Inês Domingues Oliveira

Licenciada em Ciências Biomédicas

Pós-Graduada em Gestão

Detection of *BRAF* V600E mutation in ctDNA by ddPCR: from laboratory to market

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Professora Doutora Maria do Carmo Fonseca, Instituto de Medicina Molecular da Faculdade de Medicina da Universidade de Lisboa e GenoMed S.A.

Co-orientador: Professora Doutora Fernanda Llussá, Faculdade Ciências e Tecnologia da Universidade Nova de Lisboa

Júri:

Presidente: Professora Doutora Isabel Sá Nogueira

Arguente: Doutor Pedro Silva

Vogais: Professora Doutora Maria do Carmo Fonseca

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2018

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Constituição do júri

Presidente: Professora Doutora Isabel Sá Nogueira, Professor Associada com Agregação, Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

Arguente: Doutor Pedro Silva, Diretor do Departamento de Transferência de Tecnologia do Instituto de Medicina Molecular

Vogal: Professora Doutora Maria do Carmo Fonseca, Instituto de Medicina Molecular da Faculdade de Medicina da Universidade de Lisboa e GenoMed S.A.

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Resumo

O melanoma cutâneo é a forma mais agressiva de cancro da pele e possui alta taxa de mortalidade. Os métodos atualmente usados para classificar o melanoma e detetar metástases são técnicas de imagem e biópsias de gânglios sentinela (SLNB). No entanto, estes apresentam sensibilidade limitada para a deteção de melanoma em estadio inicial e muitas vezes não fornecem evidências clínicas a tempo de recidiva da doença ou de monitoramento de terapias.

O ADN tumoral circulante (ctDNA) pode ser um biomarcador específico em doentes com melanoma que apresentem a mutação V600E no gene *BRAF*. O ctDNA é libertado pelas células cancerígenas na corrente sanguínea e, em seguida, excretado na urina, permitindo que seja analisado por biópsia líquida. Esta análise fornece um *snapshot* em tempo real da carga tumoral e representa uma alternativa não invasiva, económica e que pode ser realizada repetidamente.

Neste trabalho, uma ferramenta de vigilância foi implementada para ajudar doentes com melanoma, através da deteção da mutação *BRAF* V600E em ctDNA por *Droplet Digital PCR (*ddPCR*)*. Em primeiro lugar, concentramo-nos no desenho e otimização do ensaio para garantir um baixo limite de deteção e uma distinção entre falsos positivos e verdadeiros positivos. Em segundo lugar, testamos o ensaio mostrando que é possível detetar o a mutação V600E no gene *BRAF* em doentes com melanoma, em amostras de plasma e urina média.

Além da implementação do teste, foi explorada uma estratégia de entrada no mercado para a comercialização do teste através de um kit de recolha. Este teste é direcionado para laboratórios, clínicas e hospitais que querem fornecer aos seus médicos ferramentas para ajudar no melhor diagnóstico, prognóstico e tratamento de seus pacientes. Ao desenvolver e analisar o mercado, conclui-se que, o novo teste tem vantagens sobre os concorrentes existentes no mercado e tem um potencial para melhorar a gestão de melanoma.

Palavras-chave: biópsia líquida, ctDNA, melanoma, *BRAF* V600E, ddPCR

Abstract

Cutaneous melanoma is the most aggressive form of skin cancer and has a high mortality rate. The current methods used to stage melanoma and detect metastases are imaging techniques and sentinel lymph node biopsies (SLNB). However, these have limited sensitivity for the detection of early stage melanoma and often can't provide timely clinical evidence of disease recurrence or for monitoring therapies.

Cell-free tumour DNA (ctDNA) could be a specific biomarker in melanoma patients which present V600E mutation in *BRAF* gene. ctDNA is released by cancer cells into the bloodstream and then excreted in urine, allowing it to be analysed by liquid biopsy. This analysis provides a real-time snapshot of tumour burden and represents a non-invasive, cost-effective alternative that can be performed repeatedly.

In this work, a surveillance tool was implemented to help patients with melanoma through the detection of *BRAF V600E* mutation in ctDNA by Droplet Digital PCR (ddPCR). Firstly, we focused on design and optimization of the assay to ensure a low limit of detection and a distinction between false-positives and true-positives. Secondly, the assay was tested in order to show the possibility to detect V600E mutation in *BRAF* gene in plasma and midstream urine samples from melanoma patients.

In addition to the test implementation, a go-to-market strategy was explored for the commercialization of the test through a collection kit. This test is directed to laboratories, clinics and hospitals aiming to help doctors in the diagnosis, prognosis and treatment of their patients. After a market analysis, we concluded that the new test has advantages over existing competitors in the market and has a potential to improve the melanoma management.

Keywords: liquid biopsy, ctDNA, melanoma, *BRAF* V600E, ddPCR

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Abbreviations

ABCD	Asymmetry border colour diameter
AKT	Protein kinase B
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CT	Computed tomography
CTC	Circulating tumour cells
cfDNA	Cell-free DNA
cfRNA	Cell-free RNA
ctDNA	Circulating cell-free tumour DNA
ddPCR	Droplet digital PCR
dNTP	Deoxynucleotides triphosphate
dPCR	Digital PCR
dsDNA	Double strand DNA
DGS	*Direção-Geral da Saúde*
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal–regulated kinase
EU	European Union
FDG	Fluorine 18 fluorodeoxyglucose
FDA	Food and Drug Administration
GDP	Gross domestic product
iBRAF	BRAF inhibitor
IL-2	Interleukin 2
ISO	International Organization for Standardization
iMEK	MEK inhibitor
iMM	*Instituto de Medicina Molecular*
IRR	Intern return rate
IVD	In vitro diagnostics
KIT	KIT proto-oncogene receptor tyrosine kinase
LDH	Lactate dehydrogenase
LoD	Limit of detection
MAPK	Mitogen-activated protein kinase
MEK	MEK proto-oncogene
MPS	Massive parallel sequencing

NF1	Neurofibromim 1
NPV	Net present value
NTC	No template control
OS	Overall Survival
PD1	Programmed cell death protein 1
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PET	Positron emission tomography
PCR	Polymerase chain reaction
PFS	Progression free survival
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
R&D	Research and development
RAS	Ras proto-oncogene
RAF	Rapidly Accelerated Fibrosarcoma
RNA	Ribonucleic acid
SLN	Sentinel lymph node
SLNB	Sentinel lymph node biopsy
TERT	Telomerase reverse transcriptase
UV	Ultraviolet
WT	Wild-Type

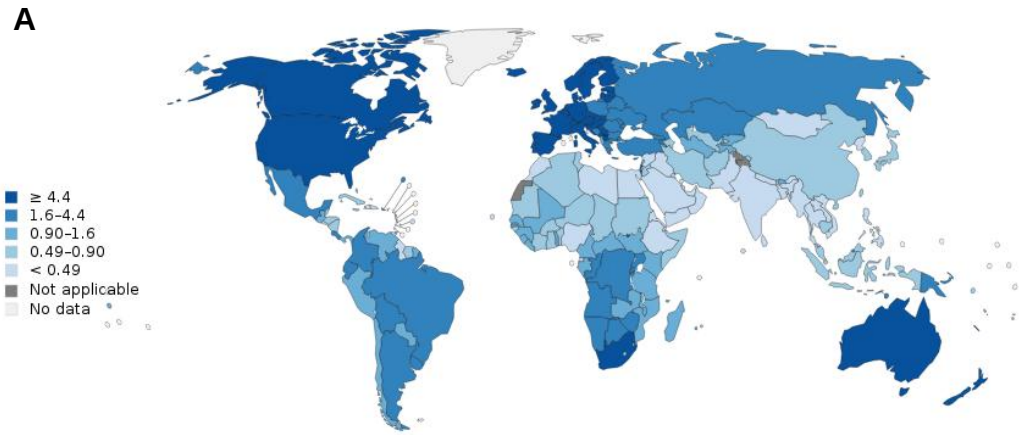
Chapter 1 - General Introduction

1. Melanoma and precision medicine

Cancer represents a great threat for the public health and it is considered one of the main causes of death. In 2013, there were recorded 14.9 million new cases and 8.2 million of deaths in the entire world. Its incidence rate is growing since 1990 and aging has contributed for this increase (20 a 43%)[1]. Cancer is considered the second main cause of deaths, only behind the cardiovascular diseases[1,2]. In 2012, in Europe, 3.45 million of new cancer cases had begun and 1.75 million of deaths occurred[3]. Some studies show that the cancer incidence should grow in the next years due to aging, population growth and the prevalence of some risk factors[1].

One of the types of cancer that has its incidence increasing is malignant melanoma. Much of this increase is believed to be associated to an exposure to intense ultraviolet (UV) light in childhood. Melanoma is a skin cancer that represents 2% of all skin cancers but causes 90% of skin cancer deaths[4]. The melanoma is an aggressive malignancy originated from melanocytes. These cells originate from the neural crest and migrate to the skin, meninges, mucous membranes, upper oesophagus, and eyes. In all these locations, melanocytes can turn into malignant transformation[5].

The World Health Organization estimates that approximately 132,000 melanomas are diagnoses per year and that increased has occurred in the last years in both men and women[6,7]. There will be approximately 50,000 annual deaths worldwide because of melanoma[5]. It is considered the fifth most common cancer among men and seventh among women. The leading cause of death in melanoma patients is the large number of metastases that can occur in lymphatic system and other organs like as lung, liver, bone and brain[4,6].



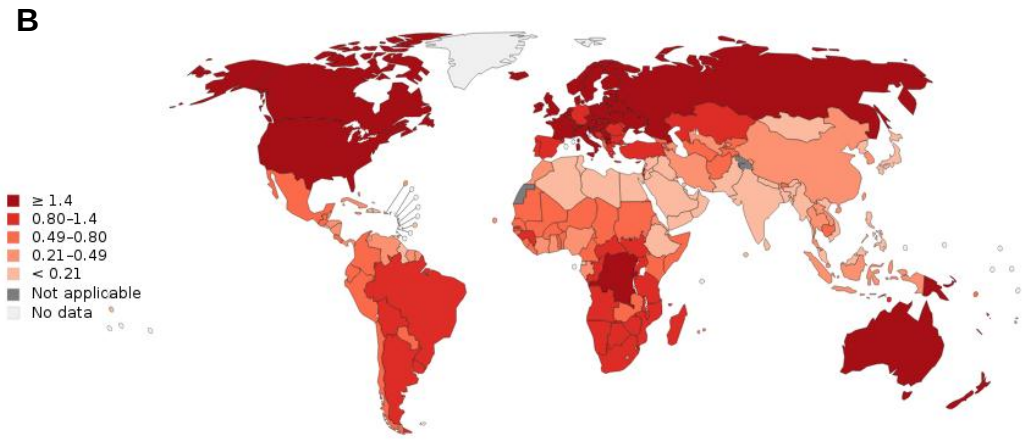


Figure 1 - Estimated age-standardized rates (World), both sexes, melanoma of skin, worldwide in 2012 (A) of incidence (B) of mortality. From GLOBOCAN *2012, World Health Organization.*

Melanoma has great variability by geographic region and ethnic group. In a study conducted only in United States, Horrel *et al* reported that the most susceptible to malignant melanoma are the caucasian[8] and is most common in Europe, North America and Oceania[8].

While incidence rate of melanoma increased since 1990 in different countries, at the same time it has been achieved a huge knowledge about its molecular genetics[9]. All cancers are mainly a genetic[2,9–12] and epigenetic[2,12] disease. Usually, it is a result of alterations that take to the inactivation of tumour suppressor genes and activation of proto-oncogenes (inherited or somatic mutations)[9,11]. These genes are responsible to regulate the cellular growth, the proliferation of cells, the cellular adhesion and apoptosis [9]. As a result, alterations in them can cause the development of a tumour.

This is a slow development that happens in many steps and that can be turned into malignant tumour. The tumour will present an uncontrolled cellular growth[10], loss of normal function and many times presents metastasis[11] leading to cancer diagnosis. Metastasis are the dissemination of tumour cells from the initial position to distant locals all over the body. This stage presents a great challenge to the medical treatments and also is responsible for the great number of deaths related with cancer[13], including malignant melanoma.

Although all tumours have different patterns related to their genetic alterations[2], there have been always some identic physiologic alterations. These changes occur self-sufficiency in growth signals, insensitivity to growth-inhibitory, dysregulation of apoptosis, unlimited replication, high angiogenesis, and tissue invasion (metastasis)[10]. However, with regard to therapies, the main important focus is checking the differences in tumours. Its aim is getting the best efficiency in treatments. Due to this need to recognize these differences, the approach of precision medicine emerges.

Inside the oncology field, the precision medicine consists of identifying specific molecular lesions that mainly results in oncogenesis followed by therapeutic strategic adoptions that could cause inhibition to the specific driver oncogene and could avoid the tumour growth. In other words, it consists in treatments directed to the individual patients needs considering the genetic characteristics of their diseases[14–18]. According to this meaning, the goal is to improve the clinic results; moreover, it takes to the reduction of the collateral effects that arise when a patient is less prone to a specific treatment. Furthermore, the patients with these identified molecular lesions get better results. On the other hand, patients without these mutations (probably because they wouldn't respond to those therapies) would stay less exposed to the expensive and toxic therapies.[15]

To identify the molecular alterations, it is necessary high throughput genomic technology, because these alterations may be punctual mutations, small deletions or insertions, amplifications, translocations, gains or losses, fusions and other structural variants. Precision medicine becomes an interdisciplinary field between the various interested parts since medical, clinical, biological, technical and biotechnological knowledge[16]. Precision medicine in melanoma can become a good tool to improve the outcome of patients. However, for this it is necessary to understand the disease molecularly and identify targets are of interest.

2. Molecular signalling in malignant melanoma

MAPK pathway

The RAS/RAF/MEK/ERK pathway is one of the most known pathways involved in melanoma progression. This pathway can be activated by several extracellular factors that bind tyrosine kinase receptors (TKIs). Then, they bind to cytoplasmic effectors and ultimately nuclear effectors that regulate cell behaviour like proliferation, differentiation, survival, and apoptosis[19].

Firstly, an extracellular signal (hormones, cytokines or growth factors) interact with membrane receptors (tyrosine-kinase – like KIT). In a second step, this signal induces the RAS-family GTPases from the inactive GDP-bound state to the active GTP-bound state activating a GTP belong to the RAS family (protein situated at the plasma membrane)[20]. Moreover, the RAS is can be regulated by NF1 negatively[21]. The RAS activation results in the activation of the serine-threonine RAF. RAF is a family of three serine threonine-kinases (ARAF, BRAF and CRAF), and the activation of BRAF is obtained after binding to RAS alone, whereas additional signals are required for activation of ARAF and CRAF. After activated, it can activate both MEK1 and MEK2 through the phosphorylation of two serine residues[22]. Lastly, the MEK can activate the ERK by phosphorylation. The ERK can translocate to the nucleus and regulate the activation of a variety of transcription factors responsible for controlling a lot of biological activities such as differentiation, proliferation and cell survival[23].

This means that the regulation of this pathway is very important to maintain homeostasis in response to extracellular signals. It has been shown that hyperactivation of this pathway may result in tumourigenesis[24].

PI3K/AKT pathway

PI3K belongs to a family of lipid kinases that can phosphorylate inositol phospholipid. Activation of PI3K can occur by RAS or receptor tyrosine kinases and causes phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). Finally, activated PI3K activates AKT and it translocate to the nucleus where many of its substrates are located. AKT regulates several targets resulting in cell survival and proliferation[22]. In addition, PTEN is a PIP3 phosphatase and regulates the PI3K-AKT pathway negatively[21].

Mutations presents in melanoma

In malignant melanoma, both RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT signalling pathways may be activated through multiple mechanisms and due to this are considered key pathways in the development and progression of melanoma. Mutations in these pathways are identified in melanomas and many often are considered target-therapeutic[21,22,25].

Inside the MAPK pathway, *NRAS* are mutated in approximately 20%[26], *BRAF* between 50-70%[25] and *KIT* in approximately 1% of melanomas[21]. However, *KIT* mutation is mostly found in mucosal and acral lentiginous subtypes[27]. On the other hand, some patients present mutation in PI3K/AKT pathway in *AKT* (≈3%) (which can be amplified) and in *PTEN* (≈12%)[25] that can be deleted or undergo epigenetic silencing. In addition, a mutation in *NF1* is in approximately 10-15%[21] of melanomas and may affect both pathways described.

Considering the prevalence of mutations present in melanoma, it can be classified genomically into four subtypes: *BRAF* mutant, *NRAS* mutant, *NF1* mutant and triple wild-type (WT). Therefore, both pathways, MAP kinases and PI3K/AKT, which promote proliferation and inhibit apoptosis, respectively, may be responsible for mutations present in melanoma.

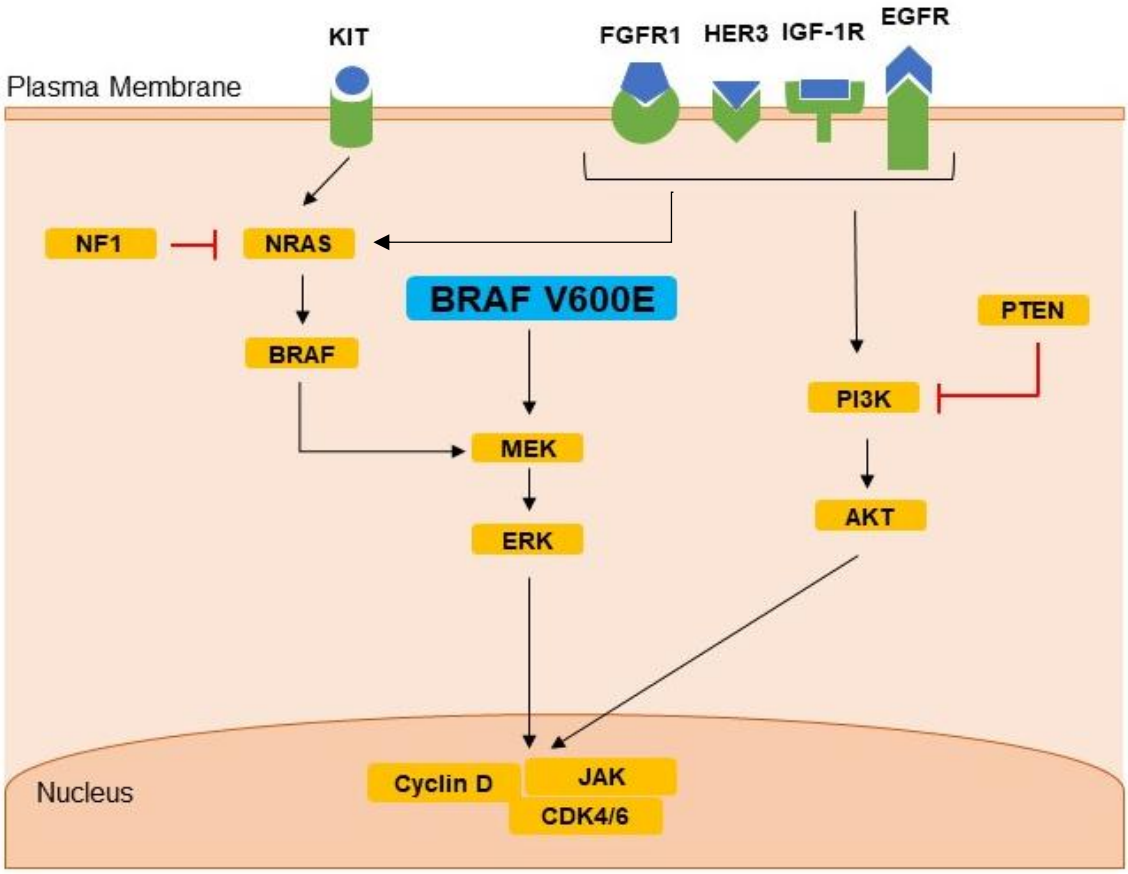


Figure 2 - Key molecular signaling to the clinical treatment of melanoma. Advanced stage melanoma can be classified according to mutational profiles. These mutations are usually in the *BRAF*, *RAS* or *NF1* genes in approximately 50%, 20% and 20% of patients, respectively. These mutations have as their main characteristic the activation of ERK signaling. In addition, they also have mutations in the PI3K/AKT pathway. Therefore, most melanomas can be corrected by therapeutic inhibition through one of these pathways. Adapted from Luke *et al* 2017.

According to cBioPortal [28], mutations in these two pathways together account for up to 80% of melanomas, showing the importance of them in the diagnosis and therapeutics of this disease.

BRAF V600E Mutation in Melanoma

The sequence *BRAF* V600E for the malignant melanoma were initially identified from Davies *et al* in 2002. His group identified a 1799T-A transversion in chromosome 7 (7q34) in exon 15 of the *BRAF* gene, that leads to the replacement of a thymine by an adenine at position 1799[24] and a substitution of Valine for Glutamic Acid. *BRAF* V600E mutation is the most common, comprising 90% of all *BRAF* mutations[29]. It stimulates the constitutive activation of the MEK/ERK pathway and can make it independent of the extracellular factor previously reported as required for activation. This happens because the substitution of Valine for Glutamic acid, it destabilizes the hydrophobic profile of protein and allows constant access to the active site. This lead directly to abnormal differentiation, proliferation, inhibition of apoptosis in melanocytes, and, finally, a tumourigenesis[23].

Moreover, there are other mutations that can be found in codon 600 from *BRAF*. For example, the substitution of a Valine for a Lysine - *BRAF* V600K - responsible for 5-12% of the *BRAF* mutations. In addition, V600R (Valine to Arginine), V600D (Valine to Aspartic acid), V600M (Valine to Methionine) mutations can occur in malignant melanoma[30].

The great advantage of detecting this mutation is the existence of a driver therapy, which results in very promising results in patients with melanoma. More specifically, the *BRAF* V600E variant is an attractive target for molecular therapy. Inhibitors against BRAF (iBRAF) and the primary downstream MEK (iMEK) has produce very promising results[4,31].

3. Clinical Trials Evidence in Melanoma

Since it was approved by Food and Drug Administration (FDA) in 1976, Dacarbazine, remained for many years the first-line treatment in melanoma advanced cases. Dacarbazine is a cell cycle nonspecific antineoplastic agent that functions as an alkylating agent after activation in the liver. This drug presented a rate of 10-20% response in the Phase I and II studies and without clear benefits of overall survival[31]. Other chemotherapies are also used in the treatment of melanoma as temozolomide, paclitaxel, albumin-bound paclitaxel (Abraxane) and carboplatin[32].

However, owing the progress in immunobiology and molecular signalling pathways in melanoma, new therapies have been developed in recent years. In the last 10 years several immunotherapies and molecular target therapies have been approved for the advanced melanoma treatment[21,31].

Relative to immunotherapies, approval of interleukin 2 (IL-2) in 1998 was the first for treatment of stage IV melanoma. However, the use of IL-2 is limited because it is substantially toxic and has a modest benefit[33]. Nevertheless, in recent years the focus in melanomas and other cancer has shifted to the use of monoclonal antibodies to increase cell-mediated immunity. This approach is known as immune checkpoint inhibitors. The first immune checkpoint inhibitors approved to melanoma treatment was Ipilumab in 2011 (anti cytotoxic T-lymphocyte–associated antigen 4) and then Pembrolizumab and Nivolumab (anti programmed cell death protein 1 – antiPD1) in 2014. These therapies have been demonstrated a significant increase in overall survival rate, and long-term benefit[34].

Regarding molecular target therapies, drug development has grown significantly with the discovery of *BRAF* mutant in more than half of the melanomas. The first *BRAF* inhibitor (iBRAF) was Vemurafenib. This drug had a high benefit in metastatic melanoma when compared to Dacarbazine. A Phase III study started in 2010 (BRIM3) showed that median progression free survival (PFS) was 5.3 months with Vemurafenib versus 1.6 months with Dacarbazine and soon

in 2011 was approved by the FDA[35]. In 2013 Dabrafenib (iBRAF) was also approved with clinical benefit very similar to Vemurafenib[36].

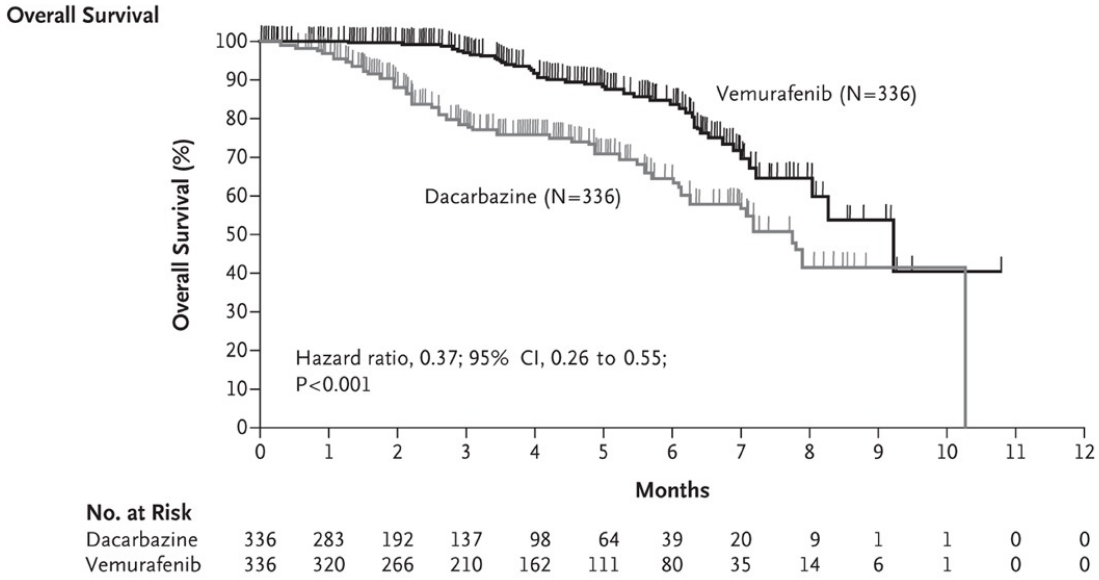


Figure 3 - Kaplan–Meier estimates of survival in patients in the intention-to-treat population. Comparison between Dacarbazine (chemotherapy) and Vemurafenib (BRAF inhibitor) for 336 days. From Chapman *et al* 2011.

With the MEK downstream of BRAF on the BRAF/ERK pathway, the development of MEK inhibitors (iMEK) have also begun to be studied for the treatment of melanoma. In 2013, Trametinib (iMEK) was approved to advanced melanoma based on the survival benefit in the phase III (METRIC) trial[36]. More recently, another iMEK was approved, Cobimetinib in combination with Vemurafenib. All these new therapies had a higher survival rate when compared to traditional chemotherapy[21].

The great advantage of *BRAF* V600E detection is to use iBRAF and iMEK when patients have this specific mutation. Wagle *et al* showed high efficiency of iBRAF after 15 weeks of treatment. However, the same patient developed resistance to therapy and this is a major challenge of molecular targets therapies[37].

