UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE BIOTECNOLOGÍA

Mutational analysis of RAS genes in metastatic colorectal cancer: concordance between tumour tissue and liquid biopsy

TRABAJO FIN DE MÁSTER EN BIOTECNOLOGÍA BIOMÉDICA

ALUMNO: BORJA LAFUENTE GUTIÉRREZ

TUTORA: ELOISA JANTUS LEWINTRE

DIRECTORA (Co-tutor externo): SILVIA CALABUIG FARIÑAS

Curso Académico: 2014/2016

VALENCIA, JULIO DE 2016

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE BIOTECNOLOGÍA

INFORME TUTOR TRABAJO FIN DE MÁSTER (para cualquiera de las modalidades)

Informe del tutor (per a qualsevol de les modalitats del treball fin de master)

TUTOR's REPORT (for any type of final Master's degree thesis)

CURSO - Curs - *Academic year* **2015-2016**

DATOS DEL ALUMNO/A - Dades de l'alumne/a - Student's identification

Máster (Màster –Master's degree): Biotecnología Biomédica

Apellidos (Cognoms - *Surname(s)):* Lafuente Gutiérrez Nombre (Nom – *Name*): Borja
DNI (*ID card nr.*): 21007055M Teléfono (Telèfon - *Phone nr.*): 662275327
Correo electr. (Correu electr. – *email*): borjalafuente.biomedicina@gmail.com

TÍTULO DEL TRABAJO FIN DE MÁSTER - Títol del Treball Fi de Màster - Title of the Final Master's degree thesis

Mutational analysis of RAS genes in metastatic colorectal cancer: concordance between tumor tissue and liquid biopsy

Tutor/a (*Tutor*): Eloísa Jantus Lewintre
Cotutor/a (*Co-Tutor*): Silvia Calabuig Fariñas

Considero que el TFM está finalizado y se puede aceptar para la defensa del mismo **SI**
Considere que el TFM està finalitzat i es pot aceptar la defensa d'aquest
I consider that the TFM is completed and can accept for the defense

Evaluación de la calidad del TFM y la labor del estudiante.
Avaluació de la qualitat del TFM i la labor del estudiant.
Evaluation of the quality of the Final Master's degree thesis and the work of the student

El trabajo presentado tiene un claro objetivo traslacional, valorando la posibilidad de usar muestras de plasma para analizar mutaciones en genes *RAS* presentes en el tumor, específicamente a través de una técnica de muy alta sensibilidad como lo es la PCR-digital. En este caso, el estudio ha mostrado una alta concordancia entre las técnicas comparadas, y por lo tanto su posible implementación en la práctica clínica. El estudiante ha participado activamente en el trabajo experimental y también en el análisis y discusión de datos, habiendo adquirido las competencias requeridas para un grado de master.

El tutor/a – *Tutor:*	El cotutor/a – *Co-Tutor:*
Eloísa Jantus Lewintre	Silvia Calabuig Fariñas
Fdo. – *Signed*	Fdo. – *Signed*

FECHA (Data) *Date*: 01 de Marzo de 2016

FECHA (Data) *Date*: 08 de Julio de 2016

DEPARTAMENTO DE BIOTECNOLOGÍA
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA Y DEL MEDIO NATURAL

AUTORIZACIÓN DEL COTUTOR PARA PRESENTACIÓN DEL TRABAJO FIN DE MÁSTER DE BIOTECNOLOGÍA BIOMÉDICA

LA COTUTORA:

D.ª SILVIA CALABUIG FARIÑAS Adscrita al organismo FUNDACIÓN PARA LA INVESTIGACIÓN DEL HOSPITAL GENERAL UNIVERSITARIO DE VALENCIA (FIHGUV) **donde se ha realizado el Trabajo Fin de Máster** titulado:

Mutational analysis of RAS genes in metastatic colorectal cancer: concordance between tumor tissue and liquid biopsy

Del que es **AUTOR:**

D. BORJA LAFUENTE GUTIÉRREZ

AUTORIZA la presentación del TRABAJO FIN DE MÁSTER para su defensa.

Valencia, Julio 2016

LA COTUTORA

Fdo.: D./D.ª SILVIA CALABUIG FARIÑAS

Universitat Politècnica de València
Departamento de Biotecnología
Edificis 3J bajo. Camí de Vera, s/n, 46022 València
Tel. +34 96 387 74 20 • Fax +34 96 387 74 29
depbtc@upv.es

DEPARTAMENTO DE BIOTECNOLOGÍA
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA Y DEL MEDIO NATURAL

RESUMEN PARA PRESENTACIÓN DEL TRABAJO FIN DE MÁSTER DE BIOTECNOLOGÍA BIOMÉDICA

EL(LA) ALUMNO (A):

D./D.ª Borja Lafuente Gutiérrez

Del **Trabajo Fin de Máster** titulado:

Análisis mutacional de genes RAS en cáncer colorrectal metastásico: concordancia entre tejido tumoral y biopsia líquida.

Aporta el siguiente **RESUMEN:**

Las mutaciones en genes RAS (KRAS y NRAS) en cáncer colorrectal confieren resistencia a los anticuerpos monoclonales anti-EGFR (cetuximab y panitumumab). Aunque el análisis estándar se realiza en muestras de tejido tumoral, la biopsia líquida ofrece un método no invasivo para analizar mutaciones específicas de tumor. El análisis de DNA tumoral circulante es un método óptimo para detectar mutaciones, y capaz de seguir la evolución del tumor. En este trabajo, hemos comparado nuestra técnica estándar para determinación del estado mutacional de RAS en tejido tumoral, la pirosecuenciación, con el análisis de DNA tumoral circulante con BEAMing, un sistema de PCR digital basado en el uso de microesferas (Beads), Emulsion, Magnetismo y Amplificación, en una cohorte de 30 pacientes. Además, hemos explorado el efecto de las mutaciones en RAS sobre variables clinicopatológicas y parámetros de supervivencia. Hemos encontrado una fuerte concordancia entre las dos técnicas. No se ha encontrado una correlación estadísticamente significativa con las variables clinicopatológicas ni los parámetros de supervivencia. En conclusión, consideramos que BEAMing es un método muy eficaz para el diagnóstico molecular de mutaciones RAS en cáncer colorrectal metastásico.

Valencia, a 8 de Julio de 2016
(lugar, fecha y firma)

EL(LA) ALUMNO(A)

Fdo.: D./D.ª BORJA LAFUENTE GUTIÉRREZ

DEPARTAMENTO DE BIOTECNOLOGÍA
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Del **Trabajo Fin de Máster** titulado:

Mutational analysis of RAS genes in metastatic colorectal cancer: concordance between tumor tissue and liquid biopsy.

Aporta el siguiente **RESUMEN:**

Mutations in RAS genes (KRAS and NRAS) in colorectal cancer are known to confer resistance to anti-EGFR monoclonal antibodies (cetuximab and panitumumab). Although standard assessment is performed on tumor tissue samples, liquid biopsy offers a non-invasive method to analyze tumor-specific mutations. Analysis of circulating tumor DNA has proven to be a proficient method for mutation detection, able to track tumor evolution. In this work, we have compared the performance of our standard technique for RAS status determination on tumor tissue, pyrosequencing, with ctDNA analysis by BEAMing, a digital PCR system based on Beads-Emulsion-Magnetics-Amplification, in a selected cohort of 30 patients. Furthermore, we explored the effect of RAS mutations in clinicopathological variables and survival parameters. We found a strong concordance between the two techniques. No statistically significant correlation of RAS mutations with clinicopathological features or survival parameters was found. In conclusion, we consider that BEAMing technology is an optimal method for molecular diagnosis of RAS mutations in advanced CRC.

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EL(LA) ALUMNO(A)

Fdo.: D./D.ª BORJA LAFUENTE GUTIÉRREZ

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1. INTRODUCTION

1.1. The Concept of Cancer

Cancer is a group of related diseases, involving two common phenomena: uncontrolled proliferation and the potential to invade surrounding tissues or even spread to other organs or tissues (metastasize). The process of carcinogenesis involves dynamic changes in the genome of normal cells, eventually leading to the transformation into tumour cells (Hanahan & Weinberg 2000). This allows tumour cells to escape from homeostatic mechanisms that control proliferation.

Hanahan and Weinberg proposed that there are ten essential characteristics, known as the "hallmarks of cancer", for the development of cancer disease (Hanahan & Weinberg 2011) (Figure 1). Tumour cells present genomic instability, resulting in the accumulation of genomic mutations. The different steps involved in tumour progression are a succession of clonal expansions produced by the accumulation of mutations that generate selectively advantageous neoplastic cells.

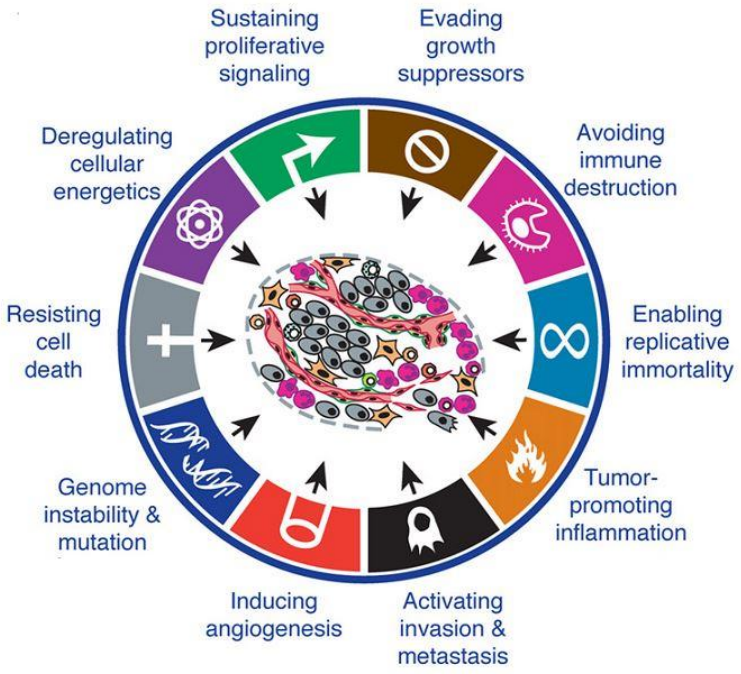

Figure 1: The ten hallmarks of cancer, modified from Hanahan & Weinberg 2011**.**

In addition to cancer cells, tumours possess another dimension of complexity: they contain a repertoire of recruited cells that are active players in the tumour "microenvironment", one of the main hallmark traits. In the microenvironment (Figure 2), epithelial neoplastic cells and the tumour-associated stroma form two well differentiated compartments, with distinct cell types and molecules that modulate tumour growth and invasiveness.

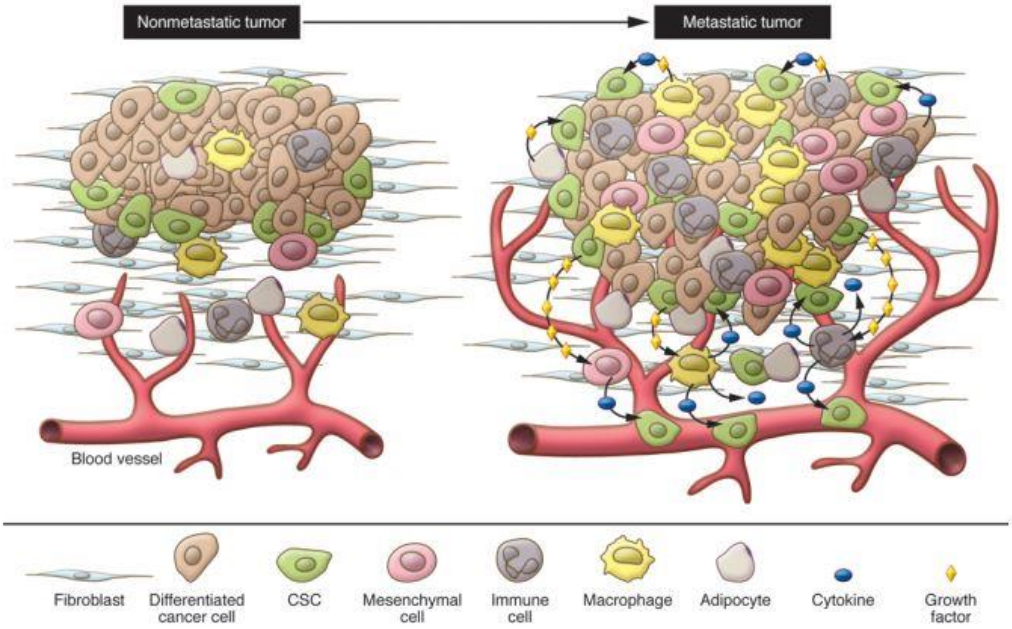

Figure 2: Influence of microenvironment in primary tumour and metastasis (Korkaya et al. 2011).

1.2.Colorectal Cancer

Colorectal cancer (CRC) is a form of cancer of the intestinal gland cells that starts in the colon or the rectum. Both environmental and genetic factors play key roles in its etiology. Genetic susceptibility ranges from well-defined inherited syndromes (familial adenomatous polyposis, Lynch syndrome, etc.), to less defined familial aggregations.

CRC arises after accumulation of acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinomas. The classic CRC progression model (depicted in Figure 3) proposed by Vogelstein and colleagues describes the transformation of normal colonic epithelium into carcinomas (Vogelstein et al. 1988). A number of key pathways are involved in the oncogenesis of colon cancer, with the clinicopathological features of specific subgroups being driven by underlying molecular changes.

