding: lung cancer, its histological classification and main characteristics; liquid biopsy, its benefits when compared to conventional biopsy, as well as the main biomarkers incorporated in this procedure; circulating tumour cells, its main features, recent enrichment technologies and its applicability in liquid biopsy and lung cancer diagnostic, prognostic and monitorization; and microfluidic technologies, how this innovative strategy has evolved the areas of liquid biopsy and tumour investigation. In chapter 3 is described the work plan carried out along the project, the main goals for the study and description of the main techniques used. Chapter 4 the results obtained along the study are portrayed, as well as a discussion about the success of the outcome of each step of step performed and comparison to what was expected based on the literature review. Finally, in chapter 5 a conclusion about the overall achievements and progress in the work executed, and if all the initial established goals were accomplished. Suggestions for future work are also mentioned.

Chapter 2

Theoretical Review

Initially, in-depth research was carried out on lung cancer, including its classification into different types and their main characteristics, as well as the main causes, symptoms and treatments currently applied to this oncological disease. Then, the main advantages and disadvantages of tissue biopsy were compared to the innovative and non-invasive liquid biopsy, as well as the biomarkers that can be used in the latter. The biomarkers incorporated in this dissertation project were CTCs, with their main priorities being highlighted, as well as the methodologies currently implemented in their isolation and characterization. Follows a description of microfluidic systems, and the advantages of their use, namely regarding their sensitivity and efficiency in the analysis of blood and cells.

2.1 - Lung cancer

Lung cancer is the cancer with the highest rate of mortality worldwide [1]. In 2020, lung cancer was the leading cause of approximately 1.8 million deaths, with over 2.2 million new diagnoses [2].

2.1.1 - Histopathological classification

There are two main types of lung cancer, non-small cell lung cancer (NSCLC) of epithelial origin, accounting for 80%-85% of the cases, and small cell lung cancer (SCLC), of neuroendocrine origin and that constitutes 15%-20% of overall cases [10, 13]. Its appearance is highly related to smoking habits and age [14], but there are other risk factors involved, such as naturally occurring carcinogens, long-term and accumulated exposure to air pollution and family history of lung cancer cases [15]. Environmental, industrial and economic conditions also have an impact in the incidence of the disease in the population [16].

One of the main causes for the high mortality rate is being usually asymptomatic in the early stages, being most of the times only diagnosed at an advanced state, and with relapse post treatment [17]. In fact, approximately 60% of newly diagnosed patients already present

locoregional or metastases formation when diagnosed [66]. Late-stage lung cancer may lead to metastatic formation that hinder treatment, as well as higher chances of mortality [18]. Cancer detection at early stages is ideal for better responses to therapy and, in turn, a decrease in incidence of recurrences [19]. The most common symptoms of this oncologic condition, appearing mostly in advanced stages of the disease, are persisting cough, difficulty in breathing, hoarseness, among others [10].

Since SCLC and NSCLC are originated from different types of cells, they also have varying clinical features, including mutations occurred. SCLC is localized in a central location of lung and is highly associated to smoking habits [20]. This type of lung cancer is more aggressive and grows at a rapid rate, with formation of necrotic tissue at tumour site, as well as higher probability of metastasis in early phases of the disease and acquired resistance to chemotherapy [21]. Resistance to a certain therapy can be classified as primary or intrinsic resistance, that is associated with patients who have no response towards the therapy applied. Secondary or acquired resistance is related to patients who temporarily respond to treatment but eventually stop because drug-sensitive tumour cells die and the subclones that gained resistance start to increasingly proliferate [22]. SCLC staging includes limited (LS) and extensive (ES) stage disease. ES is more common, which translates into approximately 65% of diagnosed cases. Over the last few years, the average overall survival (OS) for patients diagnosed with ES has been reported to be 8–12 months, while patients LS are associated to 12–20 months [23]. Despite of presenting a worse prognosis, some clinical results registered a better response to initial chemotherapy in SCLC cases. However, in case of relapse, the mortality rate drastically increases, and the frequency of the appearance mutant alleles are much greater, when compared to cases with stabilized disease [24].

Regarding NSCLC, there are different histological types, with approximately 40% being adenocarcinoma (AdC), 15% to 30% squamous cell lung cancer (SCC) and 10% to 15% of large cell carcinomas (LCC) [25, 26]. Since NSCLC is more common, there are more studies and advances in methods correlated to this type of lung cancer, including therapeutic responses to specific targets identified [27]. Survival rates usually vary according to TNM stages of the disease. The TNM staging system is used to describe the amount and spread of cancer in a patient's body, including the size of the tumour, its spread to nearby lymph nodes and existence of metastasis [28]. The five-year survival rate for NSCLC stages 0-I is 90%, while stages II and III reach 60% and 40%, respectively. However, the five-year survival is less than 20% for patients diagnosed with stage IV, metastasis or recurrence, making early detection pivotal to improve patient's outcome [29, 30].

Precise determination of histological characteristics has a major role when deciding the type of treatment that is going to be administered, as a way of ensuring the maximum efficacy and reducing toxicity of chemotherapeutic agents [31]. Table 2.1 presents a summary of the main differences between both types of lung cancer.

Table 2.1 — Classification of lung cancer.

Type	Subtype	Location in lung	Main characteristics
NSCLC (80-85%)	AdC (40%)	Peripheral	Most common in non-smokers Should test for EGFR, ALK, ROS1 and BRAF mutations for targeted therapy and proteins such as PD-L1 for immunotherapy therapy
	SCC (15-30%)	Central and peripheral	Associated with smoking habits
	LCC (10-15%)	Peripheral	Similar to AdC
SCLC (10-15%)		Central	Associated with smoking habits Fast growth Worse prognosis Best response to initial chemotherapy Treatment with poor survival in relapse

(Adapted from [10, 32])

2.1.2 - Treatment approaches

Medical treatment for oncological diseases is based on type of tumour, presence of metastasis and TNM staging [33]. The main treatments applied for oncological diseases, including both SCLC and NSCLC, are surgery, chemotherapy and radiotherapy [10, 15]. Surgical resection is considered optimal for NSCLC cases [30, 34].

Additionally, medical advances with the discovery of oncological driver mutation, in patients with NSCLC, provided a new type of treatment that is targeted to the patient. A precise and real-time evaluation of immune status of ill patients presents high importance for diagnosis and prognosis, providing a viable evaluation of the efficiency of a certain immunotherapeutic and tailoring drug treatment, according to the patient's immune characteristics [13]. Targeted therapy and immunotherapy are two methods that have been widely used in lung cancer treatment. Targeted therapy attacks specific mutations.

There have been increasingly new NSCLC genotypes discovered, that allow better understanding of therapies resistance and lead to new more effective therapeutic responses [35]. Over the years, several studies have discovered molecular mechanisms corelated with the acquisition of resistance to therapies, such as the T790M mutation, in response to first line treatment applied for the epidermal growth factor receptor (EGFR), an activating mutation that is estimated to occur in 10 to 30% of all NSCLC cases. MET amplification, epithelial mesenchymal transition, among others, have also been investigated [9, 36, 37]. For instance, a study reported a correlation regarding the extent of the T790M mutation and the degree of MET amplification. The rapid growth and proliferation of cancer is sustained by protein-specific pathways, such as EGFR and HER-2 [38]. This indicates the presence of heterogeneity and alterations related to treatment interventions, which can have a big impact in the response to a certain therapy [39]. BRAF melanoma cases are an example of acquired resistance since patients initially respond to treatment with tyrosine kinase inhibitors (TKIs), but eventually relapse and die [22]. Particularly for EGFR, the detection of sensitive mutations of this kind in ctDNA, acts as a sensitive marker guiding targeted therapy for patients diagnosed with

NSCLC, that already present metastases in the central nervous system [40]. Targeted therapies using TKIs have been applied as treatment for patients diagnosed with EGFR mutations. T790M commonly appears to nearly 48–62% of EGFR TKI-resistant patients, after first line treatment. In these cases, third generation TKI may be administered [41]. There has also been used targeted therapy for anaplastic lymphoma kinase (ALK) [42] and ROS proto-oncogene 1 (ROS1) mutations [43].

On the other hand, patients who do not present mutations, but instead have specific proteins expressed by the tumour, are selected for treatment by immunotherapy, where T cells are stimulated to fight cells that express this protein [44]. One of the proteins associated with lung cancer is programmed death-ligand 1 (PD-L1). Programmed death 1 (PD-1) receptor and its ligand (PD-L1) have been shown to regulate antitumor responses. PD-L1 binding to PD-1 inhibits T-cell function and proliferation, resulting in ineffective immune response. Analysis of blood biomarkers can also be useful in assessing the presence of proteins, particularly in exosomes and CTCs [45, 46]. A research described an improvement on progression free survival (PFS) when patients with positive expression of PD-L1 were treated with an immune checkpoint inhibitor (ICI), compared to patients with negative expression of PD-L1 that received the same treatment. These results show that by extensively analysing a patient's CTCs, a personal therapy can be selected and managed to slow down or even prevent the progression of the disease [39].

Diagnosis and discovery of specific mutations in SCLC cases may be more difficult, as well as development in studies, targeted therapies and design custom applications [47]. Besides being usually diagnosed at a later stage and highly metastatic, when discovered SCLC is considered a systemic disease, presenting a high frequency of recurrence and less survival cases [44, 48]. Chemotherapy has been for decades the number one treatment chosen for SCLC cases. There have been only a limited number of new treatments available over the last years and approved by the U.S. Food and Drug Administration, as an efficient method to treat SCLC patients, including nivolumab monotherapy and atezolizumab. Although there is an initial improvement as response to treatment, there is no significant improvement in patient's survival thus far, because relapse follows almost immediately and associated to acquired resistance [49, 50]. Tobacco-derived carcinogens are believed to be intrinsically connected to high heterogeneity and mutational landscape in SCLC cases. This type of lung cancer is also associated to high incidence of inactivating mutations of TP53 and retinoblastoma 1 gene (RB1), as well as to other with lower frequencies and in smaller populations, including amplification of MYC, MYCN, MYCL1, and FGR1 or inactivation of PTEN [51, 52]. This limited number of therapeutic options, not only for SCLC but also for NSCLC cases, arises a need to find improved diagnostic, follow-up and treatment methods for this specific pathology, that can improve patient's outcome [53].

Lung cancer, being a heterogeneous and complex disease, that may present up to six different clonal cell lines within the same tumour [34], made customized medicine emerge as a viable solution to improve oncological survival rate. This method includes target-based drug combination, mutation-guided drug design and collecting data of population genetics, to obtain better guidance on how to treat patients who cannot take genetic testing. As previously

mentioned, the increasing rate of new cases of lung cancer led to the development of innovative techniques, that are expected to raise the standard for cancer patient treatment [54].

2.2 - Liquid biopsy

2.2.1 - Screening and diagnosis

Nowadays, in oncological disease screening, imaging or pathological examination are the most used techniques to diagnose and stage lung cancer [3]. However, as in most cases, this strategy presents disadvantages, including low sensitivity and precision, leading to false positives and misleading diagnosis, especially in early-stage cases, when tumour lesions are quite small [46]. In clinical practice the most used image modalities for tumour diagnostic remain the same, such as radiography, magnetic resonance imaging (MRI) and ultra-sound, among others [55]. Tumour burden can be assessed by computed tomography (CT) scan and tumour metabolism can be measured through positron emission tomography (PET) scan [56]. From all, low-dose computed tomography (LDCT) is considered the most valuable method for lung cancer screening [57, 58]. However, this kind of monitorization presents low detection sensitivity and must be done periodically, which may have risks of radiation exposure [25].

2.2.2 - Tissue biopsy and its limitations

In case of doubt or detection of suspicious nodules, it is customary to resort to tissue biopsy. Tissue biopsy is still the golden standard method used to detect, through a morphological assessment, the presence of cancerous illness, along with radiological staging with the TNM criteria [59]. The conventional biopsy involves extraction of tissues samples to examine under the microscope after fixation and staining of the cells, as well as their genetic characterization [60]. Tumour tissue represents an important resource for research breakthroughs and are a common component of therapeutic clinical practice [61]. Tissue biopsy has been implemented for a long time which confers some experience and more knowledge about the technique, when compared to more recent methods [66]. Although tissue biopsy is still the most used method, it presents some limitations that can disable patient assessment [13]. To get the genotype of the tumour tissues, invasive procedures are performed, which comes to prejudicial risks to patients. The conventional methods of tissue biopsy may not provide sufficient tissue to determine driver mutations, thus preventing a faster application of the correct treatment. In fact, approximately 25% of lung cancer biopsies are not enough for tissue assessment. Some tumour lesions may be more difficult to perform a tissue biopsy also. This is especially true for lung cancer patients, due to the great difficulty in obtaining a tissue sample due to the location of the tumour. Additionally, tissue biopsies cannot be routinely performed and thus, cannot follow the tumour evolution [66]. Furthermore, the profiles of primary and secondary tumours are not always coincident, leading to intrinsic molecular cancer heterogeneity due to metastasis [62, 63]. The genetic heterogeneity is believed to be associated with the ability of the tumour to harbour subclones, that present metastatic abilities or mutations associated with drug resistance [64]. Metastasis may occur through the bloodstream, via blood and lymphatic vessels and are considered hostile more challenging to treat. Even if relying on strategies such as imaging biopsies, surgical resection of lesions is practically undoable [29]. Hence the importance to regularly monitor the progress of the disease [65].

2.2.3 - Liquid biopsy

Liquid biopsy emerged as a reliable option for a better monitoring to assist in the diagnosis, prognosis, and treatment of patients with cancer [13]. The liquid biopsy can detect genetic material from cancer origin, in a non-invasive test, using blood samples or other body fluids and avoiding repeated invasive procedures [60, 66]. Liquid biopsy is believed to revolutionize clinical practice in oncology, by signalling the presence of an already molecular metastatic status in an otherwise phenotypically local disease, by evaluating the result of a certain treatment in metastatic disease and by predicting tumour spread according to the persistence of minimal residual disease (MRD) after medical procedure [63]. MRD is observed in a situation where any tumour-derived material is detected in blood after treatment [67].

Liquid biopsies are not being developed with the final goal of replacing common biopsies or imaging techniques, but to provide additional information and an alternative to certain situations, including patients who cannot be subjected to biopsy or when biopsies do not have enough tissue [68, 69]. In this last case, liquid biopsy offers a mean to perform genetic testing for targeted therapy. This type of biopsy also presents advantages, mainly related to the great sensitivity necessary to determine the expression of the low levels of circulating free DNA (cfDNA) present in peripheral blood [70]. Overall, liquid biopsy with cfDNA is already implemented in thoracic oncology and being used in several medical centres [71]. The EGFR Mutation Test v2 is a U.S. Food and Drug Administration (FDA) approved diagnostic tool for two types of drugs of NSCLC treatment, these being erlotinib, that has been replacing chemotherapy as first line treatment, and osimertinib, effective as a second line treatment to fight acquired resistance. Mutations in the EGFR gene are detected in real-time from cfDNA, that ultimately plays a part in the decision of which NSCLC patients are suitable for treatment and which targeted therapy should be used [72 - 74].

Blood samples may contain information, not only about the primary tumour, but also about the metastases that are associated to nearly 90% of deaths in oncological diseases [75]. Furthermore, blood can be obtained several times and used to monitor the development of the disease, or even early detection of response and relapse to a certain therapy [60]. Other samples are studied in liquid biopsies besides blood, such as saliva, urine and cerebrospinal fluid, which carry nucleic acid fragments, that are originated from cancer cells [56, 63].