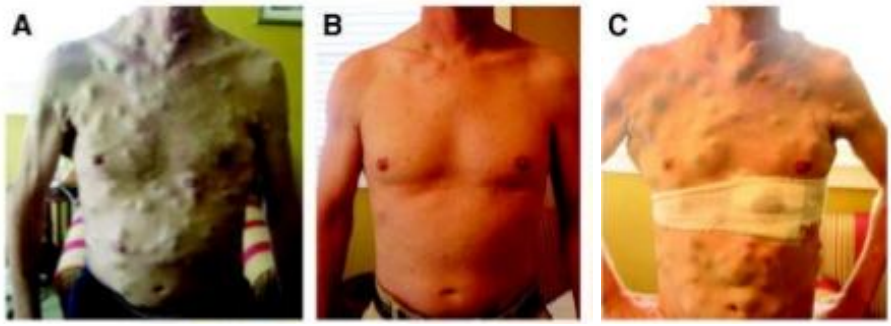


Figure 4 - The resistance to RAF inhibitor. Man, with *BRAF* mutant melanoma and miliary, subcutaneous metastatic deposits. Photographs were taken (A) before initiation of RAF inhibitor, (B) after 15 weeks of therapy with RAF inhibitor, and (C) after relapse, after 23 weeks of therapy. From Wagle *et al* 2011.

Commonly, oncogene-addicted tumours develop resistance to therapeutic inhibition. The genetic mechanisms of resistance to kinase inhibitors are normally within two categories (mutations in target kinase or gene changes within signalling life that can compensate for inhibition of the target protein). Nevertheless, the iBRAF resistance mechanisms are still not clear. One of the mechanisms used to combat this resistance is the combined use of iBRAF and iMEK. This combination can delay resistance and avoid the development of secondary malignancy. Moreover, this strategy increases the efficacy of tumour treatment. At present, several of new target therapies in combination are in different stages of preclinical and clinical trials[21].

Nowadays, the great advantage of molecular target therapies when combined iBRAF with iMEK is the fast and remarkable response of patients. On the other hand, immunotherapies present long-term results for over life. Due to these factors, interest in combining target therapies and immunotherapies has emerged. Triple combination therapy of iBRAF, iMEK and immunotherapy has already been initiated in clinical trials. For example, Dabrafenib+Trametinib in combination with Ipilimumab (NCT01767454) and combination of MPDL3280A (anti-PDL1 antibody) with Vemurafenib+Cobimetinib (NCT01656642)[31].

One of challenges of this triple combination is the hepatotoxicity of this treatment. However, it is believed that it will overcome this challenge and achieve better results in the treatment of melanoma. Initial reports using this triple combination suggest that they have tolerable responses like those observed in the combination of iMEK and iBRAF[38].

With all the possibilities of treatment, it is important to notice the need of a tool that helps to take in clinical decisions.

4. Current clinical methods to melanoma management

Malignant melanoma early detection is a very important factor in order to decrease the mortality rate. The recognition in time and fast detection and treatment are critical factor for the success of the treatment[39].

Unlike other cancers, melanoma has a cutaneous location that facilitates non-invasive diagnosis. There are several methods of diagnosis in melanoma like the ABCDs (Asymmetry, Border irregularity, Colour variegation, Diameter >6mm), and other technologies like dermoscopy, ultrasound and bioimpedance. However, current methods for genotyping require invasive biopsies of the tumour[39].

At the present, several studies are looking for genetic abnormalities in patient's tumours. Based on the genetic results, patients with advanced tumours that have stopped responding to standard therapy are enrolled into a drug that might target the specific molecular pathology driving their cancer. In other words, when the goal is the detection of genetic markers (cancer driver mutations) for the application of targeted therapy, it is still necessary to perform an invasive biopsy for its detection[16,40].

Nevertheless, the big problem is not the first biopsy in diagnosis, as it would be a single invasive procedure. The great challenge is to monitor the tumour and realize the effectiveness of the treatment without performing multiple invasive sequential biopsies and at a later stage controlling the possibility of recurrence. Moreover, melanoma is a highly metastatic disease and numerous invasive biopsies would be required to perform the diagnosis and monitoring of disease.

Currently, there are no reliable early markers of melanoma recurrence and monitoring. Sentinel lymph node biopsies (SLNB), radiologic imaging techniques[41,42], and measurement of blood lactate dehydrogenase (LDH)[43] are currently the methods used to stage primary melanomas, detect metastases, monitor the disease, and detect recurrence.

In patients with melanoma, an important predictor of survival in patients with metastases is the measurement of LDH. LDH is the only blood-based biomarker that has been incorporated in the staging system. However, LDH is non-specific and increases with many conditions and malignancies other than melanoma[43,44].

Regarding SLNB, is a valuable tool that provides prognostic information and can help identify patients at high risk for recurrence. Not all patients are subject to an SLNB, however, usually in patients with lesions>0.76mm can be performed to determine the metastatic dissemination. The sentinel node is identified by use of a blue dye and a radioisotope around primary site. After identification, the nodes are removed and sent to histological analysis where they can identify melanocytes[45,46].

Another method that is often used to help in disease staging and monitoring of response to therapy is FDG-PET/CT. Positron emission tomography (PET) with a labelled glucose analogue fluorine 18 fluorodeoxyglucose (18F-FDG) combined with computed tomography (CT) scans

(FDG-PET/CT) are usually used to determine the disease burden[42]. CT provides information about the location of lesions on any part of the body, while the PET informs details about normal and abnormal tissue metabolism. Due to the Warburg effect, tumour cells have high rates of glucose utilization[44]. Because of this, it is possible to use a labelled isotope (18F-FDG) in order to check which cells are having a high metabolic rate. Currently, this method is used to evaluate the therapeutic response of melanoma as well as other types of cancer. However, when the tumour mass is evaluated can have presence of more collagen, extracellular matrix or even greater infiltration of lymphocytes. This may cause FDG-PET/CT to become inconclusive as to the amount of tumour cells present during the monitoring of treatment. Moreover, it is expensive and subject patients to excessive radiation exposure[44].

Due to limitations, other tools have been explored to complement conventional methods. The use of this emerging tool could help to achieve timely interventions that could result in better treatment options with a positive impact on quality of life and patient survival.

Dawson *et al* evaluated 30 patients with metastatic breast cancer who compared the evaluation of liquid biopsy with CT. The results showed that ctDNA was able to detect resistance to treatment and identified progressive disease much earlier than radiological examinations, proving the credibility of using ctDNA as a surveillance tool[47].

In other words, liquid biopsy can be an important tool in melanoma monitoring. The presence of *BRAF* V600E in more than 50% of melanoma cases is a great opportunity for the development of a new surveillance tool based on liquid biopsy.

5. Liquid Biopsies

Conventional biopsies provide a diagnosis for the molecular alterations of tumour DNA. However, this procedure presents several limitations. First, it is sometimes not possible to perform biopsies because it is an invasive procedure and depends on tumour location[13,48–51], which can result in surgery complications and clinical risks[49]. In addition, another limitation is a non-detection of the tumour heterogeneity[13,52]. Cancer is heterogeneous and presents different genetic profiles in different areas of the same tumour, well as it also presents heterogeneity between the metastasis in the same patient[53]. Because of these limitations in conventional biopsies, it is necessary to develop new alternatives for a better effective and efficient application of precision medicine.

One of these alternatives is to obtain knowledge of tumour molecular profiles from liquid biopsies[54]. The tumours release into the blood circulation free nucleic acids, tumour-derived vesicles and tumour cells. Therefore, as blood contains samples like circulating cell-free tumour DNA (ctDNA)[55–59], cell-free RNA (cfRNA)[60,61], circulating tumour cells (CTCs)[30,54] and exossomes[54], it becomes a reliable tool to obtain of the necessary molecular information about the tumour. Moreover, ctDNA is the most viable and reliable alternative. The ctDNA seems to be

the best choice to research the spatial and temporal genomic heterogeneity of the tumour[47]. Dawson *et al* [47] show a direct comparison between the ctDNA and CTCs in the same patients. This comparison shows that the ctDNA levels present a higher correlation with the tumour burden, being the most sensitive biomarker and being also the first to show response to the therapy. Furthermore, Bidard and his colleagues[62] have stated that ctDNA is more abundant than CTCs in the bloodstream.

These positive characteristics suggest that the use of ctDNA can be a great alternative to identify mutations and monitor response to specific therapeutics.

Circulating cell-free tumour DNA

Cell-free DNA (cfDNA) is a blood-based biomarker of short fragments of extracellular DNA that are released from cells into the bloodstream. Most of the cfDNA in circulation is derived from normal cells, although, when the patient has cancer the tumour DNA can be released into the circulatory system and is called circulating cell-free tumour DNA (ctDNA)[63].

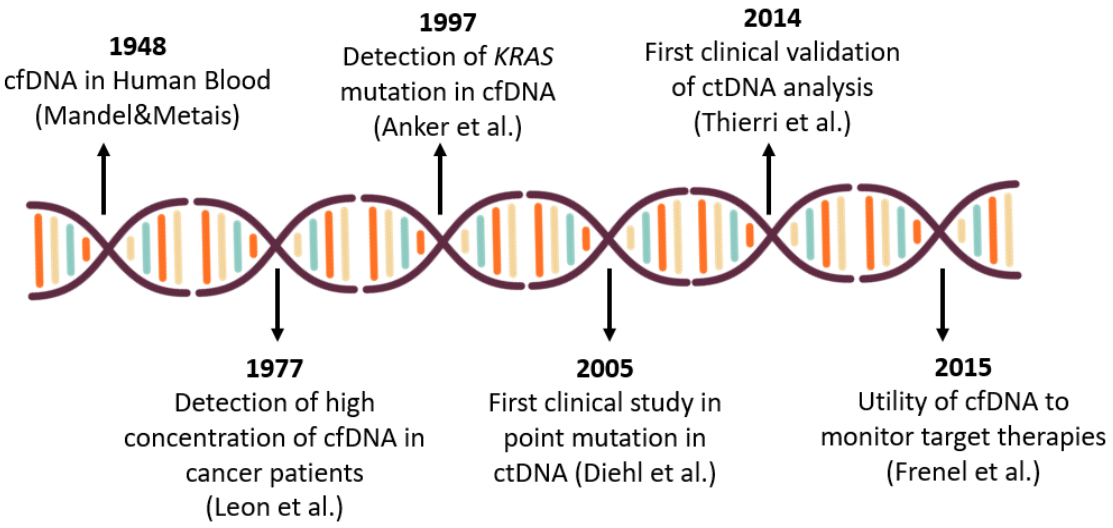


Figure 5 - Timeline of significant historical findings regarding circulating tumour DNA (ctDNA) between 1948 and 2015. Adapted from Dominguez-Vigil *et al* 2018.

The first step in discovering the potential of cfDNA for genetic testing of cancer was made with Mendal & Metais in 1948 when they published the first work about cfDNA in human bloodstream[64]. However, the origin of this DNA was still unknown. Only in 1997 Leon and colleagues show that cancer patients had higher levels than in normal control subjects[65]. From this moment, a search for studies was initiated that would elucidate this relation better; however, the technology of the time was limited. Nevertheless, other studies came to show that the origin of cfDNA was correlated with the presence of the tumour. In 1997, one group analysed patients with colorectal adenocarcinoma. Interestingly, they found that the *KRAS* mutation was present in the tumour and

also in the cfDNA of these patients[66]. From this moment the possibility of analysing the mutations present in the tumour through the blood emerged.

Hence, with the great advance of the technology in the last years, the search for the use of cfDNA as a potential tool of diagnosis and surveillance has grown.

ctDNA properties

Although the existence of ctDNA is currently fully accepted, the way that DNA tumour is release into the bloodstream is not yet fully understood, studies suggest that it occurs by necrosis, apoptosis or by active secretion[52]. Early studies showed that necrosis and apoptosis were more likely to contribute to the release of DNA into the circulation, but, García-Olmo *et al* suggest that active secretion of circulating DNA acts as a molecular message system and plays a role in the development of metastatic disease in distant sites[67].

As much the tumour increases its volume, the number of apoptotic and necrotic cells also increase. Usually, these cell debris are eliminated by nucleases, renal excretion[68] or by phagocytic cells like macrophages. Nevertheless, this mechanism of elimination doesn't occur so efficiently within the tumour mass and generates a greater release of DNA into the bloodstream[52]. The increase of this concentration may be due to abnormal chromatin degradations or the intensive proliferation of tumour cells, which inhibit their phagocytic activity. In addition, low vascularization in some tumour areas may result in increased cell necrosis[68].

Diehl *et al* estimated that ctDNA increases up to 3.3% in circulation every day and when are metastases the release of ctDNA into the circulation is greater[69]. This observation shows the ability to reproduce the tumour burden by ctDNA.

The fragments of cfDNA has less than 300bp in length in the circulation[70]. Heitzer and his colleagues believe that there may be variation in the size of cfDNA in healthy patients and in cancer patients. However, there are variations in size within patients and may relate to the way the DNA is released[71]

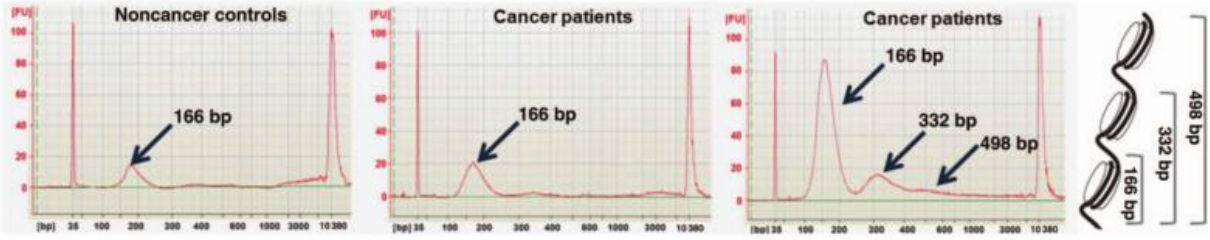


Figure 6 - Size distribution of plasma DNA samples from healthy individuals and cancer patients. From Heitz *et al* 2015.

Another important property of cfDNA is the half-life time. Between release into the bloodstream and its posterior elimination, the ctDNA has a half-life time between 16 minutes and 2.5 hours[52]. This time allows the blood test to be considered optimal for monitoring the disease stage and to provide a real-time molecular snapshot of the tumour[72].

Moreover, ctDNA mirrors tumour specific molecular lesions and especially its heterogeneity. ctDNA can be a great tool in cancer diagnosis, prognosis, residual disease detection, selection of treatment by molecular markers and monitoring disease progression.

Body fluids with ctDNA

The liquid biopsies that use the cfDNA analysis can be realized in different body fluids ranging from saliva, pleural effusion, cerebrospinal fluid, ascitic fluid, urine and blood[52]. In the blood is where we can find the highest concentration of ctDNA[73], and therefore is the principal target of study in liquid biopsies[74]. Although the ctDNA concentration in the blood is higher, it is also eliminated from the bloodstream. This elimination occurs through nucleases, liver, spleen and also undergo renal excretion[71].

As the ctDNA undergoes a renal excretion, many studies suggest a urinary ctDNA analysis, mainly because it is a less invasive procedure[68,75–77]. After ctDNA crosses the renal barrier, some ctDNA fragments appear in the urine between 150-300bp[68]. Due to the contribution of the urinary system, the concentration of DNA in the urine is greater than in the circulation[78]. This increase in the total concentration of the DNA difficult even more the detection of the ctDNA that appears in the lower proportion in the urine when compared to the blood. In both, plasma and urine, the cfDNA is in low concentrations. Due to this, the great challenge is to identify technologies capable of detection the molecular targets with great sensitivty[79]. Therefore, it would be possible to get better results in the ctDNA analysis in biological fluids, mainly those of bigger interest: blood and urine.

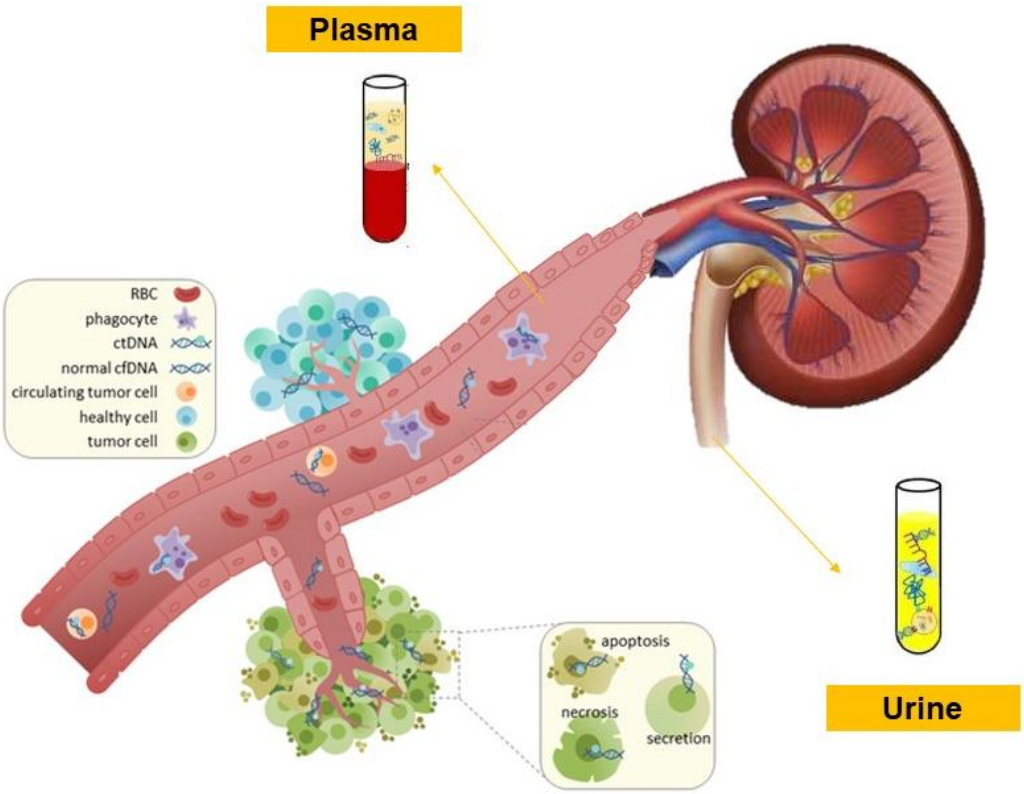


Figure 7 - Release and extraction of cfDNA from the blood and urine. cfDNA is released from healthy or diseased (cancerous) tissue from cells undergoing apoptosis, necrosis or active secretion. Adapted from Crowley *et al* 2013.

6. GenoMed and liquid biopsies

Due to the great potential of liquid biopsies and high incidence of melanoma, a Portuguese company GenoMed S.A. has decided to focus on the development of a tool capable of detecting *BRAF* V600E in ctDNA in both type of samples: blood and urine.

GenoMed – Diagnósticos de Medicina Molecular S.A. is a spin-off from *Instituto de Medicina Molecular* (iMM). It was founded in 2004 and its objectives is to transfer the scientific knowledge of iMM to clinical practice. The GenoMed's vision is to provide cutting-edge solutions for molecular diagnostics in medicine, with the following objectives:

- Maintain a leading role in diagnostic services;
- Provide a molecular diagnostic to hospitals and other health service to reduce investment costs;
- Train doctors and researchers at the interface between fundamental science and its technological application;

Its mission is to create value by applying technical and scientific expertise in the molecular biology and genetics. It promotes better diagnosis and prognosis of diseases and better evaluation and therapeutic responses monitoring. GenoMed operates in two main areas of medicine: genetic diseases and oncology.

Today, GenoMed is licensed by *Direção-Geral da Saúde* (DGS) for Genetics and Molecular Pathology (License nº 0075L/2007) and certified by the NP EN ISO 9001:2008 (2009/CEP.3390) standard within Quality Management.

GenoMed is located in the *Faculdade de Medicina da Universidade de Lisboa (*Campus *Centro Hospital Lisboa Norte*). The proximity to the Medical Academic Center of Lisbon allows a direct and unique contact with hospital clinicians, researchers and professors, which drives GenoMed to develop new solutions to the real and current problems of human health. The relationship between fundamental research and clinical practice leads the company to put science at the service of health.

The company responds to molecular diagnosis by public, private, national and international healthcare providers. To provide these answers, GenoMed has a multidisciplinary team with specialized academic training. The ability to respond to the challenges by the constant progress of medical knowledge is carried out by the Scientific Council that has members with high scientific differentiation.

To differentiate itself from other laboratories, GenoMed has a great investment in research and development. The company created GenoResearch, a research and development unit dedicated to medical research projects in partnership with clinicians and industry for the development of innovative products/test.

Currently GenoResearch is investing in the development of tests applied to precision medicine, more precisely in liquid biopsies. From this area, the development and a transfer to the market of the development of the “Val600AcidGlutamic mutation in *BRAF* gene in Circulating Tumour DNA” emerge.

7. Research Significance and Aims

In recent years it is clear the growth of melanoma incidence worldwide making even more important to develop tools that help in better clinical decisions. In addition, the use of liquid biopsies is an innovative and affordable approach, and therefore with great commercial potential for GenoMed S.A and also improving the health of melanoma patients.

We hypothesized that is possible to use a reliable tool for the detection of the *BRAF* V600E mutation in patients with melanoma in samples such as plasma and urine (in the respective ctDNA).

The first aim of this thesis was the was the exploration of the possibility of implementing a tool capable of detecting with high sensitivity the *BRAF* V600E mutation in blood and urine from melanoma patients. This study is performed in two phases. First, we will identify the best method and develop an assay to detect *BRAF* V600E in ctDNA. Second, samples of melanoma patients, known to harbour the mutation of interest, will be used to test the protocol developed.

On the other hand, since the liquid biopsies are a recent test in a poorly explored and growing market, the second aim of this thesis is the development of a go-to-market strategy for the tool describe in the previous paragraph. This tool could be commercialized as a service by Genomed developed, and here, we verify the feasibility of a new business model for the national and international market.

The main goal is offer validated tests with competitive price that can be used in the clinic to melanoma management. The development of the test and its subsequent commercialization will have positive impact to health of melanoma patients and also as has a a great value proposition that can be explored by GenoMed.

Chapter 2 - Optimization of *BRAF* V600E detection in ctDNA by ddPCR

1. Introduction

How to detect ctDNA?

The detection of ctDNA in total cfDNA requires the use of molecular methods and it is based in genetic or epigenetic differences between cfDNA and ctDNA. There are two main methods for ctDNA analysis: polymerase chain reaction (PCR) based techniques and massive parallel sequencing (MPS) based techniques[80]. The first consists of candidate-gene analysis and the second, typically, consists in deep sequencing[81].

Although the PCR-based techniques need the prior knowledge about the exact genomic modifications for research, they have many advantages over the MPS-based techniques. The sensitivity of the MPS-based techniques depends on how deep is the sequence and the instrument's error rates[81]. In addition, it requires a very specific bioinformatic analysis and these are longstanding procedures. On other hand, PCR-based techniques have highly sensitivity, don't require a bioinformatic analysis, are economically viable and are appropriate for detection of point mutations, copy number variation, short indels and gene fusions. These advantages make PCR-based techniques much more attractive and easy to implement in the clinical setting[71,82].

Within PCR-based techniques, there are some methods that stand out for ctDNA detection: quantitative PCR (qPCR), Digital PCR (BEAMing and ddPCR), Inplex and Endpoint PCR. However, Digital PCR presents less susceptibility to the qPCR inhibitors and don´t require a calibration curve or intern control[83]. Furthermore, when comparing the limit of detection (LoD) of all these methods, the best option is Digital PCR, and even so the ddPCR (LoD: 0,001%) has a lower LoD when compared to BEAMing (0,01%)[71,81,82]. Since plasma and urine samples have very low concentration of ctDNA, and ddPCR shows highly sensitive results, it is likely that this will become great tool for detection of *BRAF* V600E in ctDNA, in patients with malignant melanomas.

Droplet Digital PCR

The Polymerase Chain Reaction (PCR) was developed in 1983 by biochemist Kary Mullis, who was the Nobel Prize winner in 1993 for his pioneer work. Using the PCR it is possible to obtain millions to millardsof copies of a specific section of DNA from a small amount of samples[84] Because of this procedure, this technique innovates the way DNA is studied and has become fundamental to a large set of research. The PCR is based on the amplification of a desired sequence with a polymerase and on the use of the pair of primers that hybridize to the sequence of interest, creating a template DNA. It is required that the mix would occur in a repeated number

of the DNA denaturation cycles, primers hybridization and finally, their extension, create an exponential increase of the copy numbers in the amplified region[85,86].

When qPCR appeared, new possibilities have arose. It allowed the quantitation of the progression of amplification after each cycle using fluorescence probes. However, to estimate the concentration, this technique required external calibrators or endogenous controls, which limit their accuracy to the complete quantifications[84]. Due to these limitations of the qPCR, a new concept emerged: the digital PCR. The fundament of digital PCR (dPCR) is to quantify the total number of target present in a sample, using limited dilutions, PCR and Poisson statistics[87].

The dPCR uses the same primers and probes as qPCR, nevertheless, with a great sensitivity, accuracy and don't need calibration and internal controls (necessary in qPCR). Therefore, dPCR can merge small differences in gene expression and identify alleles that occur in one from milliards. Furthermore, the advances in dPCR, led to the development of the Droplet Digital PCR (ddPCR) causing a higher impact in the diagnostic fields. The ddPCR produces thousands of reactions partitions taking to a larger precision in the nucleic acids detection at a molecular level. ddPCR becomes a great highlight tool in the detection of punctual mutations[88] like *BRAF* V600E, in ctDNA.

Fundaments of ddPCR

The ddPCR workflow begins as another PCR assay in which the DNA is combined with buffer, dNTPs (deoxynucleotides), primers and DNA polymerase. Moreover, the ddPCR uses hybrid probes with fluorescent to detect the amplified product. In a single use cartridge, the mix is charged into individual wells followed by the oil charge containing stabilizing surfactants[89].

After, the next workflow step is the one that makes the ddPCR unique. Microfluidic technology makes it is possible that the reaction mixture to be divided into thousands of small spherical droplets made from oil surface and aqueous nucleus that contain the PCR mix reaction. This happens by application of vacuum to the outlet wells, sample and oil are drawn through a flow-focusing junction where monodisperse droplets are generated at a rate of ≈1,000 per second. The surfactant-stabilized droplets flow to a collection well where they quickly concentrate due to density differences between the oil and aqueous phases, forming a packed bed above the excess oil. Subsequently, there are no more 20 µL of the PCR mix but 20,000 reactions of 1 nL each. This step is developed in a way that all droplets contain the required standard materials (enzyme, primers and probes), nevertheless, it will be a random distribution of droplets containing DNA mutant and DNA WT targets, so that the droplets contain 0, 1, 2 or more copies of each target[90].

