Downstream methylation analysis of cell-free DNA (cfDNA) from lung cancer patients' plasma following a microfluidic-assisted filtration

Duarte Manuel Neves Seixas

Dissertation for the Degree of Master of Science in Bioengineering at Faculty of Engineering of the University of Porto and Abel Salazar Biomedical Sciences Institute of the University of Porto

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FEUP – Faculdade de Engenharia da Universidade do Porto

ICBAS-UP – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

Dissertation for Masters in Bioengineering

Supervisor: **Ângela Carvalho, PhD**

i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal

INEB – Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Portugal

Co-Supervisor: **Carmen de Lurdes Fonseca Jerónimo, PhD**

ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

CI-IPOP – Centro de Investigação do Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E, Portugal

Adviser: **Vera Inês Salvado Constâncio, PhD student**

ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

CI-IPOP – Centro de Investigação do Instituto Português de Oncologia do Porto Francisco Gentil, E.P.E, Portugal

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“Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world.”

Albert Einstein

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Resumo

Introdução: Mundialmente, o cancro do pulmão (CaP) é um dos cancros com maior incidência e mortalidade em ambos os sexos. Embora progressos tenham sido feitos no rastreio do CaP, a necessidade de uma estratégia eficiente continua a existir. As biopsias líquidas representam um teste minimamente invasivo que possibilita a avaliação de múltiplos biomarcadores de cancro. A metilação aberrante de promotor de genes manifesta-se precocemente no desenvolvimento de cancro, e a sua avaliação pode ser utilizada como método de deteção desta doença. Neste trabalho é proposto um dispositivo de microfluídica para o isolamento de células tumorais circulantes (CTCs) e filtração de plasma para posteriori análise de metilação de DNA circulante livre de células (cfDNA) para deteção de CaP.

Métodos: Após o isolamento de CTCs através do dispositivo de microfluídica, procedeu-se à extração de cfDNA a partir de 26 amostras de plasma de pacientes com cancro do pulmão, seguido de modificação sódio-bissulfito e pré-amplificação. Os níveis de metilação dos genes *NID2$_{me}$*, *ADCY4$_{me}$*, *MIR129-2$_{me}$*, *HOXA11$_{me}$* e *MAGI2$_{me}$* foram avaliados por PCR quantitativo específico de metilação em multiplex.

Resultados: A análise de dois métodos para a extração de cfDNA mostrou o kit de QIAamp de cfDNA como a melhor opção após o processamento das amostras de plasma pelo dispositivo. Amostras de estadio mais avançado apresentaram uma tendência para um maior número de genes metilados, bem como níveis de concentração de cfDNA mais altos. *HOXA11$_{me}$*, de todos os genes apresentou a maior sensibilidade individual para a deteção de cancro do pulmão. O conjunto dos genes *ADCY4$_{me}$*, *MIR129-2$_{me}$* e *HOXA11$_{me}$* demonstrou ser o melhor painel para detetar CaP com 80.77% e 61.54% de sensibilidade para todas as amostras e para as amostras em estádio precoce, respetivamente. A contagem de CTCs não mostrou nenhuma associação aparente com a avaliação do cfDNA, embora tenha sido observada uma predisposição para detetar 3 ou mais CTCs e um maior número de gene metilados em amostras de estádio avançado.

Conclusões: Foi concluído que o dispositivo de microfluídica testado demonstrou ser promissor como uma ferramenta adjuvante para o isolamento de CTCs e enriquecimento de cfDNA para a deteção de CaP. Além disso, a deteção de genes metilados evidenciou o potencial de cfDNA como biomarcador para a deteção de cancro do pulmão.

Abstract

Introduction: Lung cancer (LC) is one of the most incident and fatal cancers in both genders, worldwide. Although advances have been made in LC screening there is still a necessity of efficient strategies. Liquid biopsies represent a minimally invasive test that allows the evaluation of multiple cancer biomarkers. Since aberrant DNA promoter methylation develops early onset, it could be a valuable phenomenon to evaluate cancer detection. Herein, we purpose a microfluidic platform for CTCs isolation and plasma filtration, followed by a downstream methylation analysis of circulating cell-free DNA (cfDNA) for LC detection.

Methods: Following on-chip CTCs isolation, extraction of cfDNA from 26 LC patients' plasma samples proceeded, followed by sodium-bisulfite modification and PreAmp amplification. Promoter methylation levels of, $NID2_{me}$, $ADCY4_{me}$, $MIR129\text{-}2_{me}$, $HOXA11_{me}$ and $MAGI2_{me}$ were assessed by multiplex quantitative methylation-specific PCR.

Results: Analysis of two cfDNA extraction methods showed QIAamp cfDNA kit as the best option following samples processing in the microfluidics device. Late-stage samples exhibited a tendency for higher number of methylated genes as well as cfDNA concentration levels. $HOXA11_{me}$, from all biomarker, had the highest individual LC detection. A panel composed of $ADCY4_{me}$, $MIR129\text{-}2_{me}$ and $HOXA11_{me}$ detected LC with an 80.77%, and 61.54% sensitivities for overall and early-stage cancer samples, respectively. CTCs' count showed no apparent association with cfDNA evaluation although a predisposition to detect 3 or more CTCs in late-stage samples, with a tendency for higher number of methylated genes was observed.

Conclusions: We concluded that the tested microchip showed promise as an adjuvant tool for CTCs isolation and cfDNA enrichment in LC detection. Furthermore, the detection of promoter methylated genes evidenced the potential of cfDNA for LC detection.

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List of Abbreviations

5mC	5-methylcytosine
ACTβ	*β-Actin*
ADCY4	Adenylate Cyclase 4
ADCY4$_{me}$	Adenylate Cyclase 4
AEEA	3-[2-(2-aminoethylamino)-ethylamino]-propyluimethoxysilane
AID/APOBEC	Activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex
AJCC	American Joint Committee on Cancer
ALK	Anaplastic lymphoma kinase
APTES	3-aminopropyltrietboxysilane
ASLC	Adenosquamous lung carcinoma
AS-PCR	Allele-specific Polymerase Chain Reaction
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
BEAMing	Beads, Emulsion, Amplification and Magnetics
BEC2	Beclin2 gene
BER	Base excision repair
ccfRNAs	Circulating cell-free ribonucleic acids (ccfRNAs)
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cfDNA	cell-free DNA
CFS	Cerebrospinal fluid
CH_3	Methyl group
CNAs	Copy number aberrations
CREBBP	cAMP-response element-binding protein
CRL^+	Diluted PreAMP positive standard control
c-SCLC	Combined small cell lung cancer
Ct	Cycle threshold
CTCs	Circulating tumour cells

ctDNA	Circulating tumour DNA
ddPCR	Droplet Digital PCR
DNMTs	DNA methyltransferases
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
ETS	Environmental tobacco smoke
EV	Extracellular vesicles
$H_20_{PRE}^{+}$	Non-template control preamplified
H_2O	Water
HOXA11	Homeobox A11
$HOXA11_{me}$	Homeobox A11
IARC	International Agency for Research on Cancer
IFN-α	Interferon alfa
KMT2D	Histone-lysine N-methyltransferase 2D
LC	Lung Cancer
LCLC	Lung large cell carcinoma
LCNEC	Large cell neuroendocrine carcinoma
LDCT	Low-dose computational tomography
LUAD	Lung adenocarcinoma
LUSC	Lung squamous carcinoma
MAGI2	Membrane-associated guanylate kinase inverted 2
$MAGI2_{me}$	Membrane-associated guanylate kinase inverted 2
MBD	Methyl-CpG-binding
MBPs	Methylcytosine-binding proteins
MECP2	Methyl CpG binding protein 2
$MIR129\text{-}2_{me}$	Methylated microRNA 129-2
miRNAs	MicroRNAs
MRI	Magnetic resonance imaging
MS-PCR	Methylated-specific Polymerase Chain Reaction

NaCl	Sodium chloride
NGS	Nex-Generation Sequencing
NID2	Nidogen 2
NID2$_{me}$	Nidogen 2
NK	Natural Killer
NSCLC	Non-small cell lung cancer
NTC	Non-template control
O_2	Oxygen
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDMS	Polydimethylsiloxane
PET	Positron emission tomography
PNA-LNA-PCR	Peptide nucleic acid-locked nucleic acid Polymerase Chain Reaction
PreAMP	DNA preamplification
PTEN	Phosphatase and tensin homolog
qMSP	Quantitative methylation specific
qPCR	Quantitative Polymerase Chain Reaction
RB1	Retinoblastoma 1
RBCs	Red blood cells
Re	Reynold number
ROS1	Proto-oncogene tyrosine-kinase ROS
rpm	*Revolutions per minute*
SAM	S-adenyl methionine
SCLC	Small cell lung cancer
SERS	PCR/Surface-Enhanced Raman Spectroscopy
Si	Silicon
STD$^-$	Negative control standard control

STD^{+}	Positive standard control
STD_{PRE}^{-}	Negative PreAMP control standard control
STD_{PRE}^{+}	PreAMP positive standard control
STK11	Serine/threonine kinase 11
Tam-Seq	Targeted Amplicon Sequencing
TDG	Thymine DNA glycosylase
TEPs	Tumour educated platelets
TET	Ten-eleven translocation
TKR	Tyrosine kinase receptor
TKRI	Tyrosine kinase receptor INHIBITORS
TNM	Tumour Node Metastases
TP53	Tumour suppressor p53 gene
TRT	Thoracic radiotherapy
UICC	Union for International Cancer Control
VALG	Veteran Administration Lung-Study Group
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
WHO	World Health Organization

I. Introduction

Cancer represents one of the main human health challenges of the XXI century [1]. This health hurdle has an impact at a personal level on cancer patients and their respective families, but also in the country's socio-economic fabric. The World Health Organization (WHO) reported this disease as the second leading cause of death worldwide and projects a negative scenario for the near future [2]. The high pace booming rhythm of cancer reflects a combination of numerous factors. The global population health lifestyle (e.g., diet, exercise, stress, and smoking) and ageing, individual genetics' and epigenetics' defects and environmental factors are some of the causes of this illness [3] [4].

Lung cancer (LC) is one of the most frequent and deadliest cancer in both genders, worldwide, even though its incidence and mortality rate is much higher in men. According to the International Agency for Research on Cancer (IARC), in 2020, 2.2 million individuals were diagnosed. In that same year, LC was responsible for more than 1.78 million deaths, 18% of total cancer fatalities [5] .

LC has two main subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Lung cancer is, in general, associated with a poor prognosis (5-year survival of 17.8%). Like any other cancer, survival rates vary according to the subtype, stage of the disease, and even the treatment patients undergo [6] [7].

NSCLC represents 85% of all lung cancer cases [8] [9]. For this subtype, patients with small and localized tumours (stage I) tend to report a 5-year survival rate between 70 to 90%, while locally advanced and distant metastatic (stage III/IV) NSCLC patients have less than 10% [10] [11]. Furthermore, SCLC distinguishes as the most aggressive, with an average five-year survival rate below 7%, compared to the less than 20% of NSCLC [12] [13].

The characteristics of both LC subtypes arise from their respective molecular (genetic and epigenetic) landscape. NSCLCs display mutations in both epidermal growth factor receptors (*EGFR*) and KRAS proto-oncogene, and gene rearrangements involving anaplastic lymphoma kinase (*ALK*) gene and proto-oncogene tyrosine-protein kinase ROS (*ROS1*) [6] [9]. SCLCs, on the other hand, exhibit predominantly genetic alterations linked with tumour suppressor p53 gene (TP53), cell regulation's genes such as retinoblastoma 1 (*RB1*) and B-cell lymphoma 2 (*BCL2*), and amplification of MYC family genes' amplification [12] [14] [15]. Moreover, several epigenetic alterations, such as DNA methylation, histone modifications, non-coding RNA, and chromatin remodelling, have also been implicated in LC development. *ADCY4*, *MIR129-2* and *HOXA11* are two examples of lung cancer-associated genes, where the CpG-island promoter region is hypermethylated, leading to gene inactivation and consequent inactivation of their functional role as communication cell gene and tumour suppressor gene [16] [17] [18].

The disease is commonly diagnosed at an advanced stage, where metastasis is spread into the pleura, opposite lung, or even distant organs (e.g., central nervous system – CNS,

adrenal gland, bone, and liver) [19] [20]. The late diagnosis occurs mainly due to the lack of early lung cancer symptoms [21]. Generally, elder people (age 65 or older) are the most affected by this disease, unless the individual presents a smoking habit or is an ex-smoker, which has proven to trigger the lung carcinogenesis process at an earlier age [22]. Besides smoking, environmental effects like chemical management (asbestos) can contribute as well to lung tumorigenesis [23].

The initial LC screening implicates a low-dose computational tomography (LDCT) to rule out possible malignancy [24]. If nodules detection is positive, then a standard tissue biopsy is performed to acquire more tumour information [25] [26]. Regrettably, this standard procedure in a lung context is not always feasible since it may cause complications, and the tumour location may not be accessible for a biopsy [25] [27]. Furthermore, the lack of information on intra-tumoral heterogeneity and tumoral evolution seems intrinsic to these biopsies unless different tumour' regions and periodically tissue samples are collected, respectively [28] [29].

All these standard biopsy' limitations led to the emergence of liquid biopsies as a suitable complementary procedure. For LC, this minimally invasive technique is an enthralling tool as it may enable efficient disease monitoring throughout treatment and assesses possible relapse when patients are in a remission period [30] [31].

Liquid biopsies refer to body fluids, such as blood, urine, saliva, and cerebrospinal fluid, amenable to be tested for specific biomarkers. These proxies are extremely valuable for the information they provide, respecting the primary and metastasized tumours. Circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), extracellular vesicles (EVs like exosomes), cell-free ribonucleic acids (ccfRNAs) (e.g. microRNAs - miRNAs), and tumour educated platelets (TEPs) are some of those valuable biomarkers [30] [31] [32].

ctDNA represents a 0.01-90% fraction of total circulating cell-free DNA (cfDNA) depending on cancer's characteristics. Also known as cancer or tumour-derived cfDNA, this nucleic acid is often released from the primary tumour, metastatic lesions, or CTCs by cell apoptosis, necrosis, or active release [33] [34].

The ultimate goal of fluid biopsies testing is to obtain sensitive and cancer biomarkers to analyse and characterize the disease. However, many of the current techniques and isolation kits only allow the extraction and characterization of one biomarker at a time.

This limitation might addressed microfluidics devices, allowing the possibility of multiple biomarkers isolation depending on biomarkers' characteristics and microchips' features [35]. Microfluidics has been introduced in the field of liquid biopsies due to their advantages of reducing sample handling and reagents use, high operation control, faster analyses and results, and less environmental impact [35] [36].

A. Hypothesis, Strategy, and Aim

Circulating cell-free DNA is increasingly getting attention for its clinical biomarker potential in cancer. Presently, reproducibility, sensitive and specific assays to detect molecular alterations in this DNA represent two of the biggest hurdles that need to be surpassed to allow liquid biopsies as part of a clinical patient routine care.

This dissertation aims to develop a novel simple, and non-invasive platform for detection, and analysis of two biomarkers (CTCs and cfDNA) in lung cancer, an illness with a high impact on the worldwide population. The work developed focused on cfDNA methylation analysis from patients' plasma samples from different LC histological subtypes and stages. A PDMS size-based microfluidic device was used to filter CTCs from plasma. Following plasma filtration, cfDNA extraction and enrichment approaches were assessed and evaluated through single and multiplex quantitative methylation specific (qMSP) techniques. For that, genes with methylated promoters believed to be involved in LC were chosen (*MIR129-2*$_{me}$; *ADCY4*$_{me}$; *NID2*$_{me}$; *MAGI2*$_{me}$; *HOXA11*$_{me}$). Results will confirm if methylation might promise an LC biomarker event to cancer diagnosis.

This dissertation resulted in the preparation of a review paper, currently submitted in *Cancers*: A. Carvalho, M.G. Ferreira, D. Seixas, C. Guimarães-Teixeira, R. Henrique, F.J. Monteiro, C. Jerónimo. Emerging lab-on-a-chip approaches for liquid biopsy in lung cancer: Status in CTCs and ctDNA research and clinical validation.

II. Literature Review

1. Lung Cancer

Globally and in both genders, in 2020, lung cancer had one of the highest cancer incidence and caused the highest number of deaths [5]. Gender-specifically, men display a LC similar pattern just like the one found for both genders, while women LC's pattern shows this cancer to be third most incident and second deadliest (Figure 1). The impact of lung cancer in Portugal follows a slightly disparate incidence and fatality patterns from the one found worldwide and for both genders individually (Figure 1) [37] [38] [39].

Regarding age, LC is an illness particularly characteristic of elderly patients, where more than 65% of individuals are older than 65 years old, and the mean age of diagnosis is over 70 years [22] [23]. The occurrence in under 44-year patients with LC is uncommon (3% of deaths worldwide) [40] [41]. Tobacco smoking is a well-established etiologic risk factor for LC, and all its histological subtypes, accounting for about 80% and 50% of global LC fatalities in men and women, respectively [41].