In figure 2.1 is represented the comparison between tissue and liquid biopsy, mainly regarding advantages, limitations and the type of information that can be retrieved from each technique.

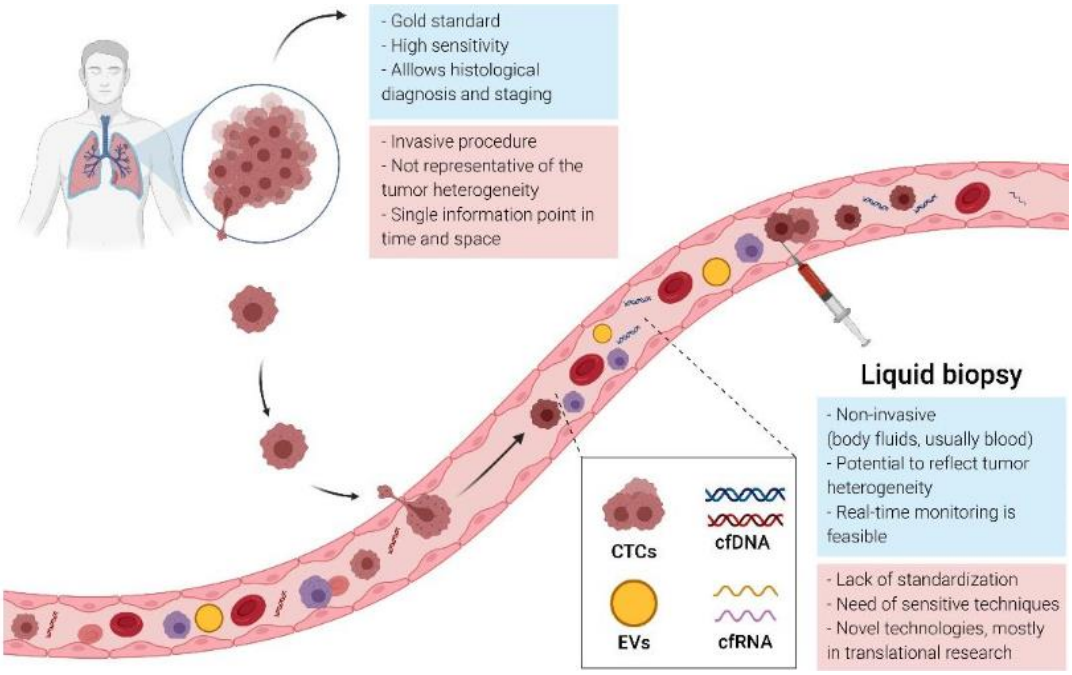


Figure 2.1 - Comparison between tissue and liquid biopsy in tumour assessment, regarding the advantages and limitations of each technique. (Adapted from [59])

While tissue biopsy only gives information about the cancer state at the exact moment of the procedure, a longitudinal liquid biopsy provides information about tumour evolution. However, it is possible that the liquid biopsy fails to ascertain the existence of pathology related to gene alterations, since circulating biomarkers can be present at a very low percentage in early stages, restricting the procedure's sensitivity [63]. The release of these biomarkers into the bloodstream is facilitated by a series of factors, including tumour location, aggressiveness and molecular properties, such as the loss of molecules that confer adhesion [70].

According to the NIH Biomarkers Definition Group, a biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [48]. These measurable biomolecules can be detected in body fluids and give information regarding the presence of a tumour and of its expected future development [19]. The main types of biomarkers collected in this process are circulating tumour cells (CTCs), tumour-educated platelets (TEP), extracellular vesicles (EVs), microRNAs (miRNA), long non-coding RNA (lncRNA) and even nucleic acids dissolved in blood samples, including circulating tumour DNA or RNA (ctDNA, ctRNA, respectively) [5, 22, 40, 69]. Simultaneous studies of several biomarkers can allow a greater range of information obtained, when compared to assays focusing on one single biomarker alone [39].

In lung cancer particularly, the two most analysed biomarkers are CTCs and ctDNA, having both different characteristics and advantages [76, 77]. Dissemination of tumour cells occurs since early stages in both NSCLC and SCLC [48]. While CTCs are released into the peripheral blood by primary tumours and provide the study of whole cells with DNA, RNA and protein-based molecular profiling; ctDNA, DNA released by tumour cells to blood, consists in a part of all cfDNA in blood and is mainly chosen for mutation analysis, copy number aberration and methylation changes in DNA [11, 60]. ctDNA methylation profile is an accessible analysis of

the early and common epigenetic alteration, which can reflect the tumour location, providing additional information, such as nucleosome positioning of the DNA sequence [67, 70].

In figure 2.2 is represented the mechanism of CTCs and ctDNA transition into the circulation.

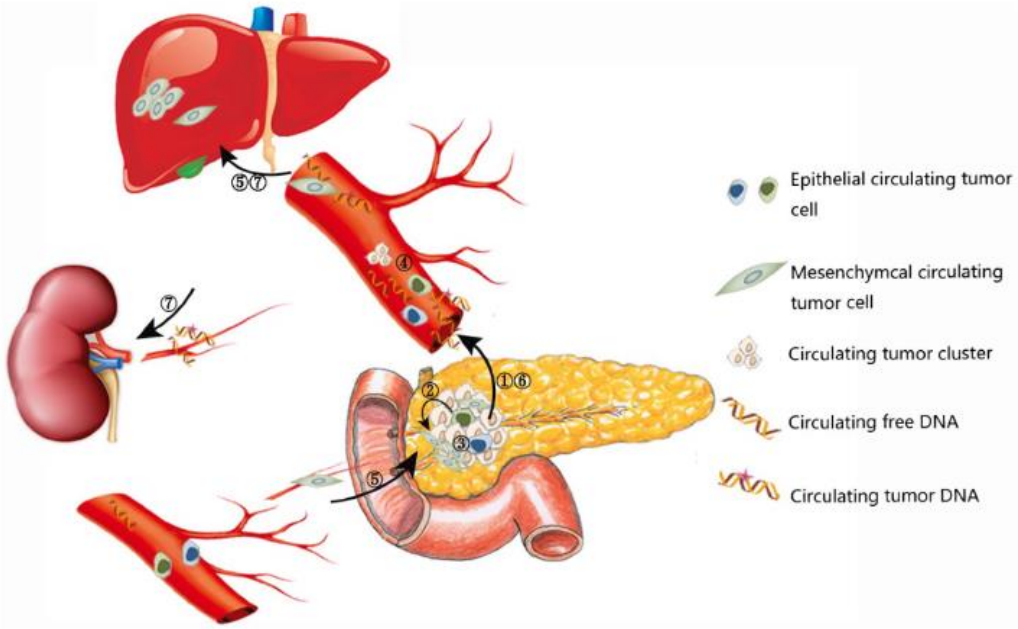


Figure 2.2 - Schematic representation of the release of CTCs and ctDNA into the bloodstream, and the associated process of metastasis. (Adapted from [77])

In figure 2.3 the applications of liquid biopsy in the treatment of lung cancer, throughout different treatment phases, are represented schematically, in early and advanced stages of the disease, respectively.

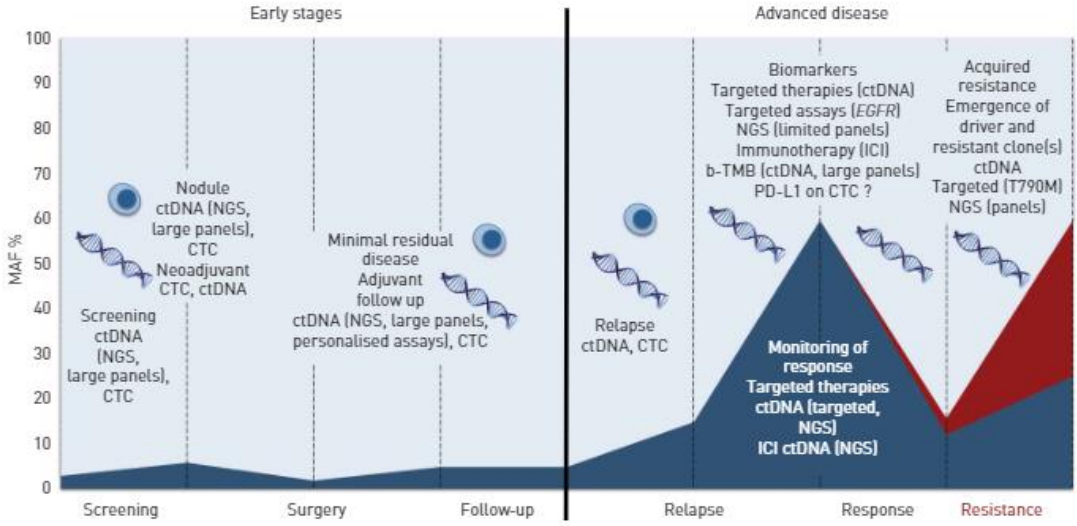


Figure 2.3 - Potential applications of liquid biopsy in NSCLC early-stage treatment and advanced stage treatment. MAF: mutant allele frequency. (Adapted from [67])

2.3 - Circulating tumour cells (CTCs)

2.3.1 - CTCs properties

The first reference to the acknowledgement of CTCs was back in 1869, when Thomas Ashworth described that a small number of cells found in a test blood from a patient, presented similarities with the cells found on the primary tumour [39, 78].

CTC is one of the responsible agents in the growth of metastasis [79], along with tumour microenvironment constituents and particular conditions, including cancer associated fibroblasts and cancer stem cells, believed to have a major role in tumour progression as well [80, 81]. CTCs leave the primary site of the cancer and circulate in the bloodstream and lymphatic vessels [75], sowing cancerous metastases in different parts of the body, promoting the formation of a secondary tumour [82]. This type of cells is believed to have a linear relation with cancer development, being possible to use their detection as a useful appliance to assess how aggressive the tumour is, and its potential of metastasize in other organs [83]. Thus, an increasing concentration of CTCs usually represents a more advanced stage of the tumour [84].

Some studies have shown the potential of CTCs incorporation in development of clinical treatment and personalized medicine [78]. There are different types of CTCs according to specific tumour surface genotypes [85]. Molecular characterization of these cells is fundamental to link gene mutation with tumour aggressiveness and find a suitable therapeutic response to target those specific cases [46, 69]. Through the study of this type of cell, it is possible to gather intel regarding their molecular genetics, epigenetics, transcriptomics and protein profile [71]. CTCs have been suggested as potential tumour markers for breast cancer, by the American Society of Clinical Oncology (ASCO) [86, 87].

This kind of cells are highly promising for liquid biopsies and monitorization of cancer development; however, they are present in very low percentage in blood at early stages of the disease, and contain heterogenous subpopulations, which makes the process of its capture and isolation more challenging [65]. To overcome this problem, it is necessary to carry out high sensitivity techniques to enrich and detect CTCs. Their genes and proteins expression characterization may be quite interesting for eventual assistance in a clinical decision for target therapies [79, 88]. Likewise, CTC isolation matched with protein profiling, can be helpful to analyse divergences between some CTCs and the primary tumour [68]. Single cell proteomic profiling may provide useful intel of what are the optimal novel drug targets, as well as the interpretation of clinical results and development of biomarkers helpful for patient stratification [89]. Quantitative proteomics gives the relative different protein abundance in both healthy and cancer patients, which translates in information about molecular interactions, signalling pathways, drug resistance and biomarker recognition. In lung cancer proteomic profiling is essential to plan target treatments, associating which therapy might present more benefits to a certain patient by analysing the molecular mechanism underneath [90].

Tumour mutations can be unique and analysis of mutations on CTCs has been reported to be useful in the identification of resistance mechanisms, although further clinical results are still needed [9, 22, 75]. Even after chemotherapy or other kind of therapy, CTCs analysis may be used to determine treatment viability [34, 46].

For the assessment of circulating biomarkers from whole blood, a variety of processes need to be considered. An example of a relevant process is the metastatic cascade, which includes the series of steps involved in the formation of metastases [44]. One of these important steps is the epithelial to mesenchymal transition (EMT), in which cells decrease their

epithelial phenotypes and adopt characteristics of mesenchymal cells [85]. During this process, CTCs suffer some changes on the molecular level, namely the loss of E-cadherin, cytokeratin, but also a significant increase in fibronectin, α-smooth muscle actin, vimentin and motility [91, 92]. These alterations endorse cells' migratory and invasive features, essential characteristics for the cells to survive the adversities, such anoikis and defence action by the immune system [5]. Other types of conditions can influence metastasis cascade. For instance, studies have demonstrated that intratumor hypoxia may lead to formation of CTC agglomerations with high metastatic capacity [93]. In the bloodstream there may be present single migratory CTCs or multicellular tumour aggregates, such as circulating tumour microemboli (CTM) or groups of CTCs, known as CTC clusters [94]. When these leave the blood, the reverse event of EMT occurs, the mesenchymal to epithelial transition (MET), in which cells seed in different tissues with favourable conditions for colonization, leading to micro and macro metastasis formation [34, 81, 95]. From the numerous cells that get released from the primary tumour into the bloodstream, only a few are capable of surviving and creating new lesions at distant sites [96]. CTCs may remain dormant at locations of metastasis and activate later in time, leading to relapse episodes [97]. It is also plausible to assume that hybrid EMT states of invasive cells help immune evasion and colonization [89]. These events are of great importance, since the high rate of mortality associated with oncological pathologies is intrinsically related to metastasis [98].

Several factors are linked to the success or not of a certain therapy for tumour treatment. One important factor is cancer heterogeneity, which has a major influence in tumour diagnose, as well as in choosing which therapeutic response should be applied. This phenomenon translates into tumour subpopulations and genetic diversity, that differ from primary tumour and between different lesions. Furthermore, tumour characteristics may change in assessments acquired, even more when samples obtained before and after treatment are compared. For instance, in the case of NSCLC, substantial intratumoral heterogeneity was found in a study involving 100 patients with early-stage cancer, in which various regions of 327 tumours were analysed. This type of study also had positive results in other types of cancer [39]. CTCs heterogeneity ultimately mean that these cells may present different characteristics. CTCs may be classified as epithelial, mesenchymal or epithelial/mesenchymal hybrids. The hybrid is considered as the most aggressive, since these cells maintain epithelial characteristics, such as cell junction properties for clusters, while also achieving the motility necessary to spread the tumour. Both epithelial and mesenchymal markers work for this kind of tumour cell [91]. The CTC population is mainly composed by cancer stem cells (CSCs), that present capacities of self-renewal and phenotypic heterogeneity regeneration, while subpopulations are thought to experience the EMT during tumour propagation to other organs. CTCs that present an EMT phenotype are believed to have the ability to get past the blood vessels, travel the body, until they find endothelial cells and attach to them, exiting the bloodstream and forming new metastasis [39].

2.3.2 – CTCs enrichment

In recent years, many of the methods that have been developed are mainly based on exploring the presence of epithelial markers on cells [81]. Affinity-based separation has been one of the first methods to be implemented; however, considering the EMT process, affinity-based separation according to certain antigens may not be so reliable [27].