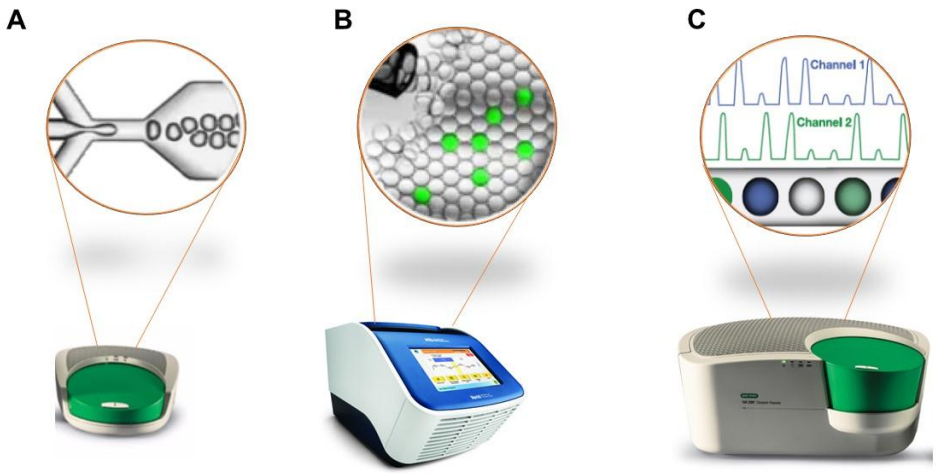


Figure 8 - Biorad QX100/QX200 ddPCR system workflow. (A) The reaction mixture is charged into a cartridge that will generate up to 20,000 droplets. The wild-type DNA and the mutant DNA are randomly distributed into the droplets (B) The droplets are transferred to a 96-well plate and subject to a PCR reaction in a thermocycler machine (C) The plate is loaded into the droplet reader. Positive and negative droplets are read, and results are analyzed in QuantaSoft.

In the next step, droplets are transferred to 96-well plate and PCR reaction is performed using a standard thermocycler. Then, the fluorescence of each droplet is read step by step continuously by a droplet reader (an instrument similar to a flow cytometer), which means that each droplet has an intrinsic fluorescence signal. Droplets that have a DNA mutant and DNA wild-type will fluoresce in the correspondent channel (positive droplets). The positive and negative droplets counting for each target are related to the target concentration in the sample by Poisson function (Equation 1)[91].

$$\lambda = -\ln(1-p) \quad \textbf{(1)}$$

Where λ is the medium number of copies by droplet and p is the proportional of positive droplets for the total droplet number. The copies per droplet, λ, can be converted for a number of copies per µL. This allows that target DNA concentration to be calculated without the use of standard samples, one of the key advantages of ddPCR[91].

These characteristics make ddPCR an extremely sensitive technique capable detecting small concentrations of DNA (like ctDNA samples) for the detection of point mutations like *BRAF* V600E in melanomas patients.

Technology EvaGreen and TaqMan in ddPCR

The sample preparation in ddPCR can occurs in a similar way to the qPCR. Namely, before the droplets generation, it is necessary to get the TaqMan hydrolysis probes which can be labelled with fluorophores reporters: FAM and HEX (or VIC). On the other hand, one can also obtain the quantification from EvaGreen intercalating dye[89]. Assays performed by the TaqMan method require specific primers for the sequence with the purpose of PCR and for labelling the oligonucleotide probe with fluorophore. The used probe has a quencher in the 3´ extremity and a fluorophore linked covalently in 5´extremity. While the probe is intact, the quencher stops the fluorescence due to its proximity. However, during the sequence amplification the polymerase synthetizes a new template, which takes to the degradation of the probe that is linked to the template strand. After that, the probes degradation takes to the fluorophore separation from the quencher and consequently, it results in a proportional fluorescence signal to the amount of the amplified sample[92,89].In addition, the ddPCR also allows the use of the intercalating dye EvaGreen. This strategy consists in the emission of fluorescence when the dye is associated to double strand DNA (dsDNA). The fluorescence amplitude variation is dependent on the amplicon length, therefore, the longer the length amplicon, the more EvaGreen molecules bind to the sequence, and thus the more fluorescence is emitted. Moreover, the dye don't inhibit the PCR which allows the use of the great dyes concentrations[92].

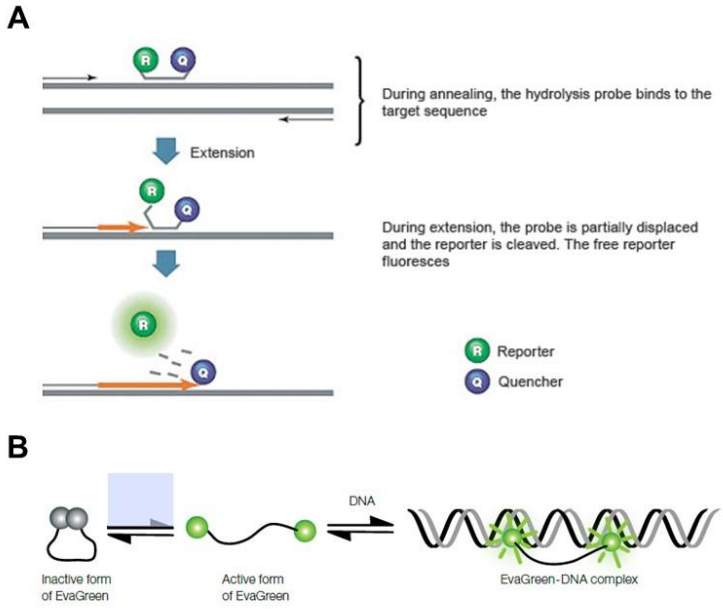


Figure 9 - Methods to ddPCR (A) TaqMan method (B) EvaGreen Method. From Bio-Rad Laboratories Guidelines.

Although the use of the hydrolysis probe has a higher associated cost, there are still more advantages in using the TaqMan method in punctual mutations like *BRAF* V600E. Some of these advantages are high specificity and the ability to perform multiplex reaction.

2. Materials and Methods

Patients

Four patients with malignant melanoma, and a positive tissue biopsy to *BRAF* V600E mutation, were studied. All biological samples to this study are collected prior to any treatment. This study was conducted in the GenoMed – *Diagnósticos de Medicina Molecular S.A..* All patients gave consent for specimen collection, clinical information collection, and biomarker analysis.

Sample Collection

For each patient, peripheral blood samples were collected in 10mL PAXgene Blood ccfDNA Tubes (Qiagen, Cat. No. 768115)), for the preservation of cell-free DNA. After drawing blood, samples were centrifuged in a time of up to 7 days at 1,600 x g for 10 minutes and after this the samples were centrifuged at 16,000 x g for 10 additional minutes at room temperature. The supernatants were collected, frozen, and stored at -80˚C until cfDNA extraction.

Urine samples were collected into 50mL corning tubes containing Urine Preservative Solution (Norgen Biotek, No. Cat. 18126), for the for the preservation of cell-free DNA at room temperature for over two years. The collection was conducted in two different moments: a) the first urine in the morning with 1st flow (urine 1st flow) b) the first urine in the morning without the 1st flow (midstream Urine). The Norgen Biotek Guidelines of Urine Preservative Solution don't recommend first flow for collection. However, both possibilities have been tested to draw our own conclusions. After collection, urine samples were centrifuged, to separate any present cells, at 200 x g for 10 minutes. Then, urine samples were centrifuged at 16,000 x g for 10minutes in Avanti^{TM} J-25 high-speed centrifuge (Beckman Coulter) at 4ºC. The cell-free supernatant was transfer to a new tube. After this, the cfDNA were extracted or, alternatively, stored at 4ºC until further use.

Controls

All PCR-based mutation detection experiments require a specific set of controls. When setting up the test we need a positive and negative control.

The positive control was obtained from Horizon Discovery. DNA standard is an EGFR T790M Reference Standard (Horizon Discovery, No. Cat. HD258**)** with an allelic frequency of 50%. However, this reference sample has allelic frequency to 66% for *BRAF* V600E. The standard was diluted using the corresponding wild-type standard to generate a lower allelic frequency. Positive controls helped us to verify the negative results, and to check that the conditions we used were able to amplify our template.

The negative control was obtained from digested genomic DNA of peripheral blood leukocytes from healthy individuals. They provided us with a way to measure our false positive rate.

Cell-free DNA extraction

Three DNA extraction kits were used for extractions: MagMAX™ Cell-Free DNA Isolation kit (Applied Biosystems™, ThermoFisher Scientific, Cat. No. A29319), QIAamp® Circulating Nucleic Acid (Qiagen, Cat. No. 55114) and QIAamp® MinElute ccfDNA Kit (Qiagen, Cat. No. 55204). The plasma samples were extracted exclusively by the QIAamp® MinElute ccfDNA Kit (Qiagen, Cat. No. 55204). Magnetic Separation Rack (OZ Biosciences, Cat. No. MSR1000) was used in the samples that were extracted through magnetic beads.

All the samples were extracted according to the kit per the manufacturer's instructions, with exception of QIAamp® Circulating Nucleic Acid (Qiagen, Cat. No. 55204). In this protocol, RNA carrier wasn't add, because it can inhibit downstream analyses.

All extraction methods tested are based on the interaction between silica and DNA. The key interactions for DNA binding to silica are intermolecular electrostatic interactions. In addition, dehydration of the surface of silica and DNA as well as intermolecular hydrogen bond also contribute to this affinity. There are several factors that affect this adsorption, from temperature, pH or ionic strength[93]. During extraction, these factors are controlled by the specific buffers of each respective kit.

The Applied Biosystems™ MagMAX™ Cell-Free DNA Isolation Kit is designed for isolation of cfDNA in human plasma, serum, and urine samples. The kit uses Dynabeads™ MyOne™ SILANE technology and extraction chemistry. Dynabeads™ MyOne™ SILANE are monosized ferrimagnetic beads, 1 µm in diameter and uniform. The beads are composed by polystyrene, magnetic material and coated by optimized silica-like chemistry.

The QIAamp® Circulating Nucleic Acid enables efficient purification cfDNA from human plasma, serum, or urine. The extraction is done in 4 steps (lysis, binding, washing, elution) and is performed using QIAamp Mini columns in a vacuum collector. After lysis, the goal is that, cfDNA binds to the silica membrane present in the QiAmp mini column where it will be adsorbed through the vacuum pressure. The salt and pH conditions ensure that there is no contamination of other biomolecules.

The QIAamp® MinElute ccfDNA kits use a two-step procedure. First, there is the pre-concentration of circulating nucleic acids onto magnetic beads. In a second step, adsorption of the cfDNA to the silica membrane occurs on QIAamp UCP MinElute column.

Cell-free DNA quantification

Qubit 3.0

The DNA concentration was performed by fluorometry using the Qubit® Fluorometer 3.0 (Invitrogen™, ThermoFisher Scientific). Based on the detection of target-specific fluorescence, this integrated system is more sensitive than UV absorbance–based quantification, making it ideal for precious samples and demanding applications as cfDNA.

The Qubit® dsDNA HS Assay (Invitrogen™, ThermoFisher Scientific, Cat. No. Q32854) and Qubit® dsDNA BR Assay (Invitrogen™, ThermoFisher Scientific, Cat. No. Q32850) quantification assays were used to quantify small and large-scale DNA preparations, respectively. In both cases they are highly selective for double-stranded DNA (dsDNA) over RNA. The first within the range of concentration 10g/µL-100ng/µL and the second between 100pg/µL-100ng/µL. All the samples were quantified according to the manufacturer's instructions. Two microliters of the DNA sample were diluted in 198 µL of HS/BR dsDNA reagent and buffer. They were then measured with respective DNA standards in ng/µL. The final concentration (ng/mL of biological sample) was calculated using the following formula.

$$[\text{Purified DNA}] = \frac{Qubit\ Concentration\left(\frac{ng}{\mu L}\right) x\ Elution\ Volume\ (\mu L)}{Extraction\ Volume\ (mL)} \quad \textbf{(2)}$$

$$[\text{Purified cfDNA}] = [Purified\ DNA]\ x\ \%cfDNA \quad \textbf{(3)}$$

Agilent 4200 TapeStation System

To measure the amount of cfDNA present in samples, DNA size distribution was analysed using Agilent 4200 TapeStation System (Agilent Technologies, Cat. No. G2991AA). The 4200 TapeStation system uses a ScreenTape gel matrix similar to an agarose gel, to separate the sample by molecular weight. Moreover, the sample is coated with an intercalating dye. From this, the percentage of DNA present at a specific molecular length (100bp-300bp) can automatically be calculated.

The Agilent High Sensitivity D1000 ScreenTape Assay kit and was used to quantify small and large-scale DNA preparations. The kit includes High Sensitivity D1000 ScreenTape (Agilent Technologies, Cat. No. 5067-5584), a High Sensitivity D1000 Sample Buffer (Agilent Technologies, Cat. No. 5067-5603) and a High Sensitivity D1000 Ladder (Agilent Technologies, Cat. No. 5067-5587). All the samples were analysed according to manufacturer's instructions.

Development of ddPCR method for the detection of *BRAF* V600E in ctDNA

Testing primers and probes

Primers and probes were obtained from Bio-Rad. Hydrolysis probes have Iowa Black Quencher on the 3' end while the 5´ end of the *BRAF* V600E probe was labelled with a FAM™ reporter fluorophore and the *BRAF* V600 WT probe with a HEX™ dye. The amplicon has a length of 91bp. To test whether the primers amplified specifically, and the probes bound reliably to the target sequences, we ran a preliminary ddPCR test.

The analysis of samples was based on the Droplet Generator and Droplet Reader QX200 manuals (Bio-Rad, Cat. No. 10031246, Cat. No. 10031246, respectively). All the primers, probes, and reaction mixes were prepared gravimetrically.

Amplifications were performed in a 20 µL reaction containing 1x ddPCR Supermix for Probes (No dUTP, Bio-Rad, Cat. No. 1863023), 1 x PrimePCR™ ddPCR™ Mutation Assay: BRAF p.V600E, Human (Bio-Rad, Cat. No. 10031246), 1 x PrimePCR™ ddPCR™ Mutation Assay: BRAF WT for p.V600E, Human (Bio-Rad, Cat. No. 10031249), 2-5 U/reaction HindIII restriction enzyme (NEB, Biolabs, Cat. No. R3104S) and 7,5 µL of sample DNA or water for NTC controls.

The reaction mixture (20 µL) was then loaded on eight-channel disposable Droplet Generator Cartridges (Bio-Rad, Cat. No. 1864008) with the respective gaskets (Bio-Rad, Cat. No. 1863009). Droplets were generated with 70 µL of droplet generation oil (Bio-Rad, Cat. No. 1863005) in the droplet generator of the QX200 system (Bio-Rad, Cat. No. 1864002). The generated droplets were transferred to a 96-well ddPCR plate (Bio-Rad, Cat. No. 12001925). After, they were sealed using the heat sealer PX1 (Bio-Rad, Cat. No. 1814000).

The plate was placed in a Veriti™ Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific, Cat. No. 4375786). Thermal cycling conditions were 30ºCx2min (one cycle) 95°C x 10 minutes (one cycle), 50 cycles at 94°C for 40 seconds and 55°C (annealing temperatures suggested by Bio-Rad Guidelines) x 1:40 minutes, 98°C x 10 minutes (one cycle) (ramp rate of 2°C per second), and then 4ºC hold. After PCR, the 96-well PCR plate was read on a QX200 Droplet Reader (Bio-Rad, Cat. No. 1864003).

Annealing temperature determination

Ideally, the temperature should be as high as possible to minimize unspecific PCR amplification while still allowing for a clear separation of positive and negative bands.

The annealing temperature was tested under the conditions described above. However, with a range of annealing temperatures from 54°C to 60°C with negative and positive control. All temperatures were analysed as technical duplicates.

Enzyme Digestion

The amplicon sequence (to detect *BRAF* V600E mutant and wild-type) was checked by NEBCUTTER v2.0 (NEB, Biolabs).

According to Bio-Rad guidelines (Bio-Rad, Cat. No. Bulletin 6407), for an optimal performance in ddPCR assay, a restriction digestion step should be performed before droplet generation and ddPCR. We next performed an on-bench HindIII restriction digestion for 0, 15 and 40 minutes at room temperature immediately prior to the addition of template DNA to the PCR reaction.

TouchDown PCR

We test an optimized ddPCR method for measurement of circulating DNA that employs a universal touchdown PCR thermocycling protocol for increased specificity.

Where thermal cycling conditions in Veriti™ Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific, Cat. No. 4375786) were 30ºC x 2 minutes (one cycle) 95°C x 10 minutes (one cycle), 50 cycles at 94°C x 40 seconds and where the annealing temperature starts at 65ºC and decreases 1ºC for each cycle to 55°C x 1:40 minutes, 98°C x 10 minutes (one cycle) (ramp rate of 2°C per second), and then 4ºC hold.

Data Analysis

The quality assessment results and the percentage of cfDNA present in the sample were analysed using 4200 TapeStation Software A.02.01 (Agilent Technologies). The concentration values are calculated using the area of the sample compared to the known concentration of the marker. The range selected as cfDNA comprised between 100bp-300bp. The software automatically calculates the percentage of DNA present in the sample corresponding to the selected size (Figure 23) according to the following equation:

$$\%\text{cfDNA} = \frac{[cfDNA]}{[Total\ DNA]}\ \text{(Measured by TapeStation Analysis)} \quad \textbf{(4)}$$

The concentrations of target alleles were calculated using QuantaSoft software 9.2.1 (Bio-Rad) based on Poisson distribution. For an optimal reaction, one would expect two clearly separated bans of positive and negative droplets in a 1D plot (x-axis: number of events, y-axis: fluorescence amplitude) and in a 2D plot (x-axis: fluorescence amplitude Ch2, y-axis: fluorescence amplitude Ch1). Briefly, a threshold was drawn for channel 1 and channel 2, respectively, to separate the

two clusters of negative and positive droplets. At the end, a 2-D plot is obtained where the droplets are classified as Negative Negative, Negative Positive, Positive Negative and Positive Positive. The threshold line was determined by the positive control samples.

To adjust our choice of manual threshold, an open source R package "twoddPCR" was used[93]. The droplet classification was based on the "k-means" method to separate droplets into 4 clusters, like in QuantaSoft. The clusters were classified based on the presence or absence of *BRAF* V600E mutation in the droplets. The droplet may contain only DNA with the mutation (*BRAF* V600E - detected on Ch1), DNA wild-type (*BRAF* V600 WT - detected on Ch2), both targets or no targets (empty droplets). The "MahanobisRain" function was used to remove ambiguous droplets between the four clusters.

After adjusting the threshold, ddPCR provides a concentration of sample to both channels in copies/µL. The final concentration (Copies/mL of biological sample) was calculated using the following formula.

$$\text{[Total DNA] (Copies/mL)} = \frac{ddPCR\ Concentration\left(\frac{Copies}{\mu L}\right) x\ Elution\ Volume\ (\mu L)}{Extraction\ Volume\ (mL)} \quad \textbf{(5)}$$

$$\text{[cfDNA] (Copies/mL)} = [Total\ DNA]x\ \%cfDNA \quad \textbf{(6)}$$

In addition, the ratio of the mutant DNA present in the wild-type DNA samples was calculated by fractional abundance according to the following formula.

$$\text{cfDNA Fractional Abundance (\%)} = \frac{[Ch1\ BRAF\text{V600E}]\ (\frac{Copies}{\mu L})}{[cfDNA]} \quad \textbf{(7)}$$

Limit of Detection

The goal of the proposed method was to quantify the *BRAF* V600E concentration in biological samples. One of the factors that needs to be evaluated in quantitative tests includes the Limit of detection (LoD). To calculate the LoD, it was first calculated the sensitivity of the assay, that is, the minimal concentration of detecting 1 unique mutant positive droplet in the experiment, according to the following equations:

$$\text{MinConcentration (Copies/µL)} = \frac{-ln(Accepted\ Droplets)}{(1+Acceoted\ Droplets)} * Vd\ (\mu L) \quad \textbf{(8)}$$

$$\text{Sensitivity (Copies/mL)} = \frac{MinConcentration\ x\ 20}{PCR\ Volume}\ x\ \frac{Elution\ Volume}{Extraction\ Volume} \quad \textbf{(9)}$$

The minimum concentration was calculated according to Poisson statistics and a droplet volume of 0.85 nL (volume incorporated in QuantaSoft for calculations) was considered. Finally, the Limit of Detection, that is, the fractional abundance of detecting 1 unique mutant positive droplet in the experiment was calculated according to following equations:

$$\text{LoD cfDNA (\%)} = \frac{MinConcentration}{MinConcentration + cfDNAWild-TypeConcentration} \quad \textbf{(10)}$$

$$\text{cfDNA Wild-Type Concentration} = DNA\ Wild-Type\ Concentration\ x\ \%cfDNA \quad \textbf{(11)}$$

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.1 (GraphPad Software). Means and standard error of mean were plotted and only the three assays with more droplets formed per extraction were considered for data analysis.

3. Results

HindIII is compatible to *BRAF* V600E detection

The first step in returning a tool capable of detecting a specific mutation is to check which restriction enzyme is to be used. NEBcutter, version 1.0, is a program accessible via a web server (http://tools.neb.com/NEBcutter) that will accept an input DNA sequence and produce a report of the restriction enzymes that will cleave the sequence[94]. In this display, only enzymes that cleave the sequence once are shown.

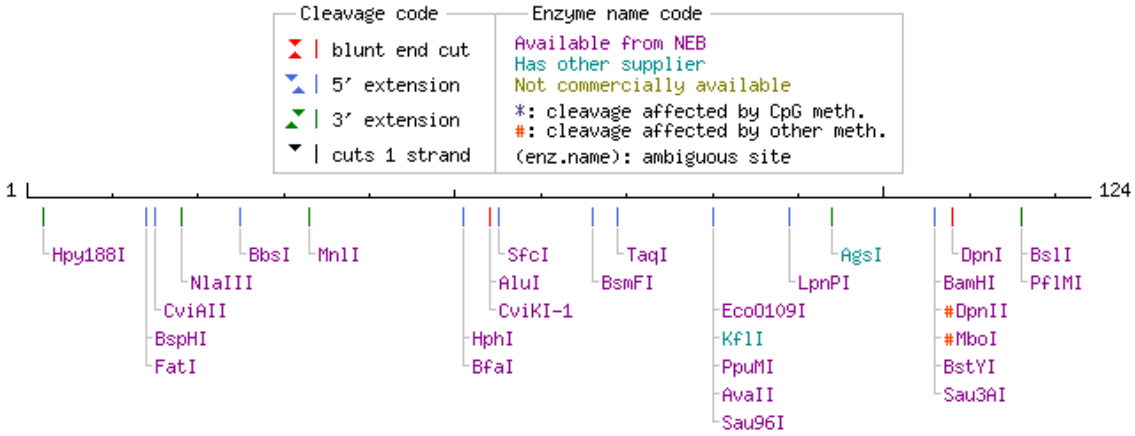


Figure 10 - The results of finding possible recognition sites for a specifics restriction enzyme with our target amplicon from NEBcutter version 1.0.

In ddPCR is necessary verification to demonstrate that the restriction site is not present within the target amplicon. In figure 10, it is possible to check all the restriction enzymes that cut the amplicon of interest. In this case, none of the enzymes that the Bio-Rad Guidelines mention as compatible (HaeIII, MseI, HindIII, CviQI) for the detection of mutant/wild-type *BRAF* V600E appears. Hence, we chose to use HindIII which is the enzyme recommended by Bio-Rad in the assay with our target amplicon.

Annealing temperature chosen is 55ºC

One recommended way for improving separation between positive and negative droplets in ddPCR is lowering the annealing temperature of the PCR. Ideally, temperature should be as maximal as possible to minimize the amplification of non-specific products while at the same time still has a clear separation of negative bands.After testing a temperature gradient with *BRAF* V600 WT *(BRAF* V600 Wild-Type*)* sample (Figure 11), it was noted that the lower temperatures led to a clear separation of the bands, whereas at high temperatures this separation became more difficult to distinguish. Lower temperatures also led to greater amplification of non-specific

products, which may result in false-positives. Because of this, assays with *BRAF* V600 WT and *BRAF* V600E *mutant* with a 1% allele frequency are also important to temperature determination.

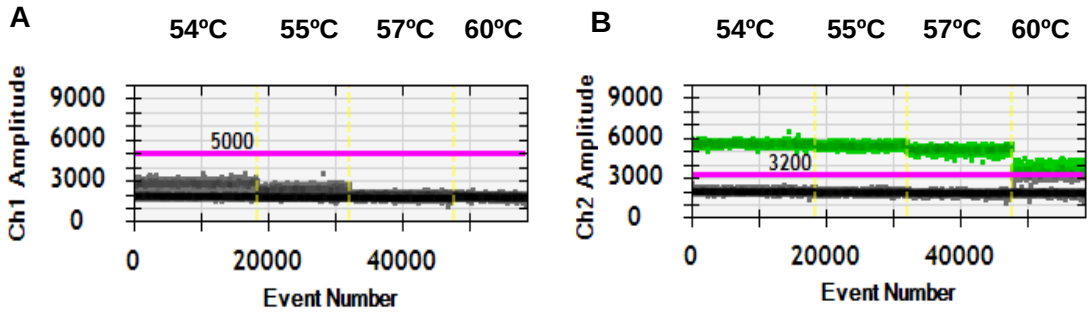


Figure 11 - QuantaSoft 1D plots. DNA *BRAF* V600 WT amplified at varying temperatures. (A) FAM signal from mutant probe binding to V600E (B) HEX signal from binding of WT probe.

Using a sample of the mutant *BRAF* V600E control, it is verified amplitude discrepant between temperatures in Channel 1 (Figure 12). At 55ºC, there are no positive droplets up to the amplitude of 8500, whereas, at 56ºC and 57ºC there is a frequent presence of positive droplets before this amplitude. All these data facilitate the finding of false positives. In addition, the delta amplitude[a] also decrease with increasing temperature, making it difficult to separate the clusters in Channel 1. Therefore, the optimum annealing temperature determined to detection of *BRAF* V600E in ctDNA by ddPCR is 55ºC.

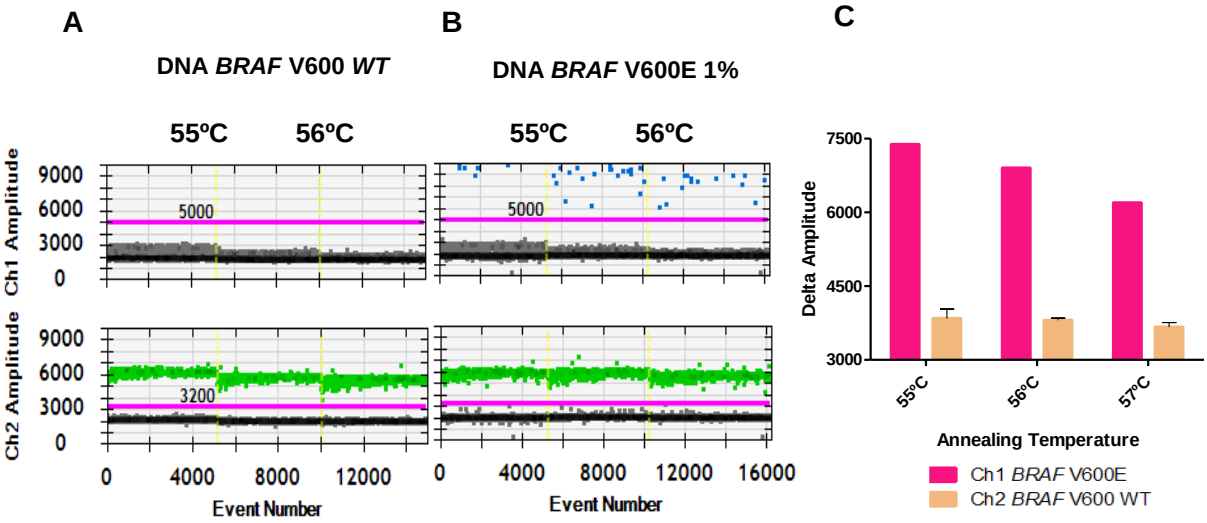


Figure 12 - DNA amplified at varying temperatures. Channel 1: FAM signal from mutant probe binding to V600E Channel 2: HEX signal from binding of WT probe. (A) DNA *BRAF* V600 WT (B) DNA *BRAF* V600E with a 1% allele frequency (C) Delta amplitude in varying temperatures in Channel 1 and Channel 2.