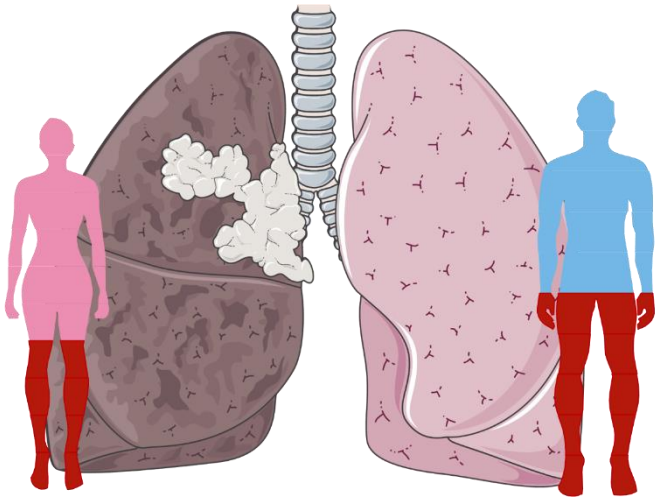

Figure 1. Worldwide and Portuguese's LC impact (incidence and mortality) on males and females in 2020 (last register performed).

However, approximately 25% of worldwide LC situations do not emerge from the consumption of tobacco smoking [23]. It is speculated that environmental exposure (e.g., outdoor air pollution, asbestos and ionization radiation) and heredity factors might be some of the possible factors involved in lung cancer of non-smokers [23] [42].

Smoking cessation is also impactive in the probability of an ex-smoker developing LC, not only the age at which that smoking termination occurs but also the duration itself. Age studies involved in this LC problematization show cumulative risks between 2-10% for men who stop smoking between the ages of 30-60, respectively. Concerning cessation duration, different perspectives have shown opposite impacts. Some investigators argue that a renounce of more than 15 years would translate into an 80-90% reduced risk of an ex-smoker having LC, while

others defend smoking discontinuance as the trigger of LC in this group of individuals [23].

The high mortality rate associated with lung cancer arises mostly because, in over 75% of cases, tumours' detection happens in advanced stages (stage III-IV) [43]. This mainly happens because LC's initial symptoms are most of the times misdiagnosed with other chronic respiratory illnesses that present similar indicators such as cough, shortness of breath, head cold and chest pain. Moreover, it has been clear that LC diagnosis requires to be more efficient and prompter so that patients could enter the treatment phase in a much earlier cancer's stage [44].

NSCLC and SCLC are the two major LC clinicopathological histological subtypes based on their biologic, therapeutic, and prognostic characteristics [8] [9].

1.1. Non-Small Cell Lung Cancer (NSCLC)

About 85% of LCs diagnosed are non-small cell lung cancer (NSCLC), making it the most prevalent lung carcinoma major subtype. It includes three different main histopathological subclasses: lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and lung large cell carcinoma (LCLC) [9] [45]. NSCLC follows a tumour node metastasis (TNM) staging defended by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) [46]. In 2010, the average 5-year survival rates for each NSCLC subclass – LUAD, LUSC, and LCLC reached 26.2%, 21.3%, and 21.1%, respectively [7].

Representing 25-30% of all LC situations, LUSC distinguishes from the other subtypes by displaying cells keratinization and intercellular bridges showed in its malignant tumour [6] [47]. The epidermoid carcinoma is originated in epithelial cells from the central lung region where the large central bronchi join the trachea (mainstem bronchus) and the mid-calibre airway branches (lobar and segment bronchi). Both areas are deeply affected by smoke [6] [47] [48]. Typically, this subtype tends to be more locally aggressive rather than metastasise to other organs. Besides, compared to the other subtypes, if surgical resection is performed in LUSC patients, it is common for the carcinoma to reoccur in its locoregional focus [47].

With a 40% frequency, LUAD is the most common NSCLC subclass. This carcinoma typically arises from a malignancy involving epithelial cells with glandular differentiation or mucin production in the outer region of lungs (terminal bronchioles and small diameter bronchi) [6] [45]. Peripheral adenocarcinoma, 15% of cases, sporadically disseminate over pleural surfaces and chest wall [47]. It also distinguishes from the other lung carcinomas for being frequent on never or ex-light smoker patients and growing slower. The slow growth might explain why there is a high chance of diagnosing adenocarcinoma patients at an early onset where the illness has not yet spread [46] [49]. LUAD is a prevalent subclass of lung cancer that, in a late-stage, tends to metastasise to distant organs. Like any tumour cells,

when cancerous lung cells assess lymphatic and hematogenous routes, distant metastases might happen to the brain and bones, two typical LUAD metastases focus [50].

In certain rare situations, 0.4-4% of LCs, and particularly in old smoker men, the carcinoma presents features of both LUAD and LUSC in different proportions. In this particular condition, the carcinoma is described as adenosquamous lung carcinoma (ASLC) and postulated to represent a transitional stage between LUAD and LUSC [51]. Usually located in the lung's periphery, the rare carcinoma may also contain a central scar [47]. Compared to the subclasses individually, ASLC is much more aggressive, demonstrated by earlier metastases and poorer prognosis.

LCLC, an undifferentiated and less typical subtype, accounts for 5-10% of LC cases [6] [52]. This carcinoma is assessed after the other LC subtypes are ruled out as possibilities. Generally, it tends to have a strong association with smoking and rapid growth and spread. Even though peripheral lung areas near subpleural regions, chest wall, or adjacent structures are prevalent, central lung locations, occasionally near lymph nodes, also display as probable LCLC sites [52] [53]. Regarding metastases, LCLC follows the pattern associated with NSCLC, where hilar and mediastinal nodes are targets, and the pleura, liver bone, and brain are other structures and organs affected [47].

LCLC can present a subtype with neuroendocrine differentiation known as large cell neuroendocrine carcinoma (LCNEC). This feature makes LCNEC carry a worse prognosis than LCLC, with an aggressiveness matching SCLC's [47] [54]. Both SCLC and LCNEC neuroendocrine features are distinguished through cytologic characteristics [54]. LCNEC accounts for 3% of LC, where patients diagnosed are generally 65-year-old smokers (90%) males (70%) with stage III-IV cancer.

1.2. Small Cell Lung Cancer (SCLC)

Small cell lung cancer (SCLC) accounts for 13-15% of neuroendocrine family tumours that manifest in the lungs [55] [56]. From all LC, this subtype is the one with the highest association with tobacco, approximately 95% of cases. This high-grade neuroendocrine carcinoma appears in a central region of the lung around the lobar or main bronchi above 90% of all situations.

SCLC aggressiveness arises due to rapid doubling time and high tumour growth, and early dissemination to distant sites [12]. Evidence reveals that more than 90% of SCLC patients show the disease in stage III (locally advanced) or IV (distant metastases). Some common metastases locations are contralateral lung, bones, liver, brain, and adrenal glands [12] [56].

Clinicopathologically, the SCLC division follows the American Veterans Administration Lung-Study Group (VALG) 2-stage system in which this LC subtype is defined as limited-stage

(LS-SCLC) and extensive stage (ES-SCLC) [57]. Because of VALG accuracy and lack of detail, SCLC's current staging system results from a combination of TNM and VALG staging [58]. LS-SCLC corresponds to stage I to III, which only involves tumour restriction to hemithorax without implicating any extrathoracic metastatic spread. On the other hand, ES-SCLC associates with stage IV, which implies possible dissemination to pleura, lung regional lymph nodes, and other organs. SCLC's five-year survival rate, as previously stated, is exceptionally low owing to its carcinoma characteristics. According to the VALG system, the five-year survival rate differentiates 10%-15% and 1%-2% for LS-SCLC and ES-SCLC, respectively [56].

In rare circumstances, SCLC might not present as an exclusively pure carcinoma but instead combined other subclasses of NSCLC [59] [60] [61]. When this occurs, the multiphasic malignant lung tumour is diagnosed as combined small cell lung cancer (c-SCLC). Anent LCLC, since both LCLC and SCLC have similar neuroendocrine nature, requirements demand a presence of more than 10% of LCLC characteristics to categorize the tumour as c-SCLC. In a study developed by Wang et al., investigators attributed an overall 5-year survival for c-SCLC patients of 15.9% [62].

1.3. Lung Cancer Genetic Landscape

NSCLC and SCLC are two distinct LC subtypes. These similar and dissimilar characteristics between the two reflect on the molecular alteration involved in each LC diseases. The same can be said regarding the three different histological NSCLC subclasses. Even though exhibiting genetic differences, a common foundation harbouring some of the same gene alterations is present with a varying frequency (Table 1) [63] [64] [65] [66] [67].

EGFR, *KRAS*, *TP53*, cyclin-dependent kinase inhibitor 2A (*CDKN2A*), and serine/threonine kinase 11 (*STK11*) genes are frequently mutated in NSCLC subclasses. ALK and ROS1 gene rearrangements have been linked with NSCLC genomic landscape [9] [6].

SCLC mainly displays mutations in genes such as *TP53*, *RB1*, cAMP-response element-binding protein (*CREBBP*), phosphatase and tensin homolog (*PTEN*), and histone-lysine N-methyltransferase 2D (*KMT2D*). Furthermore, *BCL2* and *MYC* genes have also been shown to be involved in this lung carcinoma [14] [12] [15].

Table 1. Genes association with different subtypes and subclasses of lung cancer.

Types of Lung Cancer	Genes harbouring frequent alterations	Other genes involved		
NSCLC	*KRAS* (26.66 %) *TP53* (42.13 %) *EGFR* (19.77 %) *CDKN2A* (10.74 %) *STK11* (9.36 %)	*ALK* *APC* *BRAF* *ERBB2* *KEAP1*	*KIT* *KMT2D* *MET* *NFE2L2* *PDGFR*	*PIK3CA* *ROS1* *RB1* *PTEN* …..
LUAD *	*KRAS* (29.71 %) *EGFR* (22.47 %) *STK11* (10.38 %) *CDKN2A* (- %) ** *TP53* (- %) **	*ERB2* *RET* *NRAS* *HRAS*		
LUSC *	*TP53* (63.4 %) *PIK3CA* (15.36 %) *NFE2L2* (13.22 %) *KMT2D* (- %) ** *CDKN2A* (- %) **	*ALK* *BRAF* *EGFR* *ERB2* *HRAS*	*KRAS* *KIT* *MET* *NF1* *NRAS*	*PTEN* *RET* *STK11* ….
LCLC *	*TP53* (- %) ** *KRAS* (- %) ** *STK11* (- %) ** *KEAP1* (- %) ** *CDKN2A* (- %) **	-		
SCLC	*TP53* (65.58 %) *PTEN* (8.54%) *RB1* (- %) ** *KMT2D* (- %) ** *CREBBP* (- %) **	*ATR* *BRAF* *BRCA2* *CDKN2A* *EGFR*	*ERBB2* *HRAS* *KIT* *KRAS*	*MET* *MYC* *PIK3CA* ….

* Subtypes of NSCLC; **Percentages were not displayed in the database.

1.4. Diagnosis and Screening

The most significant hurdle still concerning lung cancer refers to the late-stage diagnosis. New and improved treatments could be a feasible response but will always be entangled to the late stage's aggressiveness on which the majority of LC is detected. Therefore, optimal screening to ensure cancer detection at a much an earlier phase is essential to ensure a higher chance of survival [10].

Low-dose computed tomography (LDCT) is currently a clinical screening method for LC [68] [69]. Compared to chest X-ray (CXR) and sputum screenings, LDCT proved to be beneficial by reducing LC mortality [10] [70] [71]. Regrettably, LDCT carries a high percentage of false-positive assessments, approximately 96%, mainly because LDCT identifies both cancerous and benign non-calcified nodules. [10] [72]. Revealing LDCT's inability to be used as the sole LC screening method, instead, it should be handled as a part of a possible consortium for LC screening. Another clinically used CT procedure mostly for diagnostic and prognostic combines positron emission tomography (PET), and for that is designated as PET-CT [73] [74]. This technique has the advantage of allowing staging evaluation of nodal and metastases of different LCs types, even able to crucial assess nodal sites that otherwise might be deemed inaccessible for a biopsy retrieval. Moreover, it also permits patients' monitoring response to treatment and even assesses eventual disease reoccurrence in disease-free individuals. Even though this method is expensive, it presents value for its specificity and sensitivity compared to CT and magnetic resonance imaging (MRI). PET radiopharmaceutical tracers are deployed to differentiate tumour biology's aspects [75] [76]. The most common is ^{18}F-fluorodeoxyglucose, used for differentiating benign from malignant tissue. Besides this tracer, others exist for other functions such as hypoxia (e.g., ^{64}Cu-ATSM), angiogenesis (RGD peptides), and lung neuroendocrine tumour differentiation (e.g., ^{68}Ga-DOTA-peptides).

LC diagnosis, ultimately, relies on cytologic and histologic analysis to make the most accurate diagnosis possible. With that in mind and considering, even though lungs are a complicated organ for sample retrieval, the occurrence of tissue biopsy is standard practice. Routine or ultrasound-guided bronchoscopy, mediastinoscopy, transthoracic needle aspiration, thoracentesis, and medical thoracoscopy are some of the many examples of lung tissue biopsies [25] [77]. Clinical complications associated with tissue biopsy procedures, such as pneumothorax, haemoptysis, and pneumonia, may reach 20% of the cases. Thus, physicians must always weigh them because most patients are elderly and more health compromised [25] [27] [78]. Unfortunately, this procedure fails to guarantee absolute delivery of information. A 2010 study revealed that samples retrieved through a bronchoscopy demonstrated a limited amount of malignant cells, and only around half of the biopsies effectively contained tumour [77] [79]. Furthermore, there is no guarantee that intra-tumoural heterogeneity is represented, leading to an incomplete molecular diagnosis, which might culminate in a less ineffective treatment [27] [28] [29].

1.5. Treatment and Therapy

Any therapy involved in cancer must always be employed, considering a multivariable set. The subtype/subclass of the diagnosed cancer, tumour stage, possible nodes and metastasis

involvement, and the condition in which patients present themselves like age and health status, comorbidities, and possible survival outcomes are some of those variables. Generally, surgical resection, chemotherapy, radiotherapy, targeted therapy, and immunotherapy are the possible employed treatments for LC [9] [6] [7] [80].

1.5.1. NSCLC

In NSCLC treatment, chemotherapy is not as effective as other therapies; however, its practice could still be a part of a three-way system treatment alongside surgery and radiotherapy, as pre and/or post adjuvant treatment [9].

Standardly chemotherapy involves the combination of two drugs, cisplatin or carboplatin (platinum-based antineoplastic agents) with another drug (e.g., etoposide) [81] [82]. Nonetheless, multiple lines of treatment might be administered accordingly to the patient's response to it.

The core of NSCLC target therapy involves monoclonal antibodies and tyrosine kinase inhibitors (TKIs) and directing them to the respective tyrosine kinase receptor (TKR).

TKIs, small weighted molecules, are used for blocking the adenosine triphosphate (ATP) binding intracellular catalytic domain site of the respective tyrosine kinase receptors (TKR) and stop their downstream signalling [83]. Epidermal growth factor receptors (EGFR) show to be linked with cell proliferation, invasion, migration, adhesion, inhibition of apoptosis, and angiogenesis [37]. This specific TKIs is the most common, with three NSCLC generations existing and a fourth under progress [51]. Even though EGFR TKIs tend to work initially, resistance to TKI inevitably emerges from ongoing mutagenesis or the pre-existence of minor clones [84].

Frequently, NSCLC patients develop an EGFR mutation that results from a deletion in exon 19 or a point mutation in exon 21 (L858R) [37] [84]. During treatment with a 1st generation EGFR TKIs (gefitinib and erlotinib), a substitution of threonine residue by a methionine one (T790M) tends to establish. As a result, the affinity of the ATP binding pocket of the EGFR kinase domain to ATP increases which reduces TKIs bind efficiency [84] [85].

Other TKRs, such as ALK and ROS1, also suffer gene alterations in NSCLC (e.g., rearrangements EML4-ALK and TPM3-ROS1), for which crizotinib was developed [86] [87].

Cancer cells display the capacity of evading patients' immune cells by stimulating immune checkpoints receptor programmed death-ligand 1 (PD-L1), which are recognized by the programmed cell death protein 1 (PD-1) present in T cells [88] [89] [90]. Immunotherapy, another possible treatment for NSCLC, blocks these receptors and makes the individual's T cells recognize the malignant cells as foreign, thus stimulating an immune response.

Human immune-checkpoint-inhibitor antibodies such as nivolumab and pembrolizumab

are used for blocking the PD-1 receptor, whereas atezolizumab for PD-L1 [88] [9].

1.5.2. SCLC

Over the past decade, SCLC standard treatments have not changed. Any therapy option will depend on whether the patient suffers from LS- or ES-SCLC [12].

Surgery can be beneficial even though only for a small percentage, 2 to 5%, of SCLC patients [10] [15] [55].

SCLC chemotherapy does not differ much from the one used in NSCLC. However, there seems to be a higher inclination in using this therapy for SCLC's treatment [91].