Today, the CellSearch Veridex system is the only FDA approved method, since 2004 [34], to isolate CTCs and monitor diseased patients with metastatic colorectal, breast, or prostate cancer [79, 84]. In fact, most studies regarding CTCs analysis were carried out using this enrichment strategy [99]. The system is constituted by a capture reagent of ferrofluid and an immunofluorescent reagent. The ferrofluid reagent is based on nanoparticles with a magnetic strip core, a polymeric layer and a coating of antibodies that detect and target anti-epithelial cell adhesion molecule (EpCAM) antigen. Then, enriched cells are stained with DAPI, Cytokeratin (CK) and Cluster of Differentiation 45 (CD45). While Cytokeratin-PE is used to distinguish CTCs among nucleated cells, CD45-APC allows the identification of leukocytes [100]. Although CTCs are detected using the CellSearch system in both SCLC and NSCLC patients before medical intervention [86], it also presents some concerns regarding the dependence of the system on epithelial markers, which CTCs may have lost when undergoing the EMT process [101, 102]. The main issue found in this type of capture, is that its efficiency strongly relies on EpCAM expression, which may vary in different tumour cells, and even be absent in certain non-epithelial cancers [103], which is why complementary CTCs isolation based on physical properties or biomarkers unaffected by the EMT is so important [96]. For example, it has been reported that there is a lower EpCAM expression in lung adenocarcinoma comparing to other lung cancer subtypes [88]. It is also interesting to include more than one CTC marker as a target, so that the ones who are negative for EpCAM can be included in the final count and the therapeutic efficiency can be maximized to its fullest potential [81]. For this reason, a growing interest in label-free CTC enrichment technologies has emerged in recent times. This is a strategy that focuses on other characteristics of this type of cells that distinguish them from other blood cells. Hence, physical separation according to size and deformability is a broadly used method for isolation. CTCs are thought to range from 15 μm to 25 μm in most cancers. However, some small CTCs may present a diameter of approximately 2–4 μm [18]. Sized-based strategies focus on this range for their enrichment and separation from other blood cells. However, certain aspects such as sensitivity and reproducibility still need improvement, as there are still some problems with the efficiency of isolating CTCs, especially in their separation from white blood cells (WBCs) [84]. For instance, the Isolation by Size of Tumour Cells System incorporates a cell filtration based on the different size and deformity of CTCs, when compared to other circulating blood cells. This size selecting method is achieved with the incorporation of 8 μm pore size features for blood filtration [58, 97]. However, this strategy may present some issues regarding white blood cells contamination, since these present a size of 9–16 μm [84]. This strategy does not depend on antibodies against tumour-associated markers but is unable to detect CTCs that present very low dimensions [9, 87], besides being a time consuming and expensive method [78]. The CTC-iChip is an inertial focusing enhanced microfluidic capture platform, based on magnetophoresis, that isolates CTCs from whole blood. This method allows cytopathological and molecular characterization of both epithelial and non-epithelial tumours, which is extremely valuable especially in early stages of lung cancer [104, 105]. Other interesting method is the immunostaining-fluorescence in situ hybridization, also known as iFISH, in which aneuploid CTCs are detected and alternation of chromosomes that confer drug resistance are analysed [39].

There are many other types of CTCs isolation technologies, relying on different working methods, such as immunoaffinity-based separation, immunomagnetic-based separation, microfiltration in two and three dimensions, acoustophoresis, DEP force-based separation and

density gradient separation, among others [40, 39, 78]. CTCs enrichment can also be carried out considering whether the isolation is focused on single cells or clusters [106].

Although studies have been developed in this area, data from clinical results still needs further progression on how CTCs are intrinsically connected to various carcinomas, mainly because of their reduced concentration and phenotypic heterogeneity, that make the analysis more challenging [10, 18]. The non-existence of a general procedure to isolate CTCs is mainly justified by the disparity of results due to the heterogeneity among subpopulations of tumour cells, as well as the poor number of standard preparation protocols available, there being a great need and challenge to idealize a more tailored strategy [9, 107]. The main goal is to achieve high throughput and purity, as well as minimize as much as possible cell loss.

Despite all the limitations found in the current techniques focused on CTC isolation, these were proven to be useful in the detection of several types of cancer, including lung cancer [104]. The use of liquid biopsy, including CTCs characterization, is a promising strategy to be combined with standard screening tests and provide conditions for molecular and genetic characterization of the tumour [108]. Thus, enumeration of CTCs may serve as biomarker for cancer stage diagnosis, monitoring during and after treatment of either early, locally advanced, or advanced state of lung cancer, offering a better knowledge of how metastasis occur [10, 109]. It may also be used to monitor tumour evolution and progression free survival in NSCLC cases [85], since a higher concentration of CTCs is correlated with poorer prognosis and more advanced tumour phase, providing valuable insights into mechanisms underlying drug resistance and disease relapse [101]. Studies have shown correlation between SCLC patients and superior flow of CTCs [27, 34], as well as a significantly increase along the development of the disease (from stage I to IV) [86].

2.3.3 – CTCs characterization

Detection of CTCs in lung cancer is a difficult task due to its low concentration, when compared to other blood cells [83], and the presence of non-epithelial characteristics, which may diminish the efficiency of certain immunoaffinity strategies [110]. In fact, a concentration of one CTC in 1 mL of blood is a quite rare event in comparison to other blood cells [97, 103]. For example, white blood cells present a concentration around 5 × 10^6 cells per mL, while red blood cells may reach 5 × 10^9 cells per mL [96, 100]. Furthermore, CTCs have a very short half-life from 1 to 2.4 hours [111]. Only a few CTCs present in the bloodstream present stem cells characteristics [91]. CTC clusters detection in peripheral blood of lung cancer patients are even more uncommon, constituting between 2% and 5% of the total cells [112]. For this reason, among others, urges the necessity of developing sensitive and specific CTC detection methods, improved to assist the patient monitoring the best way possible [108].

When it comes to CTCs isolation, many methodologies have been developed, and are described above. These can be label-dependent or biochemical methods, based on detection relying on the presence or absence of specific cell surface markers, like the CellSearch system, or they can be label-independent or biophysical methods, that focus on the physical characteristics of CTCs, such as the Isolation by Size of Tumour cells (ISET) system [27, 94, 113].

Some of the main cell markers are described next. Epithelial cell adhesion molecule (EpCAM) is a type-1 transmembrane glycoprotein [81], a cell-cell adhesion molecule in epithelial cells, used to positively select CTCs [34]. This protein is expressed in NSCLC and weakly or not

at all expressed in healthy patients [26]. It is one of the most studied antigens associated to tumours [81], and usually associated with the epithelial phenotype and successfully incorporated into CTC detection kits, such as CellSearch [101]. Satisfactory laboratorial results have been registered concerning EpCAM capacity in complete membranous expression in carcinoma [26]. However, CTCs may weaken or even completely lose the expression of epithelial markers following EMT, which makes the isolation process with cell markers, such as EpCAM and cytokeratin, less effective [34, 97]. EGFR is a surface marker, commonly associated with NSCLC due to EGFR mutations [40]. Cytokeratin (CK) is also an epithelial marker commonly used to distinguish CTCs from leukocytes [105, 114]. CK, like EpCAM, is expressed by most cells derived from epithelial cancers [100] and has been used to positively identify CTCs [34]. It has been reported that benign diseases usually present lower EpCAM and CK expression when compared to patients with malignant disease [39]. Cluster of differentiation (CD) 45 is present on the surface of white blood cells [100]. It is usually used to negatively select leukocytes from the sample [34]. In the cases of SCLC patients, CD133 has been reported to identify CTCs and signalling pathways implicated in CSC proliferation [91]. CD133, also known as prominin-1, is a five-transmembrane cell surface glycoprotein, that is vastly used as a specific cell surface marker in various tumours, including lung cancer. Its high expression is believed to be correlated with worse prognosis and metastasis in lung cancer patients [115]. Vimentin is an intermediate filament type protein found in mesenchymal cells [18]. It is believed to be linked to cancer progression and later stages in different types of tumours, being a feasible target in cancer therapy and CTCs identification, with notable results regarding moderately and well-differentiated lung adenocarcinomas [92, 116]. CTCs positively marked with Vimentin are usually associated to smaller/deformable cells that are undergoing EMT and linked to metastasis [96]. Thus, this marker is considered useful to indicate EMT phenomenon in tumour spreading and to better understand this type of cells biological characteristics and clinical significance [103, 117]. Aldehyde dehydrogenase 1 (ALDH1) is a marker usually used for CSCs [118]. 4',6-diamidino-2-phenylindole (DAPI) is commonly used to stain cell nuclei [100, 119] in association with other specific markers, for both CTCs and leukocytes, such as those mentioned above. Other surface markers have also been used, such as CD9 or CD41 antibodies [120, 121]. The study of PD-L1 in tumour cells can be used to monitor response to immunotherapy in lung cancer [122]. PD-L1 expression is believed to be linked to tobacco smoke habits and, consequently, lung carcinogenesis [123]. Therefore, there has been recently incorporated the program cell death ligand I (PD-L1) inhibitors in treatment of advanced NSCLC, achieving positive results. The percentage of PD-L1 protein expression by the CTCs is associated with the type and aggressiveness of the tumour [113], and has been used as a biomarker to foresee the response to immunotherapy.

2.4 - Applicability of CTCs in liquid biopsy

CTCs are a useful tool for liquid biopsy. Their isolation from blood of a patient with a solid tumour may serve as a biopsy in real time. CTCs have already been identified in lung cancer and other several tumour types, but almost never found in benign cancers or healthy patients [21]. For that purpose, CTCs enrichment is carried out through marker dependent or independent methods, followed by detection and characterization of molecular features that can

ultimately give detailed information about the oncological disease and guidance for therapy response [62].

The analysis of tumour cells may be complementary to other types of biomarkers, such as techniques for studying already known mutations and identification of new mechanisms of resistance to treatment [60]. However, CTCs are believed to not be as useful in this context, because they represent limitations concerning the platforms for their identification and retrieval for ulterior analyses, as well as their low concentration in blood, which makes testing even more challenging because genetic material is so limited [64]. CTCs are usually more related to the process of metastasis [36]. Table 2.2 highlights the main advantages and limitations in the use of CTCs in summary form.

Table 2.2 — Main advantages and disadvantages of CTCs usage.

	CTCs
Advantages	FDA-approved assays (CTC isolation) Several components (DNA, RNA, proteins, metabolites) Associated with tumour metastasis Allow structural evaluation of cancer phenotype
Limitations	Rare and fragile High heterogeneity Undefined standard isolation method Not as useful for mutations analysis

(Adapted from [36, 69, 124, 125])

The fact that CTCs present different positives and negatives aspects regarding their use and efficiency, the complementary use of CTCs with other biomarkers may provide great advantages in the personalized study of oncological diseases, making this strategy extremely appealing and a candidate to become the gold standard for lung cancer cases, in the era of precision oncology [36, 126]. With recent advances in oncological research, the concept that a treatment is eligible for any patient is no longer accepted. Traditional methods for molecular profiling often involve image-guided tissue biopsies, which have associated risks due to invasiveness and limited number of samples, as well as risks for the patient [22]. However, developments on the continuous study of liquid biopsy have allowed to go beyond only CTC enumeration and expand the possibilities of analysis of the CTC genome, transcriptomes, proteome, epigenome, phenotypic features and other interesting components [62]. In figure 2.4 is presented a schematic representation of the main possible analysis of CTCs.

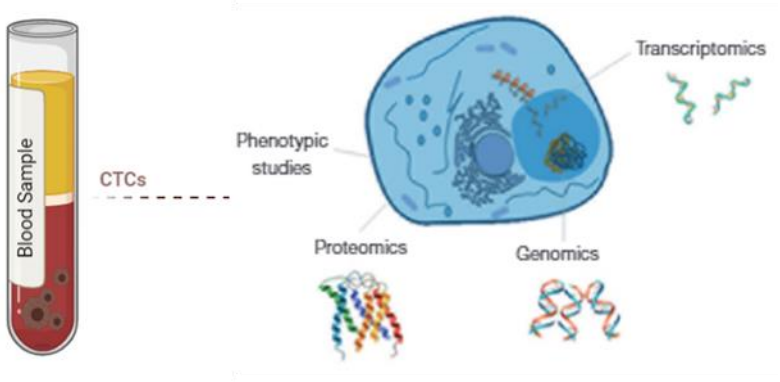


Figure 2.4 – Main purposes of the use of CTCs in blood analysis of a lung cancer patient. (Adapted from [36])

CTCs have already been implemented as liquid biopsy to study genomic profiles and try to catch up with the alterations that occur throughout tumour evolution, as well as treatment and relapse episodes. Taking colon cancer as an example, mutation identified within driver genes in primary tumour and lesions formed, matched the ones found in the corresponding CTCs. However, studies have also reported that mutation profiles of single tumour cells may not coincide with ones identified in primary and metastasis tumours, because of CTCs sub-population heterogeneity [39]. Some reports also suggest that CTC clusters may me more relevant for metastatic process and present higher prognostic value when comparing to single cells [106].

Mutations keep occurring and originate tumour heterogeneity that may cause resistance or setbacks in treatments, since the most aggressive clones are selected [111]. To study the tumour heterogeneity and specific mutations within the same patient, many techniques have been introduced, especially genomic medicine and single-cell analysis (DNA and RNA). Proteomics and genomics are quite recent techniques that have been successfully applicated in the analysis of CTCs, since it only requires a small amount of sample from the patient [39].

Individual evaluation of patients through minimally invasive liquid biopsy, is useful not only to monitor disease progression and to choose an adequate therapy, but also to prevent patients who would not benefit from immunotherapy and could even produce adverse reactions. Immunotherapy has been one of the approaches used for advanced tumour treatment. Treatment with ICIs have been increasingly used. These function as supplement for anti-cancer activity of immune cells [44, 72]. Some examples include inhibitors of PD-1 or its ligand PD-L1 and inhibitors of cytotoxic lymphocyte-associated protein 4 (CTLA-4), both approved by The European Medicines Agency (EMA) and the FDA. ICI therapy may be monotherapy or in combination with other therapy strategies, such as chemotherapy or anti-angiogenic antibodies [127].

In lung cancer, more specifically metastatic NSCLC cases, test for PD-L1 expression is used to understand which patients would benefit from immunotherapy. Studies reported that a high quantity of PD-L1 positive cells can be related to less chances of survival and mostly linked to metastases. Biomarkers, such as CTCs and exosomes are distinguished as preferable for PD-L1 expression analysis [111].

2.5 – Microfluidic devices

Currently, there are still some difficulties regarding the efficiency of liquid biopsies, such as limited sensitivity, low specificity and the fact that only one biomarker is isolated for each kit, among others. Thus, microfluidic technology appears as an innovative method.

In microfiltration systems based on cell sample processing, cells flow through arrays of a microscale geometric design incorporated with constriction to dictate the movement of CTCs and capture them according to their physical characteristics, such as size [55]. The incorporation of microfluidics chip brings several advantages, such as greater efficiency in the separation of cells, greater speed, less cost associated, less use of reagents and decreased creation of waste. Besides being an affordable method, it allows precise control and manipulation of the studying fluids which confers higher sensitivity and better cell recovery. However, it has the disadvantage that it can sometimes be unreliable as there is a wide range of sizes that CTCs can take. Hence, the combination with other techniques, such as immunoaffinity based methods, can improve the efficiency of capture and identification of CTCs [39, 128]. In future developments, it is believed that the incorporation of multiple processing capability, portability and automation features may help turn this proceeding suitable for clinical settings and routine consultations [107].

Microfluidic chips are used very often to substitute traditional methods, constituting a liquid biopsy in which immune-affinity, physical separation and immunomagnetic procedures can be used.