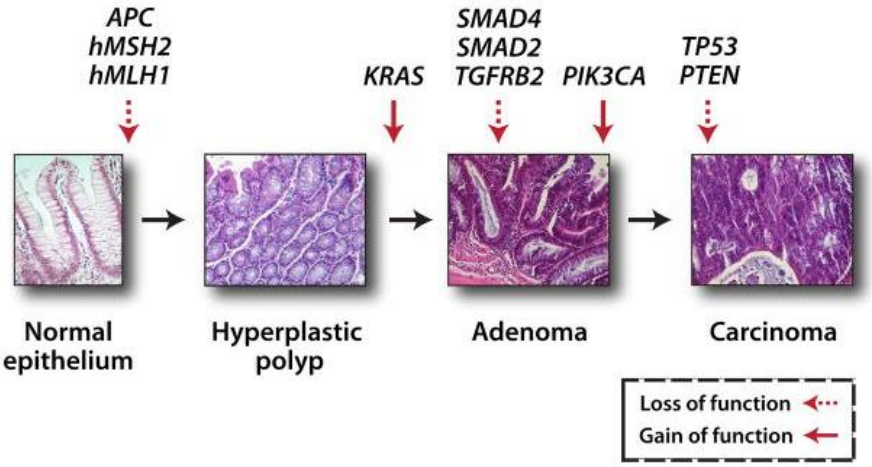

Figure 3: Molecular events underlying the colorectal adenoma-carcinoma sequence. Modified from Cho & Vogelstein, 1992**.**

1.2.1. Epidemiology

CRC is an important contributor to cancer mortality and morbidity worldwide (Ferlay et al. 2015). In Spain, the most recent data available refer to the year 2012: there were more than 19.000 new cases of CRC in men (44 cases per 100.000 inhabitants) and almost 13.000 cases in women (24.2 cases per 100.000 inhabitants). This makes CRC the third most common cancer in men (15 cases per 100.000 inhabitants) and the second in women (14.9 cases per 100.000 inhabitants). In addition, CRC is the second cause of cancer mortality in both men (13.7%) and women (15.2%). The 5-year prevalence of CRC is similar; 15.4 cases per 100.000 inhabitants in both sexes (Sociedad Española de Oncología Médica 2016).

1.2.2. Diagnosis and staging

The high incidence of colorectal cancer makes it a good candidate for screening programs. The two most important methods for CRC population-based screening are colonoscopy and fecal occult blood test. The first can detect colorectal polyps and cancer, and allows for surgical removal of the polyps, while the latter will only detect colorectal tumours, but it is easier to perform and less invasive (American Cancer Society 2016). Endoscopic removal of premalignant lesions reduces the incidence of CRC by avoiding the progression to cancer (Patwardhan et al. 2006).

Clinicopathological staging of primary tumour by TNM classification system remains the core of prognosis and treatment selection (see Table 1). The 7th edition of the American Joint Commitee on Cancer (AJCC) includes the newest TNM classification for CRC (Edge et al. 2010).

Table 1: Colorectal cancer TNM staging 7th ed., adapted from (Edge et al. 2010).

Primary Tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into pericolorectal tissues
T4a	Tumour penetrates to the surface of the visceral peritoneum
T4b	Tumour directly invades or is adherent to other organs or structures
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumour deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (for example liver, lung, ovary, nonregional node)
M1b	Metastases in more than one organ/site, or the peritoneum

1.3. CRC classification: from histology to molecular biology

From a histological perspective, adenocarcinomas constitute around 90% of CRC. The next most common histology is mucinous adenocarcinoma (around 5% of all CRCs) and signet ring cell adenocarcinoma (less than 1%). The latter is typically more aggressive and difficult to treat. There are many other types of uncommon cancers located in the lower gastrointestinal (GI) tract which together account for 5% of the CRCs (Van Cutsem et al. 2014).

One of the models proposed by Fearon and Vogelstein to explain colon carcinogenesis (Fearon & Vogelstein 1990) involves the accumulation of mutations, which leads to oncogene

activation (*KRAS*) and suppressor gene inactivation (*DCC*, *APC*, *TP53*). A second model implicates mutations in genes responsible for DNA repair, particularly *MLH1* (Rustgi 1994). In these cases, subsequent mutations tend to accumulate in microsatellite regions, hence the name "microsatellite instability pathway" (MSI pathway). Finally, a third route of carcinogenesis shows a phenotype of aberrant global hypermethylation, termed "CpG island methylation phenotype" or "CIMP", responsible for 15-20% of sporadic CRCs (Binefa et al. 2014).

Most investigators divide CRC biologically into those with MSI, that are located primarily in the right colon, and frequently associated with CpG island methylator phenotype (CIMP) and hyper-mutations; and those that are microsatellite stable but chromosomally unstable.

There were several attempts to achieve a more accurate molecular subtyping in CRC (The Cancer Genome Atlas Network 2012; Schlicker et al. 2012; Perez-Villamil et al. 2012; Marisa et al. 2013; Sadanandam et al. 2013; Budinska et al. 2013; Roepman et al. 2014; Hoadley et al. 2014; De Sousa E Melo et al. 2013). However, the results had been inconsistent, given the great variability between methods and patients cohorts. After that, researchers from different institutions decided to create a Consortium, combining their datasets and using their respective clustering methods on each other's datasets. This collaborative effort resulted in the Consensus Molecular Subtypes of Colorectal Cancer (Guinney et al. 2015). The Consortium identified four molecular subtypes (detailed in Figure 4) and was able to assign a subtype to 97% of the 4562 samples. This molecular consensus classification will surely shape the future research on CRC pathogenesis and therapeutic approaches.

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 4: Consensus molecular subtypes of CRC (Guinney et al. 2015)**.**

1.3.1. Individual somatic mutations

All cancers arise through accumulation of mutations in proliferation-related genes. In the case of CRC, the most important mutations are those found in *APC*, *RAS* and *BRAF* genes.

RAS is the name given to a family of genes that encode a class of 21 kD membrane-bound GTPases. *KRAS* (*Kirsten Rat Sarcoma*), a member of the *RAS* family, is the most frequently

mutated gene in all of human cancer. The mitogen-activated protein kinase (MAPK) pathway comprised of the *RAS*-RAF-MEK-ERK signaling cascade is deregulated in around 50% of CRC due to somatic gene mutation in *RAS*/*RAF* genes *KRAS* is a 188 amino acid protein and a downstream effector of *EGFR* (*Epidermal Growth Factor Receptor*) that signals through *BRAF* to activate the MAPK pathway (see Figure 5).

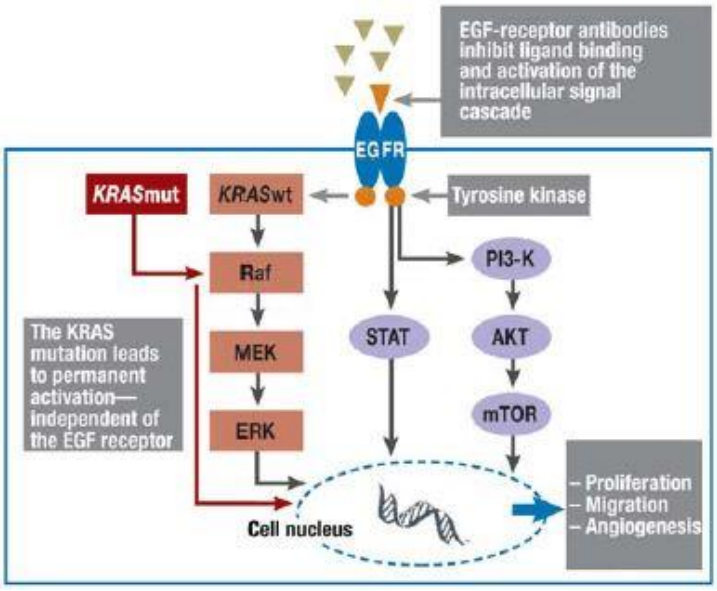

Figure 5: *EGFR* pathway components and therapeutic targets (Stintzing et al. 2009).

Mutations of *KRAS* predominantly lie in codons 12 and 13 of exon 2 and occur in 30-40% of CRCs and leads to constitutive pathway activation by impairing the ability of GTPase activating proteins to hydrolyze *KRAS*-bound GTP (Downward 2003). The reported incidence of *KRAS* exon 2 mutations in CRC has been reported in several studies. The *RAS*CAL trial, screened more than 2700 patients, reporting a 37.7% of mutated *KRAS* in metastatic CRC (mCRC) (Andreyev et al. 1998); similar frequencies have been found in posterior studies, like CALGB 89803 (Karapetis et al. 2008; Van Cutsem et al. 2011).

BRAF is another member of the *EGFR* pathway, mutated in 10-15% of CRCs, and encodes the direct downstream effector protein kinase of *KRAS*. The majority of *BRAF* mutations found in CRC specimens are V600E (change of valine into glutamic acid). *KRAS* and *BRAF* mutations are generally described as mutually exclusive, which supports the hypothesis that an activating mutation in either gene is sufficient to promote tumorigenesis. Even not very frequent, it is possible, that two concomitant mutations: in *KRAS* and *BRAF* genes appear in the same tumour (Sahin et al. 2013). Despite this, *BRAF* mutations' testing is not recommended in *RAS* mutant patients. The biological rationale for this apparent mutual exclusivity could rely on oncogene-induced senescence (OIS), as revealed by some experiments in mouse models of *KRAS* and *BRAF* tumours (Cisowski et al. 2016).

1.3.2. Detection of *RAS* mutations in CRC

Determination of *RAS* mutational status is mandatory for guiding therapy in mCRC patients. Several techniques have been employed for *RAS* mutational status analysis: Sanger sequencing, allele-specific quantitative PCR, amplification-refractory mutation system (ARMS), and pyrosequencing are some of the most widely used. Sanger sequencing is less used, as it is believed that at least 25-30% of tumour cell fraction is needed for mutation detection (Kotoula et al. 2009), a condition not usually met by resection and biopsy samples.

1.3.3. Direct Sequencing: principles of Pyrosequencing

As sequencing methods have become less expensive, direct sequencing approaches for somatic mutations detection in cancer have been incorporated into the clinical setting. These strategies have the advantage of detecting any mutation, whether it has been previously described or not. However, this can be problematic too, as the detection of unknown mutations raises questions about its clinical relevance. Pyrosequencing is a widespread technique for targeted sequencing, commonly used for RAS assessment in tumor tissue samples in molecular oncology laboratories.

Some studies had shown that assays based on pyrosequencing have a detection limit of 5% mutant alleles (Tsiatis et al. 2010; Ogino et al. 2005), whereas DxS Therascreen *K-RAS* Mutation kit (based on allele-specific PCR) has a sensitivity of 1%. However, Sundström et al reported a similar sensitivity between pyrosequencing and allele-specific PCR (Sundström et al. 2010) (see Figure 6). These results indicate that pyrosequencing was as good a choice for *KRAS* clinical mutation testing than quantitative PCR. In addition, PCR assays tended to be more dependent on high DNA quality than PyroMark® assay (Qiagen), and needed more DNA input.

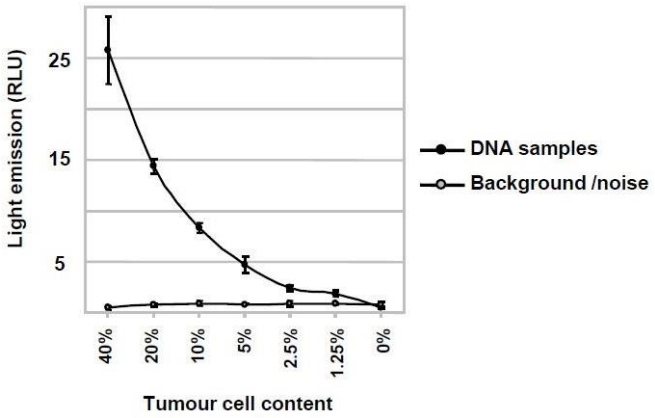

Figure 6: Sensitivity of Pyrosequencing for detection of *KRAS* mutation. Modified from Sundström et al. 2010.

Recently next-generation sequencing (NGS) technologies have opened new possibilities for *KRAS* and *NRAS* analysis. Although 454 NGS appeared to have greater sensitivity than pyrosequencing (Altimari et al. 2013), Gasparini and colleagues found that optimizing the

pyrosequencing technique allowed to detect low-prevalence mutations with equal sensitivity (Gasparini et al. 2014). It has also been found that neoadjuvant treatment of rectal cancer may deplete tumour cells and make the molecular diagnostic of *KRAS* more difficult by lowering tumour DNA quantity (Dudley et al. 2015).

In conclusion, pyrosequencing is a well-known technology with a good performance when minimal DNA quality and quantity from tumour tissue are assured. In the future, however, there will be need for more sensitive and quantitative methods that can detect small mutated cell clones, both in tumour tissue and other biological samples. Circulating tumour cells and circulating tumour DNA will most likely play an important role in *RAS* mutational analysis. A representative pyrogram showing a *KRAS* G12V mutation is depicted next (Figure 7):

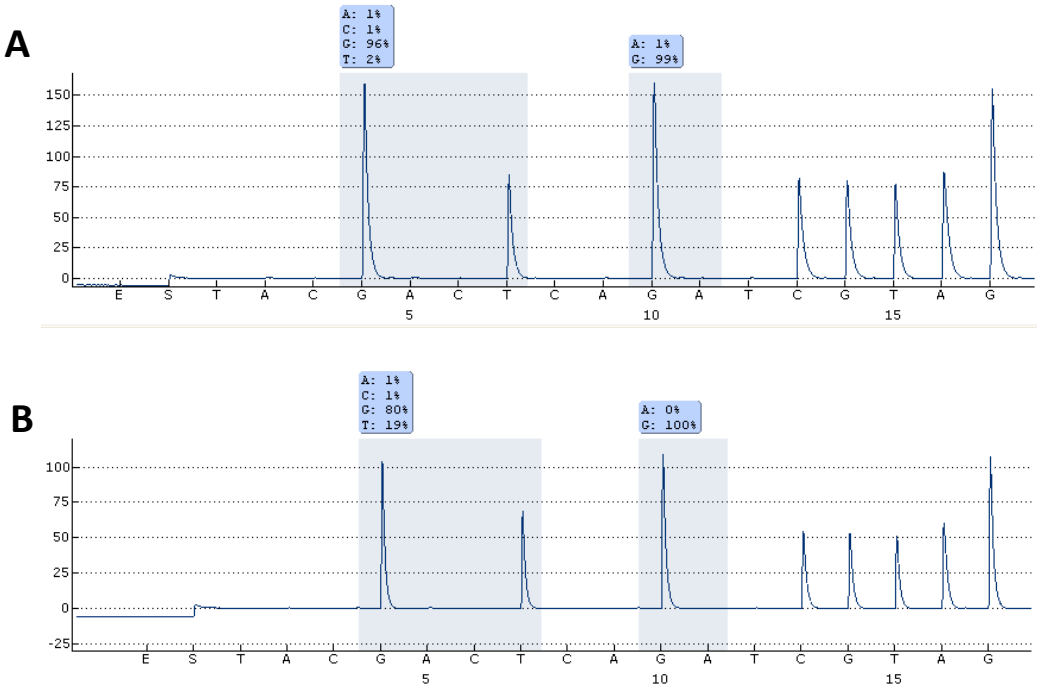

Figure 7: A) Pyrogram showing the wild-type result (GGT) for *KRAS* codon 12. **B)** Pyrogram showing a mutated result for a sample with a *KRAS* G12V mutation (GGT > GTT) with 19% mutant allele fraction.