[a] Delta Amplitude = mean amplitude of positive droplets – mean amplitude of negative droplets

Touchdown PCR improve ddPCR results

Although the temperature chosen (55ºC) is a good temperature to separate clusters, exist the risk of amplification of false-positives. Because of this, we tested a Touchdown PCR. The touchdown PCR is a method where the initial cycles have a high annealing temperature while the following cycles are gradually decreasing the annealing temperature each cycle. The purpose of this PCR is to increase the specificity of the products when using low annealing temperatures. That is, the initial temperature should be several degrees above the temperature considered optimal, which benefits more specific annealing between the primer and the template. Thus, the first amplified sequences are the ones with the highest specificity. The annealing temperature is then gradually decreased. Finally, the amplification continues using annealing temperature determined[95].

When the Touchdown PCR was used, a lower dispersion of droplets in Channel 1 was obtained showing less amplification of non-specific products. This facilitates the differentiation between false-positives and true-positive. While in the PCR with Touchdown the amplitude variation does not reach 1000, without Touchdown it reaches 3000 (Figure 13).

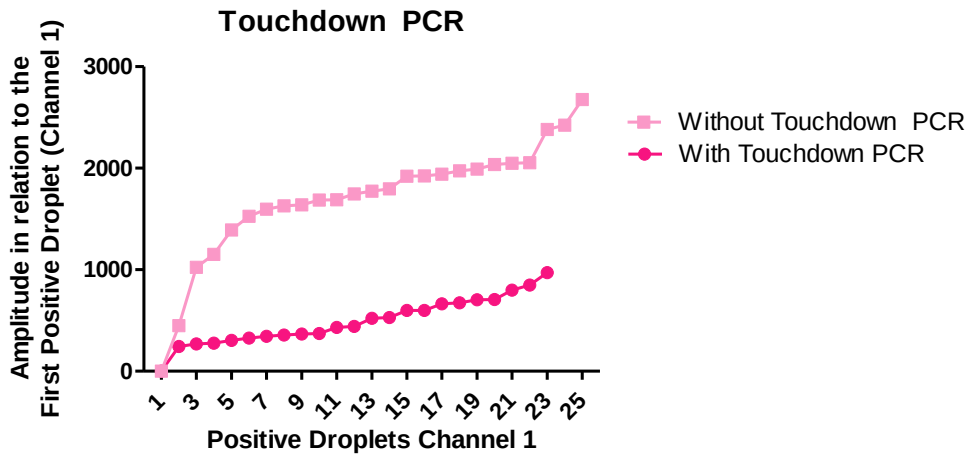


Figure 13 - Dispersion of droplets with *BRAF* V600E mutant with 1% allele frequency in Channel 1: FAM signal from mutant probe binding to V600E. Whit and without Touchdown PCR.

Incubation time chosen is 40 minutes

The incubation of the enzyme (Hind III) is a very important step for the subsequent division of DNA into 20,000 droplets. The longer incubation time, the more the enzyme cleaves the DNA, the more easily the DNA is divided by the droplets formed and the more easily the DNA is detected (better division of the DNA along the droplets may result in increased assay sensitivity). Due to this, an on-bench incubation of 0, 15 and 40 minutes was tested.

With 40 minutes of incubation the results presented larger Delta amplitude (Figure 14) the delta amplitude increased by 700. During PCR optimization, it is important that the delta amplitude be checked, because the greater the distance between the amplitude of the positive and negative droplets, the easier it becomes to distinguish between them.

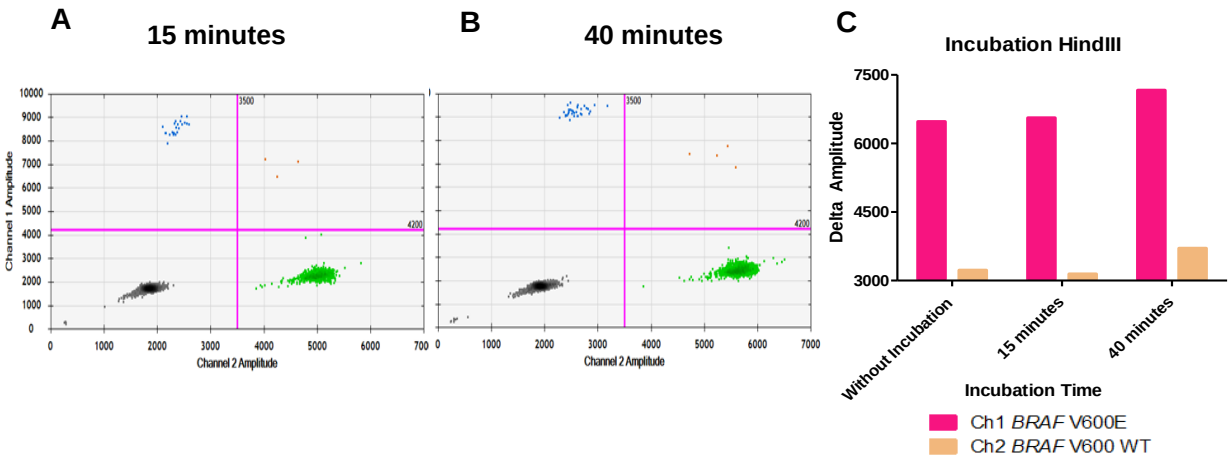


Figure 14 - DNA amplified at varying times of enzyme incubation with *BRAF* V600E mutant with 1% allele frequency. Channel 1: FAM signal from mutant probe binding to V600E Channel 2: HEX signal from binding of WT probe. (A) 15 minutes of incubation (B) 40 minutes of incubation (C) Delta amplitude in varying times of enzyme incubation.

This difference can be seen in the two graphs (Figure 14A and 14B). In the second graph, the blue cluster is clearly more distant from the grey cluster.

Finally, with 40 minutes of incubation, both, the blue cluster (DNA *BRAF* V600E positive) and the green cluster (*BRAF* V600 WT DNA positive) have a greater distance from the black cluster (double negative) indicating a functional assay.

ddPCR optimized to testing human samples

After the optimization of ddPCR it was determined which workflow will be used to test human biological samples.

ddPCR was performed using QX200 Droplet Digital PCR system (Bio-Rad). The final volume of the PCR mixture was 20µL, containing 10µL of ddPCR Supermix for Probes (No dUTP, Bio-Rad, Cat. No. 1863023), 1x PrimePCR™ ddPCR™ Mutation Assay: BRAF p.V600E, Human (Bio-Rad, Cat. No. 10031246), 1x PrimePCR™ ddPCR™ Mutation Assay: BRAF WT for p.V600E, Human (Bio-Rad, Cat. No. 10031249), 0,5 µL of HindIII restriction enzyme ((NEB, Biolabs, Cat. No. R3104S) and 7,5 µl of DNA sample. Before droplet generation the reaction mix were incubated 40 minutes. The droplets were generated by Droplet Generator QX200 (Bio-Rad, Cat. No. 1864002).

After the generation of droplets, the plate was placed in a Veriti™ Thermal Cycler (Applied Biosystems™, ThermoFisher Scientific, Cat. No. 4375786). Thermal cycling conditions were 30ºCx2min (one cycle) 95°C ×10 minutes (one cycle), 50 cycles at 94°C for 40 seconds and where the annealing temperature starts at 65ºC and decreases 1ºC for each cycle to 55°C × 1:40 minutes, 98°C ×10 minutes (one cycle) (ramp rate of 2°C per second), and then 4ºC hold. After PCR, the 96-well PCR plate was read on a QX200 Droplet Reader (Bio-Rad, Cat. No. 1864003).

ddPCR assay is functional

In order to test whether our assay was functional after its optimization, several controls were tested. Two negative controls (*BRAF* V600 *WT*) with different copy numbers, ≈200 and ≈2,000 copies/µL, a ≈200 copies copies/µL positive control (with 1% allele frequency) and an NTC control (no template control).

With the chosen conditions, the NTC (no template control) hasn't copies of WT nor *BRAF V600E* copies. The negative control has only WT copies and Fractional Abundance equal to 0. In turn, the positive control has WT copies, *BRAF* V600E copies and Fractional Abundance equal to 1.1%, as expected (Figure 15). Moreover, we tested a negative control with a higher concentration (≈2,000 copies/µL) with the aim of identifying false positives in samples with high concentrations (Figure 16).

All controls showed the expected results and therefore, the assay was considered functional for testing biological samples.

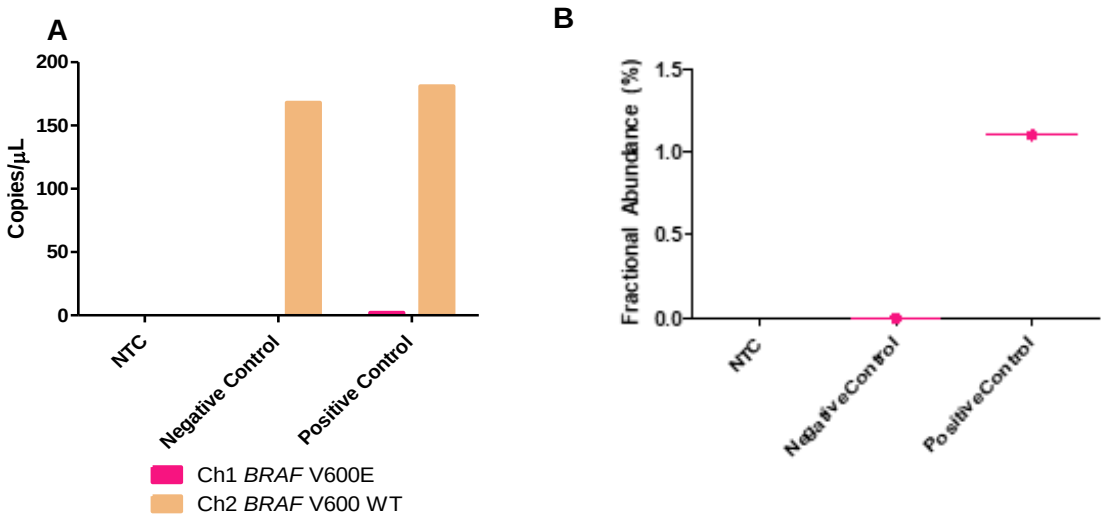


Figure 15 – Running controls with 200copies/µL with optimized assay. (A) Total number of copies that results from ddPCR (B) fractional abundance.

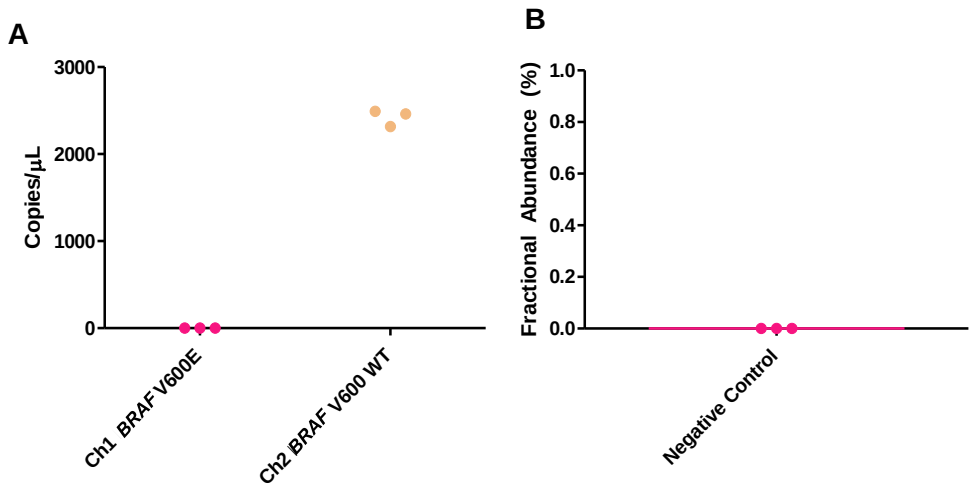


Figure 16 – Running negative control with 2,000 copies/µL with optimized assay. (A) controls concentration (B) controls fractional abundance.

cfDNA quality variation

To verify the cfDNA quality variation, the percentage of cfDNA in the sample were evaluated. As already reported, cfDNA has a size between 100bp-300bp. Therefore, not all extracted DNA is cfDNA. To obtain this information, we use the TapeStation 4200 was to calculate the percentage of cfDNA in the sample. The quality of the extracted DNA doesn´t depend exclusively on the sample concentration, but also on the amount of cfDNA extracted.

Because of this, we have decided to evaluate whether %cfDNA can influence the linearity or reproducibility of the assays, and consequently, ctDNA detection.

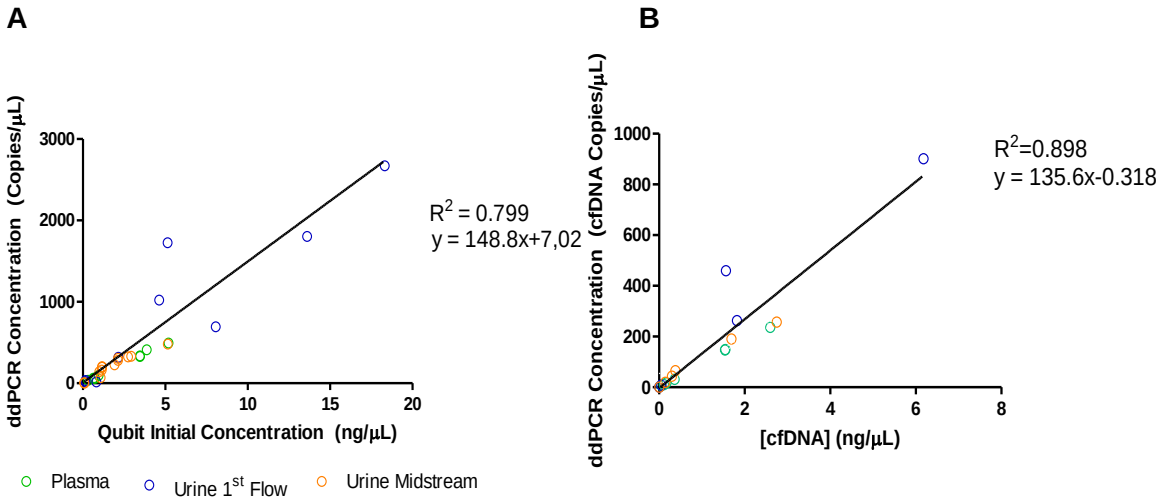


Figure 17 - Relation between ddPCR concentration (copies/mL of biological sample) and Qubit initial concentration (ng/µL) (A) total DNA (B) only cfDNA.

When analysing the ratio between the initial concentration obtained by Qubit 3.0 and the copies presented in ddPCR, it is noted that the ratio shows a much higher R^2 than when the %cfDNA obtained in the analysis of the TapeStation 4200 was considered (Figure 17). This indicates that the variation between some samples in the DNA input and the number of copies read in ddPCR is dependent on the %cfDNA of the sample. In addition, samples with less than 0.08ng/µL did not detect copies in ddPCR, so it was considered that a minimum input of 0.1ng/µL is required for droplet reading.

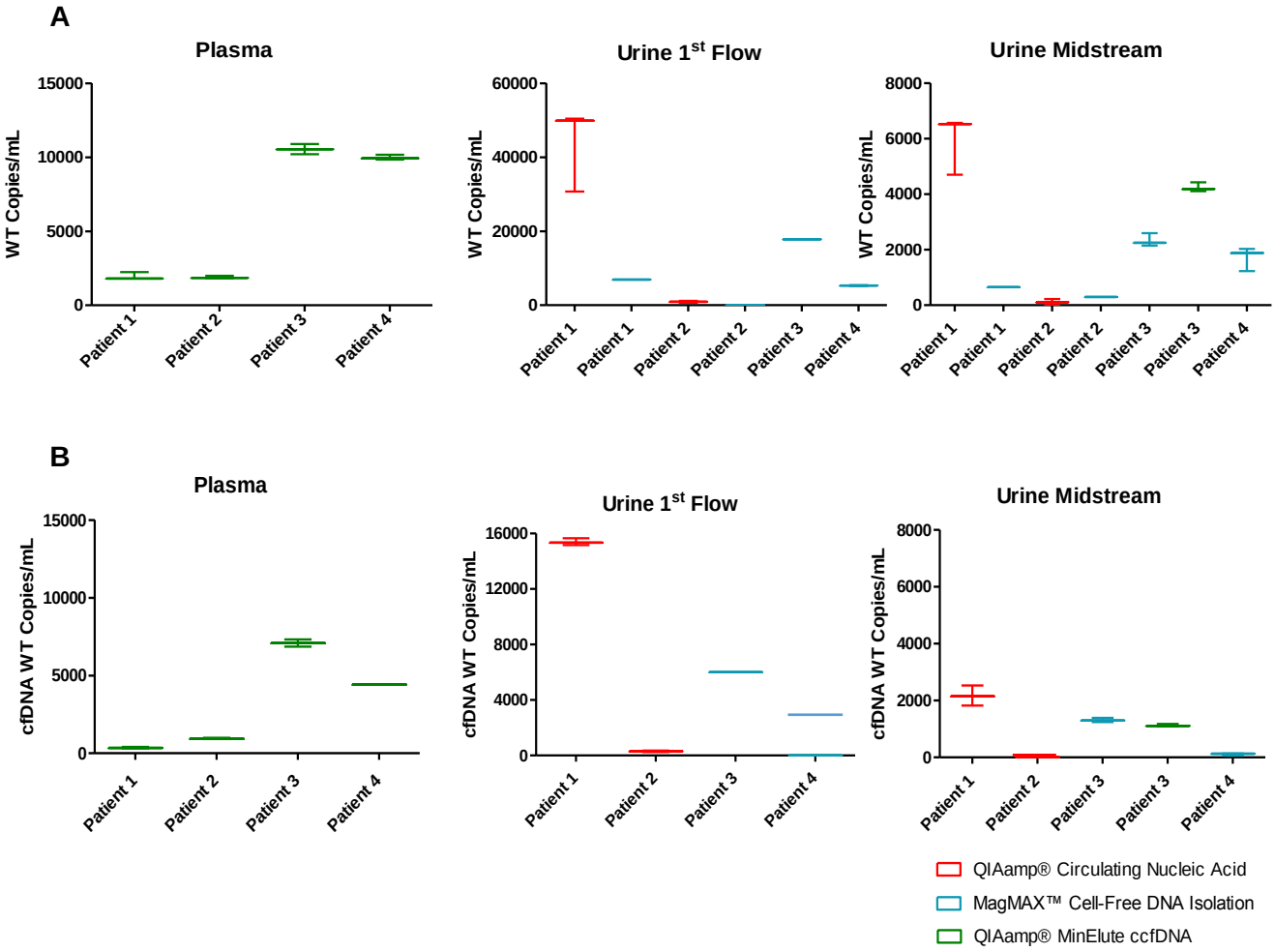


Figure 18 – Assay reproducibility of samples (A) Total number of WT copies that results from ddPCR (B) cfDNA WT copies that result from ddPCR (only samples that were analyzed by 4200 TapeStation).

Furthermore, when analysing the reproducibility of the assay, it also noted that copies/mL WT results are dependent of the %cfDNA. When the %cfDNA was considered, much lower variability was found in samples. That is, we get a better perception of the amount of cfDNA what is being analysed in the sample. It is noted that the greatest variability is in samples containing the first flow and is believed to have a high contamination of DNA that is not cfDNA (derived from urinary tract cells) (Figure 18).

Because of the evidence, it was considered that %cfDNA is important for ctDNA detection. Considering the importance of %cfDNA in the samples, the remaining results were analysed making this normalization.

BRAF V600E can be detected in patients with melanoma with our assay

Finally, the copies of cfDNA/mL present in each biological sample of each patient and their respective fractional abundance were analysed.

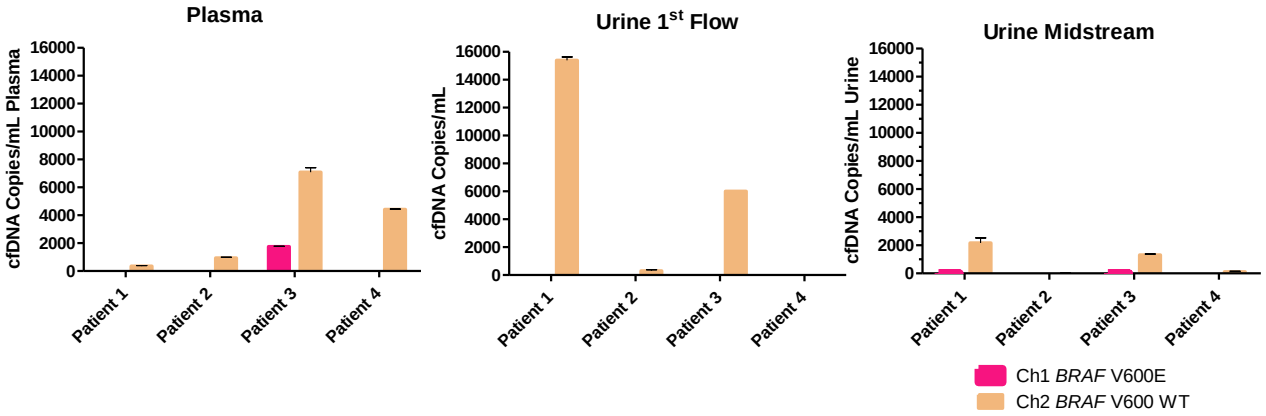


Figure 19 - cfDNA concentration for each patient. Mean of the three assays with more droplets independently of the extraction kit.

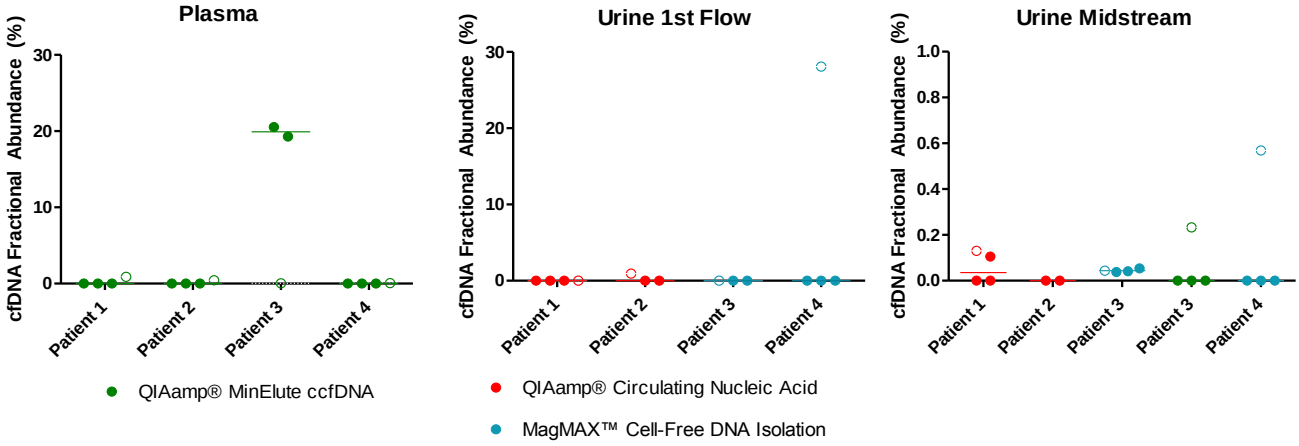


Figure 20 - Cell-free DNA fractional abundance for each patient. Mean of the three assays with more droplets independently of the extraction kit. The LoD of patient 2 in the midstream is of the plot scale.

Table 1 – Results of ddPCR assays from three assays with more droplets per sample.

	%cfDNA	*BRAF* V600E cfDNA (Copies/mL)	*BRAF* V600 WT cfDNA (Copies/mL)	Sensitivity (Copies/mL)	*BRAF* V600E Fractional Abundance (%)	*BRAF* V600E LoD (%)	Result
Patient 1							
Plasma	17.97	0	357.06±40.45	3.18±1.46	0	0.88±0.34	NEGATIVE
Urine 1st Flow	34.87±6.30	0	15389.14±246.78	2.43±0.16	0	0.02±0.01	NEGATIVE
Urine Midstream	35.75±4.26	0.89±1.54	2168.58±355.73	2.78±0.06	0.04±0.061	0.13±0.02	NEGATIVE
Patient 2							
Plasma	49.5	0	946.48±44.73	4.06±1.22	0	0.43±0.15	NEGATIVE
Urine 1st Flow	34.5±4.58	0	304.38±78.10	2.77±0.07	0	0.94±0.27	NEGATIVE
Urine Midstream	6.73±0.45	0	7.86±11.12	3.73±0.76	0	10.15±14.36	NEGATIVE
Patient 3							
Plasma	67.19	1759.50±14.85	7085.18±327.82	3.27±0.02	19.91±0.87	0.03±0.00	**POSITIVE**
Urine 1st Flow	33.74	0	6005.72±0.00	0.51±0.00	0	0.008±0.00	NEGATIVE
Urine Midstream	56.58±2.8	0.58±0.15	1309.11±73.86	0.58±0.15	0.04±0.01	0.04±0.01	**POSITIVE**
Patient 4							
Plasma	44.76	0	4423.78±20.18	2.62±0.45	0	0.06±0.01	NEGATIVE
Urine 1st Flow	0.08	0	4.24±0.13	0.95±0.44	0	28.09±1.29	NEGATIVE
Urine Midstream	6.06±1.02	0	110.27±39.12	0.57±0.042	0	0.57±0.22	NEGATIVE

Patient number 1 has a very dissimilar number of copies between plasma, 1st flow urine and midstream urine. The urine 1st flow has a high number of cfDNA copies/mL, it may be due to the sample has a high contamination of genomic DNA that has degrades and therefore presents approximately low %cfDNA (100bp-300bp). Only one positive droplet was detected in one of the three assays with the most droplets performed, however, the mean was below of the Limit of Detection (LoD) and then it was considered a false positive.

No copies of mutant *BRAF* V600E could be detected in Patient 2. Urine 1st flow displays approximately 35% cfDNA but has a very low concentration of DNA which also makes detection difficult. Already in the urine midstream, there is a little concentrated urine with little %cfDNA.

Patient 3, after extraction, showed high concentrations of DNA and high %cfDNA in all samples. In this patient, a Fractional Abundance relative to the mutant (*BRAF* V600E) of 18% - 1759 copies/mL plasma - was detected in the plasma (above the Limit of Detection) and 0.2% in the urine midstream. In addition, they are the only samples that have a %cfDNA greater that 50% (approximately 67% plasma and 57% in midstream urine) reinforcing the idea that the %cfDNA obtained in the extraction is important for the detection of ctDNA (*BRAF* V600E).

