# A Feasibility Study Evaluating the use of Circulating Tumour DNA in Genetic tests, as a Proxy for Brain Cancer Biopsy Samples

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# A Feasibility Study Evaluating the use of Circulating Tumour DNA in Genetic tests, as a Proxy for Brain Cancer Biopsy Samples

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*For my family,*

*Robbie, Josh, Fran, Mum and Dad*

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# Abstract

Circulating tumour DNA (ctDNA) in the plasma represents an exciting analyte for the diagnosis and monitoring of disease in cancer patients. In a number of solid cancers types it has been shown to have clinical utility in diagnostic and molecular profiling, tracking of therapeutic response, monitoring of resistance and tumour heterogeneity and detection of post-surgical residual disease. Such clinical utility would also be of huge value in brain cancer, providing clinical information for patients where it is not currently available and/or providing an option for less invasive biopsy-dependent testing. However, its use in glioma brain cancer patients is thought to represent a particular challenge due to the reduced permeability of the blood brain barrier.

**Aim**: This pilot study sought to investigate the practical aspects and clinical utility of using ctDNA in glioma testing in a diagnostic National Health Service laboratory; whether this analyte could be used as a proxy for the diagnostic and therapeutic decision tests performed as standard on FFPE brain biopsy samples. Additionally, the potential clinical utility of standalone metrics of cell free DNA (cfDNA) concentration was explored.

**Methods**: Peripheral blood samples were collected from a cohort of 39 high grade glioma patients comprised of anaplastic oligodendroglioma, anaplastic astrocytoma and glioblastoma tumour sub-types. cfDNA was extracted using a magnetic bead-based protocol and its quality and quantity assessed by chip-based automated electrophoresis. Where the originating cancers harboured the appropriate biomarkers, samples were run though the Laboratory assays for Isocitrate dehydrogenase gene variant analysis, *MGMT* promoter methylation status and 1p/19q co-deletion testing.

**Results**: The extraction protocol delivered cfDNA of high purity with a mean of 91% ± 5.5%, within the plasma nucleic acid fraction.

For all of the tests performed, results reflected the germline DNA profile rather than the new somatic changes of the tumour. The cfDNA analysis did not pick up the tumour biomarkers seen in the paired tumour biopsy sample. However, in all cases the yield of cfDNA was too low to meet the DNA threshold concentration for the established limit of detection for assays. It was thus not possible to fully explore whether very low levels of circulating tumour DNA could be picked up as a component of the cfDNA.

In a second part of the study, cfDNA concentrations for the glioblastoma cohort were assessed in the context of their clinical outcomes data. Within the limitations of the testing strategy, the data showed an interesting correlate, where high cfDNA concentration was independently associated with inferior outcome in terms of overall survival.

**Conclusions**: In spite of the considerable advantages of looking for glioma biomarkers within the cfDNA fraction of plasma, this was currently not possible in our routine diagnostic environment. However, high cfDNA concentration in the glioblastoma sub-cohort showed a correlation with inferior outcome in terms of overall survival. Given the simplicity of obtaining this quantifiable metric, there are grounds for further investigations as to its utility; not only with survival outcomes,

but also for correlation with the clinical assessment of tumour burden, blood brain barrier integrity and disease pseudoprogression.