Therascreen Pyro kits for *KRAS* and *RAS* extension comprise 64 mutations (see Table 2):

Table 2: Catalogue of *RAS* hotspots and mutations covered by Therascreen *KRAS* and *RAS* Extension kits for pyrosequencing determinations.

***KRAS* mutations**		***NRAS* mutations**	
Amino acid change	**Nucleotide change**	**Amino acid change**	**Nucleotide change**
Codon 12		**Codon 12**	
Gly12Asp (G12D)	GGT → GAT	Gly12Asp (G12D)	GGT → GAT
Gly12Val (G12V)	GGT → GTT	Gly12Val (G12V)	GGT → GTT
Gly12Cys (G12C)	GGT → TGT	Gly12Cys (G12C)	GGT → TGT

Gly12Ser (G12S)	GGT → AGT	Gly12Ser (G12S)	GGT → AGT
Gly12Ala (G12A)	GGT → GCT	Gly12Ala (G12A)	GGT → GCT
Gly12Arg (G12R)	GGT → CCT	Gly12Arg (G12R)	GGT → CGT
Codon 13		**Codon 13**	
Gly13Asp (G13D)	GCG → GAC	Gly13Ser (G13S)	GGT → AGT
Codon 61 (CAA)[1]		Gly13Cys (G13C)	GGT → TGT
Gln61His (Q61H)	TTG → GTG	Gly13Arg (G13R)	GGT → CGT
Gln61Leu (Q61L)	TTG → TAG	Gly13Asp (G13D)	GGT → GAT
Gln61Arg (Q61R)	TTG → TCG	Gly13Val (G13V)	GGT → GTT
Gln61His (Q61H)	TTG → ATG	Gly13Ala (G13A)	GGT → GCT
Gln61Glu (Q61E)	TTG → TTC	**Codon 59**	
Codon 59		Ala59Thr (A59T)	GCT → ACT
Ala59Thr (A59T)	GCA → ACA	Ala59Pro (A59P)	GCT → CCT
Ala59Ser (A59S)	GCA → TCA	Ala59Gly (A59G)	GCT → GGT
Ala59Gly (A59G)	GCA → GGA	Ala59Asp (A59D)	GCT → GAT
Ala59Glu (A59E)	GCA → GAA	Ala59Val (A59V)	GCT → GTT
Ala59Leu (A59L)	GCA → GTA	**Codon 61**	
Codon 117		Gln61Lys (Q61K)	CAA → AAA
Lys117Glu (K117E)	AAA → GAA	Gln61Arg (Q61R)	CAA → CGA
Lys117Gln (K117Q)	AAA → CAA	Gln61Leu (Q61L)	CAA → CTA
Lys117Asn (K117N)	AAA → AAC	Gln61His (Q61H)	CAA → CAT
Lys117Asn (K117N)	AAA → AAT	Gln61His (Q61H)	CAA → CAC
Codon 146		**Codon 117**	
Ala146Thr (A146T)	GCA → ACA	Lys117Asn (K117N)	AAG → AAC
Ala146Pro (A146P)	GCA → CCA	Lys117Asn (K117N)	AAG → AAT
Ala146Val (A146V)	GCA → GTA	**Codon 146**	
Ala146Gly (A146G)	GCA → GGA	Ala146Thr (A146T)	GCC → ACC
		Ala146Pro (A146P)	GCC → CCC
		Ala146Ser (A146S)	GCC → TCC
		Ala146Val (A146V)	GCC → GTC
		Ala146Gly (A146G)	GCC → GGC

[1] *KRAS* codon 61 is assayed in the reverse direction by Pyrosequencing. Mutations covered by OncoBEAM *RAS* CRC kit are highlighted in yellow.

1.4. Treatment of CRC

The management of CRC depends mainly on clinicopathological characteristics of the patients, tumour stage and also on the molecular alterations found in tumour cells.

The SEOM guidelines (Aranda et al. 2015) recommend, for most patients with good performance status (PS) and no significant comorbidities, the combination of 5-FU/leucovorin (5-FU/LV) with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) as backbone for first-line

treatment. Other option includes capecitabine, an oral fluoropyrimidine with similar efficacy in first-line treatment of mCRC (Van Cutsem et al. 2004).

On the other hand, first-line targeted therapies include the anti-vascular endothelial growth factor (*VEGF*) agent bevacizumab and the anti-*EGFR* drugs cetuximab and panitumumab.

An apparent lack of response to targeted therapy with anti-*EGFR* monoclonal antibodies in around 40-50% of the patients with *KRAS* exon 2 wild-type (WT) tumours observed in some clinical trials boosted the search for additional predictive biomarkers. The effect of mutations in other members of the *EGFR* signaling pathway like *BRAF*, *PIK3CA* and *NRAS* was analysed in multiple studies (Karapetis et al. 2014; Sartore-Bianchi et al. 2009).

It was found that there are around 5% of CRC patients who have mutations in *KRAS* exons 3 or 4, and a further 5% with mutations in *NRAS* exons 2, 3 or 4. These *RAS* mutations previously suggested were tested by Sanger sequencing: *KRAS* exon 3 (codons 59 and 61) and exon 4 (codons 117 and 146), and *NRAS* exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), exon 4 (codons 117 and 146) (see Figure 8).

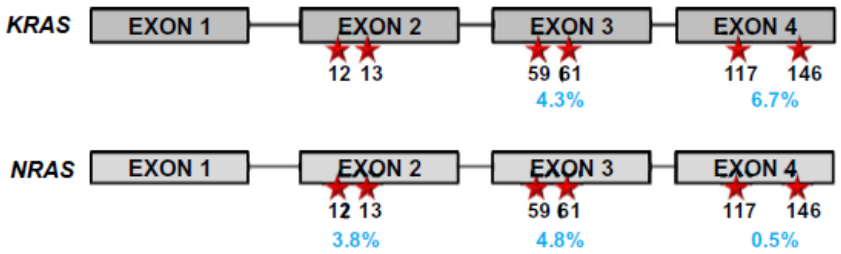

Figure 8: Frequency of *RAS* mutations beyond *KRAS* codon 2 in CRC (Hecht et al. 2015).

Retrospective analyses of several phase III trials indicated that all *RAS* mutations were regarded as a negative predictive factor of anti-*EGFR* therapy: one example was the PRIME trial (Douillard et al. 2013). These mutations were associated with inferior progression-free survival (PFS) and overall survival (OS) with panitumumab-FOLFOX4 treatment, like *KRAS* exon 2 mutations. The evidence supported the addition of all these mutations to the routinely tested *KRAS* mutation analysis, in order to further improve the selection of patients for anti-*EGFR* therapy.

As for the effect of mutations beyond anti-*EGFR* treatment efficacy, mutant *KRAS* and *BRAF* have been independently associated with worse overall survival in metastatic CRC patients. In the largest study, patients with CRC that harbored a *KRAS* mutation had a worse overall survival (OS) but similar progression-free survival (PFS) compared to patients with tumours bearing WT *KRAS* (Richman et al. 2009), as seen in Figure 9.

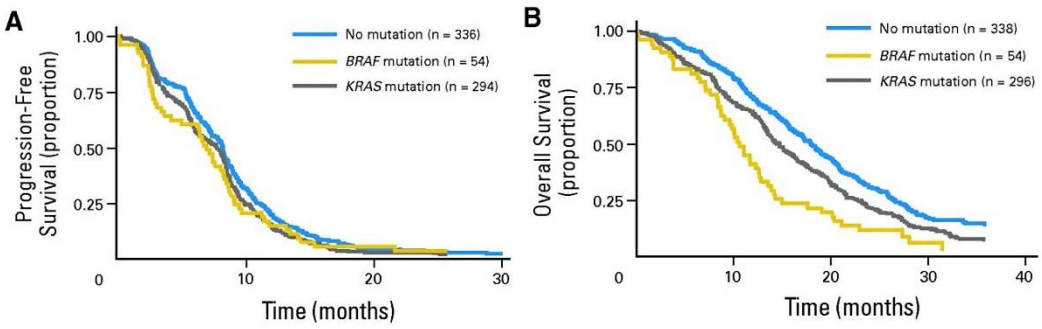

Figure 9: Prognostic impact of *KRAS* and BRAF mutations in A) progression-free survival (PFS) and B) overall survival (OS), compared to non-mutated patients, in any treatment arm. Modified from Richman et al. 2009.

1.5. Liquid biopsy

Since the advent of targeted therapies, increased survival periods and improved quality of life are achieved for patients whose cancers harbor specific molecular alterations. However, targeted therapies have brought new challenges: high costs, potential morbidity of the necessary biopsies, lack of effective drugs against most genomic aberrations, technical limitations and regulatory obstacles. In addition, almost all tumours develop resistance mechanisms through tumour heterogeneity, clonal evolution and selection. Therefore, new methodologies are needed, that allow us to overcome these difficulties. Liquid biopsies appear to be a reliable alternative to conventional biopsies. They can provide both precise molecular data useful for improving the clinical management of mCRC cancer patients, and a less invasive way of monitoring tumour behavior.

Multiple studies have shown that it is possible to reconstruct tumour genomes from plasma DNA (Thierry 2016; Goto et al. 2016). Traces of tumour DNA (circulating tumour DNA, ctDNA) can be found in the cell-free fraction of blood, together with DNA fragments from normal cells (cfDNA).

After first description of fragments of DNA existing in the blood (Mandel & Metais 1948), higher levels of so called circulating free DNA (cfDNA) were identified in cancer patients compared to healthy controls, suggesting that this correlated with malignancy and tumour stage (Leon et al. 1977).

To date, two main mechanisms for releasing circulating tumour DNA (ctDNA), “passive” and “active”, have been postulated. The passive mechanism involves the release of nucleic acids directly from apoptotic and necrotic tumour cells into the bloodstream or indirectly by necrotic tumour cells engulfed by macrophages (Diehl et al. 2005). This was further supported by measuring the size distribution of DNA fragments (Jahr et al. 2001; Heitzer et al. 2013). In contrast, fragments of ctDNA can also be “actively” secreted into the circulation, perhaps in association with a protein complex to act as an intercellular messenger of sorts (Peters & Pretorius 2012).

Considering that releasing of DNA into the bloodstream is not an exclusive process for primary or metastatic sites, ctDNA can provide a better overall representation of the malignant disease as a whole (Kuo et al. 2014). In cancer patients, ctDNA levels can vary according to tumour burden and stage, anatomical proximity to vasculature, and biological features like apoptotic rate and metastatic potential.

The clinical applications of ctDNA (see Figure 10) can be divided into three main categories: a) early diagnosis and prognosis; b) profiling and molecular characterization of tumour genomic alterations, and c) monitoring treatment response and detection of resistance mechanisms.

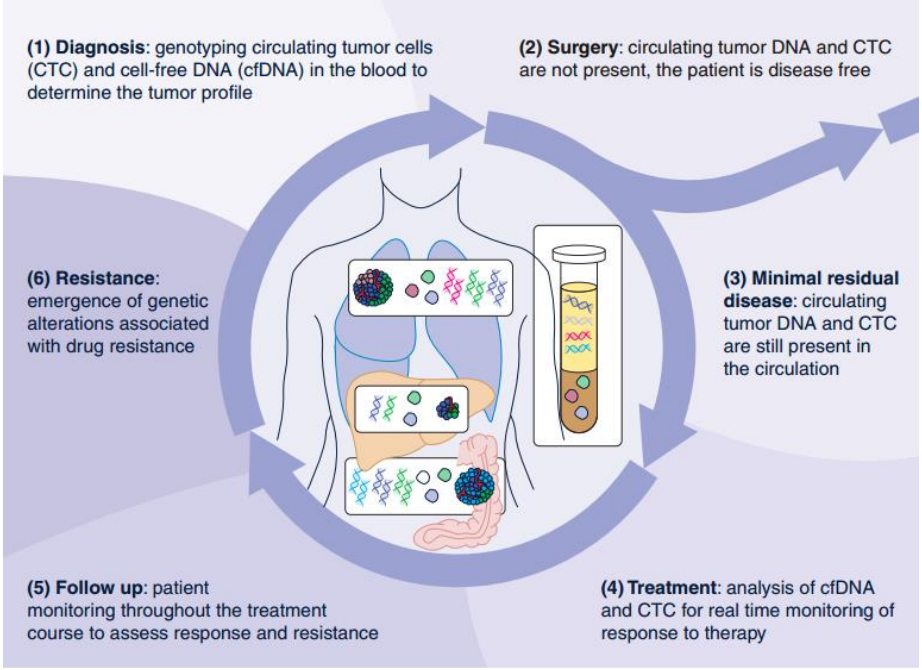

Figure 10: Uses of liquid biopsies in clinical oncology (Siravegna & Bardelli 2014).

First, it has been proposed that monitoring tumour-specific changes may be a useful tool for early cancer detection and/or prognosis. A recent example of diagnostic approach involved blood-based CRC screening test using the *SEPT9* biomarker that specifically detects a majority of CRCs of all stages and colorectal locations (Church et al. 2014). The simple measurement of the quantity of cfDNA in plasma by quantitative PCR is positively correlated with tumour burden in mCRC (Schmitt et al. 2012; Spindler et al. 2012).

Second, as a prognostic biomarker, several studies have demonstrated that circulating-free DNA levels (cfDNA) or the number of circulating tumour cells (CTCs) are positively correlated with patient outcome in mCRC. In surgically resected CRC patients, the detection of ctDNA after surgery was related to an increased relapse rate (Diehl et al. 2008). Furthermore, it was shown that high concentrations of cfDNA and *KRAS* mutation were clear indicators of poor outcome for advanced CRC patients (Spindler et al. 2012).