Radiotherapy, thru thoracic radiotherapy (TRT), has shown excellent prospects, particularly for LS-SCLC as a singular medical procedure and even better ones when deployed alongside chemotherapy [54] [55] [92].

One interesting phenomenon that highlights lung cancer plasticity is the possible evolution of a pure SCLC to a combined SCLC (c-SCLC) after receiving chemo and radiotherapy treatment. The treatment itself can function as a mutagenesis factor for cancer cells, improving their ability to resist therapy [93] [94] [95]. Moreover, disease relapse is a common SCLC's event, about 80% of LS-SCLC, and most ES-SCLC eventually develops disease recurrence despite the apparent initial success [12] [55].

Cancer cells present specific antigens that are rarely displayed in normal tissues. Acetylneuraminide α-2,8-sialyltransferase, known as GD3 ganglioside, shows approximately a 60% overexpression in SCLC tumours [90] [96]. A Bacillus Calmette-Guerin (BCG) vaccine, including beclin2 gene (*BEC2*), a monoclonal antibody responsible for inducing antibodies against GD3, has been studied for its LD-SCLC potential use as a treatment [96].

SCLC's immunotherapy also has in clinical validation interferon alfa (IFN)-α. The purpose of this immunomodulating agent is to trigger an immune cell response by activating Natural Killer (NK) cells, enhancing B lymphocytes' proliferation, and promoting an antigen presentation [96]. Evidence suggests that immunotherapy may augment the healing process when combined with chemotherapy and radiotherapy, ensuring synergetic tumour control.

2. Liquid Biopsies

Due to tissue biopsies' limitations, liquid biopsies have awakened scientist interest in exploiting this type of biopsies as a tool for cancer diagnosis, screening, and disease progression monitoring to a given therapy [97]. This type of biopsy has the advantage of being minimally invasive, easily sample retrieval, and more accurate in assessing the evolving tumour landscape, both inter and intratumour heterogeneity [98].

Liquid biopsies include blood, urine, saliva, and cerebrospinal fluid, which present themselves as a source of cancer biomarkers. Circulating tumour cells (CTCs), circulating cell-free DNA (cfDNA) and tumour DNA (ctDNA), extracellular vesicles (EVs) such as exosomes and cell-free ribonucleic acids (ccfRNAs) (e.g. microRNAs - miRNAs) are examples of circulating cancer biomarkers (Figure 2) [30] [31] [32].

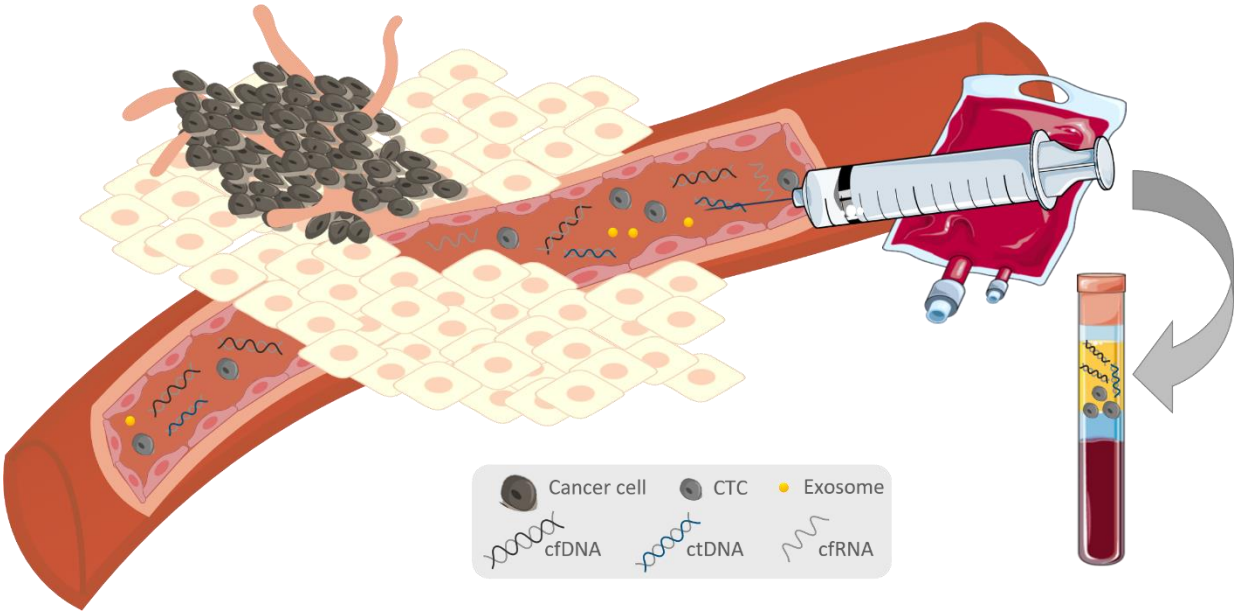

Figure 2. Blood-based liquid biopsy. Representation of cancer potential biomarker – circulating tumour cells (CTCs), circulating free DNA (cfDNA), circulating tumour DNA (ctDNA) and exosomes.

2.1. Cancer Biomarkers

2.1.1. Circulating Cell-free DNA (cfDNA) and Circulating Tumour DNA (ctDNA)

In 1948, *Mendel* and *Metais* reported circulating nucleic acids in healthy humans' blood [99]. Circulating cell-free DNA (cfDNA) results from a mixture of DNA released from cells, tissue, and even organs like the kidney, spleen, lymph nodes, and liver [100] [101].

Presently, there is no consensus about the 'main mechanism' responsible for unleashing DNA. Instead, numerous phenomena are thought to be involved. 'Cellular breakdown mechanism' (e.g., necrosis, apoptosis, mitotic catastrophe, autophagy, and pyroptosis), active DNA release, erythroblast enucleation, exogenous sources, and NETosis[1] are some of these

[1] NETosis – mechanism involved in decondensation of chromatin, cell lysis, disruption of nuclear membranes, and the liberation of neutrophil extracellular DNA traps (NETs), which is composed of extracellular DNA fibers and defence-related substances and invading microorganisms. [102]

many biological events [103].

This low molecular weighted and double-stranded nucleic acid tends to circulate free or associated with specific nucleosome or/and chromatosome units. These biologic units and structures concur with cfDNA (around 147 and 167 bp corresponding to association with nucleosome and chromatosome, respectively) [104] [105] [106]. Hence it is postulated an average distribution size range for apoptotic cfDNA between 70-200 bp [107]. Larger cfDNA fragments (>10.000 bp) typically found in cancer patients indicate a necrosis source [104].

Usually, the cfDNA half-life is relatively short, with estimations from minutes to a few hours. Even though unclear, it is believed the nucleic acid clearance of cfDNA might occur via circulating enzymes such as DNase I, factor H and FSAP, and factor VII-activating protease. Other molecules may assist in clearing cfDNA, just like extracellular vesicles and serum proteins like albumin and fibrin [100] [101] [104].

Studies have shown that these DNA levels vary depending on the health conditions of the individuals. In healthy circumstances, people show a reduced concentration due to homeostasis between DNA unleash from cells (e.g., derived from hematopoietic cell lineages - epithelial or gastrointestinal) and cfDNA clearance [101] [108]. Moreover, in conditions such as aging [109], autoimmune diseases [110], diabetes [111], sepsis [112], chronic exercise [113], pregnancy [114], and cancer [115], the nucleic acid levels are higher than normal.

Also known as cancer or tumour-derived cfDNA, ctDNA represents a cancer-specific DNA that sheds from a primary tumour, metastatic lesions, or CTCs [116] [117]. This promising clinical biomarker represents a 0.01%-90% of total cfDNA [33] [116]. Distinguishing both might prove difficult but essential for a precise assessment of minimal residual disease (MRD) and monitoring the disease progression through treatment [34].

Currently, there is no general set value stipulated for both free circulating cell free DNA basal and cancer levels since the experiments involving these biomarkers are different and dependable on both the protocols and patients' conditions [101] [115] [118]. Cancer’s subtypes and subclasses and cancer stage are two prominent variables on ctDNA levels. It has been shown that stage IV ctDNA detection levels are around 82%, while stage I shows a 47%; and stage IV ctDNA plasma concentration levels are 100-fold compared to stage I tumour. Additionally, tumour burden, accessibility to circulation, and cellular turnover also impact ctDNA levels in circulation [104] [115] [116] [119]. This circulating DNA proves crucial in producing an invaluable diagnosis and prognosis analysis by detecting significant genetic factors such as point mutations and gene amplification, deletion, insertion, fusion, and epigenetic alterations (e.g., DNA methylation) [120]. Other enthralling aspects around this non-invasive biomarker involve its potential in monitoring therapies' effectiveness by its quantitative and qualitive changes. Both ctDNA levels evaluation during the progression of the disease, as well as the track of therapy efficiency by the acquirement of mutations detected

will allow to assess MRD evaluation and possibly avoid illness relapse [34] [120] [121].

Oxnard *et al.* showed the importance of liquid biopsies to track cancer mechanisms of resistance [122]. Their study proved there were patients that tested positive for T790M mutation in plasma that were not detected in tissue samples (31%), and vice-versa. Plasma cfDNA sample analysis of the relative allele frequency of T790M could prove essential as it informs if T790M is a dominant mechanism of resistance or a subclonal phenomenon with a heterogeneous biology. In another study the same was verified as 20% of targeted variables detected in blood were not found in tissue [123].

These studies proved how important liquid biopsies were for cfDNA evaluation in lung cancer as they offered complementary information to tissue biopsy and crucial patient's cancer assessment.

2.1.1.1. Techniques for cell-free DNA characterization

Since ctDNA is highly fragmented, possibly contaminated by non-tumoral cfDNA, and could be present in low concentrations, its detection might prove to be challenging. With that in mind, efficient, sensitive, and specific analytical procedures must overcome these adversities [124] [125]. Two categories are generally stipulated to classify different platforms deployed to analyse tumoral genomic material by liquid biopsy. They are either targeted approaches to detect specific mutations for MRD based on or untargeted screening to identify copy number aberrations (CNAs) or point mutations by whole-genome sequencing (WGS) or whole-exome sequencing (WES) [125].

Real-time Polymerase Chain Reaction (PCR), also known as quantitative PCR (qPCR), is one of the most common approaches used to study quantitative levels of tumour genomic material. Real-time PCR application is made from two possible methods, thru a non-specific fluorescent dye, like SYBR Green, or fluorophore covalently attached to sequence-specific oligonucleotide probes, such as TaqMan [126] [127].

TaqMan probes are single-stranded oligonucleotides dually labelled with a fluorescent reporter molecule and quencher dye at the 5' and 3' end, respectively. These two components, when near each other, lead to suppression of fluorescence emission by the reporter. After the probe annealing to the newly replicated strand and probe cleavage by the DNA polymerase, the reporter is no longer quenched, leading to fluorescence emission that could be detected [127]. Compared to SYBR Green, TaqMan probes have better specificity since both the primers and probes need to be annealed to the target for a signal manifestation [127] [128].

A significant number of PCR variations have been developed to improve the associated general low sensitivity, Allele-specific (AS)-PCR, co-amplification at a lower temperature (COLD)-PCR, peptide nucleic acid-locked nucleic acid (PNA-LNA)-PCR clamp, and

methylated-specific (MS)-PCR) are some examples of those variations. Depending on the PCR variation technique, sensitivity and specificity range from 0.1%-1% and 79%-100%, respectively [124] [125].

In addition to qPCR based methods, Nex-Generation Sequencing (NGS), Targeted Amplicon Sequencing (Tam-Seq); Digital-PCR platforms, such as Droplet Digital PCR (ddPCR) and Beads, Emulsion, Amplifications, and Magnetics (BEAMing); and Mass-Spectrometry technologies, like PCR/Surface-Enhanced Raman Spectroscopy (SERS), are other possible techniques for cfDNA and ctDNA evaluation and characterization [124] [125].

3. Epigenetics

The term epigenetics was first introduced in 1942 by Conrad Waddington, and its definition has evolved throughout the years [129]. Presently, epigenetics refers to any mechanism responsible for affecting gene expression, during cell division, without changing the primary DNA sequence [130].

DNA methylation, histones post-translational modifications and variants, and chromatin remodelling complexes are examples of known mechanisms involved in the field of epigenetics [129] [130]. Several researchers have demonstrated these phenomena to be linked with a wide variety of diseases, and other health indicators [129] [130] [131]. However, it does not mean that the occurrence of epigenetic mechanisms automatically leads to the development of a disease. Instead, epigenetic dysregulation, translated in an unsuitable proto-oncogene due to mutations or increased expression, or tumour suppressor genes inhibition, will trigger cancer pathologies [132].

Epigenetic cancer study already proved to be essential for cancer comprehension and clinical applications to unravel its role in the disease itself. Compared to genetic studies, epigenetics proves to be a more stable and homogenous mechanism, making its study extremely valuable [131] [133].

3.1. DNA Methylation

DNA methylation, a well-known and studied epigenetic modification is the main contributor to gene expression stability in cancer settings. This epigenetic mechanism entails the covalent bond of methyl (CH_3) groups onto the C_5 position of cytosine nucleotides to form 5-methylcytosine (5mC). The transference of a CH_3 happens by the action of S-adenyl methionine (SAM), and the catalysation reaction of methylation occurs by DNA methyltransferases (DNMTs), like DNMT3a and DNMT3b. DNMT1 is another important DNMT responsible for maintaining the DNA methylation pattern throughout the replication process. Methyl CpG binding protein 2 (MECP2), by its methyl-CpG-binding (MBD) domain, and

methylcytosine-binding proteins (MBPs) recognize and bind to 5mC regions of methylated DNA. These proteins then recruit other proteins, such as DNMTs and histone deacetylases (HDACs), and form a complex responsible for modifying the chromatin structure, repressing gene transcription. A direct block interaction between transcription factors and DNA sequences is another possible mechanism [134].

The methylated cytosine generally precedes a guanine nucleotide, CpG sites, found in CpG islands (DNA 500-1500 bp long). These large clusters with a CG: GC ratio content superior to 0.6 and located at the 5' end region of the transcript resided within 70% of gene promoters [135] [136]. Usually, mammalian genomes are depleted of CpG sites due to passive or active DNA demethylation [137]. Ten-eleven translocation (TET) enzymes, activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex (AID/APOBEC), and thymine DNA glycosylase (TDG) are the three main components involved in active mammalian demethylation. Final correction is then programmed thru the induction of the base excision repair (BER) pathway [134] [137]. Passive DNA demethylation befalls when cells fail to keep methylation during DNA replication.

During carcinogenesis, genome-wide hypomethylation and regional of CpG islands hypermethylation occur. While hypomethylation tends to lead to the activation of silenced oncogenes and genomic instability, hypermethylation manifests in silencing of tumour suppressor genes [138] [139]. Moreover, DNA methylation genomic location is also crucial for the functional response that is translated; if methylation occurs in the gene body instead of the promoter, a different response might be triggered [139].

Lung cancer pathogenesis in tumour tissue samples have shown to be highly affected by epigenetics fluctuations. Hypermethylation of CpG islands in the promoter regions of genes has proved to be a highly characteristic epigenetic phenomenon in LC tumours, cell lines and patient's serum and sputum [140]. Associations between DNA methylation levels of certain genes and LC histology of tissue samples, and smoking habits of the patients have been observed, on the other hand LC stage tends to not show any. Even when the tumour is at an early-stage of tumour progression, evidence show the DNA to be hypermethylated; however, specific driving events are not yet established [141].

4. Microfluidics

Microfluidics represents a science field that allows fluid manipulation and control in a range of microliters to picoliters through a network of micrometer-scale channels [142].

Fields such as genomics, epigenomics, and proteomics, have been of particular interest in associating with microfluidics since it might allow, above all, single-cell and single-molecule analysis [143]. Through these analyses, a microfluidic device could serve as an auxiliar tool

for heterogeneity evaluation. This means, by processing a patient's liquid biopsy there is the possibility of high-throughput cancer screening and even evaluation of possible treatments.

The chance of mimicking physical conditions, with large surface-area-volume ratio, reduced reagents volume, easy handling of fluids and gases, high portability, faster results, low-cost and reduced environmental impact also contribute to the increase use of the technology [144]. Even though microfluidics shows great promising, its implementation in clinical routines is still questioned as it may entail a high level of expertise and training that protocols and kits do not comprise.

Generally, microfluidics has been more focused on CTCs, however recent developments show that cfDNA has procured interest due to its potential. Thus, many studies are trying to use microfluidics as an isolation, extraction, and analysis platform of DNA.

Some of these microfluidic chips have been designed to isolate DNA based on a solid-phase extraction though functionalized surfaces or immobilized probes to bind and capture DNA. Nakagawa *et al.* fabricated an amine silane-coated microfluidics device by using 3-aminopropyltrietboxysilane (APTES) or 3-[2-(2-aminoethylamino)-ethylamino]-propyluimethoxysilane (AEEA) [145]. Results indicated that, from the two, AEEA contributed to a more extended device surface's aminic functionalization, leading to a higher amount of captured DNA. The amount of extracted was around 10 ng and the overall recovery ratio of human genomic DNA from the whole-blood was between 27 and 40%.