Nowadays, there are several micro/nanofabrication techniques that can be incorporated in integrated microfluidic systems fabrication. The development of an integrated microfluidic system is usually done by the combination of two layers: a substrate layer, usually a glass or a polymeric substrate, and a channel layer with microchannel impressions, that may vary according to the desired application. The channel layer is commonly fabricated using Polydimethylsiloxane (PDMS), mainly because of favourable mechanical/optical properties and manufacturing by rapid prototyping. The materials used in the fabrication should provide biocompatibility with fluidic applications and transparency for optimal imaging techniques [129]. Microfluidic systems are usually used in biological assays, to perform certain functions, such as to trap, deform and sort cells [130]. These microdevices can be based on different strategies: immunoaffinity, dielectrophoresis (DEP) and size. Although there have been several advances in this technology, it is still challenging to develop a device that is not only capable of achieving high throughput, but also to capture cells taking in account purity and cell selection viability [78].

Photolithography technology is widely used in generating silicon and photoresistor masters with the negative imprints of the desired system, which includes spin coating of a photoresist film, alignment and exposure of a mask to UV light [131]. Soft lithography is the complementary technique to photolithography, used to develop the microfluidics system made of PDMS, which is poured and cured in the master mask. The combination of advanced fabrication methods with soft lithography is believed to be a promising strategy to integrate microfluidic systems due to its simplicity and low cost [129, 130].

Micro-ellipse or ellipse filters have been characterized as robust and reproducible devices for capturing CTCs. This kind of filter presents high sensitivity, viability and avoid flow by hydrodynamic forces, due to the structure and placement of the filters in series. Furthermore, the micro dimension structure of the elliptical devices allows a significant reduction in shear

stress force and gap spacing, simplifying the release procedure. The functionality of this method stands on the physical differences of size and deformity between cancer cells and blood cells. The dimension of this kind of micro ellipse filters is chosen having into account the characteristics of tumour cells, including strength and smooth flow. A study tested this isolation strategy, analysing blood from both artificial and cancer patients, by fluorescently labelling tumours cells, including nine patients with non-small cell lung cancer. In fact, the clinical results revealed that CTCs were found in diseased patients, while none were found in healthy controls. Thus, it was shown that micro-ellipse gradual filters are an efficient method to capture CTCs, allowing at the same time the separation of tumour cells from heterogeneous samples. The study also revealed an enhance of purity and anti-cancer appraise for future advances in this subject [84].

An example of a CTCs isolation technology based on microfluidic platform is the CTC-chip, constituted of a silicon chip presenting a geometric pattern coated with EpCAM. The functionalized surfaces with antibodies maximize the capture of CTCs, that can be later confirmed using stains, counted and analysed for tumour molecular characterization. This method presents high sensitivity and ability of capturing rare cell populations from whole blood, while preserving cell viability Still, only EpCAM expressing cells are captures, which may diminish the number of isolated cells [128]. The On-chip Sort is other interesting cell sorting system, which incorporates a disposable microfluidic device, that detects and isolates rare tumour cells for following molecular study. This kit also allows detection of EpCAM-negative/ CK-negative cells with the addition of an EMT marker. This system has presented more promising results than the CellSearch system, presenting a higher number of CTCs detected in lung cancer patients [88].

Droplet-based microfluidics technique is currently used in cells analysis, material synthesis, nanoparticles preparation and technology of fluorescence barcoding. Recently, droplet-based microfluidics have also been widely used for the analysis of ctDNA on droplet digital PCR strategies. This technique provides easy operation, low cost and flexibility of use in diverse samplings. It also presents limitations, such as the hardship found in simultaneously forming droplets individually and achieving high throughput. To get around these challenges, new strategies have been studied to achieve convenient sample changing and high-throughput droplet assays in droplet-based system, namely through strategies of pressure-based flow [132, 133].

Thus, microfluidics devices have shown a great promise in liquid biopsies, as an optimistic approach for circulating tumour biomarkers isolation and analyse, which further development is expected to improve the sensibility and specificity of these systems, as well as more efficient diagnosis, prognosis and monitoring of lung cancer patients.

Chapter 3

Materials and Methodologies

3.1 – Work plan

The work plan was developed as a suitable response to the proposed problem addressed in chapter 2, according to scientific basis, extensive literature research and work previously developed within the project.

The present work involves the study of the two types of lung cancer, NSCLC and SCLC. In this study, the main focus is a detailed analysis of CTCs in samples of lung cancer patients, using an advanced microfluidic system. For that, two different groups and both histopathological types of lung cancer were examined: early-stage (I/II) and late-stage (III/IV) lung cancer patients, as well as NSCLC and SCLC patients.

The work plan is divided into four different sections. Initially, the microfluidics device was prepared by lithography/soft-lithography techniques, followed by plasma cleaning to bind the polymeric device to the glass. The second phase involved the sample preparation and processing trough the microfluidic chip, followed by the isolation and characterization of CTCs, by immunofluorescence. Immunofluorescence is used to identify and quantify cells in situ. A detailed data analysis was performed to assess a correlation between the quantitative assay of CTCs and the specific characteristics of each sample, including the type and stage of lung cancer. To obtain an even more personalized tumour profile, DNA/RNA extraction was performed from the samples who presented higher CTCs count, using a DNA/RNA AllPrep Mini Kit. Quantitative proteomic analysis of CTCs was useful for the quantification of protein biomarkers on tumour cells, in this case PD-L1. The final step involved the comparison between the results obtained regarding CTC identification and the analysis of DNA methylation for specific genes. The latter was performed using cfDNA extracted from the remaining plasma after processing throughout the system.

The techniques and work plan are briefly described in table 3.1.

Table 3.1 — Description of the work plan carried out.

Step	Technique	Description
1 - System Preparation	Photolithography/Soft Lithography	PDMS device design and preparation Plasma cleaning
2 – System efficiency	Sample dilution, processing Cell spiking assays	Validation of system efficiency on CTCs isolation
3 - CTCs Characterization	CTCs Immunofluorescence	CTCs enumeration and identification of surface markers Observation under fluorescence microscope
	Data analysis	Analysis and correlation of CTC count and sample characteristics Follow-up of the disease
	DNA/RNA analysis	DNA/RNA extraction from CTCs DNA and RNA quantification
	Proteomics	Proteomic signature of lung cancer CTCs Measurement of protein abundances
4 – Comparison with cfDNA analysis	Comparison between CTC count and cfDNA methylation	dsDNA quantification Promoter gene methylation levels (ADCY4me, MIR129-2me, HOXA11me)

3.2 – Microfluidic system

3.2.1 – System description

The system used in this work is based on size separation of CTCs. Microfluidic technology was chosen because of its capacity of isolating viable CTCs with a satisfying yield for subsequent analysis. During the process of CTCs isolation, cells passed under laminar flow through a micro patterned microfluidic chamber and isolated from plasma and blood cells based on their dimension.

3.2.2 – Microfluidic chip manufactering and assembly

Following the silicon (Si) masks preparation by photolithography, the devices were prepared by a uniformly mix of PDMS and a curing agent, in a proportion of 10:1 (wt/wt) and

pouring in the Si master mask. When polymerized, after 4 hours at 60 ºC, the PDMS was cut, and inlet and outlet were punched. Then, the PDMS layer was sealed to clean glass coverslips via plasma cleaning. The process of plasma cleaning, in which oxygen is bombarded on both surfaces, constitutes as a biocompatible and versatile bonding technique for the substrates, based on the improvement of surface's affinity by removing organics contamination and introducing functional groups to the surface of exposed materials, which requires strong oxygen plasma [134].

The developed system is represented in figure 3.1.

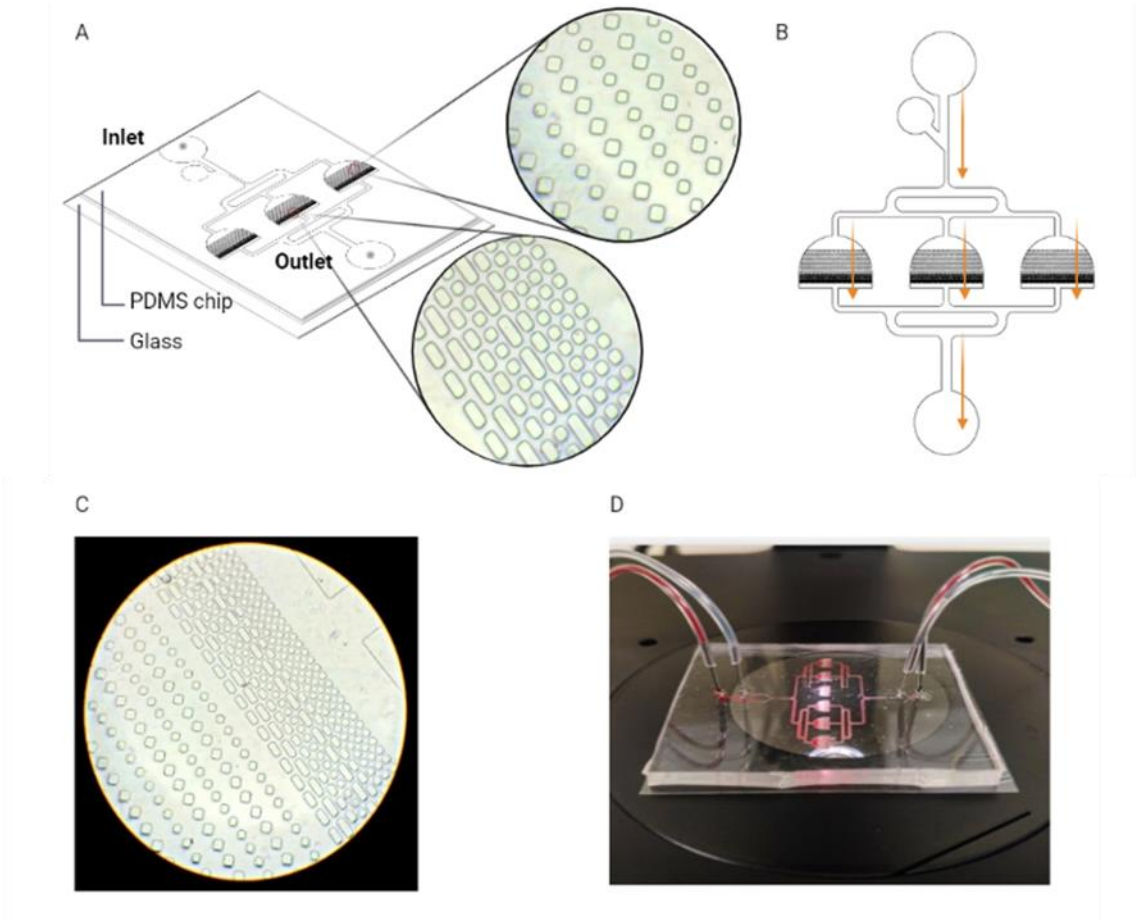


Figure 3.1 – The design and functionality of the size-based microfluidic device. A. Schematic representation of the PDMS system. The interspacing between micropatterns decreases throughout the chambers from 50 to 15µm. B. Design of the system. Illustration of the path that the cells take along the system, by induced flow. C. 20x microscope observation of system chamber. D. Experimental set-up for CTC isolation using the chip. (Created with BioRender.com)

3.2.3 – System efficiency

3.2.3.1 – Cell spiking assays

In order to assess the system efficiency for cells enrichment and characterization in-chip, a urinary transitional cell carcinoma cell line, T24, was selected. T24 cells were cultured in 25 cm^2 tissue culture flasks with high glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma -Aldrich), supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Penicillin/Streptomycin in a humidified atmosphere of 5% CO_2 at 37 ºC.

After reaching 25% of confluence, cells were washed with PBS and detached by incubation with 0.5% (w/v) trypsin for 5 minutes at 37 ºC, harvested and resuspended in culture medium. The concentration of cells in suspension was determined using a haemocytometer and cells were spiked into 2 mL of culture medium at a defined concentration of 250 cells/mL. Prior to samples processing through the chip, a solution of sodium chloride (NaCl) was flowed at a speed of 200 µL/min to clear the system of air bubbles. A flow rate of 25 µl/min was selected to guarantee laminar flow inside the isolation chambers, as well as ensuring a cellular trajectory that could maintain cells integrity during the processing. Cells' trajectory and interaction with the micropatterns was observed during processing in an optical microscope. Following isolation, T24 cells were fixed with 4% paraformaldehyde (PFA) and fluorescently labelled for quantification. Following fixation, cells were washed with NaCl and permeabilized with 0.1% (v/v) Triton solution, for 20 minutes at room temperature (RT). Subsequently, solution of blocking buffer (AbCAM) was loaded in the chip, for 20 minutes, to block non-specific adsorption of antibodies. After this, cells were stained with Alexa Fluor 488 (Life Technologies), at a 1:100 dilution, for 60 minutes, and washed with NaCl. Nucleus staining was carried out with DAPI for 10 minutes. Finally, cells were washed to remove excess dye. Cell counting was performed using an inverted fluorescence microscope (Axiovert 200M, Zeiss). Two assays were performed.

3.3 - Sample processing

3.3.1 - Clinical data - Lung cancer samples

Plasma samples were used in this study due to their greater availability and being previously characterized, allowing a proper validation of the system.

Plasma samples of patients from early and advanced-stage lung cancer, provided by the Portuguese Institute of Oncology of Porto (IPO-Porto), were used for this project. A total of 30 plasma samples were selected for this study.

All plasma samples from lung cancer patients were supplied by the Biobank & Cancer Biology and Epigenetics group from the Portuguese Oncology Institute of Porto, Portugal. The study was approved by the institutional review board (Comissão de Ética para a Saúde) of Portuguese Oncology Institute of Porto, Portugal (CES-IPOPFG-EPE 177/018), in the framework of the research project "TRIMARKCHIP - Assessing the trifecta of cancer circulating biomarkers: a combined microfluidics platform for detection of CTCs, exosomes and ctDNA". Written informed consent, in accordance with the Declaration of Helsinki ethical principles, were provided by all patients enrolled in this study.

Clinicopathological characteristics and clinical data regarding patients selected for this study are shown in table 3.2.

Table 3.2 — Clinicopathological characteristics of patients enrolled in the study of CTCs enumeration.

Parameters		Patients (n=30)
Age	Mean (range), years	67.9 [51-86]
	Median, years	68.5
Smoking Habit	No	6
	Yes	10
	Ex-smoker	14
Gender	Female	7
	Male	23
Type	NSCLC	20
	SCLC	10
Stage	I	7
	II	3
	III	6
	IV	14

3.3.2 - System set-up

The microfluidics system set-up was designed to isolate two biomarkers from the same plasma sample, CTCs and cfDNA. The sequence of steps is schematically represented in figure 3.2. A plasma sample was diluted, loaded into a syringe and placed in a syringe pump. The method for biomarkers processing focused on initial isolation of CTCs by size inside the microfluidic chip. The CTCs were then fluorescently labelled and observed by fluorescence microscopy. The remaining plasma that was processed through the chip was collected for cfDNA extraction and analysis.