Finally, one of the most widespread applications of liquid biopsy is monitoring response to therapy, particularly for those therapies with known resistance mechanisms. Several studies have reported that anti-*EGFR* resistant clones are present in the circulation months before progression was clinically obvious (Misale et al. 2014; Mohan et al. 2014).

In summary, the determination of molecular alterations enables for the selection of adequate targeted therapies for each patient, and allows the clinician to make rapid therapeutic decisions if resistant clones are detected in circulation. Although liquid biopsy is very useful and advantageous compared to tumour tissue biopsies, the detection of scarce ctDNA mixed with relatively abundant WT cfDNA requires innovative ultra-sensitive techniques, such as digital PCR.

1.5.1. Digital PCR: BEAMing system

Generally, there are two approaches for the analysis of ctDNA. A targeted approach: analysis of a small set of frequently occurring driver mutations with implications for therapy decisions, such as mutations in *KRAS* or *EGFR*. The second involves an untargeted approach without knowledge of any specific changes present in the primary tumour (Heitzer et al. 2015). Given the small proportion of ctDNA present in the total cfDNA samples, it is important to select the correct methods for its analysis; several highly sensitive techniques have been developed for the latter, ranging from real time PCR-based to more complex digital-PCR based technologies

BEAMing (Beads-Emulsion-Amplification-Magnetics, by *Sysmex Inostics*) is a targeted, quantitative digital PCR technology that employs bead-based amplification in water-in-oil emulsions, and allele-specific hybridization followed by flow cytometry, for the detection of small amounts of mutated DNA released by tumours into the blood circulation. BEAMing is highly sensitive, able to detect mutant ctDNA in very low proportion (as low as 0.01% of total DNA fragments; see Figure 11) in a backgroud of normal (WT) DNA.

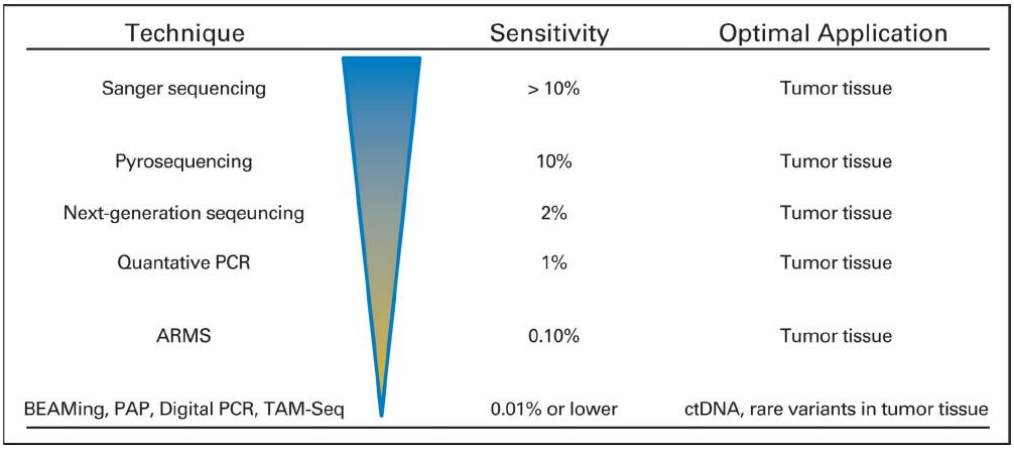

Figure 11: Methodologies for detecting circulating tumour DNA (ctDNA). *Pyrophosphorolysis-activated polymerization* (PAP); *tagged-amplicon deep sequencing* (TAM-Seq) (Díaz Jr et al. 2014).

Ideally, after preamplification, a single DNA fragment is captured in a single magnetic bead, and each bead falls into one hydrophilic droplet of the water-oil emulsion. The amplification PCR takes place with TaqMan probes designed to cover mutational hotspots in exons 2, 3 and 4 of both genes (*KRAS* and *NRAS*).

The mutant fraction cannot be interpreted as the fraction of cancer cells that harbor a particular mutation. While ctDNA quantification by quantitative PCR is positively correlated with tumour burden, mutant fraction may represent a combination of clones produced by different tumour lesions.

In the context of CRC, *RAS* WT tumours are often sensitive to *EGFR* blockade with cetuximab or panitumumab, but almost all patients develop resistance within a few months (Karapetis et al. 2008). Liquid biopsy can substitute serial tumour tissue sampling, and may provide a global and evolving picture of the disease. Several studies have reported that anti-*EGFR* resistant clones are present in the circulation months before progression was clinically obvious (Misale et al. 2014; Mohan et al. 2014). Figure 12 shows the evolution of mutant cfDNA fraction in a patient; the increase in concentration predicted the emergence of secondary resistance to cetuximab treatment (Misale et al. 2014).

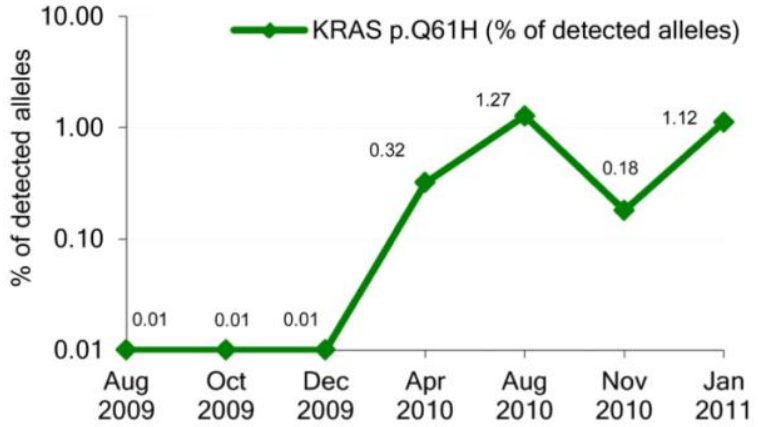

Figure 12: Quantitative analysis of *KRAS* Q61H mutation in plasma by BEAMing (Misale et al. 2014).

Proven useful in cancer research (Tabernero et al. 2015), BEAMing is now being validated for diagnostic determination of mutations in *KRAS* and *NRAS* in plasma of mCRC patients. Our laboratory had a chance to collaborate in this enterprise, and in this study we will determine whether blood-based *RAS* mutation testing is an appropriate surrogate for tissue-based *RAS* testing to assess eligibility of mCRC patients for anti-*EGFR* therapy by comparing the degree of concordance of plasma and tissue-based *RAS* testing in metastatic CRC patients.

2. OBJECTIVES

Tumour tissue is currently used for *RAS* testing in mCRC patients, but the detection of circulating tumour DNA (ctDNA) is being actively investigated as a new method for the detection of actionable mutations in plasma samples. Therefore, the main objective of this work is to evaluate the concordance of *RAS* mutational status by comparison of results from ctDNA and tissue-based testing in a cohort of mCRC patients.

The specific aims of this study are the following:

a) To analyse concordances and discrepancies in *RAS* (*KRAS* and *NRAS)* mutational status between blood samples and tissue samples in a small cohort of newly diagnosed mCRC patients (n=30).

b) To study correlations between the mutations detected in ctDNA and some relevant clinicopathological features.

c) To integrate the results and determine whether *RAS* ctDNA testing is a feasible alternative for tumour tissue-based *RAS* testing.

3. MATERIALS AND METHODS

3.1. Study design and patients

This is a retrospective analysis in 30 therapy-naïve patients with histologically or cytologically documented metastatic colorectal cancer. Patients having a history of another malignancy or having received any previous treatment (chemotherapy or targeted therapy) were excluded. We enriched our cohort with patients with detected mutations in *RAS* genes *(KRAS* and *NRAS)*, so that we could perform the concordance study.

The study was conducted in accordance with the Declaration of Helsinki, and the institutional ethical review board approved the protocol.

3.2. Biological samples: FFPE and blood

First of all, patients must sign an informed consent document, by which they are given all the information regarding the samples that they will donate, how they will be taken and the relevance that they may have on treatment and/or prognosis.

A total of 30 formalin-fixed, paraffin-embedded (FFPE) CRC samples obtained by colonoscopy or surgical resection were provided by the Pathology Service at the *Consorcio Hospital General Universitario de Valencia.* A pathology report was available for all the samples, enabling their characterisation. For mutational analysis of tumour tissue, the specimens were examined by a pathologist, and those having >5% of tumour cells were selected. Three to five 5 µM thick tissue sections were used for DNA isolation.

Blood samples were obtained at the time of diagnosis of metastatic disease, previous to any systemic treatment. 10 mL of blood were collected from each patient in K2 EDTA BD Vacutainer® tubes, and processed to obtain plasma within an hour. Briefly, a series of centrifugations are performed, at increasing speeds, in order to isolate and clean-up the plasma. The isolated plasma is stored in Sarstedt™ CryoPure tubes, at -80 °C, until cfDNA extraction.

3.3. DNA extraction

FFPE: DNA was isolated from FFPE tumour tissue sections. First paraffin was removed by incubating the samples with mineral oil at 95°C, followed by the addition of xylene. After centrifugation, the xylene supernatant was removed. Then ethanol 100% was added to clean residual xylene, followed by another centrifugation step. After that, the samples were allowed to air-dry for 20-30 minutes.

The Cobas DNA Sample Preparation Kit (Roche®) was used for the extraction of DNA from deparaffinized samples, following manufacturer's recommendations. Briefly, DNA Tissue Lysis

Buffer and Proteinase K were added. After two incubations (56 °C and 95 °C) and brief cooling, DNA Paraffin Binding Buffer and isopropanol were added, with respective incubation periods, and the content of each tube was transferred to a filtered tube. Then a series of centrifugations with washing steps in between were performed, and finally the DNA Elution Buffer was added in order to collect DNA in the definitive tube.

Plasma: DNA from plasma samples was obtained by the commercial QIAamp® "Circulating Nucleic Acid" kit, following manufacturer's instructions. This protocol is based on affinity columns for retaining nucleic acids.

FFPE samples DNA quantification was performed in the NanoDrop® 2000C system (Thermo Fisher Scientific). For plasma cfDNA, quantification was performed using Qubit® (Life Technologies) Fluorometer: A Working solution was prepared, diluting Qubit® reagent 1:200 in Qubit® Buffer. Sample DNA was diluted 1:200 in Working solution, and standard samples were diluted 1:20. The tubes were incubated at room temperature before readings.

3.4. Pyrosequencing

All tissue biopsies were analysed for *RAS* genotyping assessment using two CE-IVD marked commercial kits: “Therascreen® *KRAS* Pyro Kit” and “Therascreen® *RAS* Extension Pyro Kit” (both from QIAGEN, Hilden, Germany) according to the producer protocols (“Therascreen *KRAS* Pyro Kit Handbook”, version 1, July 2011, and “Therascreen *RAS* Extension Pyro Kit Handbook”, version 1, October 2014). From each sample, 10 ng DNA were amplified for determining mutations status in: *KRAS* 12-13, *NRAS* 12-13, *NRAS* 61, *KRAS* 59-61, *KRAS* 117, *KRAS* 146, *NRAS* 58-59, *NRAS* 117 and *NRAS* 146.

Pyrosequencing was performed using 10 µL of each PCR product with PyroMark Gold Q96 reagents (QIAGEN), Streptavidin Sepharose (GE Healthcare Bio-Science AB, Uppsala, Sweden), in the PyroMark Q24 instrument (QIAGEN). The results were analysed using PyroMark Q24 2.0.7 software (QIAGEN).

The protocol is based on template DNA immobilization on Sepharose (beaded form of agarose), PCR amplification and sequencing. The reverse amplification primer (RP) is biotinylated, and so the sequencing is performed on the forward strand (except for *KRAS* codons 59/61, as seen in Figure 13).

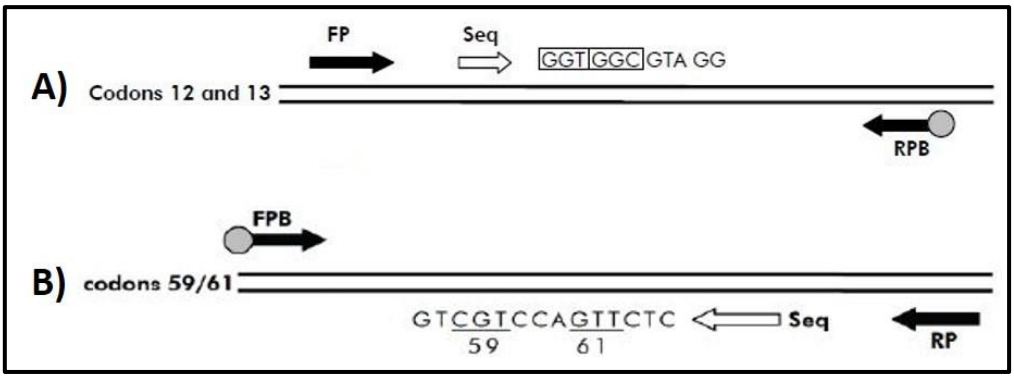

Figure 13: Amplification primers (black arrows) and sequencing primers (white arrows) for *KRAS* codons 12-13 (A) and 59-61 (B). The gray circles represent biotinylated primers.

A single nucleotide is incorporated in each sequencing step. If it is incorporated into the DNA strand, a pulse of light is generated (see Figure 14) and the intensity is registered (if more than one nucleotide of the same type is incorporated, the intensity is higher). Finally, the nucleotides are degraded and another nucleotide is incorporated, starting a new cycle.