Finally, Patient 4 presented very low %cfDNA in urine and due to the great contamination of genomic DNA it was not possible to detect DNA mutant (*BRAF* V600E).
It is confirmed that plasma and urine midstream samples coincide and that %cfDNA facilitates the detection of *BRAF* V600E.

4. Supplementary Figures

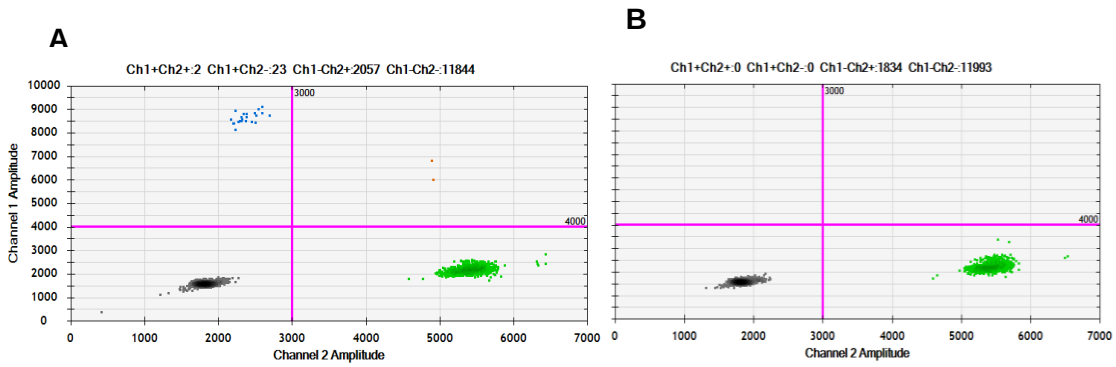


Figure 21 - QuantaSoft 2D plots - Channel 1: FAM signal from mutant probe binding to *BRAF* V600E Channel 2: HEX signal from binding of WT probe.
(A) Positive control with 1% *BRAF* V600E mutant frequency allele (B) Negative control.

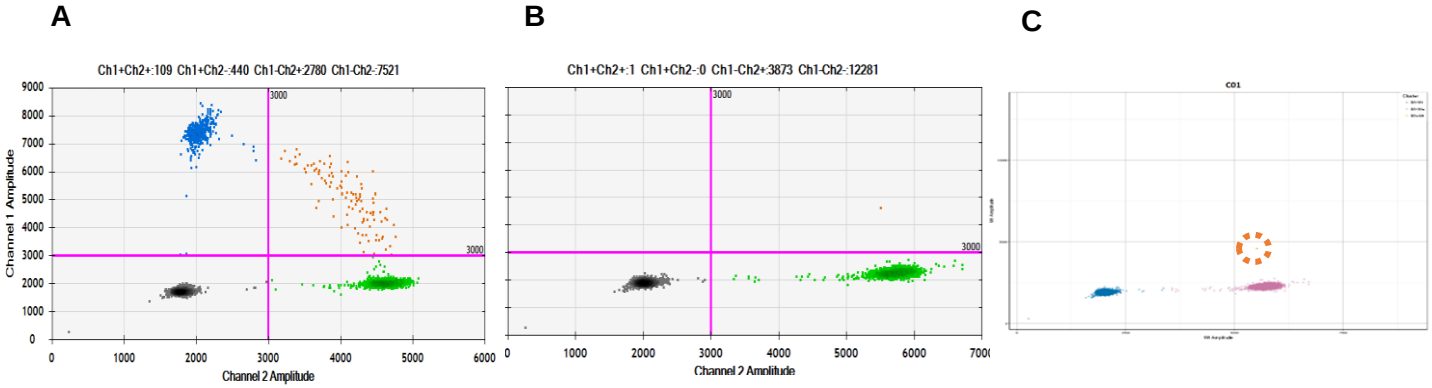


Figure 22 - Plots generated by patient 3 samples. (A) QuantaSoft 2D plot of plasma (B) QuantaSoft 2D of urine midstream (C) twoddPCR Software 2D plot of urine midstream.

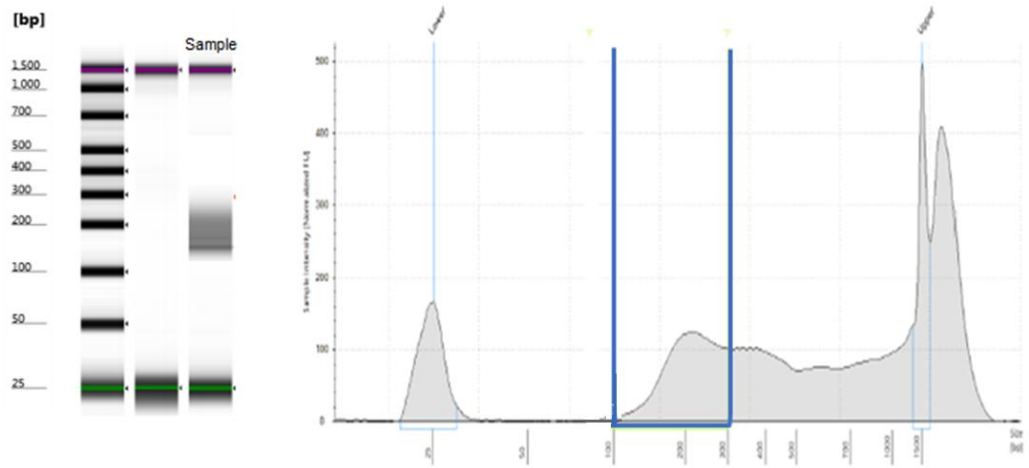


Figure 23 - Example of TapeStation Software analysis.

5. Discussion and Future Perspectives

Nowadays, numerous platforms are available for the ctDNA detection. NGS-based techniques, which allow detection of multiple changes in broader regions of the cancer genome, have been used to test ctDNA, however, are expensive, require bioinformatic analysis, and are less sensitive than PCR-based methods. On the other hand, various PCR approaches have been used, nevertheless, techniques like qPCR require calibrations curve and are susceptible to PCR inhibition. For this reason, novel methods like Digital Droplet PCR were introduced into ctDNA analyse. ddPCR is a highly sensitive and precision platform.

In this thesis, the objective was the implementation of a tool capable to detect *BRAF* V600E mutation in ctDNA in plasma and in urine samples. The goal is this study to contribute to future implementation at the clinic where it can help clinicians make better clinical decisions for melanoma patients. Here, four melanoma patients are studied and we found ctDNA with a *BRAF* V600E mutation in one of patients and the concordance between plasma and midstream urine was 100%.

The findings reported in this thesis have some limitations. The first limitation is the number of biological samples available for each patient and the number of patients available for this study (only 4 patients). The lack of samples resulted in an incapacity to compare the same biological sample with different extraction methods, affected the capacity to perform %cfDNA analysis by the 4200 TapeStation in all samples and also made it difficult to perform all desirable replicates. Tsao *et al* conducted a study which examined the ctDNA responses to therapies in only 4 patients[96] and Ashida and colleagues used only 6 patients to develop a *BRAF* V600E detection tool through the TaqMan method[97]. However, in both cases the study's results allowed to direct future investigations for clinical implementations of the assays. Besides the limitations of the number of biological samples, we have also faced other limitations like the lack of clinical information regarding the patients. However, focus of this work is the implementation of a tool capable to detect the *BRAF* V600E mutation and not the clinical evaluation of the patients.

One of the limitations of transferring liquid biopsies to the clinic is the variety of samples and molecular techniques available to identify these mutations. There is no standard method for the molecular test yet, and the sample and technique choice may influence the existence of false-positive or false-negative. In this work, the component chosen for the possible identification was ctDNA, which, although not providing information regarding the cell's morphology, has potential to fully recapitulate spatial and temporal tumour heterogeneity, which becomes a great advantage[81]. In addition, we chose ddPCR, which has a very high sensitivity and becomes more attractive for clinical application[52]. Huang *et al* also considered ddPCR as the best technique for the detection of point mutations when compared to MPS. Their choice was based on other advantages of ddPCR including low cost, easy operation, and quick turnaround time of ddPCR.

They believe that when the mutations that are to be identified are already determined, the best option to detect a point mutation in ctDNA is ddPCR[98].

A major challenge that also limits the results of ddPCR analysis in ctDNA is the pre-analytical limitation. For example, the control of the different parameters that exist from the collection of the biological sample until the ddPCR analysis in ctDNA. Thus, the many steps between these two events can strongly influence the quality and the accuracy of the data. Moreover, different methods can be used for cfDNA extraction and quantification.

The first variable in question is the use of plasma (with anticoagulant) or serum (without anticoagulant) when the sample collected is blood. In this work, the sample chosen was plasma. Several studies, such as Lee *et al* have shown that DNA is more abundant in serum than in plasma[99]. However, in 2010 Thierri *et al* demonstrated that this higher level of DNA can be derived from genomic DNA in the samples and that plasma is a much more valuable source of cfDNA than serum[100]. In addition, the PAXgene (Qiagen) tubes used for blood collection were reported by Alidousty and his colleagues as reliable for the conservation of cfDNA[101]. Regarding urine collection, Urine Preservative Solution (Norgen) was used and within our knowledge there are no data in the literature comparing the use of 1st flow or midstream in urine samples and because of that we decided to investigate that variable.

Another variable in question is the processing of samples. Herrera *et al* showed that the yield of cfDNA is higher when using a two-centrifugation protocol like used in this work[102].
One more limitation of the pre-analytical phase is the quantification of DNA. Our samples were quantified by Qubit 3.0, which is a fluorometric based method specific for double-strand DNA, making it better than Nanodrop that quantifies all nucleic acids present in the sample. However, the fluorophore can't distinguish between cfDNA, genomic, bacterial or viral DNA. To overcome this limitation, we resorted to a further quantification of the DNA by the 4200 TapeStation that can differentiate the DNA fragments by their size and where only double-strands between 100bp-300bp were counted. Thus, is possible to obtain results closer to the amount of cfDNA present in the sample.

The ddPCR has shown to be a method with high sensitivity and precision[88]. Nevertheless, like all other PCR methods it may be subject to an unsatisfactory assay design and PCR conditions that are not considered ideal. It is important to plan experiments with optimization to ensure optimal assay performance and consequently correct interpretation of results.

Therefore, optimization of the ddPCR assay is a key step in the reliable detection of the *BRAF* V600E mutation in patients with melanoma. There are several factors that may influence the ddPCR detection assay. Factors that may influence the efficiency of the assay include primer/probe design, cross-reactivity between probes and the size of the amplicon[103]. In this

study, to overcome these limitations commercial wet-lab validated ddPCR primers/probes from Bio-Rad were purchased.

The ddPCR can be considered a new technology and was launched by Bio-Rad only in 2011. Due to the recent technology, there is still a lack of standard application information. Thus, the study of this thesis was based on the manufacturers' instructions and the scarce available literature.

Optimization of the annealing temperature is a critical parameter for the specificity of the reaction. According to Bio-Rad recommendations an optimal annealing temperature could be defined as the one that results in the largest fluorescence amplitude difference between positive and negative droplets (delta amplitude) and that simultaneously avoids nonspecific amplification. Moreover, it is important that delta amplitude be checked in Ch1 and Ch2 and, for this, samples of *BRAF* V600 WT and *BRAF* V600E mutant were tested. Thus, a temperature range between 54°C and 60°C was first tested only with samples from a control without *BRAF* V600E mutation and keeping constant concentrations of primer/probe and DNA input. In this experiment it was clear that the temperature of 54°C was favouring the false positives and that of 60°C could not amplify completely. Therefore, a temperature range between 55°C and 57°C was tested later with *BRAF* V600 WT but also with *BRAF* V600E mutant. Among the three temperatures tested the 55ºC was the one that showed a higher delta amplitude and also less dispersion among the positive droplets. Therefore, 55ºC was chosen as the annealing temperature for the assay. Sanmamed *et al* also used ddPCR to quantify *BRAF* V600E in cfDNA using annealing temperature of 55°C[104] and Momtaz *et al* used the same 55ºC[105], giving support to our results in choosing 55ºC as the optimum temperature.

As one of the major challenges in detecting point mutations is the appearance of false positives. We decided to test a touchdown PCR to increase the specificity of our amplification. That is, the touchdown PCR can be considered a method that decreases the off-target initiation, and thus increases the specificity of the PCR. The PCR will start with a temperature of approximately 5°C-10°C above optimum annealing temperature. Annealing under conditions of high stringency favours the formation of perfect primer-template hybrids. In the following cycles, the temperature gradually decreases until annealing temperature is reached. Until reaching the temperature, the specific target sequence will have undergone several cycles of geometric amplification and therefore will be the dominant PCR product[95]. In our study, after determining the optimum annealing temperature for the detection of *BRAF* V600E mutant as being at 55°C, a touchdown PCR starting at 5°C above (60°C) was tested. This optimization caused less dispersion of the positive droplets of Channel 2 (corresponding to *BRAF* V600E mutant) that confirms the greater specificity of the touchdown. As the amount of ctDNA in total cfDNA is usually low, a strategy is required to prioritize target amplification.

Touchdown PCR is only mentioned in the literature, as a method of optimizing ddPCR, by Olsonn and colleagues in 2015. They used ddPCR to detect ctDNA in patients with primary breast cancer to find occult metastases that would be in low amount and support the idea that optimization with touchdown PCR can help detect ctDNA in small amounts[106].

Another important step for optimizing ddPCR is the restriction enzyme incubation time. In ddPCR, restriction digestion is useful because it separates tandem gene copies, reduces sample viscosity, and makes the DNA template more accessible[107]. According to Bio-Rad guidelines, for quantification of DNA and optimal performance, a restriction digestion step should be performed before droplet generation and ddPCR reader. This is because, the advantage that gives the highest sensitivity to ddPCR is the random distribution of DNA by the 20,000 droplets formed and this distribution is only possible if the DNA is fragmented and can be uniformly divided by the droplets. The incubation allows this DNA fragmentation to occur with better performance, increasing the sensitivity and observing a greater delta amplitude

Some authors suggest that when it comes to cfDNA (because it is already fragmented DNA with a small enough length), there is no need to incubate the restriction enzyme or even to use it. This removal of the restriction enzyme from the protocol can reduce sources of contamination for Master Mix PCR, exclude a step where human error can occur and also makes the process faster and less expensive[108]. However, although the importance of enzyme incubation in the detection of cfDNA has not yet been reported in the literature, our results suggest the importance of restriction enzyme incubation prior to generation of droplets. After three different incubation times (0, 15 and 40 minutes) the results showed that in 40 minutes we obtained a higher delta amplitude, suggesting that prior incubation with restriction enzyme plays an important role in final ddPCR results. In other words, there was better DNA fragmentation, consequently better DNA distribution by droplets, generating a greater delta amplitude. This variation of amplitudes is very important to facilitate the distinction of ambiguous droplets. Therefore, further studies should be done to confirm the importance of enzyme incubation in cfDNA analysis by ddPCR and probably this step will increase the distinction between false-positive and positive-positive, favouring the use of this technique in the clinic.

After optimizing the multiplex set-up, we performed a pilot assay with our controls to test the efficiency of assay. Both controls (*BRAF* V600E mutant – positive control - and *BRAF* V600 WT – negative control) presented the expected ratios. After the optimization, no false-positives were detected in the controls (negative and NCT). In addition, an allele frequency of 1% of our positive control (*BRAF* V600E mutant) could be detected and cross-reaction of probes was not detected. All these results are very important to ensure the functionality of our assay and confirm the possibility of using it for further testing with biological samples.

Many studies report the importance of the yield of cfDNA for downstream applications such as ddPCR. In 2012, Yuan *et al* evaluated the yield of cfDNA by Nanodrop with the aim of detecting the *EGFR* T790M mutation[109]. However, as already described, Nanodrop quantifies all nucleic acids present in the sample and does not become specific for dsDNA. In 2016, Pérez-Barrios and his team also compared the cfDNA yields in samples through Qubit 2.0[110]. Nevertheless, the main problem in analyzing the cfDNA yield by Qubit 2.0 is that the quantification does not differ from genomic DNA of cfDNA and it may be counting the contamination by genomic DNA in the yield. In our work, the performance evaluation was initially performed by Qubit 3.0 and subsequent measurement of the 4200 TapeStation's analysis. Through the 4200 Tape Station we were able to determine the percentage of cfDNA. Our results suggest that %cfDNA is an important parameter that should be implemented in clinical routine for the detection of point mutations in ctDNA. When linearity was analysed between the concentrations obtained in Qubit (ng/µL) and obtained in ddPCR (copies/µL), it was noted that linearity had a higher R^2 when we considered the %cfDNA present in the samples. This indicates that DNA that is not cfDNA may be interfering the results and reinforces the idea that %cfDNA is an important parameter. In addition, when analyzing the reproducibility of the samples, much less variability of the output of ddPCR occurs when considered as %cfDNA. Some studies already suggest the importance of evaluating the size of the DNA present in the sample as Devonshire and his collegues[111]. Moreover, Pérez-Barrios and her colleagues realized that the exclusive evaluation by Qubit was not enough and performed the analysis of samples by Bioanalyzer 2100 in order to verify the variability of the size of the DNA. In 2018, Streleckiene *et al* verified through the Agilent TapeStation (method used in our work) the distribution of DNA length after the use of several commercial cfDNA extraction kits[112]. Although these studies suggest the analysis of DNA size present in samples, none evaluates it as a quantitative method. In the implementation of our assay we suggest that the %cfDNA is always evaluated and a threshold is determined in which the extraction efficiency can be concluded and whether the sample is ready for ddPCR analysis.

Once we are going to investigate the validity of a test, it is important to define the Limit of Detection (LoD) and the sensitivity of this test. These parameters are necessary to consider a positive result or not[113]. That is, a patient sample is considered positive when the concentration of mutant DNA is greater than the determined LoD. In the literature, the LoD for ddPCR is often determined by serial dilution with a DNA control sample[79,114,115]. However, this LoD will be applied only to that concentration tested, and as our results show, there is a huge variability between the concentrations of the DNAs extracted from the biological samples.

Due to this reason, in this work, LoD was determined according to each biological sample. The LoD was considered as the presence of a positive droplet (Channel 1 – *BRAF* V600E mutant) in at least three consecutive assays. Quantitatively, it was considered as the average of the fractional abundance of the three assays if there was a positive droplet in each.

Our results presented LoD values ranging 0.0369-0.878% in plasma, 0.008-28% in urine 1st flow and 0.044-10.15% in urine midstream. It is important to remember that the sample that presented the 28% LoD in the 1st flow and the sample that presented the 10.15% LoD in the midstream are samples with very low cfDNA concentrations which makes the detection by ddPCR very difficult. When we compare our results in plasma, they are within the previously reported values. Guanshan Zhu *et al* analysed plasma *EGFR* mutations (cfDNA) through ddPCR in patients with lung cancer and considered two positive droplets to be considered positive, finding a LoD of 0.04%[79]. McEvoy *et al* aimed to develop a method for detecting mutations in the *TERT* promoter by cell free DNA (cfDNA) in patients with metastatic melanoma and detected a 0.17% LoD by ddPCR[116]. In turn, Janku *et al* wanted to determine the LoD in cfDNA of *BRAF* V600E and considered LoD above 0.107% for urine samples and 0.031% for plasma samples[55].
With these values, it is noted that ddPCR can detect the *BRAF* V600E mutant in low concentration. Probably the larger the concentration of cfDNA present in the sample, the more facility there will be in the detection through ddPCR because consequently there will be a lower LoD.

After the development of the tool to detect the *BRAF* V600E mutation by ddPCR, the methodology was tested in biological samples. The methodology was applied to a pilot study with four patients with melanoma and a positive result for *BRAF* V600E mutation in primary tumour. Plasma samples, urine 1st flow and urine midstream of each patient were evaluated.

The first check when using the biological samples was that the percentage of cfDNA is dependent on the type of biological sample.

First, it was noted that plasma samples have more %cfDNA than urine samples (except for patient 1). In addition, midstream urine samples have higher %cfDNA than 1st flow urine (with exception of patient 2) and higher amount of purified DNA in 1st flow. We propose that, the greater DNA concentration in the 1st flow but higher %cfDNA in the midstream is due to the fact that, urine 1st flow has contribution of the urinary system cells (genomic DNA), increasing the concentration of the total DNA, but with long fragments that probably are not from tumour. Concerning patient 2, the lack of agreement is probably due to the fact that urine 1st flow and midstream samples have a very low amount of purified DNA and may not be representative of the total DNA present in the urine. This low amount of DNA in the sample can be due to the low volume of extraction used in this extraction (4 mL of urine-QIAamp® Circulating Nucleic Acid kit). This proposal is in agreement Sherwood *et al* that in 2016 evaluated 15 patients with different volumes of extraction and reported that low volumes used in extractions of cfDNA can influences the extraction yield[117].

The *BRAF* V600E mutation was detected only in Patient 3 in plasma and urine midstream. All other samples from all patients were negative for *BRAF* V600E mutation. There was 100% concordance between plasma cfDNA and urinary midstream cfDNA genotype.

It is important to note that the samples with the *BRAF* V600E mutation were the samples with the highest percentage of cfDNA (plasma: 67.19% and urine midstream: 57.70% and 53.46% in each extraction). This evidence only reinforces the idea that the higher the percentage of cfDNA, the easier the mutation detection in the sample. It also reinforces the fact that samples with large amounts of purified DNA but with a high degree of contamination of the white blood cells or urinary system cells (low percentage of detected cfDNA) are more difficult to detect the mutation. This happens because there will be a smaller proportion between ctDNA/cfDNA and will be require a higher sensitivity to detect the mutation. Wang *et al* also show the importance of the presence of short fragments of DNA in samples to detect *KRAS* mutation, in other words, the presence of cfDNA[118]. Our study suggests a quantitative baseline to detect *BRAF* V600E mutation (> 50% cfDNA). However, further studies are needed to confirm this baseline

Since the percentage of cfDNA has an influence on the detection of ctDNA, we considered the best samples for the analysis that the highest percentage of cfDNA has. Thus, the best samples for analysis are plasma and urine midstream - exactly the samples where the mutation was detected. To the best of our knowledge, this is the first time that urine flow 1st and urine midstream samples were compared to ctDNA detection. Mutations in urine have already been detected in cancers other than genitourinary tract. Hyman *et al* detected the *BRAF* V600E mutation in patients with Langerhans cell histiocytosis[59], Chen *et al* detected *EGFR* T790M in patients with NSCLC and among other studies where ctDNA has already been detected in urine[119]. However, none of these studies mentions whether the urine used is 1st flow or midstream. Therefore, this study is the first to suggest the midstream urine as standard for the screen of ctDNA.

In relation to patients 1, 2 and 4, they also had positive biopsy on primary tumour for *BRAF* V600E and didn't have the mutation detected in body fluids. The first hypothesis is exactly the lowest percentage of cfDNA present in all samples of these patients. As reported above, the percentage of cfDNA has an influence on the detection of mutations. However, it is not the only explanation. In 2016, Ashida performed a quantitative analysis of *BRAF* V600E and did not detect the mutation in patients who did not have metastases in computer tomography[97] and Bidard and his colleagues considered that low tumour burden is the only clear limit for non-detection of ctDNA[62]. Nevertheless, Ashida reported detecting the mutation in a patient, 147 days after the first analysis. This may indicate that occur metastasis development or increased tumour burden over that period. Consequently, the ability to detect ctDNA depends on distinct stages (49-78% in localized tumours and 86-100% in metastatic tumours)[49]. Nevertheless, there is still a third hypothesis: the site of metastases. McEvoy suggested that, patients with brain metastasis cannot be detected[120]. Brain metastases probably cannot be detected due to the impact of the blood-brain barrier on the release of tumoral DNA into the circulation. However, non-detection of ctDNA has prognostic value. In 2015, Santiago-Walker reported that patients with undetectable levels of *BRAF* V600E mutant at baseline in plasma had the best clinical outcome based on response rate, progression

free survival (PFS) and overall survival (OS)[121]. In 2018, McEvoy confirmed this hypothesis by demonstrating that non-detection of ctDNA is associated with longer PFS in patients with metastatic melanoma[120]. Thus, it is expected that patients 1, 2 and 4 present a better clinical outcome when compared with the patient 3.

Nevertheless, it is important to note that not all patients have tumour DNA in circulation and may also be the reason for not detecting the mutation when we extract the circulating DNA. However, in patients where the tumour is a source of circulating DNA, our tool has a high sensitivity as found in Patient 3 of 0.04%. For these patients, this test may bring better clinical decisions that are likely to bring better quality of life and survival.

Therefore, the detection of *BRAF* V600E in ctDNA by ddPCR developed in our work may be a blood/urine biomarker for diagnostics, prognosis, treatment monitoring and detection of residual disease. Furthermore, this tool has a great potential that can add numerous advantages and that should be explored in clinical practice. However, we suggest that this biomarker should always be evaluated with the patient's clinical history and be considered a tool to support in medical decisions.

A first application for the test developed in our project is the use as a tool in order to choose the best therapeutic approach. The great advantage of using ctDNA with other tools is the detection of tumour heterogeneity. The use of ctDNA in plasma or urine reflects the DNA of all metastases present in the patient. At present, the standard pathway for obtaining tumour DNA is tissue biopsies which in the case of metastatic melanoma can be difficult to access and dispersed[54]. As melanoma is easily metastasized to the lymphatic system and other organs like as lung, liver, bone and brain[4,6], the use of liquid biopsy is always a good alternative. In addition, somatic mutations are tumour specific and will present better diagnostic accuracy than currently used protein biomarkers. Furthermore, melanoma patients who are positive for *BRAF* V600E may benefit from target therapy with BRAF and MEK inhibitors. If only one biopsy was performed on the primary tumour, this therapeutic decision could not be addressed if the tumour’s DNA did not show the mutation. However, there was a possibility that the mutation might be present in other metastases, making the use of the new developed tool very important. Therefore, the *BRAF* V600E test is very important for therapeutic decision in melanoma, considering that approximately 50%[25] of the melanomas are positive to this mutation.

A second application is the prognostic use of patients with melanoma. Currently the accepted prognosis for melanoma is defined by the American Joint Committee on Cancer[122]. The latter considers metastatic melanoma as the site of metastases and the serum level of LDH. Normally, LDH levels are associated with high tumour burden but are not specific for melanomas. ctDNA, however, becomes a biomarker of specific to melanoma prognosis and makes the prognosis of the patient much more accurate[43]. As previously reported, several studies have confirmed the

association between ctDNA levels and patient prognosis: OS, PFS and response rate. In other words, patients with less ctDNA will have a better prognosis. Therefore, in our study, Patient 3 will have the worst prognosis.

The third use of *BRAF* V600E detection would be the monitoring of patients with melanoma along the adjuvant or neoadjuvant therapies. This monitoring is possible because ctDNA is a quantitative measure and reflects the tumour burden. Many studies have used ctDNA to monitor therapies targeted to the mutation under study. Gray *et al* demonstrated that, ctDNA levels varied during treatments with targeted therapies and reflected tumour burden[122], confirming the usefulness of this tool.