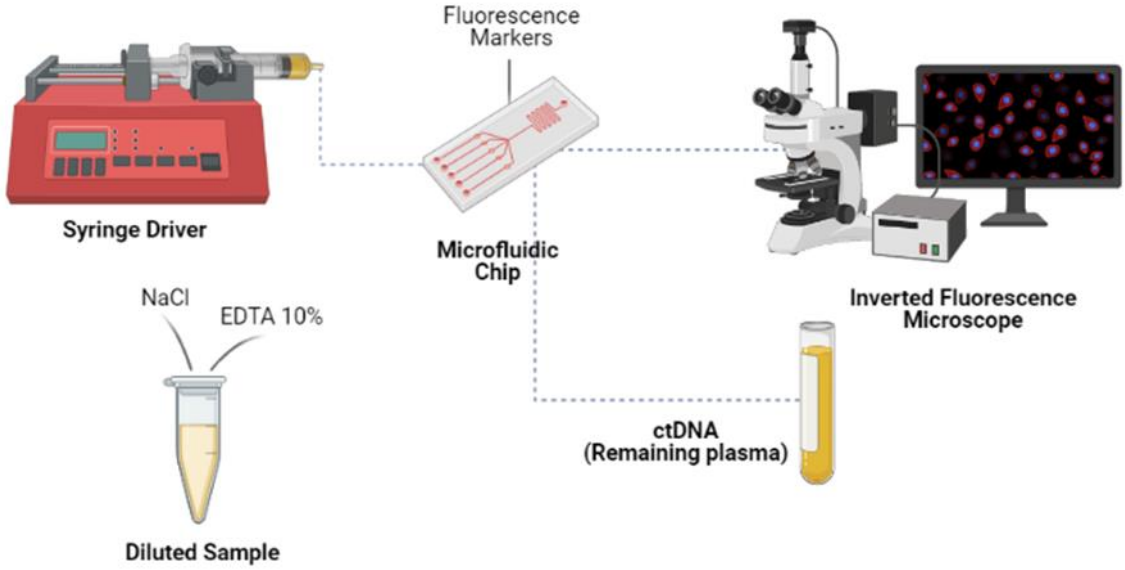


Figure 3.2 - Sequence of steps performed. The plasma sample was diluted at 1:1 with NaCl and 0.5% EDTA. The plasma was processed through the microfluidic device and fixed with 4% PFA. The isolated CTCs were then stained with fluorescence markers (EpCAM/CD133, VIM, CD45, DAPI) and the sample was analysed under a fluorescence microscope. (Created with BioRender.com)

3.3.3 - Plasma samples preparation

Defrosted plasma samples were diluted for improved processing in the system. For the dilution, NaCl and a 10% solution of ethylenediaminetetraacetic acid (EDTA) were added to the sample. The ratio of plasma and NaCl was 1:1, while EDTA was added at a 0.5% final concentration. EDTA is a commonly used anticoagulant, that is suitable for processing analysis of blood and plasma samples [56].

3.3.4 - CTCs enrichment and cfDNA retrieval

Due to the low concentration of CTCs in comparison to other blood cells, it is fundamental that the platform used is suitable for CTC enrichment and isolation.

Different flow rates were assessed for the sample processing. The fluid velocity in the chip was selected according to the viscosity of the sample studied [103]. The flow value must be carefully chosen to prevent cells from collapsing when they flow through the micropatterns, reaching a compromise between speed and purity. After preliminary tests, the flow rate was determined to 25 µl/min, following the calculation of laminar flow for the plasma samples properties, and according to previous tests with the T24 cell line (3.2.3.1). As previously mentioned, the system was designed for CTCs enrichment along the micropatterned chambers, being most of WBCs eliminated from the outlet. However, other unspecific material may be retained, such as larger leukocytes or thrombocytes.

First, a NaCl solution was flowed at 200 µl/min to clean and remove air bubbles from the chambers. The plasma samples were pumped from the inlet into the microfluidic chip using a syringe pump. After CTC capture, 4% PFA was used for cells fixation. To finish, NaCl was flowed through the system to leave the sample hydrated for following staining and analysis. The system was stored at 4 ºC until further use. The remaining of the sample was collected separately for further analysis of the cfDNA present in plasma.

3.4 – CTCs characterization

3.4.1 – Immunofluorescence

After in-chip isolation, the cells were subjected to staining with fluorescent surface markers, at room temperature, allowing CTCs identification from other blood cells that may also been entrapped in the system. Surface markers may be used to enrich CTCs samples positively or negatively [34, 103]. Some of the cell markers used include EpCAM, CD133, Vimentin, PD-L1, for CTCs identification in lung cancer, CD45 for leukocytes and DAPI for cells nuclei. This combination of antibodies targets a wider spectrum of tumour antigens, which is crucial for a more precise count and phenotypical characterization of CTCs.

Isolated cells were permeabilized with a solution of 0.1% triton for 20 minutes, followed by incubation for 20 minutes with bovine serum albumin (BSA) to reduce background interference and unspecific binding. The cells within the chip were then characterized by fluorescent staining with epithelial and mesenchymal markers. CTCs were stained for EpCAM/Vim/CD45/DAPI for NSCLC samples and CD133/Vim/CD45/DAPI for SCLC samples. Briefly, following incubation with BSA, CTCs were stained with mouse anti-human CD133 (SCLC) or mouse anti-human CD326 (EpCAM, NSCLC) at 1:100 (Biolegend), followed by goat anti-mouse IgG H&L Alexa Fluor 647 (AbCAM) at 1:400 for 1 hour at RT. Alexa fluor 488 anti-CD45 at 1:400 (AbCAM) and Alexa fluor 594 anti-vimentin (Biolegend) 1:200 were incubated for 4 hours at RT.

Cells were washed with NaCl and nuclei stained with DAPI (Biolegen) at 1:200 for 10 minutes at RT. Finally, stained cells were washed with NaCl to remove excess dye and maintain hydration until observation. Staining was performed at a lower speed than processing, approximately 10 µl/min, to ensure that cell staining was more effective and would not influence the enriched cells. After staining, the system was stored at 4 °C until further analysis.

Fluorescence images of identified CTCs were acquired with an inverted fluorescence microscope (Axiovert 200M, Zeiss) at 20x or 40x magnification and processed using Fiji (ImageJ) software. To be considered a typical intact CTC, a cell must present a round morphology, a size within the parameters for CTCs, be negative for CD45, contain a nucleus marked by DAPI and exhibit positive staining for EpCAM/CD133 and/or Vimentin. With the eventual impact against the walls of the system, the cells may deform and lose the usual round morphology [87, 135].

3.4.2 – Extraction of RNA and DNA

From the analysed samples, the ones with highest numbers of identified CTCs were selected and proceeded to the extraction of RNA and DNA from the cells, using the AllPrep DNA/RNA Mini Kit, following the manufacturer's protocol. RNA and DNA analyses may hold important information regarding intertumoral and intratumoral heterogeneity specific for each individual patient [59, 111].

First, it was necessary to lyse the cells retained in the systems. For this, a lysis buffer was introduced into the chip, followed by freezing the sample and removing the lysate with the buffer. Rapid freezing and thawing were carried out to ensure that the thermal shock, combined with the lysis buffer, generated the rupture of the cells and that the nucleic acids were released.

Lysate from the lysed cells passed through an AllPrep DNA spin to isolate DNA. Then, the remaining solution passed through a RNeasy spin column to isolate RNA. A combination of washing using specialized buffers and centrifugation was carried out, so that, through a filter membrane, DNA and RNA could be eluted. The procedure followed is schematically represented in figure 3.3.

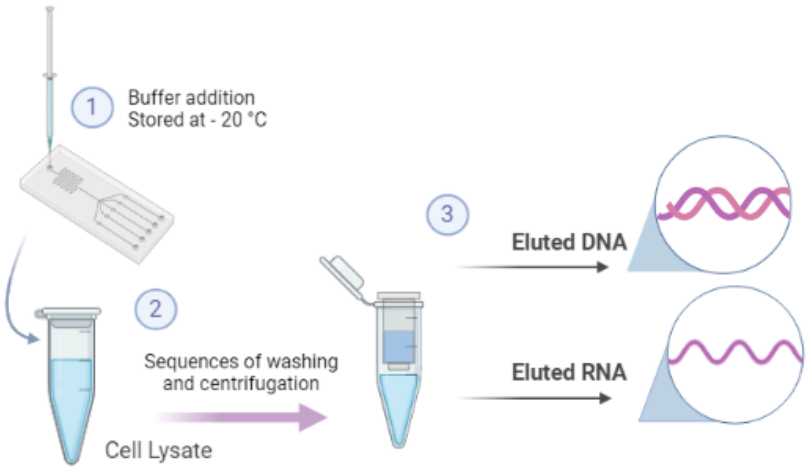


Figure 3.3 – DNA and RNA extraction and isolation from CTCs, using an AllPrep DNA/RNA Kit. (Created with BioRender.com)

After elution, DNA and RNA concentration were measured with nanodrop One (ThermoFisher Scientific), technology that allows rapid quantification and qualification of nucleic acids, using only 1.5 µL of sample. RNA was further quantified by a 2100 BioAnalyzer (Agilent Technologies) and DNA with a Qubit 3.0 Fluorometer (Life Technologies).

3.4.3 – Proteomics

The proteomics analysis was carried out to assess the possibility of PD-L1 detection in the isolated CTCs. For the proteomics study, two NSCLC III stage samples were analysed, one presenting a 90% PD-L1 expression and other with negative PD-L1 expression, as control.

After standard CTCs enrichment, a solution of 0.1% triton was flowed in the microfluidics system, in reverse flow to retrieve the isolated CTCs in suspension. Air was also pumped through the system to maximize the recovery of cells trapped in the system.

Protein identification and quantification were performed in the collected solutions. The resulting peptides were analysed by Liquid Chromatography—Mass Spectrometry (LC-MS). A 0.1 % triton sample was used as negative control.

The raw data were processed using the Thermo Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany). Protein identification analysis was performed with the data available in the UniProt protein sequence database for the Homo sapiens Proteome with 77,027 entries.

Protein functional enrichment analysis was performed with the WebGestalt (WEB-based GEne SeT AnaLysis Toolkit). The Over-Representation Analysis (ORA) was performed with the Functional Database of Biological Process.

For determination of differentially expressed proteins between the two samples in comparison to the negative control, the following filters were considered additionally: (1) the use of at least two unique peptides; (2) values of abundance ratios of at least 2.00 in ratio.

3.4.4 – cfDNA isolation and characterization

Following the in-chip CTCs isolation, the remaining plasma was used for cfDNA extraction. cfDNA was extracted using the QIAamp cfDNA kit, according to the manufacturer's instructions, followed by sodium-bisulfite modification and PreAmp amplification. Finally, multiplex quantitative methylation-specific PCR was performed for assessing promoter methylation levels of ADCY4me, MIR129-2me and HOXA11me.

cfDNA extraction and analysis was performed in 6 samples and the results were matched with CTCs enumeration. This analysis was done by the Cancer Biology& Epigenetics Group and kindly provided for comparison with the obtained CTCs results.

3.5 – Data analysis

Data analysis was performed for the obtained results.

The successful identification and enumeration of CTCs captured within the system, by immunofluorescence, was correlated with the clinical data of the patients. Comparisons between multiple groups were made. A comparison was performed between the results obtained from early-stage (I/II) and advanced stage (III/IV) samples of the NSCLC patients, as well as between the results obtained from the advanced stage samples of the two types of cancer, NSCLC and SCLC. It was also verified the variation of CTCs count in each of the TNM stages.

Follow-up monitorization of patients was also conducted, analysing the variation of CTCs count in a first assessment at the time of diagnosis in relation to a follow-up sample after treatment intervention. Graphical representations were performed using GraphPad Prism software, version 5.01. Statistical analysis was performed with the Mann-Whitney U test for the comparison between early and late-stage, and NSCLC and SCLC CTCs count.

Chapter 4

Results and Discussion

4.1 - System efficiency

4.1.1 - Experimental setting

In the experimental setting, some components for microfluidics were used. Syringes with the samples were placed in a syringe pump, which allowed to control the infusion rate. Tubing, microfluidic fittings and needles were used to connect the systems to the syringes. A bubble trap was added to the set-up to prevent air bubbles formation. In figure 4.1 the experimental setting used in sample processing is shown.

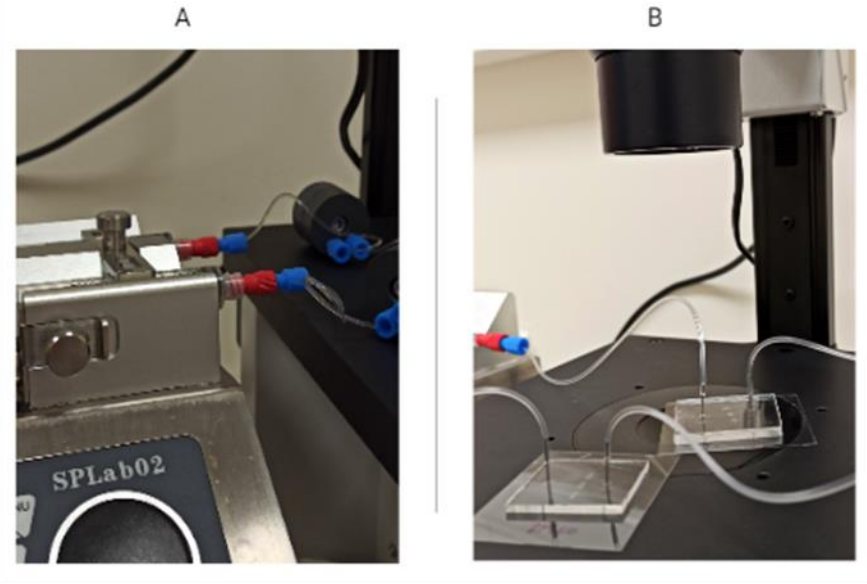


Figure 4.1 – Experimental setting used in sample processing. A. Diluted plasma samples placed in the syringe pump. B. Systems connected to the syringe and waste, through the inlet and outlet, respectively.

4.1.2 – Efficiency tests

The microfluidic system has shown to be successful in the isolation of CTCs from plasma samples, with the remaining being collected for downstream cfDNA analysis.

Visually, samples from patients with advanced lung cancer were found to have more cellular material than samples from early stages. This may be due to the greater aggressiveness of the cancer, and in turn a greater number of tumour cells and clusters of thrombi in blood, that get trapped throughout the system. Blood composition, as in the case of coagulation, can also change as a result to certain therapies. The figure 4.2 shows a sample from a NSCLC stage IV patient, before and after sample processing through the microfluidic device.

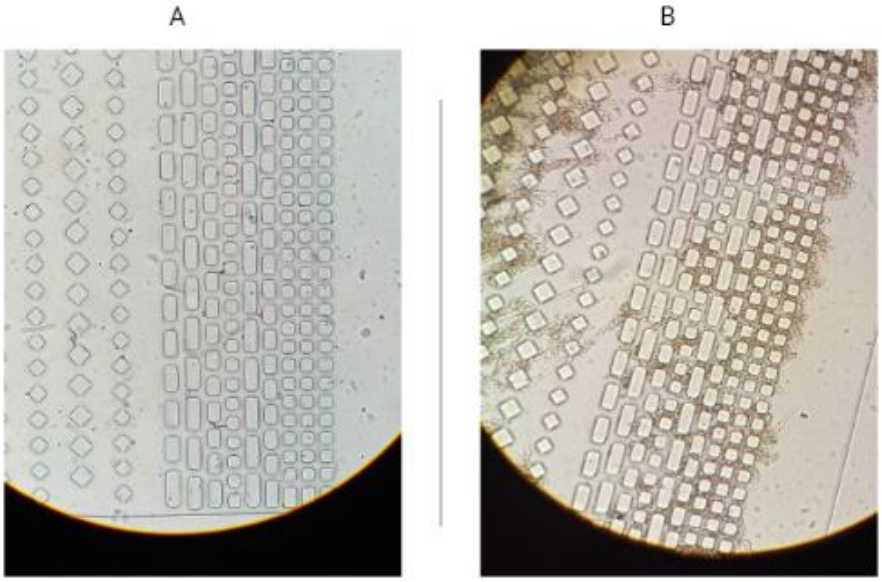


Figure 4.2 – The appearance of the system is visually different after capturing the larger constituents present in the plasma. A. System before sample processing. B. System after sample processing.