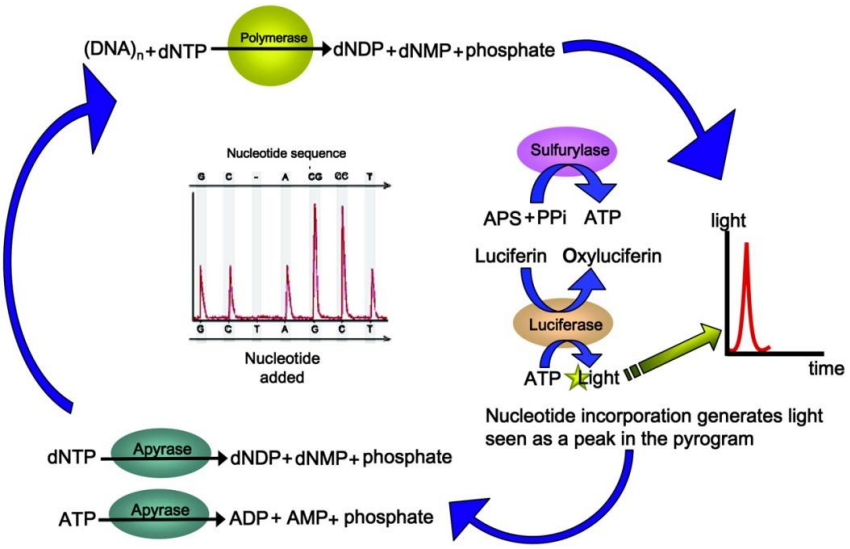

Figure 14: Biochemical basis for the generation of light by DNA pyrosequencing. ATP, adenosine triphosphate; ADP, adenosine diphosphate; dNDP, deoxy-nucleotidyl diphosphate; dNMP, deoxy-nucleotidyl monophosphate; PPi, pyrophosphate. (Petrosino et al. 2009).

3.5.BEAMing determination of *RAS* mutations

RAS mutational analysis on ctDNA was done with BEAMing digital-PCR (OncoBEAM™ *RAS* CRC Kit *Sysmex® Inostics)*, a technique based on emulsion PCR that allows detection of one mutant allele in 10000 WT alleles. As shown in Figure 15, DNA isolation and pre-amplification reactions were performed on the pre-PCR laboratory, whereas from emulsion-PCR step until the final flow-cytometry analysis the experimental work was done on the post-PCR laboratory (physically separated area), in order to avoid cross-contamination.A workflow of the different steps in the technique is shown in Figure 15:

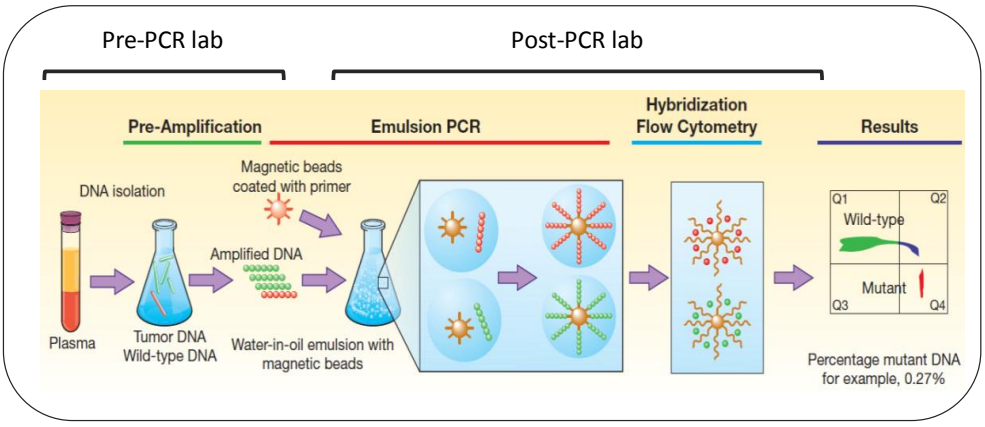

Figure 15: Workflow of BEAMing technology (Lauring & Park 2011).

RAS mutations results from plasma samples were obtained by BEAMing using the expanded *RAS* 33 mutation panel (Table 2), following the manufacturer instructions. Briefly, the steps are as follow:

1. PREAMPLIFICATION: Preamplification of target regions (*KRAS* and *NRAS* exons 2,3 and 4) is performed in a multiplex PCR (mPCR). DNA template was diluted 1:3.3 for mPCR. After mPCR, the six replicates of each sample were pooled and diluted 1:20 in another plate.

2. EMULSION PCR: Diluted DNA templates are transferred to the Emulsion PCR plate, along with Emulsion Working mixes (one for each codon). After that, EmulsiFIRE solution was added to induce emulsification, creating millions of PCR compartments (hydrophilic droplets with a single magnetic bead inside) in one single reaction. Finally, Emulsion PCR (EmPCR) was.

3. HYBRIDIZATION: After EmPCR, thousands of copies of identical DNA fragments have covered each bead. Then, emulsion was broken in order to hybridize DNA templated with specific labeled-probes(each codon was interrogated in different wells). After hybridization, everal washing steps were done, before analyzing the samples by flow cytometry

4. FLOW CYTOMETRY: The final step comprising a flow cytometry analysis of the labeled-beads. According to beads position and fluorescence intensities, different types of populations can be found. A set of plots are generated for quantification of the amount of mutant beads in each sample. . First plot shows the number of droplets containing only one bead (EmPCR compartments); in the second one the population of non-extended beads (if EmPCR failed), is separated from the population of extended beads; in the third one, the population of extended beads is isolated and then mutant and WT beads are quantified.

3.6.Statistical analysis

Concordance was determined by comparing plasma *RAS* mutation results to those from matched tumour samples, using the equations shown under Table 3:

Table 3: Cross table for the comparison of two technologies for mutation detection that allows for agreement calculation.

		Technique 1 (Tissue)		
		Positive (Mut)	**Negative (WT)**	**Total**
Technique 2 (ctDNA)	**Positive (Mut)**	a	b	a + b
	Negative (WT)	c	d	c + d
	Total	a + c	b + d	

$$\text{Overall agreement (\%)} = \frac{a+d}{a+b+c+d} \times 100$$

$$\text{Positive agreement (\%)} = \frac{a}{a+c} \times 100$$

$$\text{Negative agreement (\%)} = \frac{d}{b+d} \times 100$$

Statistical analyses between *RAS* mutational status and clinicopathological variables were conducted by nonparametric tests. Continuous variables were compared using non-parametric Mann-Whitney U and Kruskall-Wallis tests. Survival analysis was performed using the Kaplan-Meier method, and the statistical significance between survival curves was assessed using the log-rank test. Univariate survival analyses were performed by Cox's proportional hazards regression.

For all analyses, two-sided p-values < 0.05 were considered statistically significant. The statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 (Chicago, IL).

4. RESULTS AND DISCUSSION

4.1. Patient characteristics

This retrospective study included 30 patients with mCRC from *Consorcio Hospital General Universitario de Valencia.* The most relevant demographic and clinicopathological characteristics of the cohort are shown in Table 4. The median patient age was 68 years [range: 43-81], 66.7% were male and 56.7% of the tumours were located in colon.

Table 4: Characteristics of our cohort (n = 30).

Characteristics	No. of patients (%)
Age at diagnosis (years): Median [range]	68 [43-81]
Sex	
Men	20 (66.7%)
Women	10 (33.3%)
CEA at diagnosis (ng/µL): Median [range]	31 [2.7-7059.5]
Tumour histology	
Adenocarcinoma	24 (80%)
Mucinous adenocarcinoma	4 (13.3%)
Anatomic site	
Ascending colon	6 (20%)
Transverse colon	3 (10%)
Descending colon	8 (26.7%)
Rectum	13 (43.3%)
Differentiation grade	
Well differentiated (G1)	9 (45%)
Moderately differentiated (G2)	10 (33.3%)
Poorly differentiated (G3)	1 (3.3%)
Primary tumour resected	**17 (56.7%)**
Lymphatic invasion	
L0	9 (53%)
L1	5 (29.4%)
Vascular invasion	
V0	13 (76.5%)
V1	2 (11.75%)
Perineural invasion (Pn1)	
Pn0	12 (70.6%)
Pn1	2 (11.8%)
Liver metastasis	23 (76.7%)
No. of organs affected by metastasis	

One organ	14 (46.7%)
More than one organ	16 (53.3%)
Tumour size and invasiveness	
T2	2 (6.7%)
T3	8 (26.7%)
T4	7 (23.3%)
Regional lymph nodes involvement	
N0	6 (20%)
N1	6 (20%)
N2	6 (20%)

4.2. *RAS* mutational analysis in tissue and ctDNA

4.2.1. *RAS* analysis in FFPE samples

Analysis of *RAS* mutational status in FFPE samples was performed by pyrosequencing. The amounts of DNA obtained ranged from 150 ng to 1000 ng. In all 30 cases, we obtained conclusive results, with 8 *RAS* WT cases (26.7%) and 22 *RAS* mutated (MUT) cases (73.3%).

Table 5 shows the *RAS* genotype distribution in our cohort. The percentage of mutant allele found in our series ranged from 19% to 83%. Remarkably, we found a sample with a double *KRAS* mutation in exon 2, corresponding to Gly12Val and Gly12Ala.

As examples of the results obtained by pyrosequencing, in Figure 16, three pyrograms corresponding to: a *KRAS* codon12 WT case (Fig 16 A), a *KRAS* codon 13 MUT sample (Fig 16 B), and *KRAS* codon 12 MUT sample (Fig 16 C) are shown. Note that the percentage of the mutant fraction in the two *KRAS* mutated cases are 50% and 19%, respectively.

Table 5: *RAS* genotype distribution in tissue samples detected by pyrosequencing.

Codon	Amino acid change	No. of cases (n=30)	%
All WT	None	8	26.7%
Any mutation	Any	22	73.3%
Any *KRAS*	Any	20	66.7%
Any *NRAS*	Any	2	6.7%
KRAS 12	Any	15	50%
KRAS 13	Any	4	13.3%
KRAS 61	Any	1	3.3%
NRAS 13	Any	1	3.3%
NRAS 61	Any	1	3.3%
Specific mutations identified in *KRAS*			
KRAS 12	Gly12Val	7	23.3%
	Gly12Asp	3	10%

	Gly12Ser	1	3.3%
	Gly12Ala	1	3.3%
	Gly12Cys	2	6.6%
	Gly12Val + Gly12Ala	1	3.3%
KRAS 13	Gly13Asp	4	13.3%
KRAS 61	Gln61His	1	3.3%
Specific mutations identified in *NRAS*			
NRAS 13	Gly13Arg	1	3.3%
NRAS 61	Gln61Arg	1	3.3%

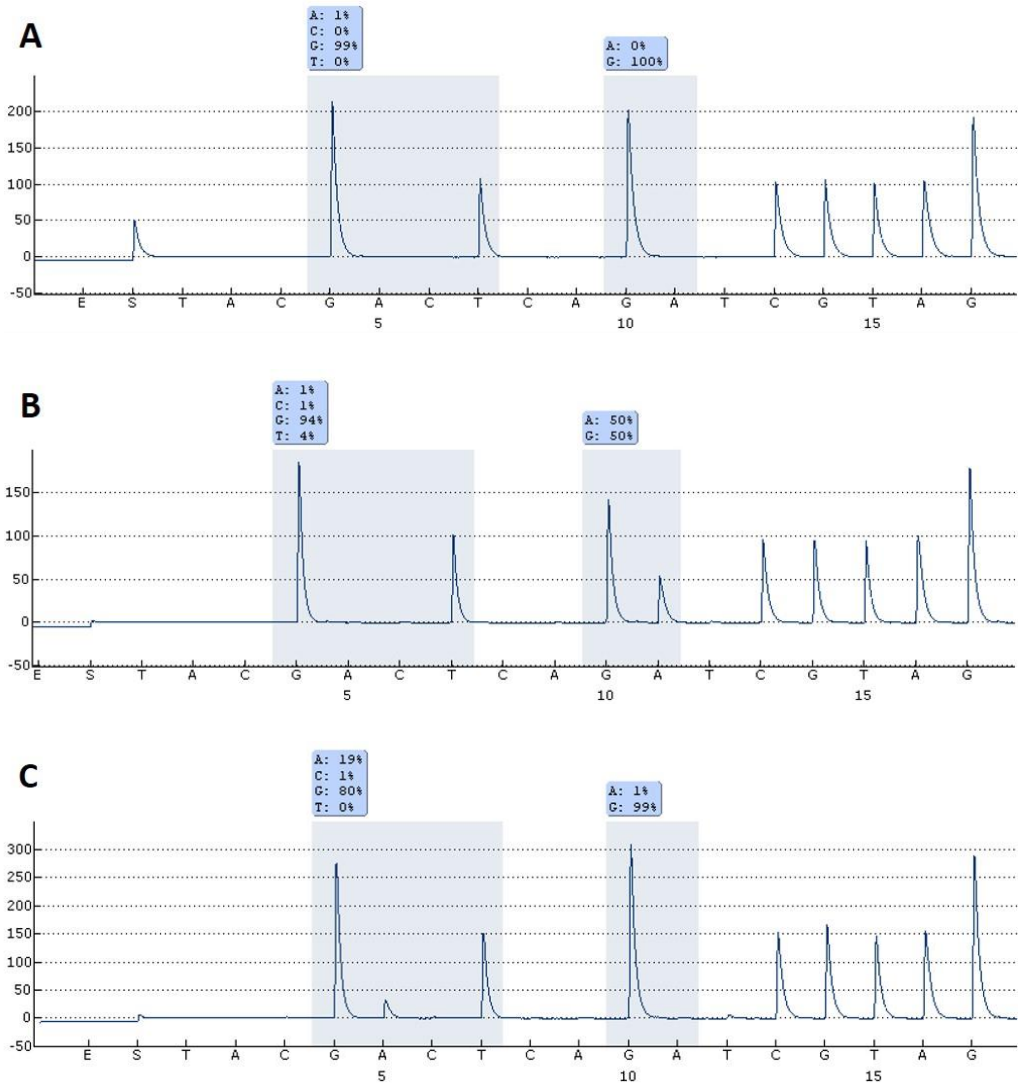

Figure 16: Representative pyrograms of our series. A) Pyrogram of a *KRAS* exon 2 WT sample; B) Pyrogram of a *KRAS* codon 13 MUT sample (50% mutant alleles); C) Pyrogram of a *KRAS* codon 12 MUT sample (19% mutant alleles).

As our patients were selected based on their *RAS* mutational status and blood sample availability, frequencies depicted in Table 5 cannot be directly compared with the ones reported in literature. In our cohort the most frequently mutated exon was *KRAS* exon 2, and

as expected the highest percentage of mutations were found in codon 12, followed by *KRAS* codon 13. In the literature, *KRAS* exon 2 mutations have a reported frequency ranging from 37% to 45% in tumour tissue samples from CRC patients (Table 6). On the other hand, in our cohort, mutations in *KRAS* exon 3 and *NRAS* exons 2 and 3 are underrepresented (3.3% each case). Even though, the Molecular Oncology Laboratory has a large experience in *RAS* mutational analysis, in CRC, with more than 4.000 samples analyzed, with frequency distributions that match exactly to the data published for the european population in other studies.