One interesting microfluidic platform involving DNA methylation was developed and reported by O'Keefe *et al.* [146]. The HYPER-melt (high-density profiling and enumeration by melt), a digital microfluidic approach for high-throughput molecular profiling liquid was applied for detection and assessment of intermolecular heterogeneity of DNA methylation. Findings showed a detection sensitivity as low as 1 methylated variant in 2 million unmethylated templates, of a tumour suppressor gene, *CDKN2A* (p14ARF). Moreover, the microfluidic approach not only showed a 20 to 300 times or more analytical sensitivity than qMSP assay, when evaluating *NDRG4* methylation in colorectal patient samples, but also a positive detection in two patients that had been negative/ nearly negative in qMSP results.

5. Gene Selection

Five methylated genes were selected to be studied in blood-based liquid biopsies of LC patients, and defined as a potential LC detection panel.

Nidogen 2 (*NID2*), one of two-known mammal nidogen proteins, is a highly conserved protein responsible for basement membrane architecture's stabilization. This stability arises by binding with laminin and collagen IV and forming a ternary complex [147]. Furthermore, this protein shows an involvement with cell adhesion, and other important cells phenomenon.

Both its downregulation expression and aberrant methylation in the promoter region have been observed in a variety of tumours, including lung cancer [148].

Adenylate Cyclase 4 (*ADCY4*) gene is part of the adenyl cyclase's family which are membrane enzymes responsible for catalysation of cyclic adenosine monophosphate (cAMP). Yu *et al.* reported high levels of *ADCY4* to be associated with longer overall survival in LUAD, believing that this gene's association with calcium signalling pathway may affect tumorigenicity and metastasis of lung adenocarcinoma cells [16]. LUAD and LUSC, two lung cancer NSCLC' subclasses, have shown this gene to be downregulated from a hypermethylation on this promoter gene [149].

MIR129-2 gene, a member of microRNA precursor family, display a tumour suppressive role in normal situations; however, in multiple cancers including LUAD this gene has shown to be hypermethylated and essential for cancer progression [17] [150].

For the Homeobox A11 (*HOXA11*) gene, its promoter hypermethylation leads NSCLC development by augmenting cell proliferation or migration [18]. The molecular mechanism of this tumour suppressor gene in LUAD is still unknown. In LUSC, *HOXA11* gene shows a tendency to be over-expressed [151].

Finally, membrane-associated guanylate kinase inverted 2 (*MAGI2*), an essential element of adherent junction, alongside *CTNNB1* (β-catenin) interacts with *PTEN*, controlling this latter gene expression and inhibiting cell migration and proliferation [152]. In some cancer, *MAGI2* has shown to be hypermethylated which causes its silence or downregulation [153]

III. Materials and Methods

1. Microfluidic Device Production

1.1. Microfluidic Design

The microfluidic system had been previously designed using CAD software per the intended purpose of assessing two potential biomarkers (CTCs and cfDNA). The first process in the system consists in the isolation of CTCs since this specific microchip coalesces a positive enrichment by relying on their physical characteristics. After the filtration process of CTCs, the remaining plasma containing cfDNA is recovered through an outlet for downstream analysis

The proposed design chip, which can be seen in the illustration below (Figure 3a), entails three different and central regions: inlets, microstructured chambers, and outlets, connected through a network of microchannels. The entrance access begins with the inlets where samples and reagents are introduced into the system. Afterwards, the fluid streams along the microchannels and splits points until reaching the microstructured chambers. In total, the microfluidic device comprises similar microchambers. Each microchamber (Figure 3b) contains multiples micropillars incrementally interspaced with decreased ranges (50 µm to 18 µm) to allow CTCs retainment between the interspaces in the liquid flow direction. These micropillars also present a repeated format pattern to maximize the interaction between pillar-cells and help in CTCs retainment. After filtration, the liquid deployed exits to one of the two outlets in the final phase. Different phenomena occur depending on the type of fluid injected

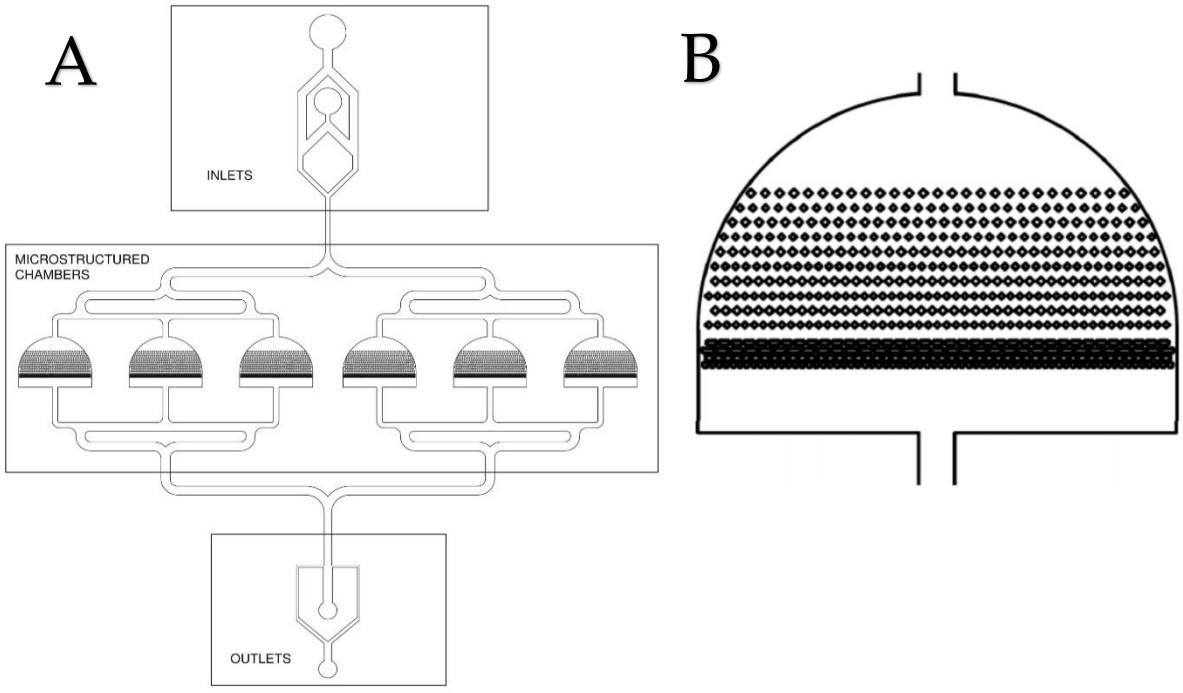

Figure 3. Microfluidic platform schematics. (A) – Complete design of the microfluidic device: inlet region, microstructured chambers, and outlets region; (B) – Micropillar pattern structured inside microchambers

into the chip (whole-blood or plasma). When processing blood, plasma was separated from the remaining blood cells by flowing through the channel with higher fluidic resistance, with the remaining cells being recovered in the waste outlet. On the other hand, when plasma was utilised, its filtration collection could occur through both outlets for subsequent cfDNA analysis.

1.2. Silicon Master Masks Production by Photolithography

Silicon (Si) masks manufacture was done using a photolithography technique in a cleanroom facility. A SU-8 photoresist (negative photoresist) was deposited on a Si wafer by spin-coating. This photomask was then aligned with the intended design and exposed to UV radiation. The master mask development process was finished with a developer solution to dissolve the areas that were not exposed to UV light.

1.3. Microfluidic Device Fabrication by Soft-lithography

Following the Si mask production, polydimethylsiloxane (PDMS) with the microfluidic design was obtained thru a soft-lithography procedure. PDMS (Sylgard 184) was mixed with a curing agent in a proportion of 10:1 (wt/wt). The mixture was then evenly poured over the Si master mask with an immediately degassing, using a vacuum pump to remove air bubbles. Next, the PDMS was incubated at 60ºC for 4h. Finally, the PDMS layer was removed carefully from the mask, cut into coverslip-like sections (50 mm x 55 mm), and holes were punched in both the inlets and outlets.

1.4. Microfluidic Plasma Treatment and Final Chip-System Assembly

The sealing of the PDMS layer to a glass coverslip to develop the final microfluidic chip was performed using plasma-activated bonding. In a vacuum chamber of plasma cleaner (Dieno Zepto low-pressure plasma cleaner), both PDMS and glass slides were placed for 2 minutes on oxygen (O_2) plasma treatment. The treatment served to remove possible contaminants and functionalize both surfaces for permitting a final irreversible and closed assembly system.

The complete process beginning in Si master mask and finishing with the final microfluidic system is summarised and outlined in Figure 4 [154].

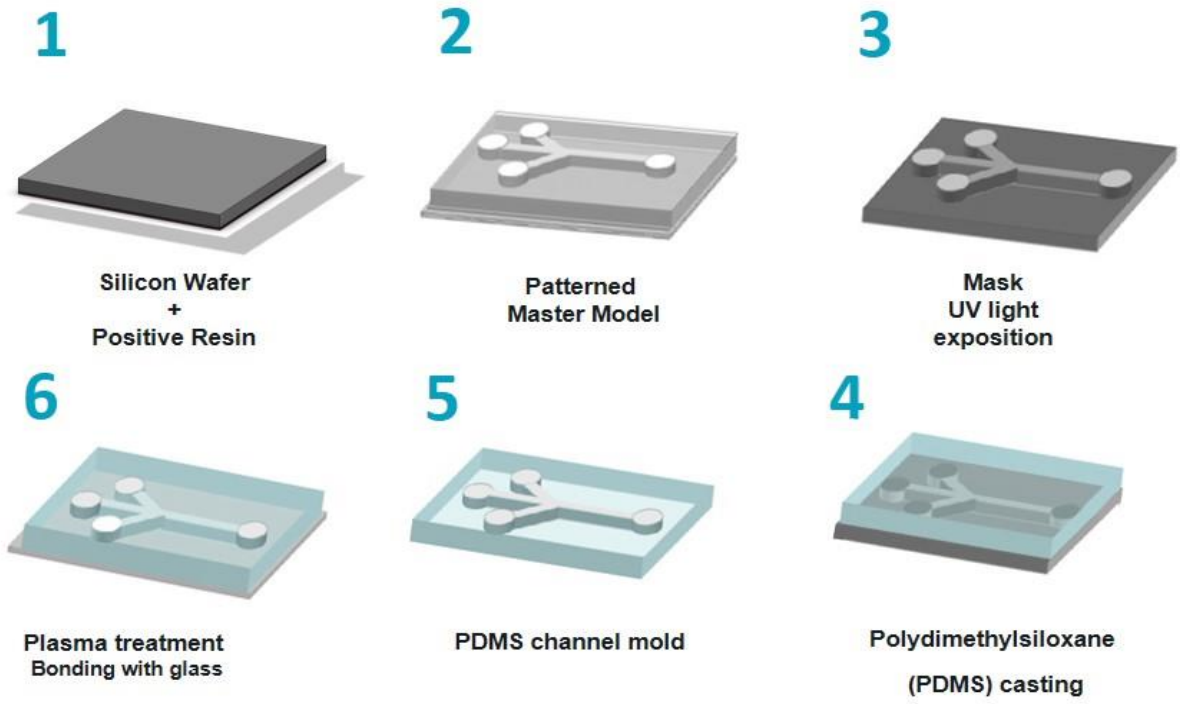

Figure 4. Schematic of the microfabrication steps. The initial photolithography technique to produce the master masks followed by soft-lithography technique for the preparation of the PDMS molds containing the microfluidics devices. Adapted from [153].

2. Lung Cancer Sample Processing

Since the presented microfluidic device serves as a tool for liquid biopsies, fluid dynamics assessments at a micro-level were required beforehand of the device's use. Plasma and whole-blood velocities were tested to determine the Reynold number (*Re*), a dimensionless variable that allows a prediction about fluid flow patterns. Laminar flow behaviour (*Re* < 2300) was intended for the microstructure chambers to ensure cellular viability.

Plasma samples from 26 lung cancer patients – 13 early-stage (stages I and II) and 13 late-stages (stage III and IV) – were selected for processing in the microfluidic platform. Afterwards, a methylation analysis of potential genes for LC detection was performed on the processed plasma. All plasma samples from LC patients were supplied by the Biobank & Cancer Biology and Epigenetics group from the Portuguese Oncology Institute of Porto, Portugal. Outlined in the research project TRIMARKCHIP - "Assessing the trifecta of cancer circulating biomarkers: a combined microfluidics platform for detection of CTCs, exosomes and ctDNA", this 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). Written informed consent, in accordance with the Declaration of Helsinki ethical principles, were provided by all patients enrolled in this study.

2.1. System Samples Preparation and Running Conditions

Whole-blood and plasma samples were diluted in multiple sodium chloride (NaCl) solution dilutions (1:1; 1:2; 1:4; 1:5), and a 2% to 5% of a 10% Ethylenediaminetetraacetic acid (EDTA) solution, previously prepared, was added to avoid coagulation. Tests were conducted at a 40 to 100 µL/min input flow rates. Different flow rates were assessed depending on the processed sample (whole-blood or plasma).

Before injecting the samples in the microfluidic system, a NaCl solution was firstly used to rinse, eliminate air bubbles, and check any possible PDMS collapsed zones in the microchip. This test was done using an input flow of 100 µL/min and a Darwin bubbletrap automation system [155] to ensure no air bubbles would enter the system. After this cleaning procedure, plasma samples were injected at a 40 µL/min for a maximum CTCs enrichment. After the CTCs filtration process, the remaining plasma was collected in the outlet region and stored for further cfDNA analysis. CTCs enrichment in the system were fixed with 4% formaldehyde, washed with NaCl, and stored at 4ºC. All these experimental procedures were accompanied by an optical microscope to monitor fluids' behaviour and cellular behaviour.

3. Plasma Epigenetic Analysis

3.1. Circulating-free DNA Extraction

For cfDNA extraction, two different approaches (kit and extractor) were used and evaluated.

3.1.1. cfDNA Extraction with an Extraction Kit

Using a QIAamp® MiniElute® cfDNA kit (Qiagen, Hilden Germany), cfDNA was extracted from a 2 mL plasma sample. 120 µL Magnetic Bead Suspension, 220 µL Protein K, and 600 µL Bead Binding Buffer were added to the recovered plasma solution at room temperature (RT). This composition was incubated for 10 minutes and slowly shaken, with a thermomixer's assistance, at 100 *revolutions per minute* (rpm) to allow cfDNA binding to the magnetic particles. Then, a brief spin (30 seconds at 140 rpm) was performed, permitting a phase partition and, by using a magnetic rack for 1-minute incubation, the supernatant was discarded, and the pellet where DNA is bound to magnetic beads were gathered. Afterwards, magnetic beads-DNA were eluted in 200 µL bead elution to separate the two components and immediately centrifugated for bead resuspension. The mixture was later transferred to a bead elution tube and incubated for 5 minutes at RT with a shake of 300 rpm. Next, the magnetic beads were removed using the magnetic rack for 1 minute of incubation until the solution was once again clear for supernatant collection and transference to a new Bead Elution Tube. It is crucial to avoid any transference of magnetic beads onto the next steps; otherwise, it might

affect cfDNA yield. Into the supernatant collected was then added 300 µL of Buffer ACB, responsible for adjusting the conditions that lead to an optimal binding of cfDNA to a membrane column. Before transferring the solution mixture onto a QIAamp UCP MinElute column, the solution was briefly vortexed and centrifugated at 4200 rpm for 1 minute. The column was placed in a 2 mL collection tube and added 500 µL of ACW2 buffer, then centrifugated at 14000 rpm for 3 minutes. Subsequently, the column was transferred to a 1.5 mL elution tube, and this set was incubated for 3 minutes at 56ºC in the thermomixer, with the lid open to ensure the membrane's dry. Residual ethanol from ACW2 buffer present in the membrane must completely evaporate before the following step. Finally, the extraction finished with a 20 µL distilled water (H_2O) elution onto the membrane for a 1-minute incubation and proceeded to 14000 rpm centrifugation. The extracted cfDNA was ready to be quantified or stored at -20ºC until its use.

3.1.2. cfDNA Extraction using an Extractor

Circulating cell-free DNA was extracted using an extractor MagLEAD 12gC (Precision System Science Co.), a fully automated nucleic acid extraction system, and a MagDEA DX SV kit according to the equipment's protocol. This equipment permits a rapid and precise nucleic extraction (DNA or RNA) from a 400 µL sample input of whole-blood and other matrixes such as plasma, urine, and cerebrospinal fluid (CFS). Since the cfDNA tends to be present in limited concentrations, 400 µL of the initial plasma sample was used. Through 25 minutes, a complete process of beads binding (magnetic beads – Magtration®), washing, and elution are performed, obtaining cfDNA that was ready to be quantified or stored at -20ºC until its use. The lowest possible sample output volume was chosen for the cfDNA extraction (50 µL).

3.2. Nucleic Acid Quantification

By using a Qubit fluorometric method, the extracted cfDNA from both previously described methods was quantified. This quantification occurs via a specific dye's fluorescent intensity that binds to the double-stranded DNA (dsDNA) [156].