When comparing the system before and after sample processing, it appears that the larger cell material was trapped throughout the system, as expected. The capture of great amounts of other plasma constituents may impair the purity of CTCs isolation. From the several samples analysed, a higher density and/or clotting was more common in the blood of cancer patient in advanced stage, rather than early. This can compromise cell isolation or even the performance of the system, obstructing the passage channels, only in very rare situations. To achieve higher purity in CTCs isolation some more parameters may be altered, such as the flow rate, and the EDTA concentration. However, since the tests were performed to allow comparison between cancer type and stage, the same parameters were applied for all samples.

The system used based on size isolation presented good results regarding CTCs capture, as well as a time saving, low cost and easy handling approach to analyse plasma samples from lung cancer patients. Cells were usually identified adhered to PDMS patterns or in equilibrium position next to the micropillars. According to the results obtained, clogging was rare and leukocytes contamination minimal, since only a few WBCs were found. Regarding immunoaffinity characterization, there were also good results. Figure 4.3 shows an observation through an optical microscope, in which it is possible to observe the biological material that got retained in the system, including possible CTCs. Screening and confirmation of identification of tumour cells was then achieved by immunofluorescence characterization.

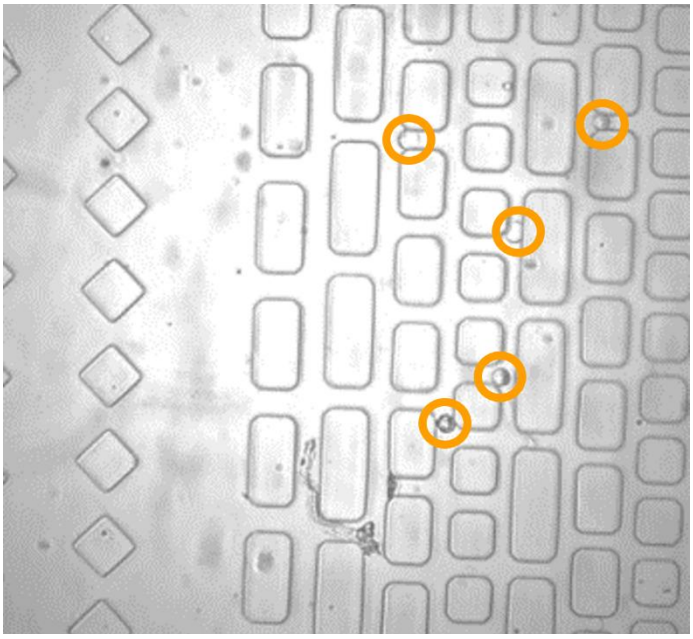


Figure 4.3 – Optical microscope observation of material retained in the system at 20x magnification. Possible CTCs candidates are marked.

4.1.2.1 – Cell spiking assays

T24 cells were successfully isolated in the microchambers of the microfluidic system. The captured cells ratio was calculated by dividing the number of isolated T24 cells per the number of spiked tumour cells, which was 250 cell/mL. From the two performed assays, the number of isolated T24 cells counted was 97 and 168, providing calculated efficiencies of 19,4% and 33,6%, respectively. T24 cells were selected considering their mean size of 18 µm [136], within the range of CTCs size, which may vary from 15 µm to 25 µm [18]. Some of the T24 cells identified are shown in figure 4.4.

Figure 4.4 - Fluorescence microscope images of T24 cells. Cell nuclei were stained with DAPI and Alexa Fluor 488 was used to stain cell F-actin.

A few issues may have prevented a proper determination of the system efficiency. First, the method of initial cell concentration calculation, performed with a hemacytometer may induced errors when defining dilutions for such low concentrations [137]. For this study, the

goal was to achieve a final sample of 2 mL with 500 cells, so a minimum dilution factor of 20 was necessary. This large-scale dilution can lead to counting errors.

Other influencing factor may be due to the size of T24 cells. The microfluidic system was designed for capture of large CTCs (≥ 15 μm). While T24 cells have been reported in the literature to present an average size of 18 μm, a study evaluating T24 cells in suspension performed measurements that revealed a mean diameter of 13.1 ± 2.0 μm [138]. This would mean that most of the tumour cells could have left the system by the induced flow force, during sample processing.

4.2 - CTCs quantification and characterization

Enriched CTCs in the isolation chamber were observed under an inverted fluorescent microscope, analysed and counted. Results from immunofluorescence staining of trapped cells within the system also confirmed the efficient capture of CTCs.

Fluorescence markers are used to improve cell counting with distinction between different cells and, mostly, for their identification and characterization [78]. The combined use of the fluorescence markers allows the identification of cells under microscope, based on their specific characteristics and interaction with the cells.

To confirm the captured cells as CTCs, the cells were labelled with DAPI, EpCAM/CD133 and VIM. CTCs were stained for EpCAM/Vim/CD45/DAPI for NSCLC samples and CD133/Vim/CD45/DAPI for SCLC samples. Cells were identified as CTCs if DAPI+, EpCAM+ and/or VIM+ and CD45- for NSCLC and DAPI+, CD133+ and/or VIM+ and CD45- for SCLC. White blood cells found entrapped in the system were identified by CD45+ and DAPI+ expression.

DAPI was used to distinguish cell nuclei. Most studies use this nucleic acid dye for this purpose [100]. EpCAM was selected for identification of NSCLC considering their epithelial origin and highly reported used in the identification of CTCs [81, 101]. However, its usefulness for CTCs identification has some complications, since tumour cells' epithelial features may be weakened or completely disappear, mostly due to EMT process during metastases. By only using this cell surface marker, tumour cell count may not be effective [102, 139]. SCLC is considered as more aggressive type of lung cancer than NSCLC, being most of the times associated to metastases formation and higher CTCs counts [21, 27, 34]. Since CTCs going through the EMT process present mesenchymal features, CD133 was chosen for CTCs identification in SCLC, instead of EpCAM. CD133 expression for CTCs immunostaining identification in SCLC cases has been evaluated as being effective [140]. Vimentin was used to identify CTCs presenting mesenchymal characteristics and possibly linked to metastasis [96]. This immunostaining marker has been used as a feasible target in cancer therapy, particularly for cancer at advances stages. Studies have shown its utility in moderately and well-differentiated lung adenocarcinomas [92, 116]. CD45 was used to identify WBCs. It is one of the most used markers to negatively select leukocytes and differentiate these haematological cells from tumour cells when analysing a sample [34, 100].

Besides fluorescence evaluation, there are other features that tumour cells may present. The cells are trapped in the flow direction, mostly between the smaller microporous channels located at the end of the chambers. The cells may lose their natural shape, becoming less round in appearance because of the force of flow and compression against the walls of the system.

Fluorescence images of identified CTCs were acquired at 20x or 40x magnification and processed using Fiji (ImageJ) software. Some representative images are shown in table 4.1 and table 4.2 of NSCLC and SCLC cases, respectively.

Table 4.1 — Representative images of identified CTCs from NSCLC plasma samples of lung cancer patients. Cells were stained with DAPI, Alexa fluor 647 + EpCAM, Alexa Fluor 594 Vimentin and Alexa Fluor 488 CD45. 20x and 40x magnification images were acquired using an inverted fluorescent microscope.

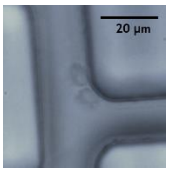
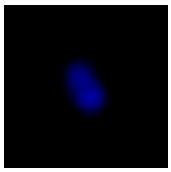
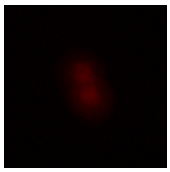
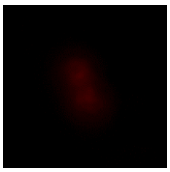
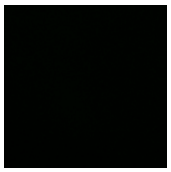
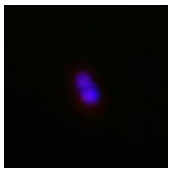
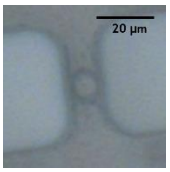
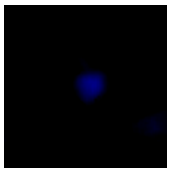
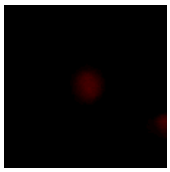
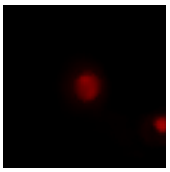
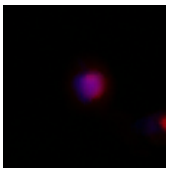
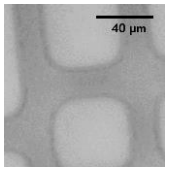
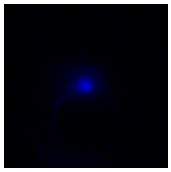
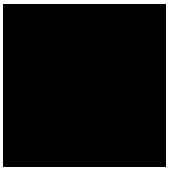
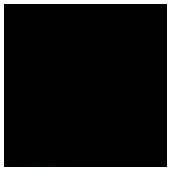
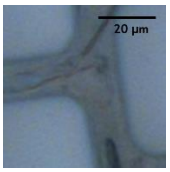
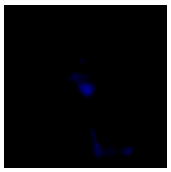
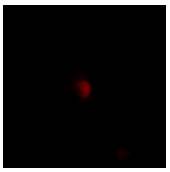
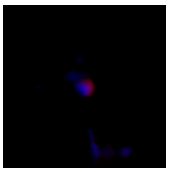
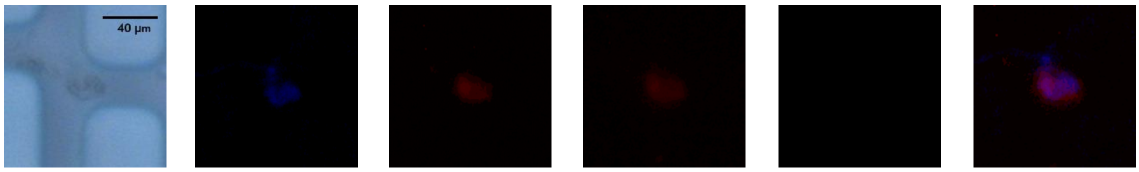
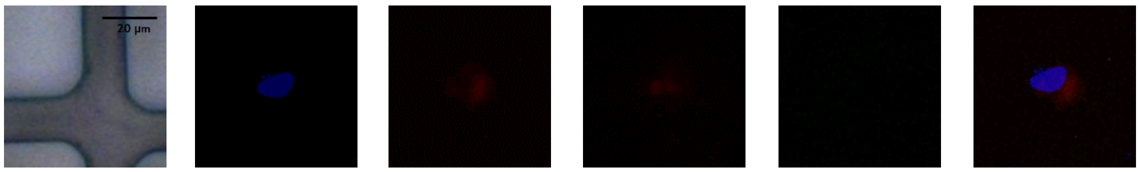
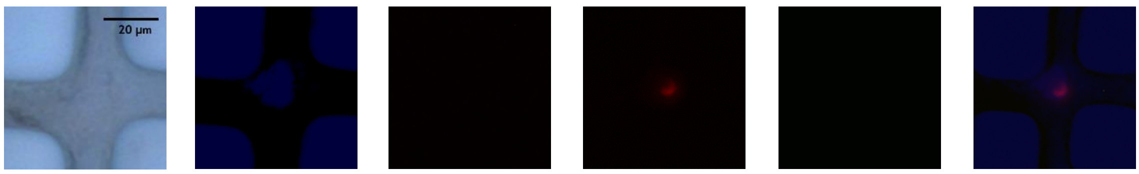
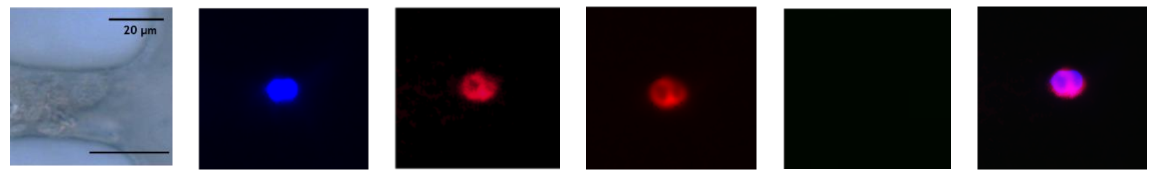
Brightfield	DAPI	EpCAM	Vimentin	CD45	Merge
					
					
					
					

Table 4.2 — Representative images of identified CTCs from SCLC plasma samples of lung cancer patients. Cells were stained with DAPI, Alexa fluor 647 + CD133, Alexa Fluor 594 Vimentin and Alexa Fluor 488 CD45. 20x and 40x magnification images were acquired using an inverted fluorescent microscope.

Brightfield	DAPI	CD133	Vimentin	CD45	Merge
					
					
					
					

Overall, the process of sample staining by immunofluorescence presented good results. DAPI marking was effective in both leukocyte and tumour cells, which is in line with expectations since this marker binds to cell nuclei.

Cells co-expressing EpCAM and Vimentin in NSCLC may be tumour cells undergoing EMT. In cases where the cells show positive fluorescence for Vimentin, and not for EpCAM, it can translate into an EMT full transition. CTCs expressing mesenchymal markers, such as Vim, are more frequently found in advanced stage disease than localized disease [101], since CTCs with mesenchymal expression are known to be associated with a more aggressive cancer [96]. Furthermore, it was observed that some Vim positive CTCs presented a smaller size than the rest, similar results to the ones Wang *et al*. reported in a study [18].

The combination of markers can provide more information about the cancer status and higher probabilities of identifying different types of CTCs, either with epithelial phenotype or under EMT, process through which cells may lose epithelial characteristics. Despite being one of the most used markers, cells may present higher or lower EpCAM expression depending on the characteristics of the tumour cells, or even be absent in non-epithelial tumours. EpCAM-based methods may fail to identify cells expressing mesenchymal proteins and lacking epithelial characteristics [96, 103]. In addition, the fact that CTCs isolation is based on the different dimensions of blood cells, also constitutes an advantage to improve the method of capturing and characterizing the cells of interest for the study [126].

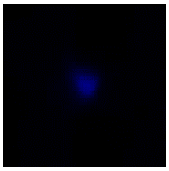
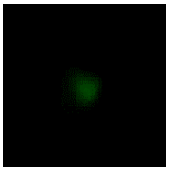
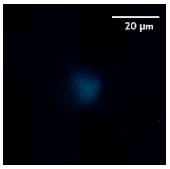
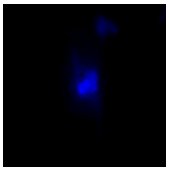
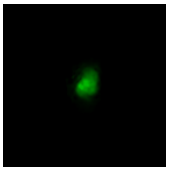
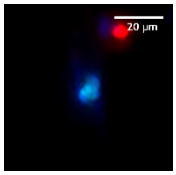
These different marker expressions suggest that tumour cells caught in the system presented different phenotypes and most likely were encountered in different stages of the pro-

cess of carcinogenesis. In some cases, more than one nucleus of CTCs or even clusters of 2 cells were identified, albeit in a smaller amount. Studies have reported higher metastatic capacity among CTC clusters [23, 126], which coincides with the results obtained, since two CTCs cluster, displayed in tables 4.1 and 4.2, refer to advanced stage samples. Furthermore, CTCs with different dimensions were found, which translates the diversity within cellular size.