Table 6: Relative distribution of *RAS* mutations in CRC patients Data extracted from Sorich et al. 2014.

Gene - Exon	Relative distribution
***KRAS* exon 2 (Codons 12 and 13)**	37% - 45%
***KRAS* exon 3 (Codons 59 and 61)**[a]	4.3%
***KRAS* exon 4 (Codons 117 and 146)**[a]	6.7%
***NRAS* exon 2 (Codons 12 and 13)**[a]	3.8%
***NRAS* exon 3 (Codons 59 and 61)**[a]	4.8%
***NRAS* exon 4 (Codons 117 and 146)**[a]	0.5%

[a] Ascertained in *KRAS* exon 2 WT populations.

4.2.2. *RAS* analysis in ctDNA

We analysed *KRAS* and *NRAS* mutations in DNA isolated from plasma samples, using the digital PCR-based BEAMing technology (mutations covered by BEAMing are highlighted in yellow in Table 2). The amount of DNA obtained in 140 µL ranged from 0.168 ng/µL (23.52 ng) to 87.6 ng/µL (12.26 µg); the median was 0.515 ng/µL (that is, 72.1 ng) of cfDNA.

A detailed distribution of the mutations found in ctDNA is shown in Table 7. We found 9 *RAS* WT (30%) and 20 *RAS* MUT (66.7%) samples. In one case, the amount of DNA obtained from plasma was extremely low, and we were unable to achieve a valid result. Therefore, this case was excluded for the concordance studies.

Table 7: Frequencies of *KRAS* and *NRAS* mutations in our cohort, as detected by BEAMing.

Codon	No. of cases	Proportion (n=30)
All WT	9	30.0%
Any mutation	20	66.7%
Invalid	1	3.3%
Any *KRAS*	18	60.0%
Any *NRAS*	8	26.7%
Total mutations identified in *KRAS*		
KRAS 12	14	46.7%

KRAS 13	3	10.0%
KRAS 61	2	6.7%
KRAS 117	1	3.3%
Total mutations identified in *NRAS*		
NRAS 12	3	10.0%
NRAS 13	1	3.3%
NRAS 61	6	20.0%
Mutations identified in one codon		
KRAS 12	9	30.0%
KRAS 13	2	6.6%
KRAS 61	1	3.3%
NRAS 13	1	3.3%
NRAS 61	1	3.3%
Mutations identified in more than one codon		
KRAS 12 + *KRAS* 117	1	3.3%
KRAS 12 + *NRAS* 61	2	6.6%
KRAS 12 + *NRAS* 12 + *NRAS* 61	1	3.3%
KRAS 13 + *NRAS* 12 + *NRAS* 61	1	3.3%
KRAS 12 + *KRAS* 61 + *NRAS* 12 + *NRAS* 61	1	3.3%

As expected, *KRAS* codon 12 was the most frequently mutation found in our cohort. In these cases, no information on the specific base substitution was available, because BEAMing is only able to distinguish between the WT form and all the MUT forms for each codon. Mutant fractions obtained in the MUT cases ranged from 0.004% (case 11), which is extremely low, to 25.045% in case 6. Notably, case 11 was one of the three cases with especially high cfDNA yield (25.8 ng/µL); this probably allowed us to detect mutations present at extremely low concentration in cfDNA. In Figure 17 some examples of high (Fig 17 A) and low (Fig 17 B and C) mutant fraction are shown.

The cutoff for *KRAS* codon 12 mutations was established at 40 mutant beads. As seen in Figure 17, case 15 (Fig 17 B) was just over the cutoff value (56 mutated beads), whereas case 3 (Fig 17 C) was just below it (22 mutated beads). In fact, case 3 was a discordant case, in which tissue sample was informed as MUT by pyrosequencing (*KRAS* Gly12Val mutation), although it showed a relatively low mutant fraction (10%).

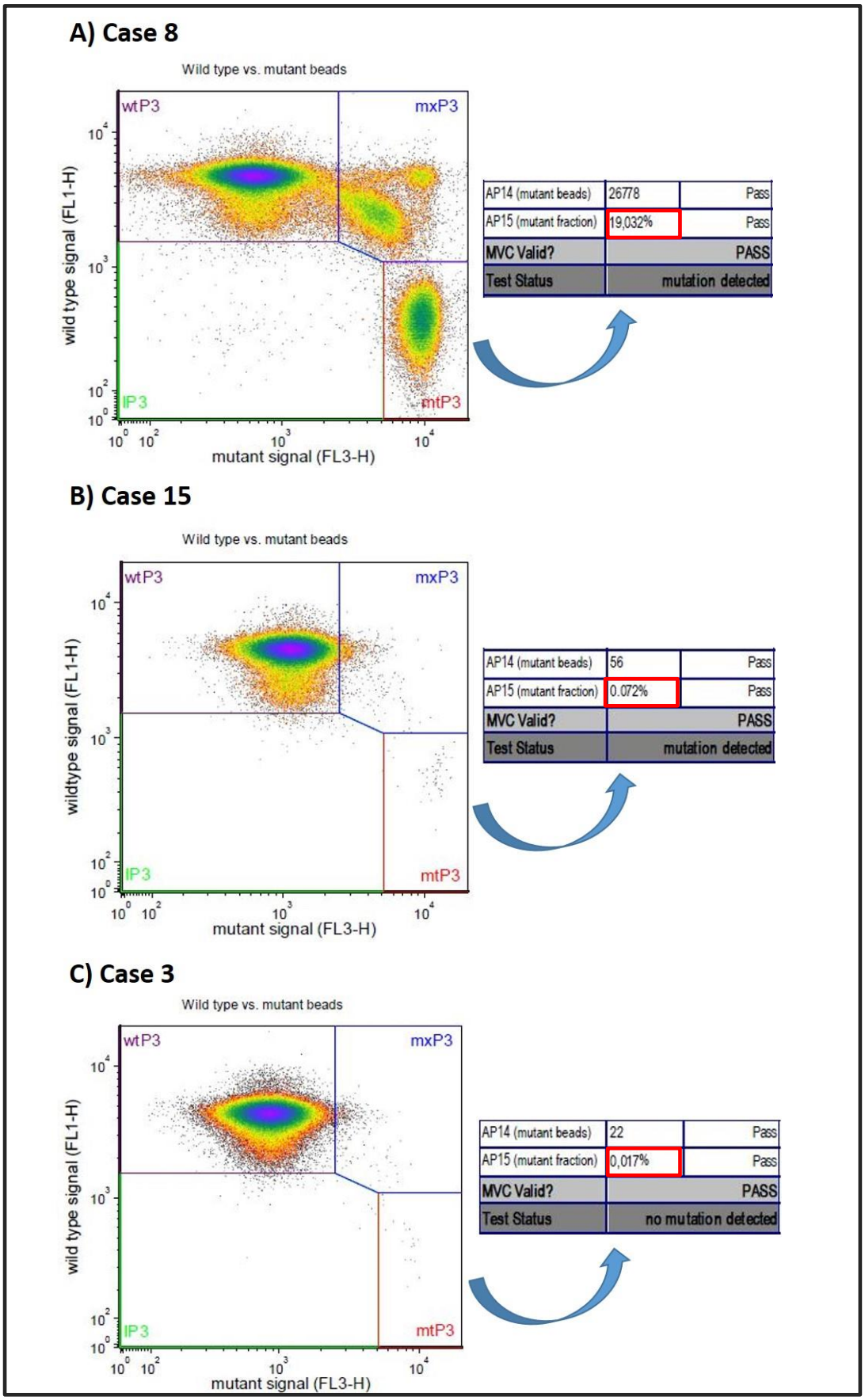

Figure 17: Examples of BEAMing results. A) Case 8, *KRAS* codon 12 MUT. B) Case 15, *KRAS* codon 12 MUT. C) Case 3, *KRAS* codon 12 WT.

Table 8 contains a summary of all the results obtained for *RAS* mutational analysis in ctDNA by BEAMing technology:

Table 8: Summary of the results for *RAS* plasma analysis by BEAMing (n=30).

Case	OncoBEAM *RAS*	[cfDNA] (ng/µL)	Mutant beads fraction (%) (same order)			
1	WT	0.370				
2	*KRAS* 13	0.554	49.442			
3	WT	1.640				
4	*KRAS* 12	1.760	28.042			
5	WT	0.782				
6	*KRAS* 12 *NRAS* 12 *NRAS* 61	1.980	25.045	0.022	0.007	
7	*KRAS* 12	0.174	2.124			
8	*KRAS* 12	0.876	19.032			
9	*KRAS* 12	0.296	0.791			
10	*KRAS* 12 *NRAS* 61	1.880	23.254	0.013		
11	*KRAS* 13 *NRAS* 12 *NRAS* 61	25.800	10.926	0.013	0.004	
12	WT	0.262				
13	*KRAS* 13	54.600	5.329			
14	WT	0.200				
15	*KRAS* 12 *KRAS* 117	0.446	0.072	0.264		
16	*KRAS* 12	0.300	13.056			
17	*KRAS* 12	0.666	10.515			
18	WT	0.168				
19	*KRAS* 12	0.294	6.575			
20	WT	0.362				
21	*KRAS* 12[b] *KRAS* 61 *NRAS* 12 *NRAS* 61	3.820	0.013	0.016	0.018	0.021
22	Invalid[a]	0.224				
23	*KRAS* 12	0.230	9.843			
24	WT	6.640				

25	*NRAS* 61	0.526	0.262			
26	*KRAS* 12	0.504	10.498			
27	*KRAS* 12 *NRAS* 61	10.500	8.061	0.006		
28	*NRAS* 13	87.600	29.580			
29	WT	0.326				
30	*KRAS* 61	0.226	0.122			

[a] Sample from case 22 reported invalid resultsdue to low DNA amount. [b] *KRAS* 12 mutation was conditioned because the beads were too dispersed.

Of interest, we found 6 MUT cases displaying coexistent *RAS* mutations in ctDNA (Table 8; cases: 6, 10, 11, 15, 21, and 27). This was not entirely unexpected due to the high sensitivity of BEAMing, but the biological and therapeutic relevance of these mutated subclones that are present in very low proportion needs to be further investigated. May be, is exactly in this point where the advantages of the high-sensitivity blood-based *RAS* testing platforms will have more relevance in the clinical practice, since it is very well known that in metastatic patients, a single tumor tissue biopsy may not represent the evolving *RAS* mutational status of the disease. In patients whose tumours show extensive heterogeneity, a comprehensive surveying of *RAS* status by testing multiple tissue samples would be desirable; however, this is impractical and unfeasible. Instead, serial blood sampling and BEAMing analysis of *RAS* status could be the solution.

Finally, it is worth pointing out that up until now, almost all studies that included BEAMing analysis had been performed centralized in the Sysmex facilities in Hamburg. The Molecular Oncology Laboratory at FIHGUV is one of the first laboratories using the BEAMing technology out of the central laboratory of Sysmex, , as part of a pilot project conducted on 8 Spanish centers.

4.3. Concordance between plasma and tissue *RAS* mutational status

Determinations of *RAS* mutational status in ctDNA by BEAMing and in tissue by pyrosequencing have been summarized in Table 9.

Table 9: Summary of *RAS* mutational status as determined in tumour tissue by pyrosequencing, and in plasma ctDNA by BEAMing.

Case	*RAS* tissue	*RAS* ctDNA	Case	*RAS* tissue	*RAS* ctDNA
1	WT	WT	**16**	*KRAS* G12V *KRAS* G12A	*KRAS* 12
2	*KRAS* G13D	*KRAS* 13	**17**	*KRAS* G12D	*KRAS* 12
3	*KRAS* G12V	WT	**18**	WT	WT
4	*KRAS* G12V	*KRAS* 12	**19**	*KRAS* G12V	*KRAS* 12
5	*KRAS* G12D	WT	**20**	WT	WT
6	*KRAS* G12V	*KRAS* 12	**21**	WT	*KRAS* 12[b]

		NRAS 12 *NRAS* 61			*KRAS* 61 *NRAS* 12 *NRAS* 61
7	*KRAS* G12C	*KRAS* 12	**22**	*KRAS* G13D	NA[a]
8	*KRAS* G12S	*KRAS* 12	**23**	*KRAS* G12V	*KRAS* 12
9	*KRAS* G12D	*KRAS* 12	**24**	WT	WT
10	*KRAS* G12V	*KRAS* 12 *NRAS* 61	**25**	*NRAS* Q61R	*NRAS* 61
11	*KRAS* G13D	*KRAS* 13 *NRAS* 12 *NRAS* 61	**26**	*KRAS* G12V	*KRAS* 12
12	WT	WT	**27**	*KRAS* G12A	*KRAS* 12 *NRAS* 61
13	*KRAS* G13D	*KRAS* 13	**28**	*NRAS* G13R	*NRAS* 13
14	WT	WT	**29**	WT	WT
15	*KRAS* G12C	*KRAS* 12 *KRAS* 117	**30**	*KRAS* Q61H	*KRAS* 61

Of the 30 paired tissue/plasma samples analysed, there was one case that had to be excluded from the analysis due to low concentration of DNA in plasma, which resulted in invalid *RAS* genotyping by BEAMing. Of the 29 remaining cases, 21 were *RAS* MUT in FFPE (72.4%) whereas in ctDNA we found 20 *RAS* MUT cases (69.0%). *RAS* mutation positive samples were called above mutant allelic fraction thresholds of 0.02% in plasma and 5% in tissue.

The agreement between BEAMing system in plasma samples and the determination by pyrosequencing in tissue samples was estimated by calculating the raw agreement and performing the concordance test. Overall agreement was obtained, along with positive and negative agreement (Table 10)

Table 10: Concordance of plasma and tissue for *RAS* mutational status.