Extracted cfDNA was quantified using the dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). First, two assay tubes were set up for standards, and one for each sample. Next, the Qubit™ working solution was prepared by combining 1 µL of Qubit™ reagent with 199 µL of Qubit™ buffer (1:200) for each sample and standards. Afterwards, reading standards were prepared by adding 190 µL of Qubit™ working solution to 10 µL of standards from the kit, while samples were formulated by 199 µL of Qubit™ working solution to 1 µL of DNA sample. All tubes are then vortexed for 2-3 seconds and incubated for 2 minutes before using the Qubit™

Fluorometer for DNA readings. All steps were performed at RT [157].

3.3. Sodium-Bisulfite Modification

Sodium-bisulfite modification, up until now, is regarded as the gold-standard procedure for DNA methylation analysis. This bisulfite method converts only unmethylated cytosines into uracil, while methylated cytosines remain unchanged. Nucleobase differentiation of both DNA is then possible using specific methylation primers and probes for a PCR reaction like qMSP. Three main chemical events are developed during this modification: sulphonation – the addition of bisulfite to the 5-6 double bond of cytosine; deamination – hydrolytic deamination to originate a uracil bisulfite derivate; and desulfonation – removal of the sulphonate group to originate a uracil residue [158].

All cfDNA samples were modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). The modification begins with 130 µL of CT conversion reagent solution adjoined to a DNA volume of 20 µL. Both DNA controls, methylated and non-methylated, and sample volumes exploited should use a volume equivalent to 1000 ng. For standard DNA control, 4 µL of DNA controls (CpGenome™ Universal Methylated and Non-Methylated DNA, Merk Millipore, Burlington, MA, USA) with concentrations of 5 µg/20µL are added to 16 µL of distilled H_2O to made up a final volume of 20 µL. For each plasma sample, 20 µL of the extracted cfDNA was used. CT conversion solution was prepared beforehand by combining 900 µL of H_2O, 50 µL of M-dissolving buffer, and 300 µL of M-dilution buffer to a lyophilize CT conversion reagent. Next, the solution was vortexed and put in a mixer rotator for 10 minutes.

Next, samples were incubated at a cycle program of 98ºC for 10 minutes (DNA denaturation), followed by 64ºC for 180 minutes (bisulfite conversion) in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). After that, 600 µL of M-Binding Buffer and each sample were added to a Zymo-Spin IC™ column in a collection tube and incubated for 10 minutes. The incubation period allowed the DNA binding of ssDNA to the column. Henceforth, between buffer additions and incubation periods, 10.000 rpm centrifugations for 30 seconds were performed until the columns were transferred to a 1.5 mL safe-lock tube. Subsequently, 100 µL of M-Wash Buffer was added and proceeded by 200 µL of M-Desulphonation Buffer to the column with an immediate 20 minutes incubation. The desulfonation buffer was used to remove any desulfonation residue and convert the nucleobase to uracil. Then the columns were washed twice with M-Wash Buffer before transferring them to 1.5 mL safe-lock tubes. The final steps involved eluting bisulfite-converted DNA in 10 µL of distilled H_2O for both the DNA controls and samples. Following a 5 minute incubation, the columns were centrifugated at 12.000 rpm for 30 seconds. This process was

repeated for DNA controls, and the samples were adapted. Since these samples have limited cfDNA, elution with distilled H_2O was not performed; instead, 10 µL of the already eluted was re-eluted. The protocol was done at RT. Finally, 20 µL of bisulfite-converted DNA control and 10 µL of bisulfite-converted DNA from each sample were obtained and stored at -80ºC until further use or immediately use for DNA preamplification.

3.4. DNA Preamplification

SsoAdvanced™ PreAmp amplification is deployed when the involved DNA sample amount is limited. The procedure is begun by preparing a preamplification assay pool, wherefrom a particular 100 µM primer stock, 2.5 µL of each primer (forward and reverse) from intended genes are added to a volume of 500 µL.

For the preparation of the preamplification reaction mix, were required per each of the 10 µL of the modified sodium-bisulfite samples - 5 µL of the preamplification assay pool, 25 µL of SsoAdvanced PreAmp Supermix (2x), and 10 µL of distilled H_2O to a final volume of 50 µL.

Then, samples were incubated at a cycle program of 95ºC for 3 minutes (polymerase activation), followed by 12 cycles of 95ºC for 15 seconds (DNA denaturations) and 58ºC for 45 minutes (annealing/extension reaction) in Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA).

The preamplification reaction was concluded by diluting all samples at a minimum of 1:5 before running a qMSP assay.

3.5. Single and Multiplex Quantitative Methylation-Specific PCR

By singleplex and multiplex qMSP analysis, promoter methylation levels of 5 genes (*MIR129-2*$_{me}$, *ADCY4*$_{me}$, *NID2*$_{me}$, *MAGI2*$_{me}$, and *HOXA11*$_{me}$) and *β-Actin* (*ACTβ*), used as the housekeeping gene, were evaluated. For each gene, primers and TaqMan probes with specific fluorochromes and quenchers (Table 2) were wielded.

Reaction preparation depended on whether a singleplex or multiplex assay was being used. Nonetheless, for each reaction: 1 µL of a sample, 5 µL of Xpert Fast Probe (GRiSP, Porto, Portugal), a mix with forward and reverse primers (10µM), TaqMan probe (10µM), and distilled H_2O were added to a final volume of 20µL. All lung cancer samples were run in triplicate in 384-well plates using an Applied Biosystem™ QuantStudio™ 12K Flex Real-Time PCR System.

The PCR program carried out was outlined as: 1 cycle at 95 ºC for 3 minutes; 40 cycles at 95ºC for 5 seconds and 60-64ºC for 30 seconds. The multiplex gene panels used are shown in Table 3; panels 1 and 2 worked on 60ºC annealing temperature, while panel 3 on 64ºC.

Table 2. Primers and probes sequences with respective fluorochrome and quencher.

Gene	Sequence (5'–3')	
β-Actin	Primers	F – TGGTGATGGAGGAGGTTTAGTAAGT R – ACCAATAAAACCTACTCCTCCCTTAA
	Fluorochrome - Probe - Quencher	Cy5 – ACCACCACCCAACACACAATAACAAACACA – QSY
HOXA11$_{me}$	Primers	F – GGAAGGTATTAAAGCGTTTCG R – CTACCTCCGACCCTAACCG
	Fluorochrome - Probe - Quencher	FAM – CCCTTCGAAACCAAAATTTAAAACCG – BHQ1
MIR129-2$_{me}$	Primers	F – GGAGTGGTGAGATTGAGTCG R –GACTTCTTCGATTCGCCG
	Fluorochrome - Probe - Quencher	VIC – CGCGTTGGGGAGATTTAGTTTGTTC – BHQ1
ADCY4$_{me}$	Primers	F – AAAGGAGACGGGATTGTTAC R –AACCGAACGCCGAATTAC
	Fluorochrome - Probe - Quencher	FAM – TTTAGGTGGGGTTCGTCGGGTC – BHQ1
NID2$_{me}$	Primers	F – TCGTAATTTCGTTATTCGTTCGC R – CCCGCAAAATTTAAAACAACG
	Fluorochrome - Probe - Quencher	VIC – CCGCAACGACGAATACGACTACTAACCTACG – BHQ1
MAGI2$_{me}$	Primers	F – GGATTTCGCGTTAGGACGTTC R – ACCTCTATACGACCGAACCGC
	Fluorochrome - Probe - Quencher	VIC – CGCGCCTAATACCACATCTCGAACTCTACG – BHQ1

Two wells of sterile distilled water were used as non-template controls of the PCR reaction, two wells of preamplified sterile distilled water used as a non-template control of the PreAMP technique in all plates and a negative preamplified control. SsoAdvanced™ PreAmp amplification subjected six serial dilutions (5x factor dilution) positive controls to generate a standard curve in each plate, permitting for relative quantification and PCR efficiency assessment. All plates displayed efficiency values above 88%, and relative methylation levels were defined as the ratio between the mean methylation level of each respective gene and *ACTβ*, multiplied by 1000.

Table 3. Multiplex gene combinations in qMSP.

Panel 1	Panel 2	Panel 3
ACTβ	$ADCY4_{me}$	$HOXA11_{me}$
$NID2_{me}$	$MIR129\text{-}2_{me}$	$MAGI2_{me}$

4. Statistical Analysis

Non-parametric tests were performed to compare methylation levels of each gene's promoter between different extraction methods of cfDNA, and to evaluate associations with clinicopathological features. Mann-Whitney U test was used for comparisons between two groups, while Kruskal-Wallis test was used for multiple groups, followed by Mann-Whitney U test with Bonferroni's correction for pairwise comparisons. Spearman non-parametric test was performed to assess correlations between methylation levels and patients' age, as well as *ACTβ* and cfDNA quantity levels. A result was considered statistically significant when p-value ≤0.05. The statistical analysis was performed using the GraphPad Prism 9.0.0 and IBM SPSS statistical softwares.

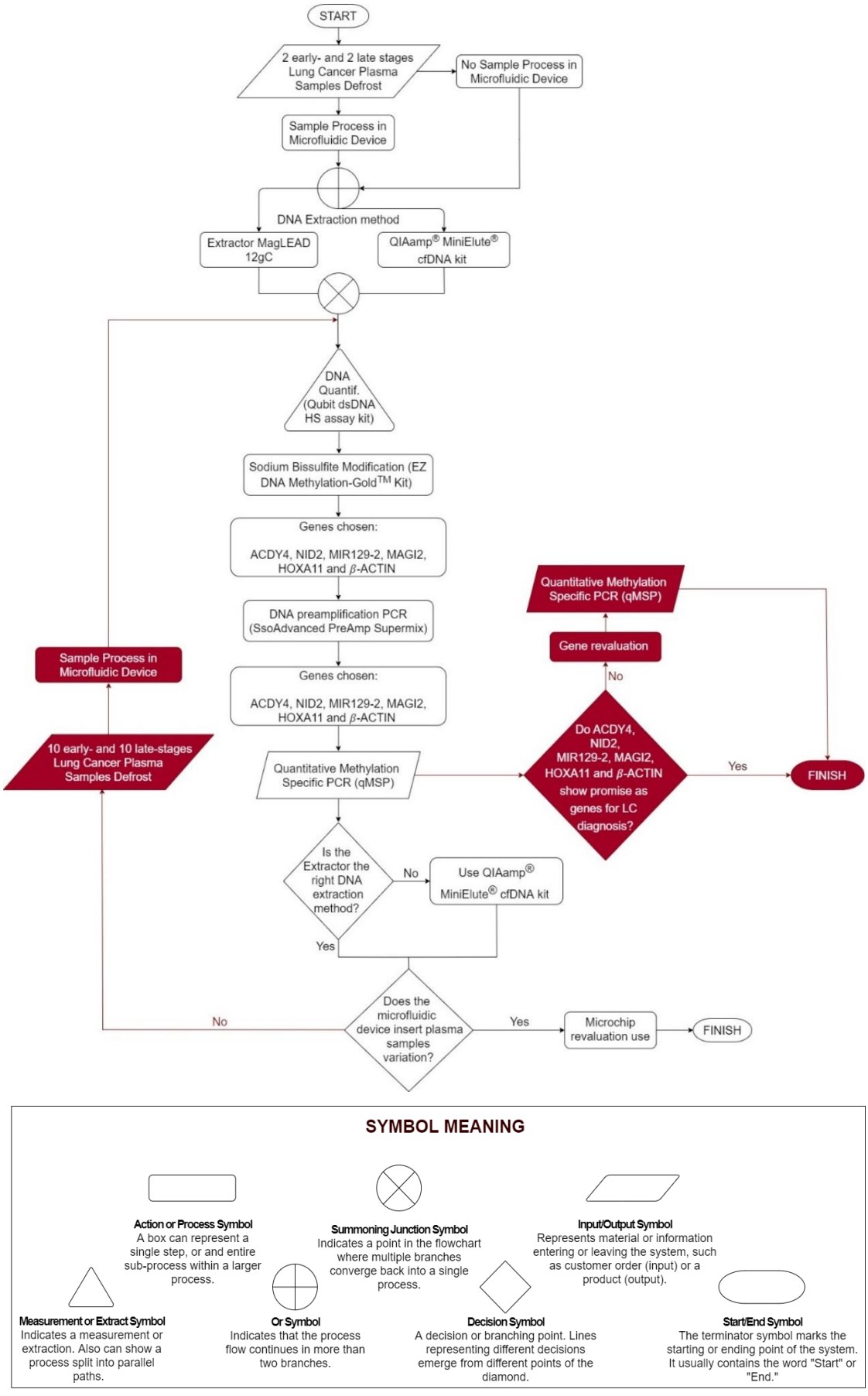

Figure 5. Flowchart of the experimental work.

IV. Results

1. Microfluidic System Experimental Setting

The microfluidics set-up was achieved successfully, with the typical set-up for sample processing displayed in Figure 6. The microfluidic chip was connected to semi-rigid Tygon tubbing with 23G needles in both the inlets and outlets. The processed samples were injected into the system using syringes that were fitted into a syringe pump, and filtered plasma samples were collected through the two outlets.

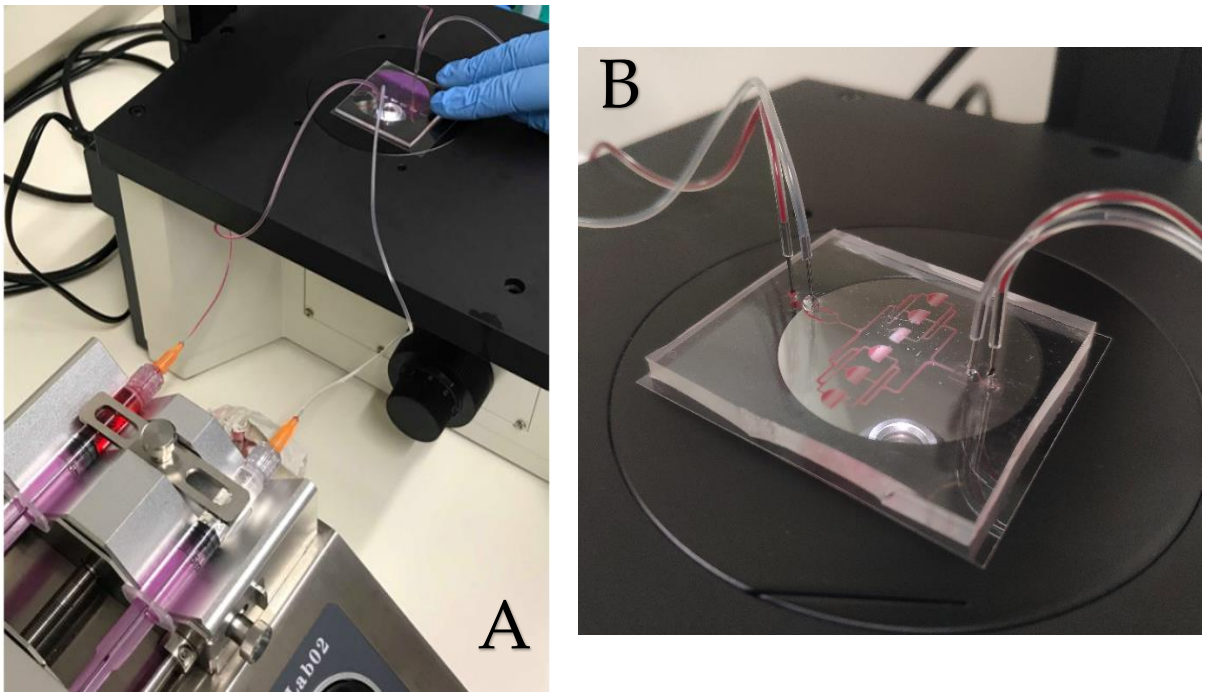

Figure 6. Experimental setup for sample running process. (A) General view of the setup; (B) Close-up view of the microfluidic system while processing whole-blood.

1.1. Whole-blood and plasma assays

Ideal set-up conditions were optimized for each type sample (whole-blood and plasma) so that efficient CTCs capture could occur without compromising its cellular integrity and viability for further study and, later cfDNA extraction, quantification, and methylation evaluation.

Two EDTA concentrations, of 2% and 5% were tested for whole-blood and plasma processing, as well as different dilutions with NaCl.

For whole-blood, it was found that dilutions up to 1:5 and 5% EDTA still caused some clogging in the microchambers (Figure 7A and 7B). Additionally, the flow rate (>100µl/min) necessary to perform plasma extraction in the partition section of the microfluidic chip could interfere with the isolation of intact circulating tumour cells. Flow rate adjustments would lead to some contaminations of red blood cells (RBCs) in the extracted plasma (Figure 7C).

These results allied with the restrictions of blood handling due to the world's current pandemic led to the system optimization to process plasma samples, which were readily available.

Different NaCl dilutions and EDTA concentrations were also evaluated for plasma, with a 1:1 dilution (NaCl volume equal to plasma's) and 5% EDTA concentration selected, which allowed obtaining cfDNA in a minimal dilution and volume possible. Figure 7D shows the aspect of a microchamber after plasma processing.