Very few leukocytes were found from immunofluorescence assay which translates in low white cells contamination (WBC count ≤ 2 in all samples). The reduced number of WBCs reflects the purity of the CTCs isolation, an extremely important characteristic for these types of systems, so that samples obtained are as pure as possible. Considering the low quantification of WBCs, there was no correlation found between the number of leukocytes identified and the type or aggressiveness of the lung cancer.

Some fluorescence images of identified leukocytes are represented in table 4.3.

Table 4.3 — Some examples of identified WBCs from samples of lung cancer patients. Leukocytes presented CD45 and DAPI expression. 20x and 40x magnification images were acquired using an inverted fluorescent microscope.

DAPI	EpCAM	Vimentin	CD45	Merge
				
				

4.3 - Data analysis

4.3.1 – CTCs count

A total of 30 blood samples from lung cancer patients were processed for CTC enumeration and characterization. 10 SCLC samples, all from III/IV stages and 20 NSCLC samples, 10 I/II stages samples and 10 III/IV stages samples. Clinical data from patients is shown in table 3.1. The enumeration of CTCs was done without taking into consideration the patient clinical characteristics.

Regarding the analysis of the results obtained in the identification and counting of CTCs, two different characteristics were compared, the stage and the type of lung cancer. This way, the results obtained from the analysed samples of NSCLC of early stages were compared in relation to those of more advanced stage. In the second group, an assessment was carried out to observe the main differences concerning the results from the analysis of SCLC and NSCLC samples, with both types in advanced tumour stages. Graphical representations of the results obtained are shown in figure 4.5 and figure 4.6.

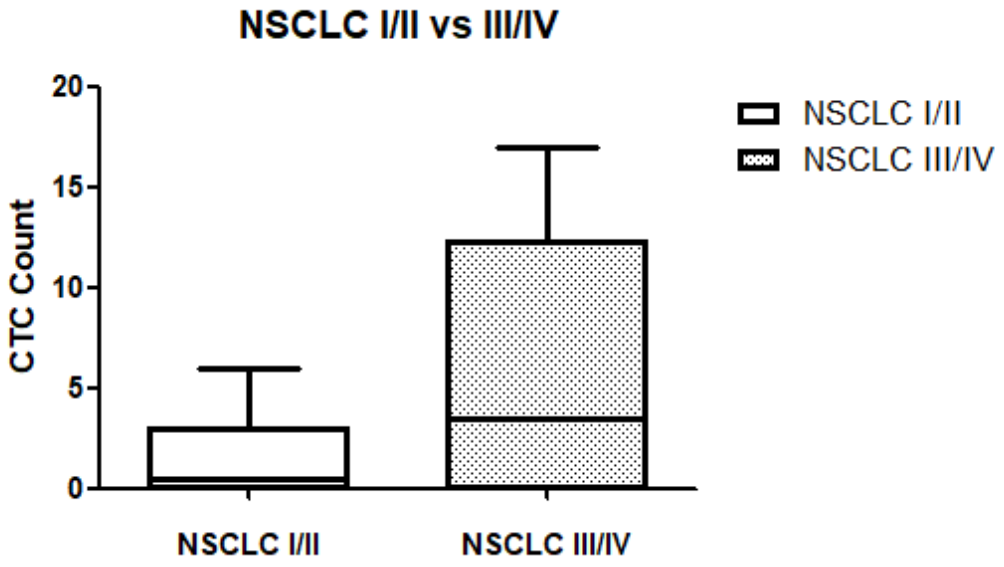


Figure 4.5 - Distribution of CTC levels in early and advanced stages of NSCLC samples. The box plot demonstrates, median, minimum and maximum values. NSCLC samples of stages I/II (n=10), mean=1.5, median=0.5; NSCLC samples of stages III/IV (n=10), mean=6.0, median=3.5. No statistically significant differences were found between CTC count in both LC subtypes ($p>0.05$). (Created with GraphPad Prism).

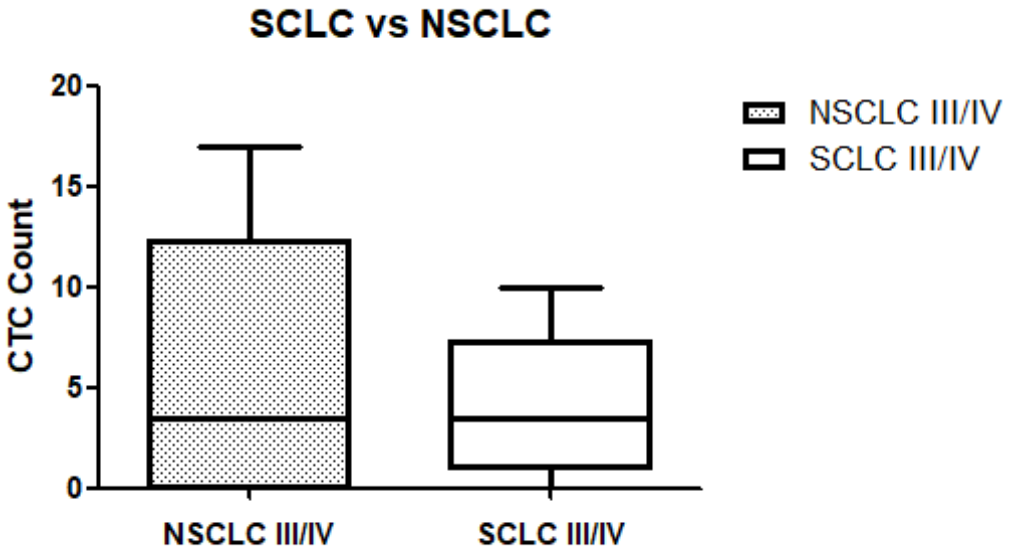


Figure 4.6 - Distribution of CTC levels in advanced stage NSCLC and SCLC samples. The box plot demonstrates, median, minimum and maximum values. NSCLC samples of stages III/IV (n=10), mean=6.0, median=3.5; SCLC samples of stages III/IV (n=10), mean=4.3, median=3.5. No statistically significant differences were found between CTC count in both LC subtypes ($p>0.05$). (Created with GraphPad Prism)

As expected, the number of tumour cells was higher in patients in a more advanced stage of the disease, as can be observed in figure 4.5. However, no statistically significant differences were found between the number of CTCs enumerated in early and advanced stages of NSCLC cases ($p>0.05$), which may be due to the low number of samples processed of each.

Most of early-stage samples analysed presented ≤ 1 CTCs. The mean value of CTC count obtained from stage I/II samples was 1.5 and the median 0.5. On the other hand, results obtained from the analysis of advanced stage samples, with a CTC count mean value of 6.0 and a median of 3.5, suggest that it is more likely to find a greater number of CTCs in these conditions, which is in accordance with the literature. Several studies have shown CTCs value as a prognostic tool for lung cancer [10, 23, 97].

In turn, when comparing the results obtained for the last-stage samples of both types, the count of CTCs in cases of NSCLC was higher than the referring cases of SCLC. According to the literature, it would be expected that since the SCLC type is more aggressive and usually associated with worse prognosis, that the CTC count would be higher than for the NSCLC type [102]. SCLC is reported to have, on average, 70-95% of the patients diagnosed presenting identified CTCs [21]. As observed in figure 4.6, the average CTC found in NSCLC stage III/IV samples was 6.0, while the mean count in SCLC stage III/IV samples was 4.3. Still, statistical analysis showed no significant differences between both subtypes, at late-stage. The higher than expected NSCLC count, when compared to SCLC, may be explained by certain conditions such as treatment or surgery, the existence of metastases, all factors that have been shown to influence CTCs number in the bloodstream.

While CD133 is commonly identified in SCLC, this marker and Vim are both representative of the mesenchymal phenotype. An epithelial marker, such as cytokeratin (CK), could also have improved the CTC count in this case, to maybe avoid losing potential tumour cells that were not in the EMT process or presenting mesenchymal markers [141].

Additionally, considering the low number of studied samples (n=10), the lower CTCs count in SCLC, may also be dependent of patients which presented higher chances of recovery, as the number of CTCs has been shown to correlate with OS. On the other hand, for NSCLC, the samples with higher CTCs count could indicate a more aggressive disease, with lower patient OS [142]. The same median values of 3.5 may also affirm these results, which reveals, despite there being differences in counts, the number of CTCs most likely to be identified is close to this value, similarly for both NSCLC and SCLC cases. The use of the median values to analyse CTC count in small sample sizes is commonly used and was considered for this analysis, identically to the strategy followed by Wang *et al.* in his work [18].

A comparative analysis was also carried out between the results of CTC count throughout the various TNM stages of lung cancer. These results are shown in figure 4.7.

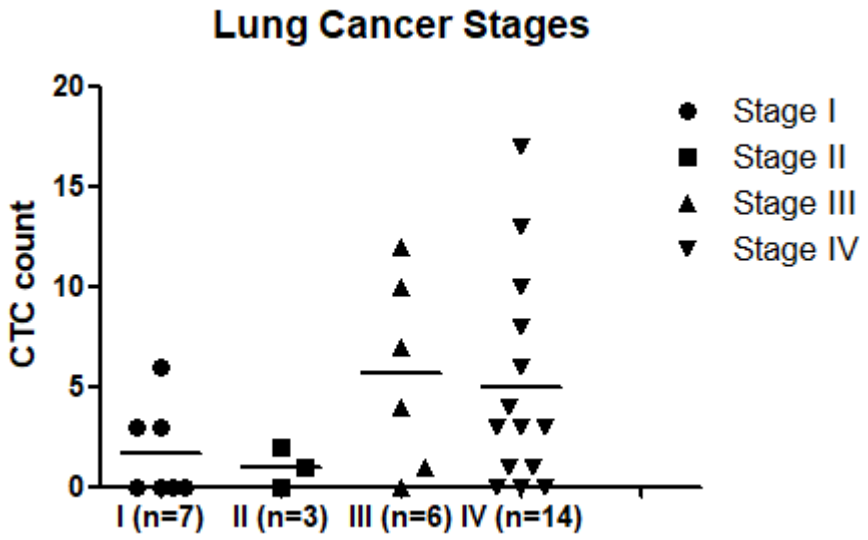


Figure 4.7 - Distribution of CTC levels in all TNM stages of both NSCLC and SCLC samples. Stage I (n=7), mean= 1.7, median=0.0; Stage II (n=3), mean=1.0, median=1.0; Stage III (n=6), mean= 5.7, median=5.5; Stage IV (n=14), mean=5.0, median=3.0. (Created with GraphPad Prism)

When comparing the number of CTCs identified between different TNM stages of lung cancer, it was observed that a greater number of tumour cells was found mostly at the more advanced stages. More advanced stages (III/IV) presented the highest values of both mean and median of CTC count, compared to early stages (I/II). Furthermore, stage I median value for CTC count was 0.0, meaning that most samples analysed presented zero CTCs identified, which was in accordance with what was expected. Several studies have shown an increase of CTC enumeration with advanced cancer state. Wang *et al.* developed a study, which enrolled 169 NSCLC and 96 SCLC patients, and verified a significant increase in CTC count parallel with higher TNM stage, tumour size and lymphatic metastasis [86]. Similar results were reported by Qiao *et al.*, that observed a higher frequency of CTCs in patients with advanced stage disease (88.0% in III/IV stages, 58.9% in I/II stages), in 103 peripheral blood samples of esophageal squamous cell carcinoma patients [143].

Further studies may be carried out in future developments, so that the same number of samples analysed for each stage is achieved, and the variation in the number of CTCs enumeration with the increase in stage and evolution of the tumour can be analysed with greater certainty.

4.3.2 – Continuous monitorization

The results obtained above prove that CTCs enumeration may be a valuable strategy for lung cancer prognostic and function as a predictive biomarker. However, the potential of CTCs as a liquid biopsy may also be used to monitor the evolution of the disease and eventually the response to a treatment [4, 22]. Thus, a comparison between samples collected from the same patient at different times was carried out, performing the analysis and identifica-

tion of CTCs from the same patient twice. The values of the CTC variation within the same sample are represented in table 4.4, as well as graphically represented in figure 4.8.

Table 4.4 — CTCs enumeration from three samples from lung cancer patients, collected on two different dates. The values presented refer to a first sample at the time of diagnosis and a subsequent follow-up sample.

Sample	CTC count	
	First assessment (at diagnosis)	Follow-up
LC_1	8	1
LC_2	6	0
LC_3	10	1

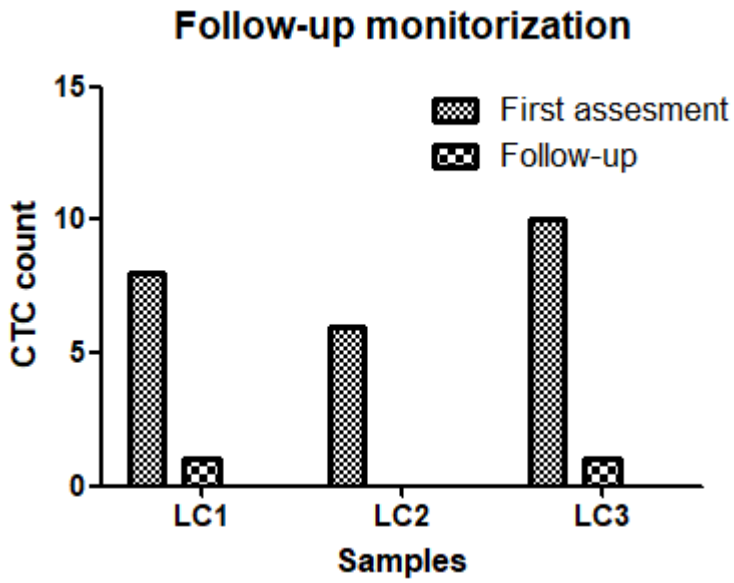


Figure 4.8 - Graphical representation of table 4.4. CTCs count from three lung cancer patients (LC_1, LC_2 and LC_3), collected on two different dates. The values presented refer to a first sample in the diagnosis phase and a subsequent follow-up sample. (Created with GraphPad Prism)

The results obtained clearly reveal a drastic decrease in the number of CTCs identified for the same patient, at follow-up. This decrease in CTC count is justified by the fact that patients were submitted to treatment for lung cancer, including chemotherapy and radiotherapy cycles, which may have led to stagnation or a regression of the tumour's evolution. This positive evolution was noticeable in the smaller number of tumour cells found in relation to the sample at diagnosis. Thus, the results obtained from the strategy of using CTCs as a predictive biomarker were consistent with the clinical follow-up. Other authors have shown that CTCs are useful as a prognostic marker, allowing the continuous monitorization of the disease development. Xu *et al.* reported changes in CTC count before and after treatment, in the course of treatment monitorization of 19 lung cancer patients [144]. Likewise, Li *et al.* shown that the measurement of alterations in CTC measurement in baseline and post-treatment

blood samples 100 patients with cutaneous melanoma, was a potential strategy for tumour evolution monitorization, drug response, and survival [145].