		Tissue *RAS* status		
Plasma *RAS* status		**Positive (Mut)**	**Negative (WT)**	**Total**
	Positive (Mut)	19	1	20
	Negative (WT)	2	7	9
	Total	21	8	29

Overall agreement = 0.896 → **89.6 %**

Positive agreement = 0.905 → **90.5 %**

Negative agreement = 0.875 → **87.5 %**

These results show a good concordance between BEAMing and pyrosequencing, with an overall agreement (OA) of 89.6%. Although there are few studies in the literature, concordance between liquid biopsy, using highly sensitive methods such as digital PCR and tumour genotyping have usually resulted in concordance. One example is a report using datasets with 76 paired tissue-blood samples from two clinical trials (OPUS and CRYSTAL), where OA between tissue (*RAS* DNA sequencing of FFPE tumour samples) and plasma (BEAMing *RAS* 33 Mutation Panel) was 93.4% (Jones et al. 2015). In other tumours, like breast and lung cancer, Higgins et al found a 100% OA in the retrospective assessment of *PI3KCA* mutations in breast cancer samples (BEAMing ctDNA vs. sequencing tumour tissue); in the prospective analysis, however, only a 72.5% of concordance was achieved (Higgins et al. 2012). Finally, Karlovich and colleagues found an OA of 67% when assessing T790M mutation by cobas® Tumour test and BEAMing plasma ctDNA on non-small cell lung cancer samples (Karlovich et al. 2016).

Although highly concordant, we had 3 discordant cases, which are summarized in Table 11:

Table 11: Discordant cases summary.

Case	Tumour site	Plasma result	Tissue result
#3	Recto-sigmoid junction	WT	*KRAS* exon 2 G12V
#5	Transverse colon	WT	*KRAS* exon 2 G12D
#21	Rectum	*KRAS* 12, 61 + *NRAS* 12, 61	WT

Among the two patients in which *RAS* mutation was identified in tissue but not in plasma, we investigated possible causes. First, we re-examined FFPE samples by pyrosequencing, confirming previous results.

Second, preanalytical procedures for plasma processing could also explain the lack of concordance. Since this is a retrospective study, characteristics of the collection tube, time from collection until processing samples and plasma storage conditions might have affected the yield and/or quality of ctDNA obtained. In both cases (#3 and #5), blood samples were collected in standard K2-EDTA tubes without any nucleic-acid stabilizing agent. Apart from this, samples were stored at -80 °C for more than 5years.

Third, there is also a possible correlation between tumour burden, number and location of metastatic disease lesions and the amount of plasma DNA recovered. Interestingly, case #3 had a unique metastatic lesion (low tumour burden), even though the amount of plasma obtained was in the median yield. On the contrary, case #5 was a patient with high tumour burden at the time of diagnosis (primary tumour and several liver metastasis, the greater measuring more than 90 mm). Consequently, we would expect higher ctDNA levels, but in this case the yield was half of that obtained in case #3.

Standardized methods are still needed in order to minimize their impact on mutation detection rates (El Messaoudi et al. 2013). Some important factors are:

- Processing the blood in 4-6 hours after drawing is essential, because half-life of ctDNA in circulation is between 16 minutes and 2 hours (Diehl et al. 2008).
- Use of Cell-Free DNA™ Blood Collection Tubes (Streck, Omaha, NE) is advantageous over other collection tubes: they contain a formaldehyde-free preservative that prevents white blood cells from breaking, avoiding WT DNA contamination up to 72 hours post-collection (Xue et al. 2009; Sherwood et al. 2016).

The other discordant case was *RAS* MUT in plasma but no mutation was detected on FFPE sample. Of interest, in this case (#21) *RAS* genotyping was performed on a FFPE sample corresponding to a liver metastasis, since primary tumour tissue was not available. Figure 18 shows the plots obtained for all four mutations (A-D):

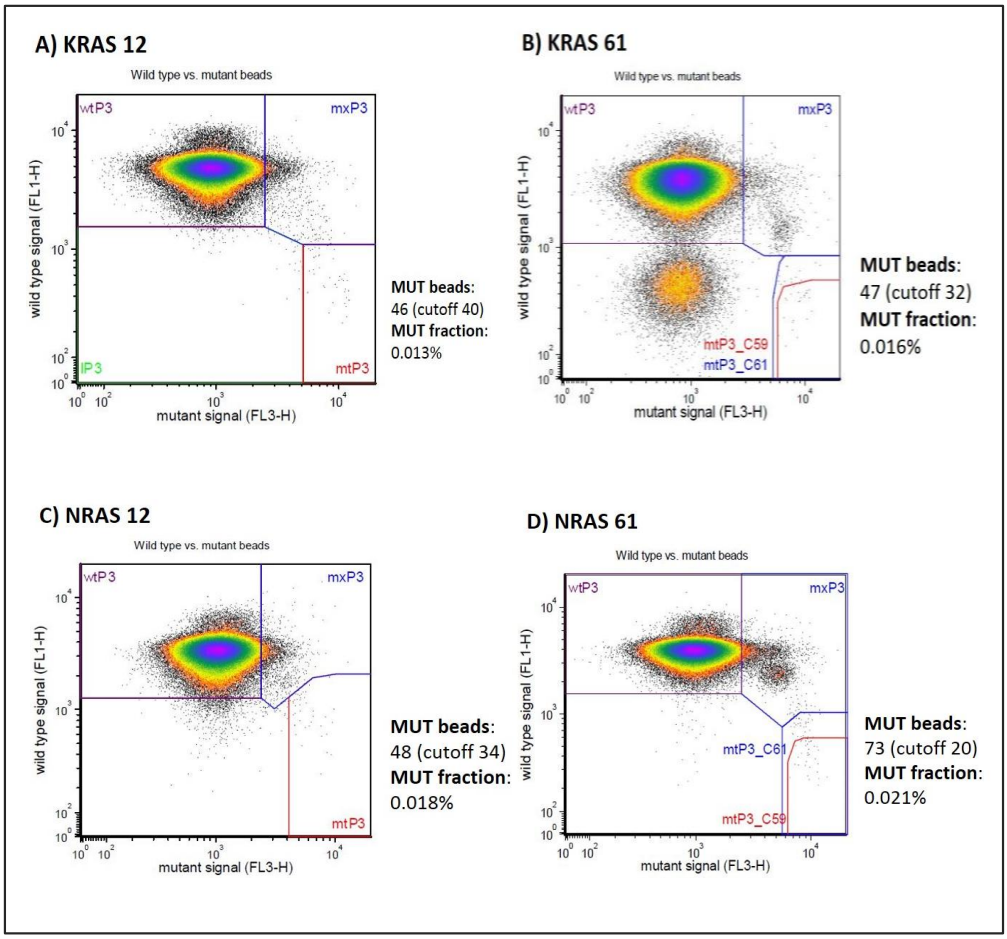

Figure 18: Plots for all four mutations detected in ctDNA of case 21, with number of mutated beads and mutant fraction.

Differences in *RAS* mutation status between plasma and tissue may be attributed to intra or inter-tumour molecular heterogeneity. For instance, *RAS* WT in tissue but MUT in plasma, such as in case 21, may arise in patients having heterogeneous distribution of *RAS* mutant clones in

the primary tumour and/or in the metastases, which are not represented in the analysed tissue sample but are detected systemically by the plasma test.

Interestingly, patient #21 was treated with a combination of FOLFOX with anti-*EGFR* agent panitumumab for 7 cycles. No response was observed, only achieving disease stabilization. The treatment was stopped and four months later, the patient progressed and a second line treatment was initiated. So, these apparently discordant results between plasma and tissue make sense when we look closely at the clinical evolution of the patient. Probably, if we had followed the patient with serial blood sampling we could have detected an increase in the mutant fraction during treatment.

Although not a discordance, there were 5 cases in which BEAMing detected the mutation observed by pyrosequencing and additional mutations present in low proportion in ctDNA. The relevance of these results is still unknown, and they should be checked; analysing tissue samples using BEAMing technology could reveal if those mutations were present in the tissue in such low proportion that pyrosequencing could not detect them. This is being performed right now on our laboratory.

Finally, case #22 did not yield any results because of low DNA concentration and subsequent failure of emulsion PCR. The concentration of cfDNA after nucleic acid extraction was 0.224 , which is just over the value we estimate to be sufficient to detect at least one mutation in ctDNA. Therefore, the best course of action would be to repeat cfDNA extraction from the same plasma sample (if still possible), increasing the amount of input plasma: there is evidence that increasing the amount of input plasma can improve ctDNA mutation detection if the processing of the plasma is optimal (Sherwood et al. 2016).

Clinical validation of BEAMing has arrived first to tumour tissue analysis. Over 1200 patients from different clinical trials had tumour tissue samples tested using the BEAMing platform in a retrospective manner:

- In OPUS trial sample set (Bokemeyer et al. 2015), mutation frequency was broadly concordant with those reported in similar studies using pyrosequencing (Douillard et al. 2013; Schwartzberg et al. 2014; Stintzing et al. 2012).
- In the CRYSTAL study, *RAS* mutations were found in 14.7% of evaluable patients.
- Finally, results from the subgroup analysis of CALGB/SWOG 80405 have not been published yet. Preliminary results show that new *RAS* mutations were identified in 15.3% of analysed patients.

In conclusion, liquid biopsy could hold the key to earlier detection and treatment of relapsed disease, and ultimately improve the outcome of a patient. As blood serial sampling is much less invasive and safe than metastasis or tumour biopsies, ctDNA analysis for *RAS* mutational

status assessment represents a potential surrogate for solid biopsies. Therefore, sensitive techniques such as BEAMing system able to detect minimal quantities of ctDNA carrying actionable mutations are desirable and will become an essential tool for molecular oncology diagnostics. Moreover, BEAMing technique is versatile, able to analyse DNA from both plasma and tissue samples: an invaluable resource for molecular oncology researchers and oncologists.

4.4. Association of *RAS* mutational status with clinico-pathological characteristics

We analysed association between clinicopathological variables and *RAS* mutational status as determined by both techniques. *RAS* status was dichotomized as follows: "*RAS* WT" (meaning no detection of mutated DNA) and "Any *RAS* MUT" (meaning that at least one *KRAS* or *NRAS* mutation was detected). First, results from tumour tissue pyrosequencing are shown in Table 12; no statistically significant correlation was found between *RAS* mutational status in tumour tissue and these characteristics:

Table 12: Correlation analysis between *RAS* mutational status (tumour tissue) and clinicopathological variables of interest in CRC using Mann-Whitney U test.

Characteristics		All (n=30)	All *RAS* WT (n=8; 26.7%)	Any *RAS* mutation (n=22; 73.3%)	p-value
Age at diagnosis (years)	Median [range]	68 [43-83]	67 [43-71]	69 [47-83]	*0.270
Sex	Man	20 (66.7%)	6 (20%)	14 (46.7%)	†0.682
	Woman	10 (33.3%)	2 (6.7%)	8 (26.7%)	
CEA (ng/mL) at diagnosis[a]	Median [range]	31 [2.7-7059.5]	19.9 [6.5-1000]	36.1 [2.7-7059.5]	*1.000
Primary tumour localization	Ascending colon	6 (20%)	1 (3.3%)	5 (16.7%)	†0.650
	Transverse colon	3 (10%)	0 (0%)	3 (10%)	
	Descending colon	8 (26.7%)	2 (6.7%)	6 (20%)	
	Rectum	13 (43.3%)	5 (16.7%)	8 (26.7%)	
Primary tumour histology[b]	ADC	24 (85.7%)	7 (25%)	17 (60.7%)	†1.000
	MUC	4 (14.3%)	1 (3.6%)	3 (10.7%)	
Grade of differentiation	Well diff.	9 (45%)	2 (10%)	7 (35%)	†1.000
	Moderately diff.	10 (50%)	3 (15%)	7 (35%)	
	Poorly diff.	1 (5%)	0 (0%)	1 (5%)	
Tumour size and invasiveness (T)	T2	2 (11.8%)	1 (5.9%)	1 (5.9%)	†1.000
	T3	8 (47.1%)	3 (17.6%)	5 (29.4%)	
	T4	7 (41.2%)	3 (17.6%)	4 (23.5%)	
Regional lymph	N0	6 (33.3%)	2 (11.1%)	4 (22.2%)	†0.350

nodes involvement (N)	N1	6 (33.3%)	4 (22.2%)	2 (11.1%)	
	N2	6 (33.3%)	1 (5.6%)	5 (27.8%)	
Lymphatic invasion (L)	L0	9 (64.3%)	2 (14.3%)	7 (50%)	†0.580
	L1	5 (35.7%)	2 (14.3%)	3 (21.4%)	
Vascular invasion (V)	V0	13 (86.7%)	4 (26.7%)	9 (60%)	†1.000
	V1	2 (13.3%)	1 (6.7%)	1 (6.7%)	
Perineural invasion (Pn)	Pn0	12 (85.7%)	4 (28.6%)	8 (57.1%)	†1.000
	Pn1	2 (14.3%)	0 (0%)	2 (14.3%)	
Liver metastasis	No	7 (23.3%)	3 (10%)	4 (13.3%)	†0.345
	Yes	23 (76.7%)	5 (16.7%)	18 (60%)	
Organs affected by metastasis	1 organ	14 (46.7%)	6 (75%)	6 (31.6%)	†0.101
	>1 organ	16 (53.3%)	2 (25%)	13 (68.4%)	

*Kruskal-Wallis test; †Mann-Whitney U test. [a] CEA levels only available for 17 patients. [b] ADC = Adenocarcinoma; MUC = Mucinous adenocarcinoma.

Second, the results obtained from *RAS* mutational status by ctDNA analysis are shown in Table 13:

Table 13: Correlation analysis between *RAS* mutational status and clinicopathological variables of interest in CRC using Mann-Whitney U test.