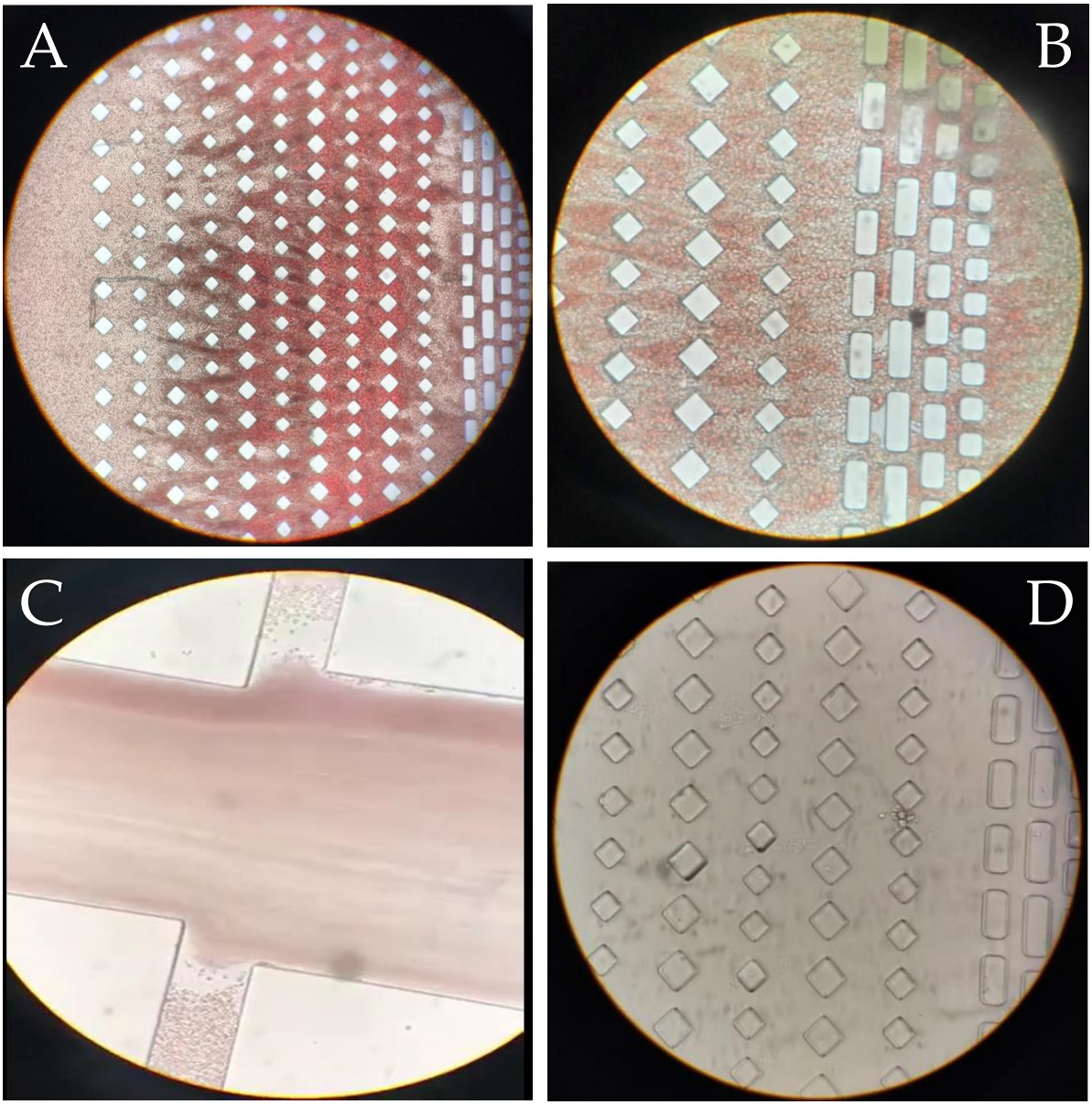

Figure 7. Plasma and whole blood set-up conditions and phenomenon displayed. (A) and (B) - Blood coagulation effect after a period of time on a blood sample diluted in 1:5 NaCl and with a 5% EDTA concentration; (C) - Partition phenomenon of plasma and RBCs in the intersection of two channels close to the outlets; (D) - Plasma sample process diluted in 1:1 NaCl series and with a 5% EDTA concentration.

2. Circulating-cell free DNA Methylation Analysis

For cfDNA analysis, a set of 5 genes (*MIR129-2*, *ADCY4*, *NID2*, *MAGI2*, and *HOXA11*), shown to be methylated in lung cancer, were tested in LC plasma following a microfluidic-assisted filtration. *ACTB* was also used as a DNA input control for a posteriori gene normalization [159].

2.1. PreAMP Validation

DNA preamplification (PreAMP) implementation was required throughout the work developed since the plasma samples may have a limited cfDNA input. Nevertheless, it was essential to demonstrate this technique's implementation did not increment samples' variability; thus, PreAMP validation was done.

In total, 15 genes (*ADCY4*, *CD01*, *MAGI2*, *MIR129-2*, *NID2*, *HOXA11*, *CELF2*, *CHFR*, *FLOT1*, *ACTβ*, *ALBUMIN*, *MAL*, *CADM1*, *FAM19A4*, and *MIR124-2*) were used to make a preamplification primer pool. For PreAMP's validation, a multiplex qMSP involving *ACTβ, ADCY4* and *MIR129-2* genes were assessed in three test condition groups. The control standard group (STD^{1+}: STD^{5+}), defined as a 1:5 dilution series of positives controls that were not preamplified; the PreAMP Standard group (STD_{PRE}^{1+}: STD_{PRE}^{5+}) which involved the PreAMP reaction of STD^{+} samples after a 1:5 dilution series had been performed; and the Diluted PreAMP Standard group (CRL^{2+}: CRL^{5+}), a 1:5 series dilution from a STD_{PRE}^{1+} sample.

Each non-preamplified and preamplified positive standard analysis were assessed alongside a non-template control (NTC and H_2O_{PRE}, respectively) and negative control (STD^{1-} and STD_{PRE}^{1-}, respectively). Table 4 shows the different cycle threshold (Ct) amplification for each gene in each respective sample.

For every gene included in the multiplex panel, a constant difference of 6-8 cycles between pre-amplified samples and non-pre-amplified was obtained (Table 5). Furthermore, in the two group conditions where the series dilution was made beforehand (STD_{PRE}^{+}) and afterwards (CRL^{+}) the PreAMP reaction, there was no major Ct difference for the three tested genes [$p(ADCY4)=0.825$ and $p(MIR129\text{-}2)=p(ACT\beta)=0.897$].

Table 4. Standard groups qMSP evaluation (Ct amplification) of methylated *ACTβ*, *ADCY4* and *MIR129-2* genes in circulating cell-free DNA in three different test condition groups (Control, PreAMP, and Diluted PreAMP).

Cycle threshold (Ct) Amplification																
Conditions		**CONTROL TEST GROUP**					**PreAMP TEST GROUP**					**DILUTED PreAMP TEST GROUP**				
Samples		STD^{1+}	STD^{2+}	STD^{3+}	STD^{4+}	STD^{5+}	STD_{PRE}^{1+}	STD_{PRE}^{2+}	STD_{PRE}^{3+}	STD_{PRE}^{4+}	STD_{PRE}^{5+}	CRL^{1+}	CRL^{2+}	CRL^{2+}	CRL^{2+}	CRL^{2+}
Genes	*ACTβ*	24.95	27.27	29.67	31.51	34.1	18.18	20.77	22.76	25.65	27.58	18.18	20.94	23.27	25.44	28.04
	ADCY4	24.91	27.42	29.87	32.35	34.86	17.31	19.63	22.30	24.76	27.40	17.31	20.23	22.47	24.83	27.40
	MIR129-2	26.08	28.54	30.73	32.88	34.73	18.98	21.13	23.61	26.20	28.27	18.98	21.60	23.81	26.11	28.48

SAMPLES		NTC	H_2O_{PRE}	STD^{1-}	STD_{PRE}^{1-}
Genes	*ACTβ*	37.67	-	24.95	18.48
	ADCY4	-	-	37.69	33.65
	MIR129-2	-	-	-	-

Table 5. Ct Differences of methylated *ACTβ*, *ADCY4* and *MIR129-2* genes in circulating cell-free DNA between the respective preamplified test and control groups.

Cycle threshold (Ct) Differences Between Sample Tests Groups											
Comparisons		**CONTROL - PreAMP TEST GROUPS**					**CONTROL – DILUTED PreAMP TEST GROUPS**				
SAMPLES		STD^{1+} - STD_{PRE}^{1+}	STD^{2+} - STD_{PRE}^{2+}	STD^{3+} - STD_{PRE}^{3+}	STD^{4+} - STD_{PRE}^{4+}	STD^{5+} - STD_{PRE}^{5+}	STD^{1+} - CRL^{1+}	STD^{2+} - CRL^{2+}	STD^{3+} - CRL^{3+}	STD^{4+} - CRL^{4+}	STD^{5+} - CRL^{5+}
Genes	*ACTβ*	6.77	6.5	6.91	5.86	6.52	6.77	6.33	6.4	6.07	6.06
	ADCY4	7.60	7.79	7.57	7.59	7.46	7.60	7.19	7.4	7.52	7.46
	MIR129-2	7.1	7.41	7.12	6.68	6.46	7.1	6.94	6.92	6.77	6.25

2.2. Circulating cell-free DNA Extraction Methods and Microfluidic Device Possible Sample Variation Evaluation

The next step was to determine which cfDNA extraction method should be applied (QIAamp® MiniElute® ccfDNA kit or extractor MagLEAD 12gC) and assess if the microfluidic device adds variations on cancer plasma samples. A total of 4 samples, two early-stage (LC_1 and LC_2) and two late-stage (LC_{14} and LC_{15}), were chosen for evaluating four different test condition: extractor and kit extraction for unprocessed samples (controls), and processed plasma' samples with the microfluidics device.

Immediately after nucleic acid extraction, dsDNA quantification was performed for each sample in the different test conditions, and then proceeded a qMSP analysis. Findings showed in control conditions, cfDNA extraction by kit to display a higher Qubit's dsDNA concentration compared to extractor. However, this same behaviour was not verified between samples processed by the microchip. In addition, when comparing the same cfDNA extraction methods on processed and unprocessed samples, once again, no constant pattern was displayed alongside the four LC samples.

The results exhibited valid Ct amplification in the genes present in the multiplex panels 1 and 2, except for the LC_{15} sample on extractor in which *MIR129-2* gene presented a Ct amplification after the 35 cycles (which in a 40-cycle reaction is the limit imposed for a valid amplification). Contrarily, for both genes included in panel 3, the amplifications either were false positives, as the Ct amplification were higher than the respective gene's negative control (STD^{1-}) or Ct > 35 cycle. Hence, panel 3, was excluded from this evaluation.

ACTβ Ct amplifications showed no major difference in each LC sample between the four conditions, as it was never more than 2.5 cycles (Table 6).

Interestingly, sample LC_{14}, the only sample that showed a biomarker gene amplification in all four conditions (panel 2 genes), like dsDNA quantification, exhibited a different Ct amplification through all four conditions. Also, results showed not many Ct differences between the different conditions for the genes evaluated in qMSP, except for *MIR129-2*.

Concerning the outcomes, no statistical evidence revealed a prevalence of a method in favour of another (*p=0.058*). Nevertheless, the condition where samples were processed by the microchip and then extracted cfDNA using the kit showed slightly better results as it was the only situation where the four LC samples showed an amplification on one of the tested genes. Therefore, kit cfDNA extraction was used as the preferential DNA extraction method.

Table 6. Extraction Methods: double-stranded (ds)DNA quantification and qMSP evaluation (Ct amplification) of promoter methylated *NID2*, *ADCY4*, *MIR129-2*, *HOXA11* and *MAGI2* genes in circulating cell-free DNA of four chosen samples.

Sample - Stage	Extraction Method	Qubit dsDNA quantif. (ng/µL)	Target Genes in a 40 cycle qMSP					
			Multiplex panel 1		Multiplex panel 2		Multiplex panel 3	
			ACTβ	*NID2*	*ADCY4*	*MIR129-2*	*HOXA11*	*MAGI2*
LC_1 - Early	Extractor	too low	23.77	-	-	-	-	-
	Extractor Micro.	0.324	25.46	-	-	-	-	-
	Kit	0.124	24.11	-	-	-	-	-
	Kit Micro.	0.252	23.11	-	-	33.12	-	32.72*
LC_2 - Early	Extractor	too low	23.74	-	-	-	-	-
	Extractor Micro.	too low	24.88	-	-	-	-	-
	Kit	0.138	24.19	-	-	-	-	-
	Kit Micro.	0.306	22.70	-	33.47	-	-	-
LC_{14} - Late	Extractor	0.228	24.65	-	30.31	34.54	-	-
	Extractor Micro.	0.354	23.08	32.06	29.21	28.96	30.12*	-
	Kit	0.254	23.31	30.71	29.56	29.45	29.82*	-
	Kit Micro.	0.390	24.15	31.76	28.79	33.23	29.36*	-
LC_{15} - Late	Extractor	0.120	25.08	-	-	-	-	-
	Extractor Micro.	too low	27.41	-	-	-	33.48*	-
	Kit	0.240	24.75	-	-	31.25	31.94*	-
	Kit Micro.	too low	25.64	-	-	32.75	-	32.61*

*Ct displayed in blue colour were not credited due to amplification after negative control (STD^-).

2.3. Multiplex qMSP panels for Lung Cancer sample analysis

Except for the *MAGI2* gene, all genes showed valid Ct amplifications; therefore, no association was evaluated between $MAGI2_{me}$ levels and patients' clinicopathological features.

After performing the respective cfDNA extraction, quantification of dsDNA was executed (Table 7). Because LC_{15} sample (late-stage) showed a nucleic acid quantity too low to be determined and considering its respective value as zero, there were no significance difference ($p=0.0568$) between late- and early-stage samples. Still, compared to early-stage samples, late onset patients tend to display higher dsDNA quantification. A strong association between *ACTβ* quantity levels and dsDNA quantification was evident (R=0.867; $p<0.0001$).

Table 7. Double stranded(ds)DNA quantification and *ACTβ* quantity levels of each evaluated LC samples (n=26).

Stage	Samples	Qubit dsDNA quantification (ng/µL)	*ACTβ* Quantity
Early	LC_1	0.252	859.90
	LC_2	0.306	1047.85
	LC_3	0.362	1201.77
	LC_4	0.400	1039.86
	LC_5	0.278	1061.29
	LC_6	0.414	1717.77
	LC_7	0.476	1490.00
	LC_8	0.294	856.60
	LC_9	0.212	910.74
	LC_{10}	0.738	2247.25
	LC_{11}	0.190	928.00
	LC_{12}	0.290	1296.53
	LC_{13}	0.102	432.6282
Late	LC_{14}	0.390	467.99
	LC_{15}	too low	161.33
	LC_{16}	0.406	1818.11
	LC_{17}	0.608	1990.80
	LC_{18}	0.706	3063.89
	LC_{19}	1.560	5053.67
	LC_{20}	1.320	3737.87
	LC_{21}	0.316	1013.96
	LC_{22}	0.228	645.16
	LC_{23}	0.514	1563.20
	LC_{24}	0.756	1618.08
	LC_{25}	0.498	1177.84
	LC_{26}	0.248	833.51

2.3.1. Clinical and Pathological Data

Clinical and pathological features of the selected patients are described below in Table 8. No correlation was found between methylated level genes and patients' age.

Table 8. Patient clinical and LC clinicopathological information.

LUNG CANCER (LC)				
Patients (n=26)				
Age median (range)			68.5 (46-86)	
Gender	Male		18 (69.23%)	
	Female		8 (30.77%)	
Clinicopathological features				
Tumour stage	Early	I	13 (50%)	8 (30.77%)
		II		5 (19.23%)
	Late	III	13 (50%)	4 (15.38%)
		IV		9 (34.62%)
Pathological type	NSCLC*	LUAD	21 (80.77%)	11 (46.15%)
		LUSC		7 (26.92%)
		ASLC		1 (3.85%)
		LCNEC		1 (3.85%)
	Neuroendocrine	Typical carcinoid	2 (7.69%)	1 (3.85%)
		Atypical carcinoid		1 (3.85%)
	SCLC		3 (11.54%)	
Smoking	Smoker		14 (53.85%)	
	Non-smoker		8 (30.77%)	
	Ex-smoker		4 (15.38%)	
Metastasis	YES		13 (50%)	
	NO		10 (38.46%)	
	NA**		3 (11.54%)	

*NSCLC – for 1 patient no information about cancer's subtype was determined; **NA – not available;

2.3.2. Association Between Promoters' Methylation Levels and Clinicopathological Features

2.3.2.1. Lung Cancer Stage

The main goal was to test the biomarker potential for LC detection from a methylation-based analysis of cfDNA after the plasma samples had been processed by a microfluidic device. $NID2_{me}$ (*p=0.0005*), $ADCY4_{me}$ (*p=0.0003*), $MIR129\text{-}2_{me}$ (*p=0.0002*), and $HOXA11_{me}$ (*p<0.0001*) levels were significantly higher in cell-free DNA's of late-stage LC patients than early-stage ones (Figure 8). For each biomarker LC detection sensitivity was assessed and showed from all four biomarkers, $HOXA11_{me}$ to have the highest and $NID2_{me}$ to display the lowest detection of LC (61.54% and 50% of sensitivity, respectively) (Table 9).