Currently, monitoring of melanoma during treatment is performed by blood biomarkers such as LDH or by imaging methods. However, as previously reported LDH has limited specificity and imaging methods such as PET and CT are very expensive and cause patients to be exposed to radiation[44]. In addition, due to the short half-life of ctDNA, it reflects the various moments of the disease very accurately[49]. Santiago-Walker and his team correlated ctDNA levels with LDH levels and with the classification of imaging methods. They showed that, ctDNA is related to changes in other markers and may be used independently[121]. Still, Dawson and her colleagues reported the finding of significant changes in ctDNA approximately five months prior to imaging methods in metastatic breast cancer patients[123], showing the earlier detection through the use of ctDNA as a biomarker. The rapid change that occurs in ctDNA levels makes it possible to detect resistance to treatment early and enables an alternative treatment to be introduced at a time-point where therapeutic success is still achievable.

In addition, ctDNA can be used to monitor patients beyond targeted therapy. Many studies have shown promise in immunotherapeutic treatments in patients with metastatic melanoma[124,125]. Currently, are studies within this scenario that evaluate the possibility to use ctDNA monitoring in two moments. First, as an indicator tool for resistance of the target therapy and decision-making to initiate an immunotherapy and secondly to monitor the immunotherapy itself. Seremet and his team concluded that ctDNA with *BRAF* V600 mutation may be a monitoring tool in melanoma patients treated with anti-PD1[126]. In addition, Lee *et al* also confirmed this possibility[125]. The evaluation of ctDNA in patients performing immunotherapy becomes very advantageous when compared to imaging methods. This is because, by imaging methods when tumour loads are analysed, pseudo-progression or late regression of the tumour is often found which is in fact characterized by infiltration of lymphocytes into the tumour[125]. Thus, with ctDNA it is possible to distinguish between patients who are responding to therapy or not, making monitoring of the *BRAF* V600E a good tool.

However, imaging and liquid biopsy present different information and it would be beneficial to use it simultaneously. Nevertheless, the use of the liquid biopsy can and must be done more often

than is standardized for the imaging tests, allowing the possibility of detecting changes in the tumour in advance.

One last use of *BRAF* V600E detection in patients with melanoma is the detection of residual disease[5] after curative measures like excision of the primary melanoma or after lymphadenectomy. As already reported, currently, the strongest predictor for recurrence is the sentinel lymph node biopsy (SLNB) and is considered a reliable outcome after treatment. However, studies have reported that, patients with SLNB-negative may present relapse[127]. Here, we propose that ctDNA analysis can be used at first to distinguish patients who require adjuvant therapy or not. Second, ctDNA is a tool with great potential for patient monitoring, ensuring that any change will be detected soon. Currently, this residual disease monitoring is performed by the same methods of monitoring the therapies. However, the *BRAF* V600E detection in liquid biopsy to monitor patients after curricular measurements presents another advantage than those already reported: the practicality of the test. The samples can be collected anywhere and is a cost effective and non-invasively test.

We believe that this tool can be used mainly in early stage melanoma patients who are considered disease free or don't present high risk for recurrence. Due to costly prices, invasiveness, and radiation exposure, these patients are not routinely assessed by SLNB or FDG-PET/CT. Here, liquid biopsy can be used as an additional monitoring regimen to physical exams that can result to timely interventions.

In conclusion, we present an initial evidence demonstrating that is possible to detect the *BRAF* V600E mutation in ctDNA in some melanoma patients using ddPCR both in plasma and urine samples. Although not all patients have ctDNA, those who present can benefit greatly from the complementary information provided by the tool. However, the detection rate needs to be increased and the clinical significance should be better evaluated with more patients.

Moreover, the findings of our study contribute to the development of a standardised ctDNA analytical protocol to clinical implementation and it is hoped that soon it will help melanoma patients.

Chapter 3 - Go-to-market strategy of *BRAF* V600E detection by ddPCR

1. Introduction

The development of nations is related to the capacity for technological innovation, transfer and application of these technologies. Already in 1996 Lawrence *et al* mentioned that up to 50% of economy growth may derive from technology[128]. Today, with the high competitiveness of the market, innovation is a crucial point not only for development, but also for the survival of companies. Looking at this trend, Vinicius *et al* said that the new economy can be called "the economy of perpetual innovation"[129]. In this context, technology transfer plays an extremely important role.

According to Association of University Technology Managers, technology transfer can be termed as the transfer of scientific discoveries from one organization to another company/to another department of the same company for development and commercialization purposes. Because biotechnology is an emerging industry, the term "bioentrepreneur" has gained some ground in recent years. The rapid growth in biotechnology has led to a plethora of bioentrepreneurship opportunities for life scientists[130]. Within biotechnology, the health sector has been a priority for many scientists in the application of science and technology to human health.

Currently, cancer is the second leading cause of death in the world. Because of this, latest advances in medical biotechnology have been dedicated to gaining knowledge related to the molecular events of cancer and consequently the development of new diagnostic and treatment methodologies that bring new hope to patients. This thesis focus on the study of the detection of cutaneous melanoma which is an aggressive skin cancer that has increased its incidence in the last 50 years worldwide and is responsible for approximately 90% of all skin cancer deaths. However, early detection and the best clinical decisions for these patients have been increasing the five-year survival rates[4].

The need for better tools for treatment and monitoring of melanoma, GenoMed has implemented a new liquid biopsy for detection of the *BRAF* V600E mutation in melanoma patients (present in approximately 50% of melanomas)[25]. GenoMed's new liquid biopsy has a huge commercial potential and can be used to complement other tools like PET/CT and LDH analysis. This technology will enable patients with melanoma to make much more accurate clinical decisions from clinicians, ranging from: prognosis, treatment and detection of residual disease. Improving the melanoma management and bringing better outcomes to melanoma patients.

However, there must be an efficient transfer of this test to the market in order to have an impact in the lives of millions of individuals that suffer melanoma skin cancer. Therefore, a good business model is important to make the service viable and this evaluation can be made from a business plan.

Teece considers that a good business model relates strategy, innovation and economic theory[131]. Other researchers consider that, a good business model becomes a competitive advantage. They take into account all internal and external factors: relationship with customers, suppliers, competitors and the whole environment[132].

A business plan is a very important tool in companies already in the business for several reason: it is like a roadmap in an unknown territory, helping also in raising money and in investment decisions. investment decisions. Barrow and Brown confirm this idea by saying that when it is necessary to invest in a new project, the business plan is the most important step of it[133]. A good business plan has such indispensable elements as product/service description, market and competition analysis, marketing and sales plan, human resource requirements and financial analysis.

Due to these evidences, a business plan was developed that focuses on the transfer to the clinic of the liquid biopsy for melanoma patient. The objective is that from this plan, check the feasibility of continuing to invest in in validation and future commercialization of this new service.

The following business plan is a hypothetical proposal developed without interference from Genomed S.A.

2. The Liquid Biopsy Market

According to Alexander Vadas and Brian Baranick, in 2016, the global market size of liquid biopsy was valued at US$23.49 million. The revenues are projected to expand at a Compound Annual Growth Rate of 21.7% during the forecast period 2019-2026 and reach a value of US$ 2,893.7 million by the end of 2026[134].

There are now countless successful tests with promising results. Moreover, already approved targeted therapies encourage the use of liquid biopsies. As already mentioned, the liquid biopsies have several applications and consequently each one has a growing market opportunity.

Table 2 – Liquid Biopsy market size per application. Adapted from Alexander Vadas and Brian Baranick 2018.

Liquid biopsy application		Size of opportunity	Benefits	Challenges
Early detection	Routine screen for presence of cancer-derived particles in healthy individuals	$10Bs Large patient base (100M+) Low-cost recurring test	Earlier intervention Definitive localized therapy	Rare-event detection with low false-positive rate
Diagnostic acid	Test to inform diagnosis in suspected cases	$100Ms Niche patient base (100K) Moderate cost, one-time test	Supports diagnosis in biopsy-constrained situations Diagnoses metastatic disease	Tissue-based testing is gold standard
Prognostic	Single test to support prognosis		Informs aggressiveness of intervention	Tissue-based testing is gold standard
Intervention outcome monitoring	Test to track response to intervention or detect minimal residual disease (surgery, radiation, adjuvant therapy)	$1Bs Large prevalent pool (10Ms) Low-cost, recurring test	Lower cost/risk to imaging	Displace imaging with compelling cost / benefit data
Surveillance and recurrence monitoring	Longitudinal monitoring of remission patients to detect recurrence earlier		Early detection and intervention (many recurrences are metastatic today) Reduced imaging (cost / toxicity)	Demonstrate ability to downstage recurrence detection and improve patient outcomes Displace imaging with compelling cost / benefit data
Therapy guidance	Tests to inform therapy decisions	$100Ms Small patient pool (100Ks) High-cost, one-time test	Enables personalized medicine in biopsy-constrained situations Increases predictability if heterogeneous cancer or metastatic disease	Tissue-based testing is gold standard
Monitoring	Longitudinal test to monitor response or resistance to therapy	$100Ms Small patient pool (100Ks), moderate cost recurring test	Reduced imaging (cost / toxicity)	Displace imaging with compelling cost / benefit data

The table above shows the great commercial potential of liquid biopsies in the market. Having said so, it is very important to design the best strategy for transferring to the market the innovative GenoMed's detection test of *BRAF* V600E mutation using liquid biopsies.

3. The choice of the market: Germany, Belgium, the Netherlands and Luxembourg

The surrounding environment is determinant in designing the company strategy, that is, considering the characteristics and signs of the environment, companies must adjust their

behaviours to ensure their survival and success. An important point to achieve this success is the identification of the target market.

Currently, GenoMed operates mainly in Portugal. However, our country has a small market with a low incidence of melanoma when compared to other countries[3]. Therefore, the market strategy will address the need to export the GenoMed service in detecting *BRAF* V600E mutation (in plasma and urine) to other markets. Because the liquid biopsy is an emerging market that is still under-explored, it may be a great opportunity to expand GenoMed's business to international market.

As the new GenoMed liquid biopsy to detect *BRAF* V600E mutation is focused on melanoma patients, the first expo criteria to choose the market will be the melanoma incidence. Figure 1 in Chapter 1 shows that one of the areas of high incidence and mortality is Europe. Choosing countries within the European Union (EU) tor start selling the service has several advantages, such as proximity, European common market, absence of currency risk and no customs barriers.

The melanoma incidence within the EU countries (Figure 24) shows that that there is great variability within Europe[135]. Considering only countries that use Euro (€) and analyzing their respective incidence of melanoma, it is noted that countries like Spain, althoug having a large dimension, are not interesting for our service internationalization since there is no need so accentuated of the same (low melanoma incidence).

Considering only countries that use Euro (€) and analyzing their respective incidence of melanoma, it is noted that countries like Spain, althoug having a large dimension, are not interesting market for our service since it has low melanoma incidence.

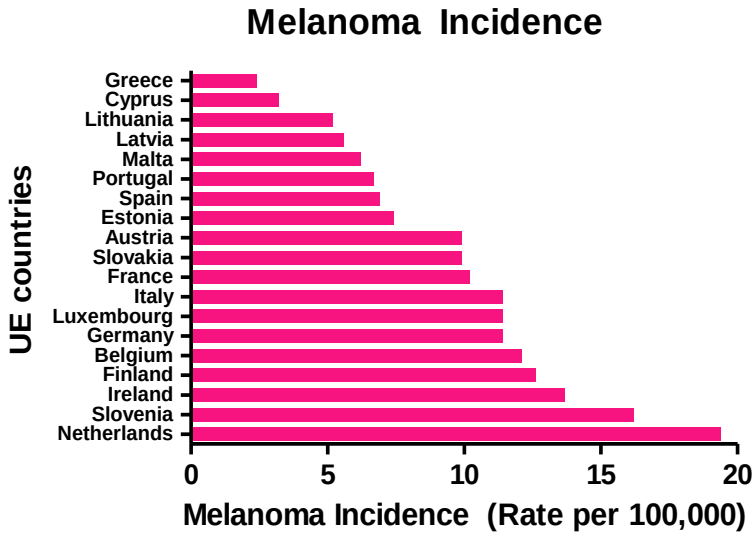


Figure 24 - Estimated standardized rates of incident cases, melanoma of skin, Europe in 2012. Data from GLOBOCAN *2012, World Health Organization.*

It is interesting to also take into account the commercial history of relations between these countries with Portugal, stability and economic growth, service need, ease of distribution channels, size of the market and reception of medical/biotechnological innovations in the countries.

In Figure 25, the incidence of melanoma was related to GDP (Gross domestic product)/per capita[136]. Among the six countries considered to most interesting markets in terms of melanoma incidence and GDP.

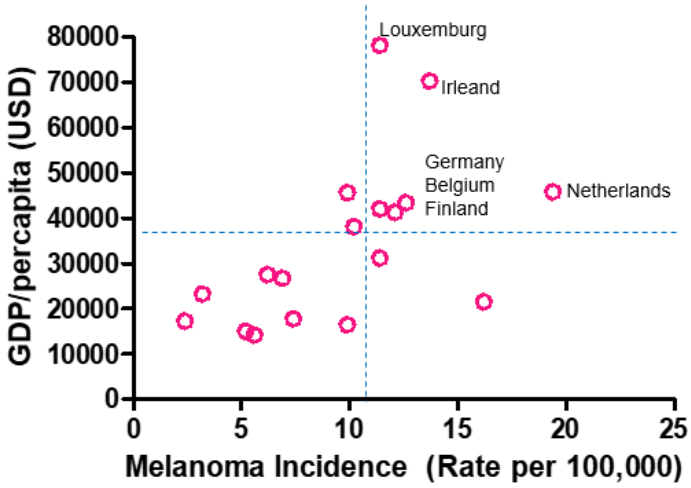


Figure 25 – Relation between melanoma incidence and GDP/per capita (USD). Data from GLOBOCAN *2012, World Health Organization* and from PORDATA 2018.

Therefore, after analyzing mentioned characteristics, following countries were chosen to start activities beyond Portugal: Germany, the Netherlands, Belgium and Luxembourg. the objective is to first consolidate sales in Portugal and subsequently sell to the international market. The four countries have a very integrated distribution chain, which is why Luxembourg was also chosen despite the smallest market size. The export of service is very important for its success, since, in addition to a small size Portugal is also not among the highest rates of melanoma incidence in Europe.

However, this choice of countries is not a restrictive choice. It is where GenoMed should focus in order to have necessary certifications to operate and sell the tests as well as its commercial strategy should focus on these countries. Also, if there are distributors from other regions interested in working with GenoMed detection test of *BRAF* V600E mutation, these opportunities should be explored.

4. Test Validation

In order to provide a safe and convenient service for physicians and patients, our test must be validated and complete before clinical use. The elements that form part of this process are analytical validation, clinical validation and clinical utility. Analytical validation includes analytical sensitivity, analytical specificity, intra-laboratory and inter-laboratory precision, and robustness of the assay (reproducibility between operators, reagent lots and instruments). Clinical validation includes sensitivity and clinical capacity. The goal is to assess whether it improves clinical outcomes compared to existing methodologies.

Another important step is verification. In Europe, the verification should generally be appropriate for CE marking. The CE mark is a mandatory conformity mark for certain products sold in the European Economic Area and is a declaration that the product meets the essential requirements of the applicable EC directives.

For a *BRAF* V600E detection in ctDNA test to become a tool in the Europe market, a CE-IVD marking is required. A performance evaluation report is required within the technical documentation required for the CE marking. The report should include a detailed explanation of the project, intended use, risk assessment, performance assessment plan and results of the clinical performance study showing compliance with 98/79/EC.

The process for obtaining a CE-IVD mark is an expensive process and therefore, the viability of the business plan must be properly evaluated. However, it is possible that the chosen market together with its opportunities will become an opportunity for the international growth of GenoMed.

5. Macro Environment

In a previous session we tried to define the best markets for the sale of the detection test of *BRAF* V600E mutation. In this session we intend to study the macro environment of these countries with the objective of identifying the opportunities and threats.

Political-Legal

The countries of the European Union are regulated by Directive 98/79/EC on IVD (in vitro diagnostics) medical devices and stipulate essential requirements for molecular testing that are intended to be implemented in market.

In our country, where GenoMed is installed, government instability can be considered a negative factor. This instability may lead to changes in public health policy (some of which have already been made in the *Memorando de Entendimento* like a reduction of contributions[138]) that may directly affect GenoMed as Public Sector in one important customer.

In addition, currently the company is subject to several laws that relate specifically to clinical laboratories, such as: data protection; safe working conditions; laboratory practices; purchase, storage, circulation and disposal of hazardous waste. The *Portaria* that establishes the legal regime of *Licenciamento de Laboratórios de Genética Médica* is a *nº 167/2014* of August 21, 2014[137] and it is also necessary to have the ISO 9001:2008 certification. In Portugal, liquid biopsy test may be requested by physician or oncologist.

In Germany, Belgium, the Netherlands and Luxembourg the test also must be prescribed by a doctor.

Economic

From the economic point of view, in recent years, Europe is experiencing some instability. The positive vote on Brexit in 2016 called into question the credibility and foundation of the European Union's existence.

However, there are still policies aimed at encouraging investment and, consequently, economic growth. The Central Bank also has a positive outlook on GDP, which is a favourable scenario for Europe. In addition, at European level, the unemployment rate reached, in 2016, 9.8%. Continued labour improvements, increased private consumption, increased exports and improvements in financing will also lead to an increase in inflation in Europe, which points to positive signs of recovery in the economy. Moreover, as the entire target market is part of the European Union there is no exchange risk.

According to the European Diagnostic Manufacturers Association, the in vitro diagnostics industry in Europe generated 10.7 billion euros of sales in 2012, representing 0.8% of total expenditure in health at European level. Adding Portugal, Germany, Belgium and the Netherlands we have approximately 30% of the European IVD market[138] (Figure 26).

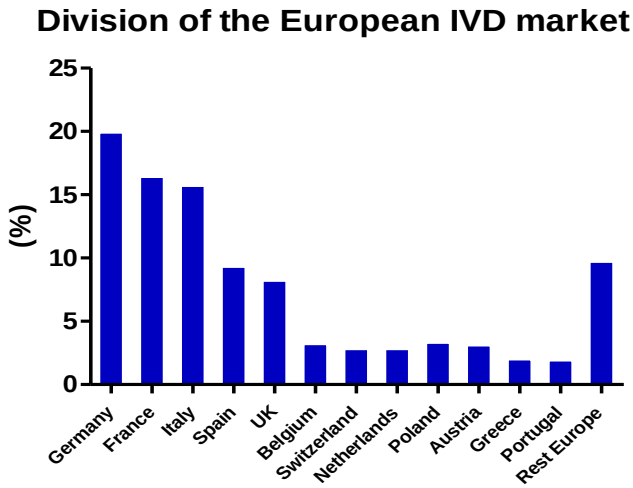


Figure 26 - Division of European in vitro diagnostics market in value. Data from Apifarma 2013.

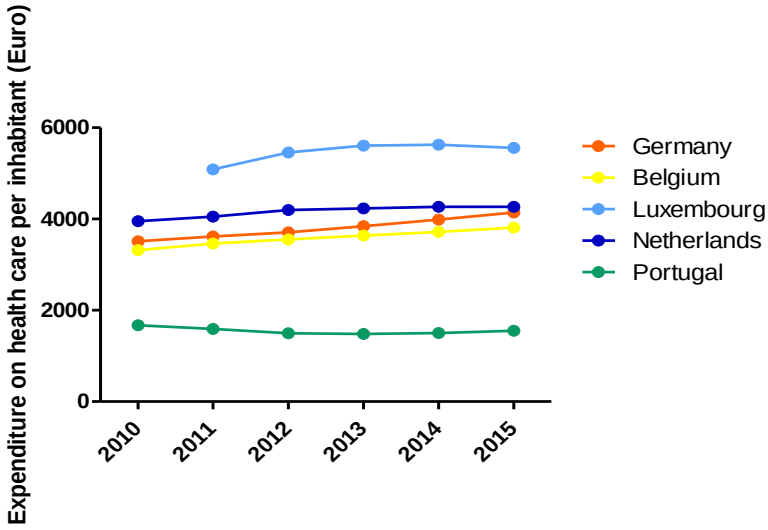


Figure 27 - Expenditure on health care per inhabitant in Euro. Data from PORDATA 2018.

It is noteworthy in Figure 27[139] that, with the exception of Portugal, spending on health has been increasing and is much larger than our country. Luxembourg in turn, despite having a trend of stability already has the highest spending in Europe.

Portugal

Regarding the Portuguese Economy, the prospects presented (Bank of Portugal and European Central Bank) indicate the recovery of our economy. The outlook indicates GDP growth in the

coming years, coupled with a decrease in the budget deficit. GDP growth in Portugal in 2016 was 1.2%, not so different from the European average growth of 1.6%.

In turn, the in vitro diagnostic industry in 2012 accounted for approximately 0.04% of the national GDP generating around 69 million euros in terms of gross value added[138]. About the health sector, the debt of the *Serviço Nacional de Saúde* shows an accelerated growth. During the year 2016, arrears increased by about 27.2 million per month. This implies that the diagnostic clinics are in arrears[140].

Germany

Germany is a country that has a very large market dimension with approximately 82.3 million inhabitants. Moreover, in terms of Gross Domestic Product (GDP), the largest economy in Europe and the 4th largest in the world. Germany accounted for 8.4% and 6.5% of the value of world exports and imports in 2016, which gives it the status of both the 3rd largest exporter and importer in the world and the largest European market and one of the most competitive in the world[141].

Portuguese exports of goods and services to Germany in the upward cycle registered an average annual growth rate of 3.3% in the period 2012-2016. In relation to Portugal, Germany has a long tradition of trade, occupying the 2nd position, both as a customer and as a supplier. In 2011, German imports of health products were around € 47 billion, making Germany the second largest market for health products in the world. In 2011, Portugal exported around € 135 million in health products to the German market, which corresponds to about 22% of Portuguese exports of health products. The German market is thus the most important foreign market for Portugal. The average annual growth rate of Portuguese sales of health products to the German market was 7.1% in the last fifteen years[141].

Belgium

Belgium is a country with 11.4 million inhabitants was the 25th world economy in 2015, according to the World Bank and the 9th European Union. In 2016, it was considered within the 12th largest importer in the world. In this year, Belgian GDP grew 1.2%, driven mainly by increased exports and investment, and public and private consumption. Belgium is more attractive to investors in the service sector than in industry, and the Belgian Government is trying to diversify sources and investment sectors, particularly in the areas of information and communication technologies, logistics and biotechnology. Belgian demand for biopharmaceuticals increased in 2016. Moreover, Belgian households spent 4.6% of their average expenditure on health products and services (€ 1,571/year), with per capita health expenditure in that year being € 3,871.8[142].

Netherlands

The Netherlands has 17 million inhabitants and was considered the 17th world economy in 2015, according to the World Bank, and the 6th economy of the European Union. In terms of services, it

is the 7th importer worldwide. It is a highly open economy, very dependent on the global and European economic environment, international trade and the financial sector. The country has a high income per capita.

In addition, there is a strong increase in Dutch imports of pharmaceuticals, the 6th group of imported products (+ 12% in 2015 compared to the previous year). The Netherlands, because of their geographic location, function as a medical / biotechnology equipment distribution center, re-exporting 20% of what they import. There are about 100 medical device trading companies in the Netherlands, including four or five large ones. Sales to local hospitals contribute more than 40% of total business volume[143].

Luxembourg

Luxembourg is a small country, approximately 576,000 inhabitants. However, it is a country with high purchasing power presented in 2016 the highest GDP per capita in the EU (76 600 €). It is a country very dependent on its neighbours like Germany and Belgium. In terms of evolution, Luxembourg imports of goods from Portugal grew by 8% in 2015 compared to the previous year[144].

Social

The European Union shows a trend towards demographic aging. The enlargement at top of the age pyramid corresponds to increase in the elderly population resulting from an increase in life expectancy[145]. This aging of the population leads to greater demand in health services, especially in oncology diagnosis.

In addition, Europe also has a high level of education[145] which leads to greater health concern and greater demand for innovations that bring improvement in this sector. European physician also has easy access to information and new technologies, which makes it possible to sell innovative diagnostic tests in Europe.

Technological

In 2017, according to PORDATA, Portugal has only 0.6% of our GDP are invested in Research and Development (R&D), against, for example, 2.3% in countries such as Germany and Sweden[146]. Although Portugal hasn't yet reached the expected level, trends are that they continue to grow in the coming years. The technological environment is increasingly present in life of the world society, and Internet access is becoming easier. In this way, it is important to use digital platforms to communicate with our customers. At the beginning of 2018, GenoMed launched a new website to add value to its image. In addition, it also has a strong stake in the LinkedIn platform, which has grown by 18% in 2016.

Germany also has a strong stake in the technology sector and regards the biotechnology area as a highlight. The main products of medical biotechnology are biopharmaceuticals, DNA tests and personalized medicine[141].

6. Micro Environment

Customers

In order to communicate with our target audience and be effective in its approach, we must know our potential customers well, so we divide them into three main groups: patients, clinics/laboratories and insurance companies.

Patients

Patients who developed melanoma are potential customers of our service, "Screening for the V600E mutation in the *BRAF* gene in circulating tumour DNA". They can be referred to GenoMed S.A. after medical appointment with their Oncologist. The collection of the sample will be performed where it is best suited to the patient in the hospital, clinics or if possible at GenoMed.

Clinics and Laboratories

The clinics and laboratories were chosen according to the credibility and size of the establishment, geographical location and focus of the establishment (whether in oncology or genetics). Regardless of where patient collects the biological sample, a medical prescription is always required. Our sales focus will be on oncologists, cancer treatment clinics, and also labs that are willing to collect the samples and earn some percentage through service intermediation. However, it may also be sold to general practitioners and clinics. It is important to note that if the clinic/laboratory acquires the sample collection kit and wants to pay for service directly to GenoMed, S.A., it is possible that it adds a value to sale to the patient.

In Portugal, where the company is headquartered, GenoMed already has some long-standing partnerships[147] that can request the new test (Confidential Appendix – Table 26).

In addition, a list of possible partnerships specialists in oncology in Portugal with GenoMed has been identified.

Table 3 - Possible partners for the GenoMed S.A. specialist in oncology in Portugal.

Client	Localization
*Institute 1**	*Porto*
*Institute 2**	*Lisboa*
*Institute 3**	*Carnaxide*
*Institute 4**	*Coimbra*

*Institute 5**	*Lisboa e outros*
*Institute 6**	*Porto*

*Confidential Appendix

Moreover, one possibility was to establish a partnership with Company A (Confidential Appendix)[149,b]. One of the great advantages would be to reach public in all places. Another advantage would be that, by Company A being a company of small laboratories, does not have large investment in R&D and usually resort to outsourcing for these services. Therefore, they would probably be willing to adhere to the GenoMed test and bring a large customer portfolio to the company.

GenoMed can also accept requests from public hospitals. In this case, the payment can be made by the hospital itself or the patient can choose to make the payment in order to avoid delays. Currently, the lab has a longstanding relationship with Client A (Confidential Appendix) facilitated by proximity.

Finally, possible partnerships were identified for the internationalization market: Germany, Belgium, the Netherlands and Luxembourg. However, while GenoMed may directly contact potential customers, customer acquisition in these markets will be highly dependent on the distribution channels of the collection kit.

Table 4 - Possible partnerships of GenoMed S.A. in the foreign market.