Characteristics		All (n=29)	All *RAS* WT (n=9; 31%)	Any *RAS* mutation (n=20; 69%)	p-value
Age at diagnosis (years)	Median [range]	68 [43-83]	68 [43-81]	69 [47-83]	*0.850
Sex	Man	20 (69%)	6 (20.7%)	14 (48.3%)	†1.000
	Woman	9 (31%)	3 (10.3%)	6 (20.7%)	
CEA (ng/mL) at diagnosis[a]	Median [range]	36.1 [2.7-7059.5]	17.25 [2.7-1000]	41.45 [2.7-7059.5]	*0.302
Primary tumour localization	Ascending colon	6 (20.7%)	1 (3.4%)	5 (17.2%)	†0.933
	Transverse colon	3 (10.3%)	1 (3.4%)	2 (6.9%)	
	Descending colon	7 (24.1%)	2 (6.9%)	5 (17.2%)	
	Rectum	13 (44.8%)	5 (17.2%)	8 (27.6%)	
Primary tumour histology[b]	ADC	24 (88.9%)	8 (29.6%)	16 (59.3%)	†1.000
	MUC	4 (11.1%)	1 (3.7%)	2 (7.4%)	
Grade of differentiation	Well diff.	9 (47.4%)	3 (15.8%)	6 (31.6%)	†1.000
	Moderately diff.	9 (47.4%)	3 (15.8%)	6 (31.6%)	
	Poorly diff.	1 (5.3%)	0 (0%)	1 (5.3%)	
Tumour size and invasiveness (T)	T2	2 (12.5%)	0 (0%)	2 (12.5%)	†0.621
	T3	8 (50%)	4 (25%)	4 (25%)	
	T4	6 (37.5%)	3 (18.8%)	3 (18.8%)	

Regional lymph nodes involvement (N)	**N0**	6 (35.3%)	3 (17.6%)	3 (17.6%)	†0.473
	N1	5 (29.4%)	3 (17.6%)	2 (11.8%)	
	N2	6 (35.3%)	1 (5.9%)	5 (29.4%)	
Lymphatic invasion (L)	**L0**	8 (61.5%)	3 (23.1%)	5 (38.5%)	†1.000
	L1	5 (38.5%)	2 (15.4%)	3 (23.1%)	
Vascular invasion (V)	**V0**	13 (92.9%)	5 (35.7%)	8 (57.1%)	†0.429
	V1	1 (7.1%)	1 (7.1%)	0 (0%)	
Perineural invasion (Pn)	**Pn0**	12 (92.3%)	5 (38.5%)	7 (53.8%)	†1.000
	Pn1	1 (7.7%)	0 (0%)	1 (7.7%)	
Liver metastasis	**No**	6 (20.7%)	4 (13.8%)	2 (6.9%)	†0.056
	Yes	23 (79.3%)	5 (17.2%)	18 (62.1%)	
Organs affected by metastasis	**1 organ**	13 (44.8%)	6 (20.7%)	7 (24.1%)	†0.226
	>1 organ	16 (55.2%)	3 (10.3%)	13 (44.8%)	

*Kruskal-Wallis test; †Mann-Whitney U test. [a] CEA levels only available for 17 patients. [b] ADC = Adenocarcinoma; MUC = Mucinous adenocarcinoma.

We found no significant association between plasma *RAS* mutational status and clinicopathological characteristics of the patients. Given the small number of patients, the results were not unexpected. The only variable that showed a trend was the presence of liver metastasis associated with *RAS* MUT status (p-value = 0.056).

There is some evidence supporting this correlation in the literature: Modest et al found correlation between *KRAS* exon 2 mutations and metastatic sites (liver and lung) (Modest et al. 2011). In contrast with our findings, Yaeger et al found that mutations in *RAS* were independent predictors for metastatic risk in lung, bone and brain, but not liver (Yaeger et al. 2015).

4.5. Survival analysis according to *RAS* mutational status

To examine the prognostic role of *RAS* mutational status as an independent factor, we performed a survival analysis: progression-free survival (PFS, from date of metastatic disease diagnosis to first relapse) and overall survival (OS, from date of metastatic disease diagnosis to death) according to *RAS* status in FFPE samples and ctDNA, separately.

Of the 29 mCRC patients included in the study, there were 20 progression events and 11 deaths during the follow-up. We used the Kaplan-Meier method to compare the two groups (*RAS* WT vs. any *RAS* MUT) for PFS and OS (Figure 19 for FFPE samples and Figure 20 for ctDNA).

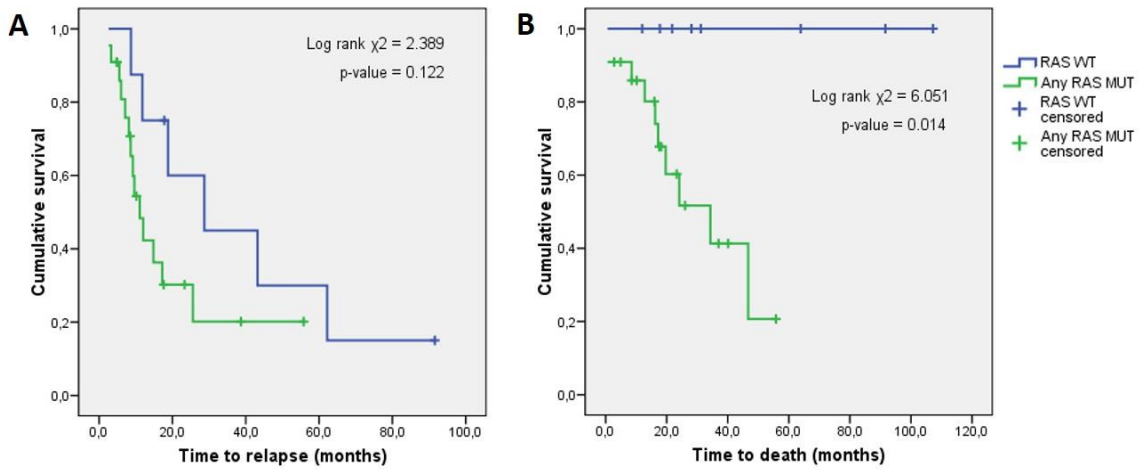

Figure 19: Kaplan-Meier curves according to *RAS* mutational status in FFPE samples. A) Progression-free survival (PFS). B) Overall survival (OS). Comparison based on Log rank test.

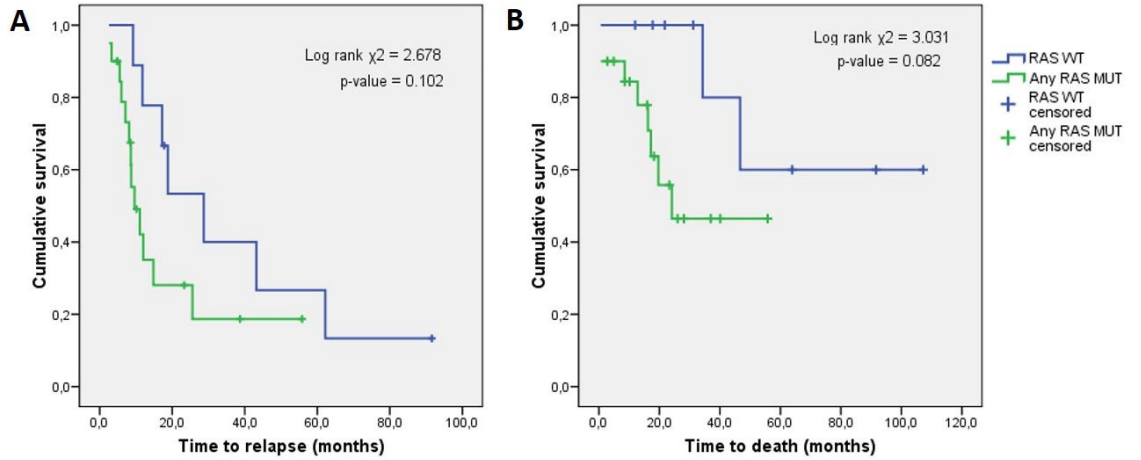

Figure 20: Kaplan-Meier curves according to *RAS* mutational status in ctDNA. A) Progression-free survival (PFS). B) Overall survival (OS). Comparison based on Log rank test.

As we see, none of the curves achieved statistically significant differences. Probably all these results have been even more influenced by the low number of patients, because the curves can be clearly separated and the *RAS* mutant group arrived to the median of survival whilst the WT group did not. However, as we selected our population to enrich it with mutated samples, no conclusions can be drawn from this analysis.

Our results, despite having little statistic relevance, are generally concordant with those of many other research groups that have not found independent prognostic value for *RAS* mutations in CRC (Akman et al. 2016; Ogino et al. 2009).

However, in the *RAS*CAL II study (Andreyev et al. 2001), on a cohort of 3439 patients, it was found that *KRAS* mutation G12V had statistically significant impact on failure-free survival (HR = 1.3; p-value = 0.004) and overall survival (HR = 1.29; p-value = 0.008). In the same line, in the MRC FOCUS trial (Richman et al. 2009), it was found that mCRC patients with mutations in

either *KRAS* or BRAF had a worse overall survival (HR = 1.40; p-value < 0.0001), on a cohort of 711 patients.

There is some evidence suggesting that *RAS* mutational status as detected by liquid biopsy could have more prognostic value that the detected in tumour tissue biopsies (Spindler et al. 2015). They found that *KRAS* status in plasma was an independent prognostic factor for OS (HR = 2.26, p-value < 0.0001) and PFS (HR = 1.69, p-value = 0.01), contrary to tumour tissue *KRAS* status that only showed prognostic value for OS (HR = 0.61; p-value = 0.02). Our results do not allow us to support these findings, but they favor our hypothesis that *RAS* assessment in plasma ctDNA can be a proficient substitute for primary tumour and metastases biopsy.

In summary, we have found a high overall agreement of plasma and tissue *RAS* testing results, thereby demonstrating that blood-based *RAS* mutation assessment is a feasible and advantageous alternative to tissue-based analysis. In the clinical context, where *RAS* status is mandatory for determining elegibility of mCRC patients for anti-*EGFR* therapy, these new approaches could improve markedly our speed in molecular diagnosis of *RAS* status, and our ability to track tumour evolution and therefore make a better therapeutic management of the patient.

5. CONCLUSION

In the present study we aimed to evaluate the BEAMing system as a suitable platform for liquid biopsy *RAS* mutational testing in the clinical setting, by comparing it with our previous standardized technology, pyrosequencing on tumour tissue FFPE samples. We intended to find possible clinicopathological correlations with *RAS* mutational status too.

1. There is a high overall agreement of plasma and tissue *RAS* testing results, demonstrating that blood-based *RAS* mutation testing is a viable alternative to tissue-based testing, when highly sensitive methods, such as BEAMing are used.

2. *RAS* mutational status determined in tissue or plasma samples shows no significant association with clinicopathological or prognostic variables in our cohort. Unfortunately, our limited cohort was insufficient to draw relevant conclusions.

3. Circulating tumour DNA analysis by BEAMing is a feasible approach for mutation detection and has many advantages: it is non-invasive, allows for detection of multiple *RAS* mutations at once and it is suitable for tracking disease evolution and earlier detection of emerging resistances.

Altogether, the results presented in this work suggest that BEAMing analysis of ctDNA in advanced colorectal cancer is a sensitive technology for *RAS* mutational status analysis. In our context, it represents the beginning of an innovative approach to molecular diagnostics of cancer, based on liquid biopsy and systemic assessment of the disease, rather than focusing on individual lesions, that only provide a snapshot of the disease in a particular time and location.

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7. APPENDICES

7.1. Communications derived from this study

VI Meeting of Young Researchers (RTICC). Salamanca, Spain 2016.

Mutational analysis of RAS genes in metastatic colorectal cancer: Concordance between tissue and liquid biopsy.

Borja Lafuente Gutiérrez, Alejandro Herreros Pomares, Silvia Calabuig-Fariñas, Marais Mosqueda, Eva Escorihuela, Sandra Gallach, María José Safont, Eloisa Jantus-Lewintre, Carlos Camps**.**

BACKGROUND: Mutations in proto-oncogenes RAS (KRAS and NRAS) are routinely tested in metastatic colorectal cancer (mCRC). Tumors with activating mutations in RAS genes do not respond to anti-EGFR targeted therapy (cetuximab and panitumumab). In consequence, these drugs are restricted to patients with *RAS* wild-type tumors. Eventually tumors develop resistance by selection of RAS mutant subclones. Thus, serial sampling for mutational analysis is desirable, but it entails multiple biopsies (invasive and expensive). Liquid biopsy systems, like BEAMing, allow for continuous mutational analysis in a non-invasive, ultrasensitive manner.

OBJECTIVE: We aim to compare our standard technique for *RAS* assessment in tumor tissue, Pyrosequencing, with a "liquid biopsy" approach based on ctDNA analysis: BEAMing system.

METHODS: We used TheraScreen® *KRAS* Pyro Kit and *RAS* Extension Pyro Kit for pyrosequencing of tumor tissue DNA, and Sysmex® OncoBEAM CRC *RAS* kit for BEAMing ctDNA analysis. Concordance was determined by the number of cases reported as “mutant” or “wild-type/no mutation detected” in each system.

RESULTS: We tested *RAS* mutational status in tumor tissue and plasma samples of 30 patients. We found an overall agreement of 89.6% (**Table 1**), with three discordant cases (**Table 2**). One case could not be analyzed by BEAMing because of low DNA input:

Table 3: Concordance table of plasma and tissue results for RAS mutational status.

		Tissue *RAS* status		
		Positive (Mut)	**Negative (WT)**	**Total**
Plasma *RAS* status	**Positive (Mut)**	19	1	20
	Negative (WT)	2	7	9
	Total	21	8	29

Table 4: Summary of discordant cases, type of discordance, plasma and tissue results.

Case	Discordant type	Plasma result	Tissue result
#3	False Negative	WT	KRAS G12V
#5	False Negative	WT	KRAS G12D
#21	False Positive	KRAS 12, 61 + NRAS 12, 61	WT

The false negatives were investigated (**Table 2**), pointing towards ctDNA degradation over time and preanalytical suboptimal processing as the main factors involved.

In case 21, the presence of RAS mutant ctDNA in plasma before anti-*EGFR* treatment could have predicted early disease progression, which occurred just four months after treatment. Probably, *RAS* mutant subclones began to proliferate and sustain the tumor, generating the secondary resistance to anti-EGFR treatments.

CONCLUSIONS: Our results indicate that BEAMing analysis of RAS mutations in ctDNA has a high concordance rate when compared to pyrosequencing tumor tissue. Therefore, BEAMing is an optimal technique for molecular diagnosis of RAS mutational status of mCRC patients. In addition, it is very useful in disease monitoring as a non-invasive, specific method for detecting low prevalence RAS mutations in plasma, allowing earlier interventions to modify and improve treatments.

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