According to LC stage, and as expected, all genes showed higher sensitivity in late onset patients. Moreover, the similar pattern found in the overall analysis for LC was shown for both stages, with the particularity that a similar sensitivity (approximately 31%) was shown by both $HOXA11_{me}$ and $MIR129\text{-}2_{me}$ in early-stage patients.

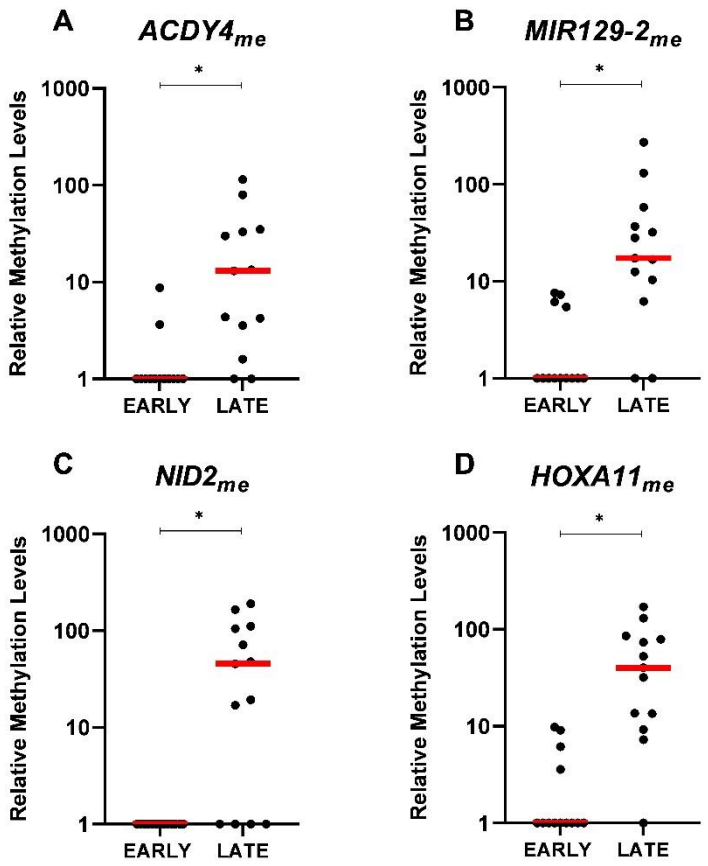

Figure 8. Distribution of (A) *ADCY4*, (B) *MIR129-2*, (C) *NID2* and (D) *HOXA11* relative methylation levels in early-stage and late-stage lung cancer (LC) samples. The y axis is in log10 scale. Mann Whitney U Test between early-stage and late-stage, *p<0.05. Red horizontal lines represent median methylation levels.

Besides the sensitivity obtained for each gene individually, it was also evaluated the best gene biomarker panel for LC detection and according to cancer's stage as well. Herein, higher number of evaluated genes tended to increase the sensitivity. Moreover, the different combination panels with two and three genes demonstrated identical sensitivities values for late-stage LC detection.

The panel composed of $ADCY4_{me}$, $HOXA11_{me}$ and $MIR129\text{-}2_{me}$ proved to be the best, as for all three analysed sensitivities it was obtained the highest values, demonstrating the addition of $NID2_{me}$ did not increment the LC detection in this specific situation.

Table 9. Biomarkers performances of *NID2*, *ADCY4*, *MIR129-2*, and *HOXA11* promoters' methylation levels, (according with the number of) for detecting Lung Cancer (LC), as well as discriminating among early-stage and late-stage patients in circulating cell-free DNA.

LUNG CANCER (LC) - Sensitivity %							
Nº. of Promoter Gene Methylation		**1**					
		$NID2_{me}$	$ADCY4_{me}$	$MIR129\text{-}2_{me}$	$HOXA11_{me}$		
Stage Sample	Early	0	15.38	30.77	30.77		
	Late	69.23	84.62	84.62	92.31		
Overall		34.62	50	57.69	61.54		
Nº. of Promoter Gene Methylation		**2**					
		$NID2_{me}$ / $ADCY4_{me}$	$NID2_{me}$ / $MIR129\text{-}2_{me}$	$NID2_{me}$ / $HOXA11_{me}$	$ADCY4_{me}$ / $MIR129\text{-}2_{me}$	$ADCY4_{me}$ / $HOXA11_{me}$	$MIR129\text{-}2_{me}$ / $HOXA11_{me}$
Stage Sample	Early	15.38	30.77	30.77	38.46	46.15	30.77
	Late	92.31	92.31	92.31	92.31	92.31	92.31
Overall		53.85	61.54	61.54	65.38	69.23	61.54
Nº. of Promoter Gene Methylation		**3**				**4**	
		$NID2_{me}$ / $ADCY4_{me}$ / $MIR129\text{-}2_{me}$	$NID2_{me}$ / $MIR129\text{-}2_{me}$ / $HOXA11_{me}$	$NID2_{me}$ / $ADCY4_{me}$ / $HOXA11_{me}$	$MIR129\text{-}2_{me}$ / $ADCY4_{me}$ / $HOXA11_{me}$	ALL	
Stage Sample	Early	38.46	53.85	46.15	61.54	61.54	
	Late	100	100	100	100	100	
Overall		69.23	76.92	69.23	80.77	80.77	

Supplementary complete information regarding Ct amplification of the 26 LC's samples qMSP evaluation is presented in appendix (Appendix I: Supplementary Table 1 and Table 2).

2.3.2.2. Lung Cancer Histological Subtypes, Smoking Habits and Metastases Dissemination

Circulating *HOXA11$_{me}$* levels showed to be statistically significant in NSCLC *vs* SCLC patients (*p=0.0035*) (Figure 9), Smoker *vs* Non-Smoker (*p=0.0235*) (Figure 10) as well as M0 *vs* M+ (*p=0.02595*) (Figure 11). On the other hand, all other gene' biomarkers, for the those same three clinicopathological analyses did not show any statistical significance.

The sensibility for each evaluation was determined and is presented in Table 10. In NSCLC *vs* SCLC, *MIR129-2$_{me}$* showed to be the best methylated gene promoter for detecting NSCLC. It is important to mention that for SCLC, since the number of cases is only 3, sensitivity might not be representative.

In M0 *vs* M+ and Smoker *vs* Non-Smoker analysis, for all genes, a tendency showed M0 and Non-Smoker, respectively, to be less detected in comparison with their analysed counterparts. The other two analysis, M0 *vs* M+ and Smoker *vs* Non-Smoker, showed *HOXA11$_{me}$* just like in early *vs* late sensibility as the methylated gene with the highest percentage for detecting metastatic and smoking LC individuals.

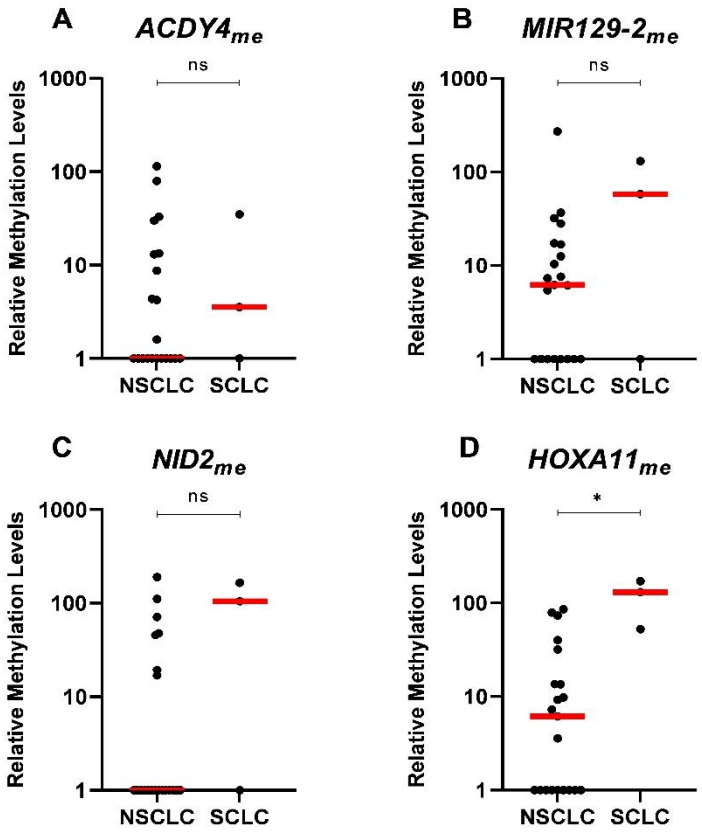

Figure 9. Scatter plot of (A) *ADCY4*, (B) *MIR129-2*, (C) *NID2* and (D) *HOXA11* promoter's methylation levels according with Histological Subtype [Non-Small Cell Lung Cancer (NSCLC) (n=21) and Small Cell Lung Cancer (SCLC) (n=3)]. The y axis is in log10 scale. Mann Whitney U Test, *p<0.05 and ns>0.05. Red horizontal lines represent median methylation levels.

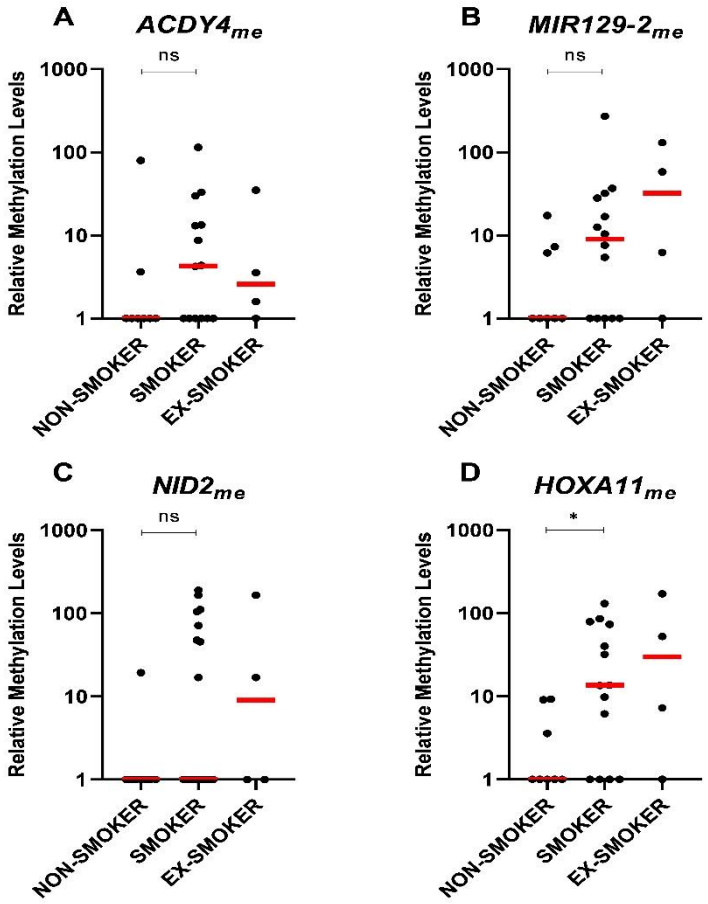

Figure 10. Distribution of methylation levels in lung cancer (LC) according with smoking habits. (A) *ADCY4*, (B) *MIR129-2*, (C) *NID2* and (D) *HOXA11* promoter's methylation levels in non-smokers (NO) (n=8), smokers (YES) (n=14) and ex-smoker (n=4). The y axis is in in log_{10} scale. Mann Whitney U Test between non-smokers and smokers, *p<0.05 and ns>0.05. Red horizontal lines represent median methylation levels.

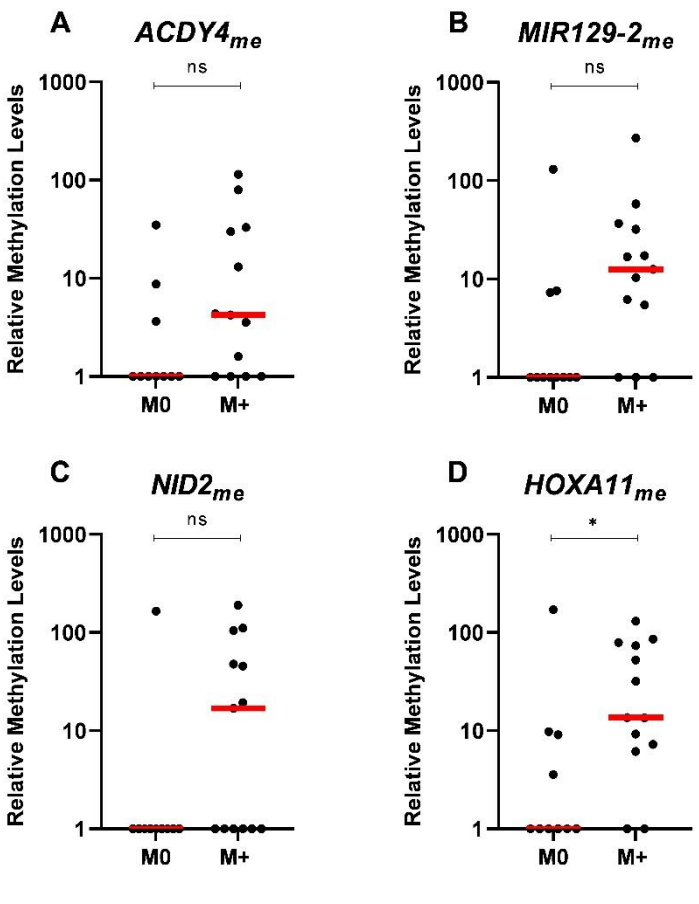

Figure 11. Distribution of methylation levels in lung cancer (LC) according with metastatic dissemination. (A) *ADCY4*, (B) *MIR129-2*, (C) *NID2* and (D) *HOXA11* promoter's methylation levels in non-metastatic (M0) (n=10) and metastatic (M+) (n=13). The y axis is in log_{10} scale. Mann Whitney U Test, *p<0.05 and ns>0.05. Red horizontal lines represent median methylation levels.

Table 10. Biomarkers performances of *NID2*, *ADCY4*, *MIR129-2*, and *HOXA11* promoters' methylation levels, for detecting Small Cell Lung Carcinoma (SCLC) and Non-Small Cell Lung Carcinoma (NSCLC); Metastatic and Non-Metastatic individuals; and Smoker and Non-Smokers in circulating cell-free DNA.

LUNG CANCER - Sensitivity %					
Genes		NID2$_{me}$	ADCY4$_{me}$	*MIR129-2*$_{me}$	*HOXA11*$_{me}$
Histological Subtype	NSCLC	33.33	47.62	61.91	57.14
	SCLC	66.67	66.67	66.67	100
Metastatic dissemination	M0	10	30	30	40
	M+	53.85	69.23	76.92	84.62
Smoking Habits	NO	12.5	25	37.5	37.5
	YES	42.86	57.14	64.29	71.43

3. Comparison evaluation between detection of cfDNA and CTCs

Since the microfluidic device platform developed and used throughout the experimental work is specific for CTCs' isolation, potential comparison between the two cancer biomarkers, CTCs and cfDNA was considered (Table 11). The number of CTCs, detected by immunofluorescence staining, showed no direct association with the dsDNA quantification, each promoter gene methylation levels and the total number of gene promoter levels detected in each LC sample.

Tendency shows a higher number (3-6) of CTCs detected in late-stage patients compared to early onset. Also, in those respective samples the dsDNA quantification was higher (>0.3 ng/µL**)** as well as total number of promoter methylated genes with three or more genes counted, except for LC_{14}. Even though in early LC cases there were three samples with CTCs counting (LC_2, LC_7 and LC_{12}), LC_7 and LC_{12} showed no detection of any gene promoter's methylation, while LC_2 only displayed one methylated gene. The remaining early LC samples presented a CTCs count of zero and a lower gene promoter methylation count as well with never more than a total number of two promoter methylated genes.

Table 11. Counted CTC, dsDNA quantification and biomarker performances of *NID2*, *ADCY4*, *MIR129-2*, and *HOXA11* promoters' methylation levels in circulating cell-free DNA in fifteen chosen samples for biomarkers association.