Customer	Location
*Laboratory A**	All Germany
*Laboratory B **	Germany (Heidelberg)
*Laboratory C**	Germany (Tubingen)
*Laboratory D**	Germany (Bielefeld)
*Laboratory E**	Germany (Essen)
*Laboratory F**	Germany (Berlin)
*Laboratory G**	Germany (Friburgo)
*Laboratory H**	The Netherlands (Zuidplas)
*Laboratory I**	The Netherlands (Den Helder)
*Laboratory J**	The Netherlands (Amsterdam)
*Laboratory K**	The Netherlands (Amdsterdam)
*Laboratory L**	Belgium (Bruxelles)
*Laboratory M**	Belgium (Genk)
*Laboratory N**	Belgium (Mortsel)
*Laboratory O**	Belgium (Tienen)
*Laboratory P**	Luxemburg
*Laboratory Q**	Luxemburg
*Laboratory R**	Luxemburg
*Laboratory S**	Luxemburg

*Confidential Appendix

[b] Company A is a company of national, autonomous and independent Clinical Analysis Laboratories. It thus constitutes an integrated network of Laboratories that provide a proximity service. This network has dozens of laboratories throughout Portugal.

Health Insurer

Another important customer in health field is the insurance companies. It is decisive for the success of the service that there is adherence by them. Insurers will benefit from sharing the service as patients get better results as they can target their treatment with *BRAF* inhibitors and also because it is a great tool in monitoring and detecting residual disease. Thus, there will be a decrease in expenses in treatment of this patient for benefit of the insurer. In addition, about Life Insurance, they also benefit from our test because they also cover disability due to serious illness. If the treatment is shorter and the response is shorter, the patient will have less work absenteeism. Therefore, the partnership between our company and the insurers is an advantageous for both parts.

Table 5 - Possible partner insurance companies for GenoMed S.A. in Portugal and in other countries.

Insurer	Location
*Insurance A**	Portugal
*Insurance B**	Portugal
*Insurance C**	Portugal
*Insurance D**	Portugal
*Insurance E**	Portugal
*Insurance F**	Portugal
*Insurance G**	Portugal
*Insurance H**	Portugal
*Insurance I**	Germany
*Insurance J**	Germany
*Insurance K**	Germany
*Insurance L**	Germany and Belgium
*Insurance M**	Germany and Belgium
*Insurance N**	Germany
*Insurance O**	Germany
*Insurance P**	Germany
*Insurance Q**	Belgium
*Insurance R**	Belgium
*Insurance S**	Belgium
*Insurance T**	The Netherlands
*Insurance U**	The Netherlands
*Insurance V**	The Netherlands

*Confidential Appendix

Insurance A (Confidential Appendix – Table 25*)* is currently the majority holder of *Shareholder A*, which in turn is the majority shareholder of GenoMed. So, in Portugal *Insurance A* is a possible partner of GenoMed. This connection facilitates the partnership between GenoMed S.A. and the insurers. Presently, GenoMed already has an established partnership with this insurer, which will facilitate the introduction of the new test.

Health insurance (Krankenversicherung) is mandatory in Germany and may be legal or private. If it is legal the amount to be paid is proportional to person's salary and so everyone can access it. GenoMed will first try to establish partnerships with private insurance companies and secondly with legal ones, presenting its value proposition to insurers that adhere to test. In Germany and Belgium, the GenoMed S.A. strategy could be to start a partnership with the insurance company

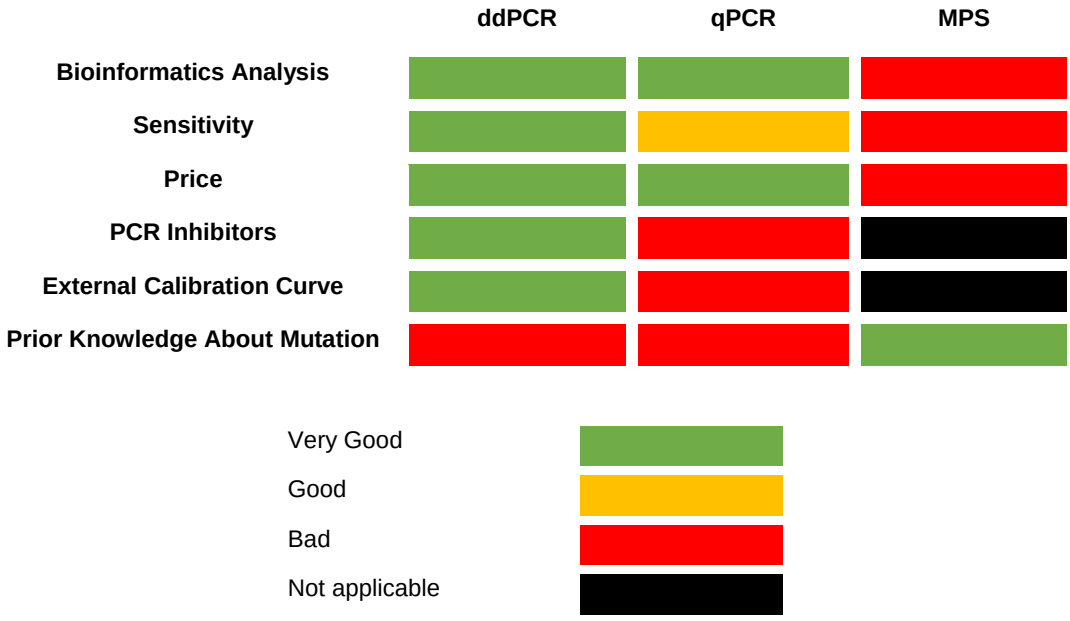
Insurance L (Confidential Appendix – Table 25). It is hoped that this partnership will be made easier by fact that the company is owned by the same investors as *Insurance A* in Portugal.

Insurance is mandatory in the Netherlands and Luxembourg. However, in Luxembourg 98% of the population is covered by mandatory public health insurance and to enter that market it would be important to join the public health insurance sector[150].

Competitors

The identification of competitors in the market is very important to identify the advantages of our service. For detection of the *BRAF* V600E mutation, several techniques can be used that provide different information and have different advantages. The techniques were compared in Table 6.

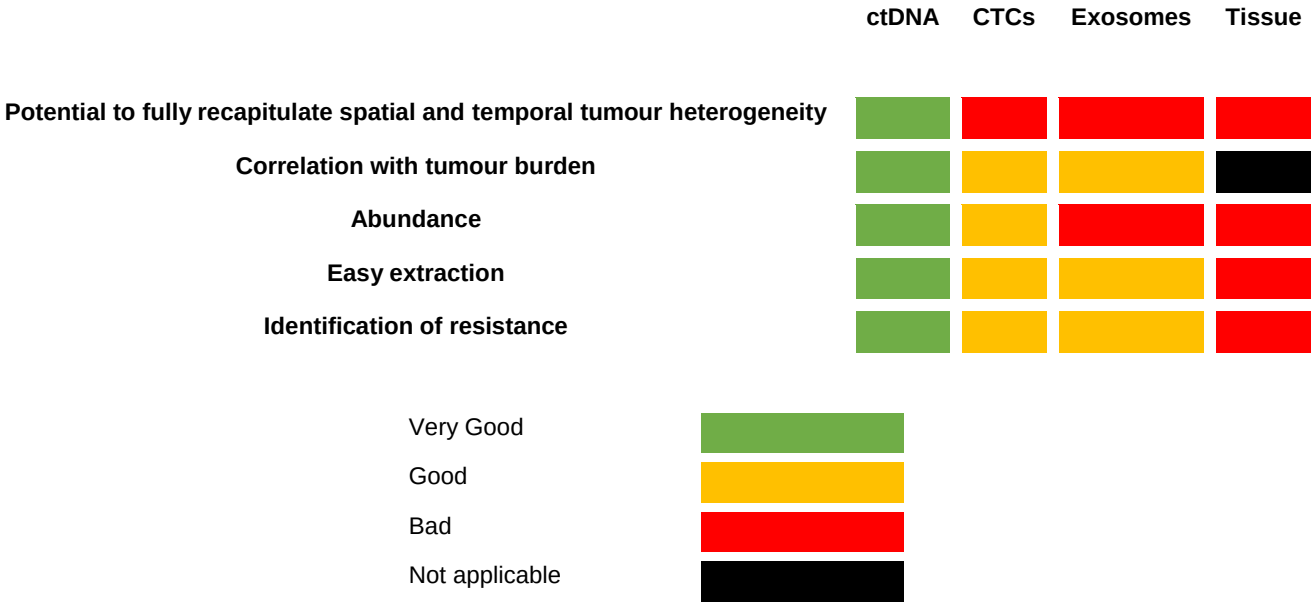
Table 6 - Comparison of technology platforms for *BRAF* V600E mutation detection in circulating tumour DNA. Adapted from Wan 2017.



Within platforms that can be used for the detection of circulating cell-free tumour DNA, the ddPCR is the one that presents / displays greater sensitivity, making the technology unique. In addition, it does not require bioinformatic analysis and has an affordable price.

In addition to the technique, detection of *BRAF* V600E may also differ according to the analysed component (tissue or liquid biopsies). However, there are several ways to perform a liquid biopsy. A range of tumour components can be distinguished from the fluids and molecular analysis of these different components (Table 7).

Table 7 - Comparison between the applications and advantages of ctDNA, CTCs, exosomes and tissue analyse. Adapted from Siravegna 2017.



Siravegna *et al*[81] considers the analysis of ctDNA is the only one able to completely reproduce spatial and temporal tumour heterogeneity. Moreover, it is the one that presents greater abundance and more facility in the extraction.

After verifying the comparison between techniques that can be used for detection of the *BRAF* V600E mutation, it is noticed that the one that has more advantages is the ddPCR and in turn, the best sample would be ctDNA (present in blood and urine) which previously described in the introductory section of chapter 2. Therefore, GenoMed S.A. is distinguished using a non-invasive sample for the detection of *BRAF* V600E mutation (ctDNA present in plasma or urine) and an extremely sensitive technique (ddPCR). This information was compared to companies in the Portuguese market that present some tests that provide similar information (Table 8).

Table 8 - Competitors in Portuguese market.

	Sample	Technique	Mutation
GenoMed	Blood/Urine (ctDNA)	ddPCR	*BRAF* V600E
Competitor A*	Tissue/Blood	qPCR	*BRAF* V600E
Competitor B*	Tissue	MPS	BRAF
Competitor C*	Tissue/Blood	MPS	BRAF
Competitor D*	Tissue/Blood	qPCR	BRAF

*Confidential Appendix

To our knowledge, in Portugal, GenoMed is the only company to offer *BRAF* V600E mutant detection service through a non-invasive sample and by ddPCR technology.

Competitor A and Competitor D are the biggest groups in clinical analysis in Portugal. However, they continue to use qPCR technology, which, in liquid biopsies has several disadvantages. When using qPCR, the main disadvantage is a lower analytical sensitivity when compared to ddPCR, which results in false negatives in patients who have low sample concentrations present in the blood or urine.

Competitor C is a company of the Competitor A group and is highly focused on liquid biopsies. However, the use of MPS for the performance of it assays like the use of qPCR has a lower analytical sensitivity. Moreover, it is not advantageous in patients monitoring situations because prior knowledge of the mutation is enough to monitor only this mutation and not being restricted to this mutation makes the test more expensive and more difficult for patients to access.

Table 9 - Competitors in other countries.

	Sample	Technique	Mutation	Location
Competitor E*	Tissue	Sequencing	*BRAF*	Germany
Competitor F*	Tissue	Sequencing	*BRAF*	Germany
Competitor G*	Tissue	qPCR	*BRAF* V600E	Germany
Competitor H*	Blood (ctDNA)	MPS	*BRAF* V600E	Germany
Competitor I*	Tissue	MPS	*BRAF*	Germany
Competitor J*	Blood	qPCR	*BRAF* V600E	Germany
Competitor K*	Tissue	MPS	*BRAF*	Germany
Competitor L*	Blood	Not referenced	*BRAF* V600E	Germany
Competitor M*	Tissue	MPS	*BRAF*	The Netherlands
Competitor N*	-	MPS	*BRAF* V600E	The Netherlands
Competitor O*	Tissue/Blood	ddPCR	*BRAF* V600E	Belgium
Competitor P*	Tissue	MPS	*BRAF*	Belgium

*Confidential Appendix

In the Belgian market, a Competitor A offers the same type of service as GenoMed. However, the business model of the company assumes that the laboratory/hospital/clinic must have collection tubes themselves and then, send the samples to the company. Moreover, it is a test offered without CE marking, that is, it is marketed as an LDT (Laboratory Developed Test) and therefore it can only be used in the health institution itself. On the other hand, the GenoMed business model which is explained in the value chain section below, in the logistics process collection tubes supply is part of model. This supply of collection kits to customers is a competitive advantage presented by providing greater confidence and convenience to our customers. Another advantage over foreign competitors is that the price of the test may be more affordable when using similar technologies because the cost of production in Portugal is much lower when compared to the target markets due to cheap skilled labour. Moreover, some competitors submit qualitative reports, and GenoMed's strategy will be to present the number of copies per millilitre of sample.

Finally, it is also important to emphasize as indirect competitors the imaging methods and the LDH serum levels in patients with melanoma. However, these methods present some disadvantages described in the discussion section of chapter two. The main goal is that the liquid biopsy is a complement to these traditional methods creating a market where previously it did not exist.

Suppliers

GenoMed has several suppliers in biotechnology sector. For "*BRAF* V600E mutant detection in ctDNA" test there are some specific consumables (Table 10) and others mutual to other tests in GenoMed. These materials have been previously described in the Materials and Methods section of Chapter 2.

Table 10 - Specific materials suppliers for the GenoMed S.A.

Sample Collection
Supplier A*
cfDNA Extraction
Supplier A and B*
DNA Quantification
Supplier B and C*
ddPCR detection
Supplier D and E*

*Confidential Appendix

Some of products reported are also used in other specific tests found on GenoMed. In addition, micropipettes tips, microtubes and non-specific reagents (ethanol, isopropanol, etc.) are purchased from GenoMed's common purchases. As far as *BRAF* is concerned, it is noted that it is a test dependent largely on the supply of Supplier D: a stable American company that probably will always be available for supply. All supply companies are outside Portugal, however, almost all of them have representatives in Portugal, which makes trading easier.

Community

GenoMed is a company that has a very large community involved in its activity and evolution. Being a join-stock company, part of that community is the shareholders themselves.

The *Shareholder B* owns 18.75% of the shares of the company. In addition to financial interest, *Shareholder B* has personal interests in the development of GenoMed.

Moreover, another prominent shareholder is the *Shareholder A* that owns a large portion of the shares (37.5%). Other shareholders with large shares of the company would be *Shareholder C* (30%) and *Shareholder D* (7.5%)[148].

In addition, the laboratories and hospitals GenoMed has partners with are interested in the company's activity. An important client is the *Client A* where GenoMed offers several tests. Also, the proximity to the hospital facilitates the integration of the procedure and some investigations of GenoResearch are also made in conjunction with the same. In the liquid biopsy developed by GenoMed an important supplier is a Supplier D. For the commercialization of a test that uses your equipment and reagents, is necessary to obtain a license from the company.

7. SWOT Analysis

After analyzing the micro and macro environment, we identify the opportunities and threats of the environment as well as the strengths and weaknesses of the company (Table 11).

Table 11 - SWOT analysis for the detection service of the *BRAF* V600E mutation in circulating DNA by ddPCR.

Strengths	***Opportunities***
-Company with 14 years and good reputation; -Non-invasive, low price and high sensitivity test; -The test is accessible anywhere; -Good partnerships with suppliers; -Partnerships already established with customers in other tests; - *Shareholder A* as stakeholder; - Production cost in liquid biopsy;	- The market for liquid biopsies is growing; - Size of Benelux+Germany market (30% European IVD); - High incidence of melanoma in Europe; - Ageing population; - Interest in new technologies by doctors; - High concern for health by population; - Existence of drugs for target therapies; - Increase in health expenditures;
Weaknesses	***Threats***
-Patent Inexistent; -Small size of the company; -Lack of experience in the international market;	-Convenience of using only traditional diagnostic methods; -Increased investment in melanoma liquid biopsies by existing or new competitors;

The small size of the company and the possibility of market competitors to can be overcome by taking advantage of the good relationship with suppliers and the great size present in the market for liquid biopsies for melanoma. This will allow GenoMed S.A. to achieve good prices and achieve a certain market share. Another alternative is to bet on a first-mover advantage strategy that together with good prices will allow to gain a good quota market and bet on loyalty of these customers. Another advantage in the international market is the low cost of production in Portugal because of the low price of skilled labour.

In addition, physicians are currently interested in innovative technologies and will be willing to give small business opportunities that meet needs of doctors and patients. Despite the company

small size, GenoMed S.A. is already a credible company in Portuguese market (14 years in the market) and therefore will not have difficulty in introducing its new test in market, which already sells other tests. Lastly, this credibility in national market can help to achieve good partnerships in the international field and gain trust from new customers.

The lack of experience in international market and lack of knowledge of Benelux+Germany market will have to be compensated with local partners. In the case of exporting the service to detect *BRAF* V600E mutation, the best alternative is that partners are distributors with relationships already established (described in the distribution section).

The lack of a patent is a weakness in the transfer to the market of this test. Due to this, GenoMed S.A.'s focus will be on differentiating itself by its business model, know-how and trade secret (described in the trade secret and business model sections).

Another threat in this business is the ease in using conventional technologies for clinical decisions. Here, the goal of GenoMed S.A. will be a strong commitment to communicate directly to the prescribers of the test (doctors) in which it will rely on clinical evidence showing all the innovation of the test, along with its good price and practicality (described in the communication section). It is necessary to show all the advantages already described in Chapter 2 in use liquid biopsy with the imaging methods. However, the big bet to overcome this threat is a quality communication by the GenoMed and its partners.

8. Strategic Analysis

In addition to strategy resulting from SWOT analysis, there are other strategies that need to be delimited, for the success of GenoMed’s new service.

Nowadays, innovation is a focal point for the success of technology companies, however, a very positive commercial feedback is needed in these innovations in order to compensate for the great investment in research. In case of innovations, such as the test developed in GenoMed S.A., the strategy adopted according to Porter reflects mainly the differentiation. That is, the adoption of this strategy implies that the company makes big investments in the image of the service with the objective of making a great differentiation of the service in front of the competitors. However, if similar technologies appear on the market, GenoMed has the advantage of also being competitive because of low production costs in Portugal.

For the success of service sale, it is necessary to actively invest in quality and marketing. Being that, a company may choose to cooperate with other companies to achieve these goals. Companies considered medium-sized, usually use outsourcing to perform certain services

necessary for success. In the case of GenoMed S.A., its key partners will be laboratories/clinics/hospitals/distributors that already have credibility in the market.

Regarding international expansion, the ideal is that it is a direct export, where GenoMed S.A. can export its service having a domestic partner to assist in the commercial part, namely the distributors mentioned later in Table 13. Through the strategy of international expansion, it is important to make three basic decisions: the type of export, where to enter and the timing of entry into these markets.

When the internationalization of a company begins, there are several ways of internationalization that can be adopted through company profile. Being the test focused on a restricted niche of patients, the licensing and franchising strategies only for this service are not adequate through investment. In this case, the best way to enter the market would be direct export, using the distribution of products in domestic markets by local partners. However, all this logistics must be following by a person of GenoMed S.A. in order to avoid losing control over the value chain. Furthermore, distributors must be very well chosen because, in addition to having to be reliable, it is also necessary that there be good prices for the service to reduce distribution costs that are usually high.

The market chosen for internationalization is also very important to success of the company. It needs to include a market that needs and is willing to pay for this service. Through these factors and others already described in the section on the choice of the market, four countries were chosen in addition to Portugal: Germany, The Netherlands, Belgium and Luxembourg.

On the other hand, the timing of entry into a specific market is an essential decision that should be well considered. Rapid entry into the market early with low competition capacity brings rapid cash-flows. Local partners can also help in this input more quickly and effectively. GenoMed S.A. intends to start commercializing its liquid biopsy initially exclusively in Portugal, later, when in Portugal the test is already well established and commercialized, it will begin to expand to German market. From the German market, the service will gain more credibility and even better recognition by the neighbouring countries that recognize the valorisation of quality by Germans. Later, when it is already established in the German market, it will begin to expand to the Netherlands, Belgium and Luxembourg. It is hoped that this expansion will have the help of the German distributors themselves that have partnerships with their neighbours.

9. Trade secret

In the case of detection of the *BRAF* V600E mutation, the test does not have as a product, any patent right. Being this a weak point of business, one solution would be to use a trade secret. Trade secrets are intangible assets that offer a competitive advantage in the market but do not

have any legal consecration. Within this category are strategic plans, marketing techniques, manufacturing formulas, computational processes, lists of customers, suppliers and materials or even process know-how. In the case of GenoMed, the company could choose to protect both its business model and know-how of performing genetic testing with a trade secret. Confidentiality agreements are usually used to protect business secrets. The purpose of this agreement is for the genetic testing information to be protected between the disclosing party and the receiving party. In this way, the party receiving does not acquire any additional right to the information. It is critical that GenoMed make these confidentiality agreements with everyone who has access to information to maintain its competitive advantage in the marketplace.

10. Marketing Mix

Service

The new service offered by GenoMed consists of detecting a mutation present in more than 50% of melanomas: *BRAF* V600E. This detection is done through innovative technology with high sensitivity (ddPCR) and is detected in non-invasive samples (blood and urine). The applications of this service have already been discussed previously in the discussion section of chapter 2.
The service has four crucial phases: the pre-sale service, the sending of the sample, the liquid biopsy, and finally, after-sales service. In addition, it is important to note that the service will follow the value chain described in the subsequent business model section.

The pre-sales service provided by GenoMed S.A. consists of the availability of specialized staff to clarify any doubt regarding the test by our customers or patients.

Sample shipment takes place through a specific collection kit supplied by GenoMed S.A distributors. The kit is very important because it adds a greater convenience to the customer, so you do not have to worry about buying special tubes for sample collection. After receiving the kit and collecting the sample, it is sent by shipping company to GenoMed S.A. This kit becomes a competitive advantage of GenoMed because it transmits greater confidence to customers.

In the GenoMed, the genetic test itself consists of detecting the *BRAF* V600E mutation through 3 steps: extracting cfDNA, quantifying cfDNA and detecting mutation through ddPCR. The whole process and optimization corresponding to this aspect was discussed in chapter 2.

Finally, the test report is made available to our customer and after-sales service is available. After-sales service consists, for example, of clarifying doubts regarding doubts in interpreting the report. It is also possible to conduct multidisciplinary discussions (with our specialized staff responsible for test and doctors involved in the treatment of patient). The area of oncology is an area that is

easily incorporating the integration of several professionals into a final clinical decision. This possible interaction between GenoMed S.A. and its partners will bring better outcomes for patients with melanoma.

Moreover, all reports and collection kits will be made available in the languages of the target markets.

Price

The service price is essential for the market, since the consumer expects the benefits. The price service of detection of the *BRAF* V600E mutation through ddPCR for this business plan was defined in € 199.00 when the sample to analyse is blood and € 319.00 when the sample is urine. The collection kit is sold separately and will have a price of € 19.00 to plasma samples and € 29.00 to urine samples.

Test costs include cfDNA extraction, cfDNA quantification and ctDNA detection. All cost prices were based on the prices of products available in the market. This final price is based on competitors, on all production costs and consider the amount that will be paid to Bio-Rad for use of the equipment and reagents. (Confidential Appendix – Table 27 to 31).

Moreover, the price may be lower when purchases are made in large quantities.

Distribution

In order to transpose the service to external markets and explore suitable positions to the specific characteristics of the product in relation to the target consumers, it is necessary to create distribution channels, guaranteeing a quality offer. In addition, many distributors are also responsible for commercial section of the service, which will facilitate sales mainly about service internationalization. Therefore, we must focus on distributors in Portugal and the other target markets of GenoMed, S.A.

In the case of liquid biopsy to *BRAF* V600E detection, is distributed only the sample collection kit, and the same is then sent by shipping company back to GenoMed and is no longer the responsibility of the distributor.

During service internationalization a local partnership is very important to ensure the success of sales. Each market has its peculiarities and these partnerships help to overcome the lack of knowledge of the new markets.

In this case, it is necessary to establish partnerships with distributors who already have contact with the target market of our liquid biopsy. It is important that partners ensure the

commercialization and logistics of the collection kit and the genetic test. According to the partnership agreement, the responsibilities and contributions of each of the parts for the introduction and commercialization in the given market would be defined. The contract would imply support in commercialization of the service from the availability of leaflets, technical and scientific documentation and participation in medical symposia, web marketing and delegates of medical information. All this communication is necessary to reach the doctors who are the class with great influence in the sales of our service, since, the service only happens after a medical prescription. Only with all these conditions would it be possible to ensure the image and notoriety of the service, through quality, efficiency and utility level, thus making it credible and capable of spreading in the market.

Below are the distributors that can be GenoMed S.A.'s partners with these responsibilities. In Portugal (Table 12) and in the market that we intend to internationalize (Table 13).

Table 12 - Leading distributor in Portugal with potential to be GenoMed S.A. partners.

Distributor	Country
Distributor A*	Portugal
Distributor B*	Portugal
Distributor C*	Portugal

*Confidential Appendix

In Portugal, GenoMed itself will also be responsible for the commercial part of the service and therefore may resort to partners who often only carry out the storage and distribution of the collection kit. Mainly in partnerships already established by GenoMed in previous phases.

Distributor A is a company with many years in the Portuguese market that distributes medicines, medical devices and also products in the area of biotechnology. It can be a viable alternative for current GenoMed customers.

The Distributor B and C in turn carry out the distribution, but also take responsibility for the commercial section, including availability of contracting of medical information delegates. These partnerships will be interesting to further disseminate the test and expansion in the Portuguese market through the strategies presented above.

Furthermore, distributors have been identified in the remaining market, which will be focus of GenoMed S.A. It is important to remember that Germany is the distribution center of target market (The Netherlands, Belgium and Luxembourg). In addition, distributors already have an interconnected distribution network between these countries. Local partners are very important because each domestic market has its own particularity and will help GenoMed S.A. determine the best strategies.

Table 13 - Leading distributor in in the demographic regions considered in this study with potential to be GenoMed S.A. partners.

Distributor	Country
Distributor D*	Germany
Distributor E*	Germany
Distributor F*	Belgium
Distributor G*	The Netherlands

Distributor D is a pharmaceutical and biotechnology wholesaler. It main business is the import and export of branded pharmaceuticals, medical devices and other innovations in the market. They operate as wholesaler for hospitals and laboratories to grant the availability of medical standard range. Mainly, innovations that are not available in the destination countries. The company usually provides several countries in region and can be an entry point to GenoMed's new service.

Within the distribution channel, it is very important that partners have access to the channels of interest as customers - laboratories, hospitals and clinics. The differentiation in the policy of communication by the distributors is extremely relevant, because, it will be necessary the senbilization and training as to the level of utility of our new service. Distributor B, C and D have characteristics that can cover all these requirements.

It is important to note that, these distributors are possibilities within the target market. However, during the service presentation at scientific fairs, other opportunities may arise, with other partnerships, other distributors and other markets and all these possibilities will be evaluated.

Communication

It is important that there is differentiated advertising for each type of customer or partnership. It is necessary to present different communication strategies, for example, hospitals, insurance companies, distributors and doctors (who are prescribers and fundamental for the sale of service).