4.4 – RNA and DNA analysis

Six samples presenting higher numbers of CTCs (CTCs ≥ 6) were subjected to RNA and DNA extraction. Once isolated, the nucleic acids were quantified by a NanoDrop Spectrophotometer. The results obtained are presented in table 4.5.

Table 4.5 — Measurement of nucleic acid concentrations extracted from CTCs. The samples analysed were among the ones with the highest numbers of CTCs.

Sample	Type/Stage	CTC count	RNA (ng/µL)	DNA (ng/µL)
LC_4	NSCLC IV	13	2.2	9.1
LC_5	NSCLC IV	17	1.5	12.0
LC_6	NSCLC III	10	0.7	21.1
LC_7	SCLC III	7	1.5	7.2
LC_8	NSCLC I	6	0.2	3.8
LC_9	NSCLC III	12	1.8	6.9

Analysing the results obtained, DNA and RNA extraction from the isolated CTCs in the microfluidic chip were successful. Overall, there seems to be a correlation between DNA concentration, RNA concentration and the number of CTCs. Furthermore, taking into consideration the type, stage and CTC count of each sample, the lower DNA and RNA values of the sample LC_8 are justified due to the early stage and lower CTC count characteristics that demark it from the remaining samples. On the other hand, samples with the highest CTC count, LC_4 and LC_5 coincided with the highest concentrations of both DNA and RNA.

The higher DNA concentration values, when compared to RNA, are due to the fact that the DNA extracted and isolated from lysate solution, was subjected to SpeedVac vacuum concentrator to concentrate the DNA sample, removing part of the buffer, while maintaining the sample's integrity.

Since dealing with low sample quantities, that are obtained by lysis inside a microfluidic chamber, RNA samples were further analysed by the BioAnalyzer and DNA by Quibit. These techniques can provide improved concentrations measurements, as well as purity and integrity of the extracted nucleic acids.

4.4.1 – RNA characterization

A more specific study of isolated RNA was carried out, using a 2100 BioAnalyzer (Agilent Technologies). The results from the six samples analysed are shown in table 4.6.

Table 4.6 — Measurement of RNA concentrations using the BioAnalyzer and Nanodrop.

Sample	Type/Stage	CTC count	BioAnalyzer RNA (pg/µl)	NanoDrop RNA (ng/µl)
LC_4	NSCLC IV	13	20	0.651
LC_5	NSCLC IV	17	29	1.515
LC_6	NSCLC III	10	20	0.664
LC_7	SCLC III	7	30	3.288
LC_8	NSCLC I	6	29	1.843
LC_9	NSCLC III	12	13	3.033

A more specific analysis of RNA showed that samples displayed integrity and purity, with lower concentration values than those obtained with the nanodrop measurement. A following step could focus on the concentration of the RNA samples with the SpeedVac, as well. Still, considering the purity of the sample and the RNA integrity, the range of concentration would allow a future molecular characterization of the tumours.

4.4.2 – DNA characterization

Similarly, a more quantitative assay of the DNA concentration was carried out, using a Qubit 3.0 Fluorometer (Life Technologies). Prior to the sample concentration, all six analysed samples presented very low concentration values when analysed by Qubit. One possible reason for these results was due to the higher sample dilution in buffer following the nucleic acids extraction from the CTCs present in the samples.

Thus, an attempt was made to increase the DNA concentration. As mentioned above, the SpeedVac Vacuum Concentrator was used, and the DNA final concentration was evaluated with nanodrop. The results are displayed in table 4.5. In a future analysis using Qubit, higher DNA concentrations are expected.

Other authors have showed the successful extraction of nucleic acids from CTCs for tumour analysis. Ribeiro-Samy et al reported on the application of a microfluidic cell filter to isolate CTCs from the blood of colorectal cancer patients. CTCs enriched in the chip were lysed and molecularly characterized by droplet digital PCR, disclosing a mutation in the APC gene for most of the analysed patients' samples, showing the potential application of CTCs for molecular characterization of cancer [107].

4.5 – CTCs proteomics profile

Proteomics is a complex method that focuses on the study of protein structures, location and functional status, as well as interactions [146]. It responds to continuous alterations of environment, therapy treatment, and post-translational modification. Recently research in

this field, more specifically in lung cancer, has been mainly focused on classification of the tumour, correlation of gene and protein expression, and recognition of new molecular targets [90]. Proteomic identification of biomarkers present in blood, has been used on heterogenous or homogenous immunoassays based on antibodies. This type of tests is useful to obtain information for diagnosis and a better understanding of immune and infectious diseases [45].

Recent advances in immunotherapy have shown improved outcomes when applied to patients with PD-L1 expression. The pair of immune coinhibitory molecules PD-1 and PD-L1 is expressed in a wide range of cancers and mediates tumour immune suppression by inhibiting T cell cytotoxicity. In fact, drugs targeting these two molecules have been approved by FDA and implemented in NSCLC relapse treatment [46, 73, 76]. Immune checkpoint inhibitors (ICIs) when combined with chemotherapy are being implemented as a first-line strategy to treat advanced NSCLC [123].

Mass spectrometry analysis was conducted with the intent of assessing CTCs value for detection of PD-L1 expression. Table 4.7 shows the characteristics of the samples studied.

Table 4.7 – Information about the samples enrolled in the proteomics analysis.

Sample	Type/Stage	Metastases	PD-L1 expression
LC_{10}	NSCLC III	No	Negative
LC_{11}	NSCLC III	Yes	> 90%

Results from the protein profile of both samples showed that PD-L1 was not discovered, most likely due to the low number of recovered CTCs, resulting in low protein amount.

Nevertheless, it was possible to identify several proteins from both analysed samples, which proves that the method of collecting the CTCs, as well as their lysis and analysis, were successful. To determine differentially expressed protein, the filters described in the methodology were applied (3.4.3). Data analysis from mass spectrometry was carried out, focusing on protein expression of each sample compared to the negative control. The resulting number of identified proteins, after filter selection, were 66 and 94 in a total of 1029, for LC_{10} and LC_{11}, respectively.

Functional enrichment analysis was performed regarding the selected proteins, using the WEB-based GEne SeT AnaLysis Toolkit. The enrichment results from geneontology functional database in biological process were similar for both samples. The gene sets included the following groups: platelet degranulation, protein activation cascade, acute inflammatory response, blood coagulation, haemostasis, vesicle-mediated transport, with similar enrichment ratios between the two samples. The biological processes found exclusively for LC_{10} were: regulation of protein processing, regulation of protein maturation, coagulation and immune response. On the other hand, in LC_{11} the following biological processes were identified: wound healing, regulated exocytosis, exocytosis and defence response.

In overall analysis of the results, it was observed that two sets of proteins, SAA1 and SAA2 (Serum amyloid A proteins), were present in sample LC_{11} but not in LC_{10}. The presence of this type of proteins is usually associated with the metastasis process, which can justify why these proteins were only present in sample LC_{11}, since the patient presented metastasis at the time

of diagnosis. Sung *et al*. reported that SAA1/2 expression was higher in lung cancer cells and that this protein promotes cancer metastasis [147].

This study is preliminary and only incorporated the analysis of two samples, only one of which contained PD-L1 expression. Thus, a larger cohort of samples to understand the effectiveness of this methodology in identifying the PD-L1 protein from isolated CTCs should be explored. Other authors have incorporated similar strategies in their studies using mass spectrometry, such as Zhu *et al*. that analysed proteome profile of CTCs in prostate cancer [148] and Nigro *et al*. that investigated protein expression in lung cancer tissue specimens [149].

Furthermore, peptide profiling may be hampered because of the very low concentration levels in comparison to proteins of high concentration in plasma, such as albumin and immunoglobulins, which could have masked other compounds [150].

Still, an objective of this initial study, which focused on extracting CTCs captured by the system and analysing their proteomic profile, was achieved successfully. Additionally, the preliminary results allowed for a comparison of protein expression between both samples, displaying the intrinsic heterogeneity of lung cancer.

4.6 – Analysis of cfDNA methylation

As already mentioned, the oncological study by liquid biopsy can certainly become more interesting when incorporating more than one biomarker that, by providing different information, can complement overall findings. CTCs are believed to be more promising in proteins assessment and single cell analysis, namely in DNA and RNA molecular profiling. On the other hand, cfDNA is more useful in assessing mutations, copy number variations and DNA methylation [23]. DNA methylation alterations are common and have direct effects on carcinogenesis, diagnosis, and prediction of lung cancer [151]. Therefore, a possible correlation was analysed, between the number of CTCs found in some samples and the results of DNA methylation, obtained from cfDNA analysis of the respective samples. This type of comparative study has been carried out for other types of cancer [152].

In this study, two early-stage and four late-stage lung cancer (LC) samples were included. Table 4.8 shows the results regarding CTC count and respective double-stranded DNA (dsDNA) quantification, as well as biomarker performances of ADCY4, MIR129-2, and HOXA11 genes promoters' methylation levels in circulating cell-free DNA, of six lung cancer samples analysed.

Table 4.8 — CTC count and respective dsDNA quantification, as well as biomarker performances of ADCY4, MIR129-2, and HOXA11 promoters' methylation levels in circulating cell-free DNA, of six lung cancer samples analysed.

Stage	Sample	CTC count	dsDNA quantification	Genes		
			Qubit (ng/μL)	ADCY4me	MIR129-2me	HOXA11me
Early	LC_{12}	0	0.306	3.642	-	-
	LC_{13}	0	0.362	8.756	7.607	-
Late	LC_{14}	1	0.394	114.475	271.198	79.126
	LC_{15}	3	too low	-	36.863	-
	LC_{16}	0	0.608	13.057	16.859	31.923
	LC_{17}	3	0.316	3.568	58.172	52.552

While the samples were diluted for processing in the chip, cfDNA concentration was not affected by the chip processing, when compared to the respective unprocessed samples.

For the selected samples, no direct correlation was found between the CTC count and the dsDNA quantification, each promoter gene methylation levels or the total number of gene promoter levels detected in each sample.

All samples, regardless of CTC count, presented a dsDNA quantification higher than 0.3 ng/μL, except for LC_{15} which result was too low. A trend was observed for the late-stage samples, LC_{14}, LC_{16} and LC_{17}, which presented higher total number of promoter methylated genes. Early-stage samples, LC_{12} and LC_{13}, which had a count of 0 CTCs, presented one or two methylated genes, with a general lower gene promoter methylation count than the late-stage samples.

While no corelation was found between both biomarkers for this sample set, the combinatorial assessment of both CTCs and cfDNA could provide the possibility of tumour characterization from at least one of the biomarkers. Additionally, the selected samples for matched analysis showed a low CTC count, while with a larger sample set, could potentially showcase different results.

Chapter 5

Conclusions and Future Work

Lung cancer is one of the diseases with the highest associated mortality worldwide. This phenomenon is largely associated with the late-stage detection of the disease, when the symptoms are more evident and the tumour metastatic propagation makes the likelihood of recovery and survival smaller. Other factors associated to the poor survival rate of lung cancer patients derived from the limitations of tissue biopsies in providing a complete characterization of the tumour heterogeneity and the lack of minimally invasive tools for detection of residual disease and early recurrence.

Liquid biopsy appears as a non-invasive and effective method for the early diagnosis and continuous monitoring of lung cancer, expected to be implemented in routine consultations and increasing the chances of survival. CTCs have emerged as promising biomarkers, not only for cancer development assessment but also to provide information regarding which therapeutic treatments are more promising for a specific clinical case, to avoid unnecessary exposure of the patient to drug-related toxicities. The continuous improvement of liquid biopsy technologies provides higher sensitivity parameters, allowing the detection of minimal residual disease and even of early-stage cancer in asymptomatic populations. However, many of the laboratory procedures and protocols for liquid biopsy still lack standardization and more research. This highlights the importance of developing this type of studies, which ultimate goal involves the analysis of circulating tumour cells using a microfluidic system, contributing to the improvement of early diagnosis and more effective methods in the study of the tumour cells subpopulations heterogeneity present in lung cancer.

The microfluidic system has proven to be efficient in the isolation of CTCs, as well as in allowing the identification and distinction between CTCs, leukocytes and other type of blood components retained in the same system. In addition, the system also showed good results in the immunofluorescence characterization of the trapped cells.

A cocktail combination of cell surface markers was used so that a greater variety of tumour cells could be identified, which is important since CTCs present differences and highly heterogeneous subpopulations. Using epithelial marker EpCAM and mesenchymal markers, Vimentin and CD133, it was possible to detect CTCs at different stages of the carcinogenesis

process and the EMT process, considering the different origins of both subtypes of lung cancer. Besides, CD45 marker allowed the distinction between leukocytes and tumour cells.

Given the results of CTCs count, it was possible to correlate them to the specific characteristics of the samples. As expected, when analysing NSCLC cases, a greater number of CTCs was found in patients diagnosed with advanced stage of lung cancer, compared to early-stage ones. By analysing the tumour cell counts between the two types of lung cancer, both at advanced stage, a higher mean of CTC per sample was observed in the NSCLC cases, when compared to the SCLC ones. However, the median value was the same for both types. According to the literature, SCLC is more aggressive and usually presents a worse prognosis, so it was expected that samples of this type would have a higher number of CTCs. The characterization of a larger patient cohort could assist in confirming these results, as well as the incorporation of an epithelial marker such as CK, which has been identified in SCLC CTCs. CTCs counts varied with increasing TNM stage. Stages III and IV presented higher values of CTCs identified when comparing to the ones observed in stages I and II.

When carrying out a follow-up of patients between the first assessment and the samples acquired after treatment, there was a decrease in the number of CTCs, which coincides with a regression in the development of the disease. This latter analysis confirms the usefulness of CTCs for monitoring treatment.

Regarding the analysis of CTC nucleic acids, there was a positive correlation between the concentration of DNA and RNA with the number of CTCs in the samples.

Proteomic study allowed the analysis of the protein profiles between the two samples studied, as well as confirmation that the extraction of CTCs from the system for posterior characterization was successful.

To complete the study of the CTCs profile, the enumeration of CTCs was compared with the methylation of cfDNA, extracted during sample processing. A correlation between the sample stage and dsDNA quantification was found in most samples, as well as the promoter gene methylation levels.

Taking in account all the results obtained, the main conclusion taken from the project is that this is an effective non-invasive approach for rapid analysis of CTCs in clinical diagnosis and cancer prognosis prediction, as an alternative for invasive procedure, such as surgical or tissue biopsy interventions. Further developments in this area and the establishment of a general recognized strategy to analyse tumour cells in routine medical consultations may help in the early diagnosis of oncological cases, which improves the effectiveness of a treatment applied, when the tumour is still in early stages and not metastasized to other parts of the body. Overall, we are looking at a health condition that affects people all over the world, which translates in the importance of achieving a good preventive screen, that will undoubtedly have a major impact on the living conditions of many oncological patients, personalized oncology and in general on the advancement of the world of medicine and science.

Future developments in the project should proceed with the analysis of a larger patient cohort to represent even more accurately the population affected by lung cancer. Regarding the obtained results, following the extraction of nucleic acids, RNA and DNA should be further characterized to perform mutational analysis and assess gene promoter methylations in CTCs. Furthermore, in future works, an in-depth study should be carried out to correlate longitudinal changes in CTC counts and the conditions in which samples are collected and individual clinical cases of patients, namely details on metastases, types of treatment to which the patient was subjected to, remission of the tumour, local recurrence, between others.

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