Stage	Samples	Number of detected CTCs	dsDNA quantification	Genes			
			Qubit (ng/µL)	*NID2*$_{me}$	*ADCY4*$_{me}$	*MIR129-2*$_{me}$	*HOXA11*$_{me}$
Early	LC_2	3	0.306	-	3.642	-	-
	LC_3	0	0.362	-	8.756	7.607	-
	LC_5	0	0.278	-	-	6.131	-
	LC_6	0	0.414	-	-	-	9.774
	LC_7	5	0.476	-	-	-	-
	LC_{12}	1	0.290	-	-	-	-
Late	LC_{14}	0	0.394	189.667	114.475	271.198	79,126
	LC_{15}	3	too low	-	-	36.863	-
	LC_{17}	3	0.608	-	13.057	16.859	31,923
	LC_{18}	3	0.706	16.936	1.597	6.227	7,277
	LC_{19}	4	1.560	165.880	34.985	130.46	171.26
	LC_{21}	3	0.316	-	3.568	58.172	52.552
	LC_{24}	6	0.756	19.326	79.811	17.297	9.256
	LC_{25}	4	0.498	71.550	13.403	28.058	40.137

V. Discussion

Due to the high incidence and mortality rates in lung cancer worldwide, sensible, affordable, and minimally invasive screening procedures are required. Even though advances have been achieved in treatments, accomplishing accurate early lung cancer detection remains a necessity.

In various studies, blood-based liquid biopsies have demonstrated their clinical utility for serving as a reservoir source of potential biomarkers for analysis [30-32]. Circulating cell-free DNA and, more specifically, ctDNA are two known biomarkers being studied for their possible use as a diagnostic and prognostic tool. Since aberrant DNA methylation of cancer-related genes develops very early during tumorigenesis, by cases of hypermethylation and hypomethylation, its evaluation might be used as a complementary procedure for LC diagnostic subtyping [160]. Coupled with liquid biopsies the field of microfluidics has been increasingly used since microplatforms have shown potential to improve cancer biomarkers extraction and enrichment in biological fluids [120].

Hence, a methylation evaluation of cfDNA from LC patient's plasma, using multiplex qMSP was assessed following a setup that involved a microfluidic-assisted filtration of circulating tumour cells, a cfDNA kit extraction, sodium-bisulfite modifications, and PreAMP procedure.

A total of six genes - *ACTβ*, *NID2*, *ADCY4*, *MIR129-2*, *HOXA11* and *MAGI2* - were selected and tested, based on research performed by the Cancer Biology & Epigenetics research group and others published studies, for their potential in LC detection and screening.

When dealing with liquid biopsies cfDNA quantity tends to be limited which constitutes a challenge even with techniques' advances in sensitivity and specificity of DNA detection [125]. To overcome this drawback PreAMP reaction was deployed, however it was important to ensure that it did not increment variability between each sample and genes [161].

Cycle threshold differences along the three genes analysed (*ACTβ*, *ADCY4* and *MIR129-2*), and between preamplified samples and controls were constant as they were never more than 1 cycle. Furthermore, the Ct difference was always between 6-8 cycles, which in total asserts the claim that PreAMP does not add any variability when used [161].

Since the proposed work was to access the potential use of a microfluidic platform as coadjuvant tool to assess circulating cancer biomarkers in LC detection, choosing the most adequate cfDNA extraction method was required. No statistically significant differences were found among the four methods, much likely due to the low number of initial tested LC samples (n=4). Nonetheless, the kit cfDNA extraction used on samples previously processed in the microfluidic device was chosen henceforth, as it was the only condition from the four tested that exhibited an amplification in all four tested LC samples.

Since the cfDNA extraction of the extractor occurs in a 50µL elution volume, which is higher than the output volume of the kit (20µL), we would assume that the DNA quantity would be lower which would translate in a later *ACTβ* cycle threshold (Ct) amplification. Interestingly,

this was not observed consistently throughout the four samples and four conditions.

Also, emphasis should be made regarding dsDNA quantification as no constant behaviour was displayed when the samples were previously processed in microfluidic device compared to control (no sample device process). Apart from what displayed in LC_{15}, every other sample when used with the platform plasma's process displayed an equal (too low) or superior dsDNA quantification. This might occur due to DNA stabilization when added NaCl; the use of EDTA, a well-known chelating agent responsible for preventing DNA degradation; or plasma filtration of unwanted material when samples were processed in the microchip [162] [163].

After defining the cfDNA extraction method, a dsDNA quantification and qMSP analysis of the 26 LC samples was followed. Firstly, the quantification showed no statistical significance between late-stage *vs* early-stage samples ($p=0.0568$). Considering that the amount of circulating cell free DNA has shown to increase with the disease progression, one can assume that late-stage LC samples have a higher concentration of dsDNA [164]. Yet, one of the late-stage samples (LC_{15}) did not display Qubit dsDNA quantification as the nucleic acid quantity was too low. Without considering this sample, however, this onset late stage samples display a higher dsDNA quantity than early-stage ones ($p=0.0160$).

Overall promoter gene methylation levels were detected in all tested genes, with $HOXA11_{me}$ displaying the highest single gene sensitivity (61.54%) and $NID2_{me}$ the lowest (34.62%). Furthermore, when evaluating a possible combination gene panel for LC detection we found that $MIR129\text{-}2_{me}$, $ADCY4_{me}$ and $HOXA11_{me}$ were the best option as they showed an 80.77% sensitivity. As anticipated, the evaluated samples showed promoter methylated genes to be stage-dependent which meant that late-stage LC patients presented higher methylation levels compared to early-stage individuals ($p<0.05$ for all genes). The high circulation methylation levels are mostly detectable in more late onset cancer stage because of increased tumour burden and metastatic dissemination. While Feng *et al*. showed that most of the plasma $NID2_{me}$ detection occurred in a middle/late cancer stage, our results showed methylation in this gene exclusively in late cancer stages [148]. A justification for this dissimilarity might reside in the sample size as in their study they evaluated a higher number of plasma samples, a difference in the disease stage classification and, also in our cohort there were in total five samples that were not NSCLCs. The highest sensitivity for early-stage cases was obtained once again for *MIR129-2, ADCY4* and *HOXA11* genes (61.54%), and expected since individually for $NID2_{me}$ did not occur any LC detection.

Association between other clinicopathological features and circulating methylation levels of the respective gene were also evaluated to unveil the possible existence of methylation patterns. From all features, even though histological subtype evaluation was in concordance with the distribution of diagnosed cases of NSCLC and SCLC (85% and 15%, respectively), showed a sample sized discrepancy that was considered. In smoking habits, the promoter

methylation levels were determined in three classes (smokers, non-smokers, and ex-smokers), however statistical analysis was only performed between smoker and non-smoker patients. Since no information was available regarding the period of smoking cessation, individuals with extended time difference of smoke cessation might be present and not constitute an exact evaluable class.

Interestingly, only *HOXA11*$_{me}$ presented statistically significant results regarding LC histological subtypes, smoking habits, and metastases dissemination, while the others candidates' genes showed none. Just like in previous findings, once again *NID2*$_{me}$ presented the lowest sensitivity for all these three clinicopathological features, and highest sensitivities were recorded mostly by *HOXA11*$_{me}$ except in NSCLC's. Regarding NSCLC's promoter gene methylation, *MIR129-2*$_{me}$ established with the highest detection rate (61.91%), and *NID2*$_{me}$ displayed the lowest (33.33%) which was lower than the 45.65% attained by Feng *et al*. [148]. The value obtained for *HOXA11*$_{me}$ (57.14%), like *NID2*$_{me}$, was also lower than the 69% obtained in a study involving *HOXA11* hypermethylation association with NSCLC's progression [18].

Even though these specific tested genes were not been studied regarding involvement of respective DNA methylation and smoking habits and metastases dissemination, other genes have been tested. Indeed, Fasanelli *et al.* study demonstrated that tobacco smoking alter DNA methylation patterns in CpG regions of *AHRR* and *F2RL3* genes [165]. Regarding metastatic spread a tissue study showed *OSR1*, *SIM1* and *HOXB3*/*HOXB4* as potential biomarkers for LUAD due to significant hypermethylation shown [166].

Lastly, comparisons between the isolated CTCs by the microfluidic platform and samples' nucleic acid quantification, and individual and total number of promoter gene methylated were evaluated. Findings, except for one sample (LC_{15}) showed for every other late onset LC patient a tendency of three or more CTCs detected.

It is important to mention that CTCs count evaluation fluctuate depending on the conditions carried-out in the respective investigation [167]. Most studies operate with blood and a 7.5ml input volume, while in our carried out work, 2 ml of plasma samples was used [168]. Added the number of samples used (n=15), which was also low compared with most researches, and these set of differences might explain why the CTCs count was lower in the obtained results. Moreover, SCLC patients tend to show a higher count of CTCs compared to NSCLC' individuals; however, this was not observed in our results as LC_{18} and LC_{19}, both SCLC, showed a similar count as the other NSCLC samples. Once again, the previous justification for the low CTCs count observed in our results might also explain the non-existent difference found for the histological subtypes.

Overall, the lack of healthy individuals to serve as asymptomatic controls (AC) was a limitation, as specificity of the methylation levels of our gene promoters were not attained. This

meant false positives and true negatives could not be assessed, and consequently a cut-off value was not calculated. This requirement was not accomplished in this study due the involved control restriction employed during current world's pandemic. Furthermore, a large patient cohort, in future work would be needed since the small sample size (n=26) in some length affected the results.

VI. Conclusion and Future Perspectives

In conclusion, the microfluidic assisted strategy successfully allowed the detection and analysis of two circulating cancer biomarkers (CTCs and cfDNA). The system processing did not compromise posteriori DNA extraction and qMSP analysis from plasma samples.

The cfDNA promoter's gene methylation analysis of *ADCY4*, *MIR129-2* and *HOXA11* revealed a potential for these genes to be used in a qMSP analysis for LC diagnosis (approximately 81% of sensitivity). Late-stage patients displayed a higher number of methylated genes than early-stage individuals. Furthermore, only $HOXA11_{me}$ levels associated with tested clinicopathological features (histological subtype, metastatic dissemination, smoking habits) showed statistical significance.

No association was found between CTCs, and dsDNA quantification and qMSP of selected genes. Nonetheless, late-stage individuals tended to show higher number of CTCs with increase dsDNA quantification and high number of promoter methylated genes. Overall, both biomarkers might bring complementary information that could be crucial for lung cancer assessment, particularly regarding an early on-set LC detection.

Considering the preliminary results obtained, as future work we propose to:

- Increase the patient cohort and include healthy donor (asymptomatic controls) for the study validation.
- Develop a methylation-specific droplet digital PCR (ddMSP) assay with selected genes, to overcome the limited sensitivity of qMSP.
- Assess the device efficacy for cancer monitoring, by patient follow-up.

VII. References

Uncategorized References

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VIII. Appendix

Supplementary Table 1. qMSP evaluation (Ct amplification) of methylated *ACTβ*, *ADCY4*, *MIR129-2, HOXA11* and *MAGI2 genes* in circulating cell-free DNA of standard groups.

Target Genes in a 40 cycle qMSP	Multiplex panel 1 (60ºC)				Multiplex panel 2 (60ºC)				Multiplex panel 3 (64ºC)			
	ACTβ		*NID2*		*ACDY4*		*MIR129-2*		*HOXA11*		*MAGI2*	
NTC	-	36.49	35.98	35.70	-	-	-	-	-	-	-	-
H20 PREAMP	31.85	31.69	-	-	-	-	-	-	-	-	-	-
CRL $^{1-}$	15.18	15.23	34.98	35.34	34.25	33.95	-	-	35.79	35.64	27.16	26.94
CRL $^{1+}$	15.76	15.83	20.27	20.37	16.59	17.88	18.02	19.12	17.14	17.48	17.50	17.65
CRL $^{2+}$	18.01	18.02	22.72	22.90	19.14	19.28	20.54	20.65	19.66	19.83	20.04	20.19
CRL $^{3+}$	20.37	20.64	25.13	25.23	21.78	21.50	22.91	22.77	21.97	21.97	22.32	22.36
CRL $^{4+}$	22.82	22.79	27.57	27.36	24.04	24.04	25.01	25.01	24.48	24.53	24.86	24.85
CRL $^{5+}$	25.51	25.29	30.07	30.02	26.80	26.81	27.69	27.61	27.51	27.41	27.61	27.47
CRL $^{6+}$	28.68	28.51	33.29	32.88	29.99	29.88	30.61	30.51	30.03	30.43	30.48	30.48

Supplementary Table 2. qMSP evaluation (Ct amplification) of methylated *ACTβ*, *ADCY4*, *MIR129-2, HOXA11* and *MAGI2 genes* in circulating cell-free DNA of each evaluated LC samples (n=26).

Target Genes in a 40 cycle qMSP		Multiplex panel 1 (60ºC)						Multiplex panel 2 (60ºC)						Multiplex panel 3 (64ºC)					
		ACTβ			*NID2*			*ACDY4*			*MIR129-2*			*HOXA11*			*MAGI2*		
EARLY LUNG CANCER SAMPLES	LC_1	23.04	22.99	22.99	-	-	-	37.28	34.49	35.10	32.77	33.08	33.16	33.49	32.62	32.64	32.32	32.94	33.06
	LC_2	22.69	22.73	22.66	-	-	-	32.93	32.76	32.79	34.31	35.82	35.07	-	-	-	-	-	-
	LC_3	22.36	22.65	22.45	-	-	-	31.50	31.10	31.12	31.98	32.13	31.92	-	-	-	-	-	-
	LC_4	22.81	22.72	22.61	-	-	36.23	35.05	36.25	38.07	-	38.62	-	32.97	34.25	33.35	-	-	-
	LC_5	22.73	22.61	22.69	-	-	-	35.93	34.79	34.21	32.17	32.94	35.42	38.22	37.96	37.42	-	-	-
	LC_6	21.96	21.94	21.86	-	-	-	33.70	39.65	38.47	36.27	36.28	37.10	30.77	31.36	30.98	-	-	-
	LC_7	22.14	22.15	22.15	35.70	-	-	35.03	38.53	-	37.20	36.87	39.78	-	-	-	-	-	-
	LC_8	23.05	23.06	22.93	-	-	-	34.59	-	35.72	-	-	36.92	-	-	-	-	-	-
	LC_9	22.97	22.93	22.85	-	36.31	-	-	39.49	-	38.50	-	38.28	31.81	31.96	32.88	-	-	-
	LC_{10}	21.48	21.67	21.36	-	-	-	34.51	-	35.09	-	-	-	36.76	37.05	36.68	-	-	-
	LC_{11}	22.85	22.94	22.87	37.53	-	-	35.03	35.48	-	32.51	32.68	32.19	-	-	-	-	-	-
	LC_{12}	22.31	22.53	22.26	37.57	-	-	-	39.39	35.10	38.15	36.90	38.03	38.20	37.94	37.90	-	-	-
	LC_{13}	24.03	24.12	24.11	35.44	34.39	35.74	34.39	34.75	34.74	39.38	37.52	37.12	37.47	37.16	38.31	-	-	-
LATE LUNG CANCER SAMPLES	LC_{14}	23.94	24.12	23.85	31.33	30.97	31.23	28.76	28.68	28.57	27.98	28.25	28.12	29.58	29.81	29.88	-	-	-
	LC_{15}	25.65	25.65	25.60	-	-	-	35.44	-	36.60	32.96	32.43	32.58	36.44	-	-	-	-	-
	LC_{16}	21.87	21.93	21.70	31.00	31.53	31.34	31.46	31.51	32.15	30.47	30.91	30.62	30.27	30.56	30.37	38.11	36.79	36.24
	LC_{17}	21.73	21.74	21.60	-	-	-	29.92	29.84	29.67	30.20	30.10	29.56	28.98	28.84	28.85	30.73	30.70	30.24
	LC_{18}	21.13	20.99	20.92	32.59	31.60	31.97	32.88	32.50	32.04	30.74	30.98	31.05	30.38	30.55	30.79	-	-	-
	LC_{19}	20.21	20.21	20.26	27.49	27.73	27.81	27.19	26.86	26.41	25.97	25.69	25.43	24.65	24.73	24.73	27.49	27.54	27.45
	LC_{20}	20.70	20.63	20.76	33.95	-	-	27.26	27.33	28.05	36.39	36.40	35.29	26.08	26.40	26.44	27.11	27.11	27.23
	LC_{21}	22.80	22.66	22.79	-	-	37.68	33.39	32.44	33.05	29.34	29.33	29.09	29.33	29.15	29.04	30.68	29.99	30.16
	LC_{22}	23.42	23.61	23.36	31.42	31.67	31.71	34.11	-	-	-	-	-	28.34	28.50	28.47	29.40	29.13	29.31
	LC_{23}	22.13	22.09	21.99	30.14	30.20	30.01	32.25	30.91	34.40	30.85	30.36	35.39	28.11	28.04	27.69	28.71	28.85	28.06
	LC_{24}	22.11	21.95	21.98	33.41	32.12	33.17	27.23	27.33	27.28	30.28	30.41	30.37	31.74	30.90	31.08	-	-	-
	LC_{25}	22.62	22.59	22.34	30.97	31.86	31.06	31.18	30.57	30.18	30.45	29.95	29.97	29.44	29.29	29.36	30.00	30.11	30.18
	LC_{26}	23.09	23.04	23.04	32.00	33.15	32.34	29.40	29.77	30.04	30.06	30.62	30.64	31.45	31.64	31.92	-	-	-