Prior to the launch of GenoMed's new service the detection of the *BRAF* V600E as a liquid biopsy, seminars, medical congress participations and lectures will be performed to disseminate the "VALUE" of our service. These will target healthcare professionals and especially in the prescribers of this genetic test: doctors. Example: A doctor, after attending the lecture, may recommend to his patients perform a test to also be accompanied by them.

There will also be advertisements in magazines linked to biotechnology, molecular diagnostics and oncology.

We will also have a strong stake in medical information delegates (particulars or from our partnerships). These professionals will be trained to be proficient regarding all the valences of the

BRAF V600E in liquid biopsy. The goal is that, they can show doctors (mainly oncologists) and laboratories the great value proposition of our service. In addition, part of our advertising will be done by our own distributors, who already have credibility in the market.

In seminars, in magazines or through medical information delegates, the major goal is to sensitize the medical class to the usefulness of our new service. It is necessary that the value proposition be very clear so that there is a greater diffusion of the target market. If doctors are convinced of the various uses and advantages of our test (described in the discussion section of chapter two), it will be easier to achieve great partnerships - such as insurers and hospitals.

In Portugal, partnerships with insurers and hospitals will be largely made directly with GenoMed S.A. Already abroad, it is expected that distributors may also take charge of the commercial side and establish such relationships. During an internationalization, a local partnership is very important for success due to the partner's local knowledge of the local market. The value proposal described later must be very clear during the communication.

Firstly, the focus will also be to pass the information of the new service that brings a lot of benefits to melanoma patients to all the long-standing partnerships from GenoMed S.A.. Already knowing the company by its quality will be much easier the adhesion to a new test.

About distributors, the communication will be made directly, addressing the great advantages of our service, or through the participation of fairs that cover biotechnology.

Table 14 shows the next fairs with greater interest in the dissemination of the new GenoMed service that will improve the lives of patients with melanoma.

Table 14 - Survey of the main international fairs in the biotechnology/health area.

Fair	Location	Date
Biotechnica 2019[(a)]	Hannover (Germany)	May 21-23 2019
Analytica[(b)]	Munich (Germany)	April 1-4 2020
The European Human Genetics Conference 2019[(c)]	Gothenburg (Sweden)	June 15-18, 2019
MedTech Summit[(d)]	Brussels (Belgium)	June 18-21 2019
Pharmaintech[(e)]	Bolonha (Italy)	April 10-12 2019

[(a)] http://www.labvolution.de/

[(b)] https://www.analytica.de

[(c)] https://www.eshg.org/

[(d)] https://lifesciences.knect365.com/medtech-summit/

[(e)] http://www.pharmintech.it/ita/home

Fairs are great opportunities to establish partnerships with distributors and possibilities should be explored. From this first contact, a future partnership can arise. The responsibilities and contributions of each of the parts for the introduction and commercialization in each market would

be defined. GenoMed S.A. shall ensure that the commercial partner has the greatest ability to provide product penetration and will use its medical information delegates to provide the training and argumentation necessary to communicate effectively and efficiently with the medical (prescribing) class. Only in this way can you ensure the image and notoriety of the product, through its quality, efficiency and level of utility provided, making it credible and capable of being widely disseminated, capitalizing on all references through direct and digital marketing as well as public relations (sponsorship of events, symposiums, medical congresses, among others).

In short, public relations and sales force are the most important variables in the marketing mix of communication since this is a mostly business-to-business market.

11. Business Model

Value proposition

As we previously reported, detection of the *BRAF* V600E mutation by ctDNA through ddPCR implemented by GenoMed has been found to fill a gap in the market. The test is ideal for melanoma management. Therefore, the value proposition of the test is based on the fact that it is not an invasive test and is a tool that allows physicians to make more accurate clinical decisions with high sensitivity, at an affordable price and a fast response time. The uniqueness of test the high sensitivity that allows changes in clinical decisions to be executed quickly in melanoma patients. This results in better quality of life and survival for melanoma patients.

Melanoma is responsible for the highest number of deaths in skin cancers. Early detection of cancer changes has a major contribution to improving patient outcomes. That is, it is necessary that a better management of melanoma is performed and for this, it is necessary that there are tools that can help this management. The tool developed in this work has the capacity to contribute to these decisions in a cost-effective and non-invasive way, improving the patient's survival. The test is useful for guiding therapies, prognosis, monitoring and detection of residual disease (more details about applications in discussion section in Chapter 2).

Europe is one of regions with the highest incidence of melanoma (Figure 1). It is believed that in the long run these rates can be lowered by adherence to our test. Concerning to melanoma, early diagnosis is critical to a successful treatment. The fact that it is a non-invasive and low-cost test makes it much easier to perform it in a larger range of patients, and more often in each patient, resulting in an early diagnosis and better treatment. That is, GenoMed will be selling health and years of life to patients.

GenoMed S.A. also presents a quantitative monetary value proposition. The idea is that, in addition to the clinics and laboratories to benefit from a tool to better serve their customers, they can also add a price range and purchase revenues from sale of our service.

For insurers, it is important to emphasize that if the patient is treated better, the patient will visit less the doctors and so the insurer will spend less money on this patient. In addition to also having less time of incapacity for work.

Therefore, our innovative service with several benefits brings a strong value proposition. This proposal integrates their patients, but also for the clinics, laboratories and insurers that want to acquire the service.

Value Chain

The value chain of in vitro diagnosis (IVD) differs greatly depending on how the sample is collected, analyzed and delivered. Here, a proposal was made for an innovative value chain for the commercialization of the new liquid biopsy: detection of *BRAF* V600E in circulating DNA through ddPCR.

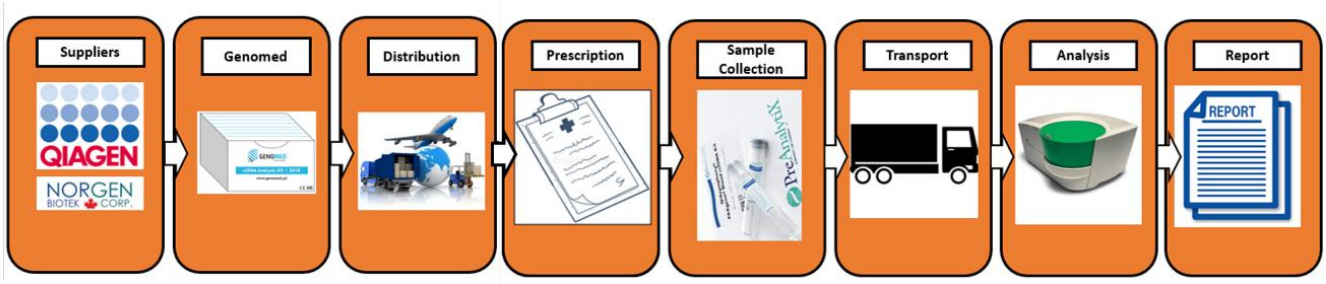


Figure 28 - Representation of value chain for the sale of the *BRAF* V600E mutation detection service in liquid samples (blood and urine).

1- The value chain begins with the arrival of the necessary equipment to assemble the sample collection kit and necessary reagents to ddPCR analysis, through the suppliers.

2- In a second moment, the special collection kit is customized and packaged by GenoMed, S.A.

3- The distribution of the kit happens through established partnerships or directly.

4- Subsequently, the prescription/request of the analysis by the physician who intends to use the test to aid in a better choice of patient's treatment or for monitoring the disease course happens. That is, the patient obtains a prescription to be able to collect the sample for analysis.

5- The collection is done through GenoMed's own kit. This collection can be carried out by laboratories and clinics that have specialized personnel for the function.

6- After collection, the sample must be transported to GenoMed S.A. laboratory. The sample (blood or urine) must be sent together with the medical requisition, and can be done individually or with group, and will be carried out via shipping company and respond to the rules for transportation of biological samples.

7- The analysis will be performed as previously described. All the analysis takes place within the facilities of the GenoMed S.A. that are highly advanced. In addition, the team involved is also highly specialized, ensuring efficient and safe operation.

8- After analysis of the patient's biological sample, GenoMed S.A. transmits the information received to clinician to support the patient's treatment within 5 business days. Information sharing can be done with the help of information systems in order to ensure greater efficiency and avoid loss of data.

This value chain by providing the customer collection kit brings much more convenience and becomes a competitive advantage of GenoMed S.A.

Revenue Model

The recovery of the investment made in the implementation of *BRAF* V600E detection test in ctDNA by ddPCR and the capture of value implies the success of implemented strategy and commercialization. The higher the number of tests sold, the higher is the volume of revenue and higher is the value created by the company. In this way, the objective is to progressively increase the number of consumers within the territorial area where it operates, creating long-term and reliable relationships with entities delegates to test distribution. The revenue model is based on continuous value creation and high-quality betting.

To this end, we propose a revenue model that consists of the national and international commercialization of genetic testing, increasing revenues with the value created by the company. These revenues can come in different ways.

First, nowadays the revenues mostly come from patients who need the test and make the private payment directly to GenoMed S.A..

Second, it is possible that in the near future laboratories, clinics and hospitals that purchased the kits, pay the test directly to GenoMed S.A.. In the case of laboratories, it is normal for them to acquire the kits, send the tests with a large number of samples (reducing shipping costs), pay

GenoMed S.A. directly for the service and add a monetary margin to its patients for the service rendered as an intermediary. Regarding to public hospitals, they often buy the test from GenoMed S.A., do all the intermediary role and patients do not pay for the service.

Third, revenue can also come from insurers who can include this test in the range of tests provided by each.

It is important to remember that in all situations payment occurs for the service. On the other hand, the sale of the kit is done as part of the diagnostic service, being also, a revenue entry, usually proportional to the number of services provided.

12. Social Responsibility

The launch of a liquid biopsy in GenoMed S.A. to improve the melanoma management will contribute to this character of social responsibility. The primary goal of test is better treatment and less invasive monitoring during patient therapy. Therefore, the company always values the interests of patient. In addition, it will also offer tests where during the treatment of some patients in poorer regions.

13. Economic-Financial Plan

The financial projections, specifically sales projections, cash flow and profitability will be a vital element in determining the viability and attractiveness of the company project and a reference to present to partners and potential investors. A financial plan was developed for six years of activities related to commercialization of the *BRAF* V600E mutation detection in ctDNA by ddPCR service by GenoMed S.A. The pioneering research of this service took place during 2017 and 2018 and its commercialization will begin in 2019. The projections made during the implementation of this financial plan are only estimated values and not precise values. The presented model is an adapted and simplified version of IAPMEI *(Instituto de Apoio às Pequenas e Médias Empresas e à Inovação*) model and begins with the establishment of assumptions for the financial analysis. The local currency unit was used: euro (€). Model assumptions of new GenoMed's project The values presented are based on GenoMed S.A. Financial Report (2014-2016)[148]. Moreover, the sector opportunity rate is based in the financial profitability of GenoMed in last three years. (Confidential Appendix – Table 32).

The revenues from this service can originate in four different ways (Table 15). Detection of blood or urine mutation and sales of the respective collection kit. Sales prices were based on the cost prices previously described in the Price section of the Marketing Mix in this business model.

Table 15 - Costs of test and kit to *BRAF* V600E mutation detection

	Cost	Sale
Detection in plasma	25,50 €	199,00 €
Detection in urine	71,25 €	319,00 €
Kit to plasma collection	10,50 €	19,00 €
Kit to urine collection	18,00 €	29,00 €

Sales of the detection service of the *BRAF* V600E mutation in ctDNA by ddPCR are predicted to begin in 2019 (Table 16).

Table 16 – Sales, revenues and costs projection of new GenoMed's project in the next 6 years.

Amount Sold	**1 year**	**2 year**	**3 year**	**4 year**	**5 year**	**6 year**
Detection in plasma	180	234	351	527	737	952
Detection in urine	100	130	195	293	410	532
Kit to plasma collection	200	260	390	585	819	1065
Kit to urine collection	120	156	234	351	491	639
Sales Growth (%)		30%	50%	50%	40%	30%
Sales and cost of sales	**1 year**	**2 year**	**3 year**	**4 year**	**5 year**	**6 year**
Total sales detection in plasma	35 820 €	46 556 €	69 849 €	104 774 €	145 683 €	190 688 €
Total sales detection in urine	31 900 €	41 470 €	62 205 €	93 308 €	130 631 €	169 820 €
Total sales plasma kit	3 800 €	4 940 €	7 410 €	11 115 €	15 561 €	20 229 €
Total sales urine kit	3 480 €	4 524 €	6 786 €	10 179 €	14 251 €	18 526 €
Total sales	75 000 €	97 500 €	146 250 €	219 375 €	307 125 €	399 263 €
Total costs detection in plasma	4 590 €	5 967 €	8 951 €	13 426 €	18 796 €	24 435 €
Total costs detection in urine	7 125 €	9 262 €	13 893 €	20 841 €	29 177 €	37 930 €
Total costs plasma kit	2 100 €	2 730 €	4 095 €	6 143 €	8 599 €	11 179 €
Total costs urine kit	2 160 €	2 808 €	4 212 €	6 318 €	8 845 €	11 498 €
Total sales costs	15 975 €	20 768 €	31 151 €	46 727 €	65 418 €	85 043 €

We propose that sales in the first year will be supported by GenoMed's existing partnerships in Portugal. During this year, the company will work on establishing new partnerships as all described during this work. In the second year, it will begin its sales in new Portuguese partnerships and gain more credibility in the market.
The 50% growth in the third year of the marketing of the service marks the entry into the German market and the 50% growth in the fourth year represents the entry into the Dutch, Luxembourgish and Belgian markets.

This number of sales is supported by Table 2, which shows market potential, market growth expectations and also the incidence of melanoma present in Portugal and other countries. It is also expected to sell more collection kits than tests to detect *BRAF* V600E in ctDNA by ddPCR, because laboratories, clinics and hospitals may want to make stock to ensure prompt customer service.

With regard to the specific external services and supplies for our new service, the biggest expenses will be with advertising and marketing. One of the strategies of GenoMed S.A. to achieve expected sales is the use of efficient communication that informs our value proposition to doctors and customers.

Table 17 – Expenses with external services and supplies of new GenoMed's project in the next 6 years.

External services and supplies	1 year	2 year	3 year	4 year	5 year	6 year
Adversiting and Marketing	10 000 €	10 000 €	12 000 €	14 400 €	17 280 €	20 736 €
Fees	1 000 €	1 000 €	1 000 €	1 000 €	1 000 €	1 000 €
Gift items	1 000 €	1 000 €	1 000 €	1 000 €	1 000 €	1 000 €
Travel and stays	3 000 €	3 000 €	3 000 €	3 000 €	3 000 €	3 000 €
Total	15 000 €	15 000 €	17 000 €	19 400 €	22 280 €	25 736 €

Table 18 - Human Resource Costs of new GenoMed's project in the next 6 years.

Personnel Expenses	Number of employees	Value	Months	1-6 Year
Salary – Laboratory Technician	1	1 000 €	14	14 000 €
Food Allowance	1	91 €	11	1 001 €
Salary – Commercial employee	1	1 200 €	14	16 800 €
Food Allowance	1	91 €	11	1 001 €
Total remuneration subject to *Segurança Social*				30 800 €
Total remuneration exempts to *Segurança Social*				2 002 €
***Segurança social* 23,75%**				7 315 €
Work accident insurance 1%				328 €
Total Personnel Expenses				40 445 €

The human resource costs for commercialization of our new test include two employees devoted exclusively to the new test. One who will be responsible for the execution of the test with respect to the laboratory part (other GenoMed S.A. employees can also carry out the new test). And the second, will be responsible for monitoring and organizing the entire commercial part of the new service. We believe that it is necessary to invest in communication to achieve the sales that we want, so it is necessary to have someone who turns all that investment.

It is important to note that distribution costs are not accounted for because the margin of distribution must be adopted according to the interest of each partner in our marketed final value.

In order to verify the viability and interest of the project, we used the preparation of a Profit and Loss Statement. This demonstration was prepared with estimates without the new service, with the new service, and the difference between two (Confidential Appendix – Tables 33 and 34). Through the Profit and Loss Statement, we were able to clearly perceive the net profitability of the project, constituting this as the first approach to the viability of our new test for patients with melanoma.

Table 19 – Profit and Loss Statement estimated of GenoMed between 1-6 year (values with the difference between the project and the project)*.

Operating Results	1 year (Delta)	2 year (Delta)	3 year (Delta)	4 year (Delta)	5 year (Delta)	6 year (Delta)
Sales of products and services provided	75 000 €	97 500 €	146 250 €	219 375 €	307 125 €	399 263 €
Costs of materials consumed	15 975 €	19 170 €	28 755 €	46 727 €	65 418 €	85 043 €
Gross margin	59 025 €	76 733 €	115 099 €	172 648 €	241 707 €	314 220 €
Operating Subsidies	-	-	-	-	-	-
External Supplies and Services	15 000 €	15 000 €	17 000 €	19 400 €	22 280 €	25 736 €
Personnel Expenses	40 445 €	40 445 €	40 445 €	40 445 €	40 445 €	40 445 €
Impairment of Debts Receivable	-	-	-	-	-	-
Other Income and Gains	-	-	-	-	-	-
Other Expenses and Losses	-	-	-	-	-	-
EBITDA /RADJI	3 580 €	21 288 €	57 654 €	112 803 €	178 982 €	248 039 €
Depreciation and amortization	36 €	36 €	36 €	36 €	36 €	36 €
EBIT / RAJI	3 544 €	21 252 €	57 618 €	112 768 €	178 947 €	248 003 €
Similar Interest and Expenses Supported	0 €	0 €	0€	0 €	0 €	0 €
Profit before Taxes	3 544 €	21 252 €	57 618 €	112 768 €	178 947 €	248 003 €
IRC	744 €	4 463 €	12 100 €	23 681 €	37 579 €	52 081 €
EBIT with tax effect	2 800 €	16 789 €	45 518 €	89 086 €	141 368 €	195 922 €
Depreciation and amortization	36 €	36 €	36 €	36 €	36 €	36 €
Operating Cash Flow	2 836 €	16 825 €	45 554 €	89 122 €	141 404 €	195 958 €

* More details in the Confidential Appendix – Tables 33 and 34.

Although Genomed already shows positive growth trends, the new project is expected to increase net results. Therefore, will allow that in addition to being able to realize the division of dividends for the stakeholders, that also incorporates money in the company to guarantee more investment in R&D, for example.

In view of the activity expected in the previous tables, it is important to clearly define the resulting working capital requirements, that is, the cash flow needs exclusively that GenoMed S.A. will need to be able to operate for the *BRAF* V600E mutation detection in ctDNA by ddPCR services.

Considering a APP and a ACP with a difference of days (Confidential Appendix – Table 32), and a 30-day stocking time, we have the following working capital needed.

Table 20 - Working Capital Required for the next 6 years to the new GenoMed's project.

Working Capital Required	0 year	1 year	2 year	3 year	4 year	5 year	6 year
Financial Requirements							
Cash Reserve	0 €	---	---	---	---	---	---
Stocks	1 331 €	1 731 €	2 596 €	3 894 €	5 451 €	7 087 €	0 €
Customers	0 €	72 391 €	94 108 €	141 162 €	211 743 €	296 440 €	385 371 €
Total Requirements	1 331 €	74 121 €	96 704 €	145 056 €	217 194 €	303 527 €	385 371 €
Financial Resources							
Suppliers	1 637 €	25 782 €	30 103 €	40 635 €	55 619 €	73 409 €	85 208 €
State	-306 €	3 012 €	3 673 €	5 050 €	7 155 €	9 688 €	12 670 €
Total Resources	1 331 €	28 794 €	33 776 €	45 685 €	62 774 €	83 097 €	97 878 €
Working Capital Required	0 €	45 328 €	62 928 €	99 371 €	154 420 €	220 429 €	287 493 €
Investment in WC	0 €	45 328 €	17 600 €	36 443 €	55 050 €	66 009 €	67 064 €

Although GenoMed has a good term for payments, it still has a longer term for receipts, implying the need for capital to cover this difference and also the existence of stock.

In order for this project to be completed, an initial R&D and other investments are also required.

Table 21 - Initial investments to the new GenoMed's project.

Investment Plan for Fixed Capital	0 year	Depreciation (%)	Depreciation 1 year – 6 year	IVA (%)	IVA (€)
R&D Investment					
Total R&D Investment	**12 095 €**	**---**	**0**	**---**	**2 782 €**
Other investments					
CE and IVD Certification	100 000 €	0,00%	0	23%	23 000 €
Other investments	356 €	10,00%	36 €	23%	82 €
Start Advertising	30 000 €	0,00%	0	23%	6 900 €
Total other investments	**130 356 €**	**----**	**36 €**	**---**	**29 982 €**
Total Fixed Capital	**142 451 €**	**----**	**36 €**	**---**	**32 764 €**

* More details in the Confidential Appendix – Table 35.

All pioneering investment in R&D has been made during the years 2017 and 2018. In addition, the equipment necessary for the test but absent from the investment is because the company itself had them bought before deciding to implement this new service.

The initial investment in advertising will be used to make our new service known to a wide range of potential customers. The investment will be used for the actions already described previously

in the Marketing Mix section. In addition, an investment in advertising will be made each year as reported in Table 19.

Finally, a cash flow map was carried out and feasibility indicators for the project were evaluated.

Table 22 - Cash Flow Map for new GenoMed's project for the next 6 years.

Cash Flows Map	0 year	1 year	2 year	3 year	4 year	5 year	6 year
Resources							
Operating Cash Flows	-	2 836 €	16 825 €	45 554 €	89 122 €	141 404 €	195 958 €
Residual Operating WC	-	-	-	-	-	-	100 000 €
Total resources (inflows)	-	2 836 €	16 825 €	45 554 €	89 122 €	141 404 €	295 958 €
Requirements							
Fixed Capital Investment	142 451 €	-	-	-	-	-	
WC Investment	0 €	45 228 €	17 600 €	36 443 €	55 050 €	66 009 €	-
Total requirements (outflows)	142 451 €	45 228 €	17 600 €	36 443 €	55 050 €	66 009 €	-
Cash Flow Total	-142 451 €	45 228 €	17 600 €	36 443 €	55 050 €	66 009 €	295 958 €
Update coefficient	*1,00**	*0,90**	*0,81**	*0,73**	*0,66**	*0,59**	*0,53**
Update Cash Flow	-142 451 €	-38 281 €	- 630 €	6 662 €	22 444 €	44 743 €	158 231 €
Accrued update Cash Flow	-142 451 €	-180 732 €	-181 362 €	-174 700 €	-152 255 €	-107 512 €	50 719 €

*(1+Sector opportunity rate)^{year}

Table 23 – Viability indicators of new GenoMed's project.

NPV	50 719 €
IRR	16,29%
PayBack	5 years and 8 months

The cash flow map of new GenoMed S.A. project shows that after five years and eight months we have achieved payback. In addition, it has an Intern Return Rate (IRR) of 16.29% and a Net Value Profit (NPV) of 50 719 €, making it a viable and attractive project.

After all the market research conducted in this work and corresponding financial plan, we believe that this can be a great opportunity for Genomed S.A. to start exploration in the international market. We hope that by the end of these six years we can start a new project where we will explore sales to other countries like Nordic or Great Britain.

Final conclusions

Melanoma has been the focus of this thesis since it remains the most common cause of death from skin cancer. Patients are often diagnosed in advanced stages of the disease, where there is a poor prognosis. There is a great need to develop and commercialize tests capable of diagnosis of melanoma through biomarkers, for use in clinical practice and routine clinical trials. The test would be useful in melanomas for early diagnosis, prognosis and patient stratification for different therapies and especially for monitoring treatment response and detect residual disease. Hence, the central aim of this project was to develop and delineate a go-to-market strategy for a tool that could be used recurrently, cost effective and non-invasively to support in medical decisions for these patients.

To do this, our first aim was implemented a novel assay to detect the most common mutation found in melanoma patients (*BRAF* V600E mutation) in ctDNA samples using ddPCR. With the *BRAF* V600E mutation assay designed and with the test in biological samples from patients with melanoma, it was possible to verify the possibility of detecting the mutation in midstream urine and blood samples. However, limitations were identified as the percentage amount of cfDNA present in the samples. Due to this limitation, we demonstrate that the analysis done by the 4200 TapeStation is very important to identify the baseline defined by us as possible detection (above 50% cfDNA). In addition, we also find for the first time that midstream urine is a better choice for *BRAF* V600E mutation analysis and has 100% agreement with plasma samples from melanoma's patients. Therefore, we demonstrated the high sensitivity of ddPCR to detect *BRAF* V600E mutation. In addition to all the benefits of being a non-invasive test, its high sensitivity allows it to be an alternative to when there is no access to sentinel lymph node biopsies and fine needle aspiration biopsies of metastatic sites. All these findings make the applicability of liquid biopsies very close to reality. This innovative test will mainly change the way of monitoring the patients with melanoma. The possibility of constant, non-invasively and cost effective, patient monitoring will bring much better results for patients. It will allow melanoma patients who are geographically limited to imaging methods to have access to a monitoring method.

Before the test was effectively implemented in the clinic, it would still be interesting to develop a quality control system within ddPCR for routine clinical use and a study with a greater number of patients with melanoma to provide more information. Thus, there would be much greater credibility of the test in the market.

Being a test with great commercial potential, the second objective of this thesis was the design of a go-to-market strategy. The growth of the market for liquid biopsies, high rates of melanoma incidence and the economic evaluation of the countries, were important for the selection of four target markets for the sale of the new net biopsy for melanoma patients: Germany, Belgium, the Netherlands and Luxembourg. These integrated countries have market size, purchasing power

and service needs, thus making it possible the success of high service sales. In addition, many possible partnerships have been identified for this success. From potential customers to distributors who can act as local and essential partners for the successful launch of the service. Moreover, existing partnerships will be used to increase the credibility of the service in the market. Competitors were identified in the internal and external market, and it was verified that GenoMed's great competitive advantage would be the application of the business model proposed in this work: the commercialization of a sample collection kit. This strategy will allow great convenience to the customers, besides providing greater credibility. Furthermore, in the international market GenoMed S.A. is favoured by the production costs practiced in Portugal. Financially, the project presents good indicators. With an investment of 142 451 € the business will have an IRR of 16.29%, NPV of 50 719 € and a PayBack of 5 years and 8 months. Currently, GenoMed S.A. is already a company with almost 15 years in the market and with a solid structure, which will allow to enjoy the benefits of implementing this innovative test without many critical risks.

Here, we have identified that this project is a great opportunity for the international market to be exploited by GenoMed. Liquid biopsies are a new market, little explored, and there are very few certified tests in world that offer ddPCR detections. Moreover, we believe it is a feasible project and it is worth continuing to invest in all validation of the test and future commercialization.

In conclusion, melanoma and mainly its recurrence are of great importance for public health. There is a great to obtain better alternatives for that patients with melanoma can be better diagnosed and monitored regularly, cost effective and non-invasively, in order to improve survival. Here we present an innovative *BRAF* V600E ddPCR mutation assay in non-invasive samples (blood and midstream urine) for the management of patients with melanoma.

Finally, the implementation of this tool for melanoma management in GenoMed S.A. will improve the conditions of patients bringing benefits to their health but will also be a commercially attractive project. Therefore, the implementation of this tool should happen as quickly as possible to bring all the benefits expected to company and to society.

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