# Abbreviations

| | |
|---|---|
| AIF | allelic imbalance factor |
| ATRX | Transcriptional regulator ATRX |
| BBB | blood brain barrier |
| bp | Base pairs |
| CNS | Central nervous system |
| CSF | Cerebral Spinal Fluid |
| cfDNA | Circulating cell-free DNA |
| Ct | Threshold cycle |
| ctDNA | Circulating cell-free tumour DNA |
| COLD PCR | co-amplification at lower denaturation temperature-PCR |
| DNA | Deoxyribose nucleic acid |
| ddPCR | Droplet Digital PCR |
| EGFR | Epidermal Growth Factor Receptor |
| EPR | electronic patient record – CHARTS at University Hospital Southampton |
| FFPE | formalin-fixed paraffin embedded |
| GBM | Glioblastoma (multiforme) |
| H&E | Hematoxylin and eosin |
| HRM | High resolution melt |
| IDH | Isocitrate Dehydrogenase |
| KM | Kaplan Meier |
| LOD | Limit of detection |
| LOH | Loss of heterozygosity |
| MDT | Multi-Discipline Team |
| MGMT | $O^6$-methylguanine-DNA methyltransferase |
| miRNA | MicroRNA |
| MRI | Magnetic Resonance Imaging |
| MS-HRM | methylation-specific High Resolution Melt Analysis |
| NGS | Next generation sequencing |
| NTC | Non-template control |
| OS | Overall survival |
| PCR | Polymerase Chain reaction |
| PFS | Progression free survival |
| QC | Quality Control |
| RT | Room temperature |
| SNV | Single nucleotide variant |
| TKI | Tyrosine Kinase Inhibitor |
| UMIs | unique molecular indices |
| UHS | University Hospital Southampton |
| WHO | World Health Organization |

# 1 Introduction

Gliomas are the most common type of tumours originating from the central nervous system (CNS). They are primarily classified according to the cell type that gave rise to them, but now molecular parameters are added to the histology to fully define tumour identity. Currently, the laboratory tests which aid both the diagnosis and treatment of brain cancer are carried out on highly invasive biopsy samples. Cell-free circulating tumour DNA (ctDNA), shed from a solid tumour, can be used to look at cancer mutational characteristics (Francis and Stein, 2015; Wan *et al.*, 2017). ctDNA represents an exciting new analyte for molecular pathological investigations for cancer diagnostics and monitoring. Potentially it can better represent a fuller frame shot of a cancer aetiology and offers benefits over a cancer biopsy specimen, which is subject to the inherent heterogeneity of a sampling process. Also, as it is isolated from blood plasma, testing is relatively non-invasive and represents an opportunity to evaluate real time tumour dynamics through the course of treatment.

However, yields of ctDNA from glioma are low compared to other tumours type, purportedly with the blood brain barrier a physical obstacle preventing ctDNA from reaching the peripheral circulation (Bettegowda *et al.*, 2014). This initially muted the enthusiasm for the value of plasma ctDNA studies for glioma, but encouragingly, the recent literature reports better yields and 'actionability' from glioma plasma ctDNA studies (Zill *et al.*, 2018; Bagley *et al.*, 2019; Piccioni *et al.*, 2019). The genomic profiling of glioma samples obtained from brain biopsy may not always be clinically feasible and there remains risk of morbidity or mortality (Yong and Lonser, 2013; De la Garza-Ramos *et al.*, 2016). Additionally, glioblastomas exhibit marked molecular heterogeneity (Patel *et al.*, 2014). Thus, the pursuit of ctDNA as a substrate in glioma molecular analysis remains a worthy goal for attaining better patient care.

In this project, we asked if we were able to detect ctDNA from the plasma of patients with glioma brain cancers and if it could be used in our conventional laboratory molecular tests. These tests provide both diagnostic genetic information and DNA gene methylation detail that guides choice of therapy. Potentially, ctDNA analysis could provide clinical information for patients where it is not currently available and/or provide options of less invasive testing. The project also sought to investigate the potential clinical utility *per s*e of cfDNA burden, in the context of survival outcomes data and to augment other clinical investigations glioma patients receive, such as image-based response monitoring.

## 1.1 Gliomas - their clinical, histological and molecular classification

Gliomas are brain tumours that start in glial cells, the supporting cells of the brain and the spinal cord. There are three types of glial cells:

- Astrocytes – tumours that start in these cells are called astrocytoma or glioblastoma.
- Oligodendrocytes – tumours that start in these cells are called oligodendrogliomas.
- Ependymal cells – tumours that start in these cells are called ependymomas.

Brain tumours are categorized in to groups according to their malignancy and presumed level of differentiation. There are 4 groups, WHO grades I-IV (1-4), so the more normal the cells look, the lower the grade and the more abnormal the cells look, the higher the grade. Grade I cells look like normal cells; they are slow growing and less likely to spread. A Grade I brain tumour usually only requires surgical treatment. Grade II cells look less like normal cells; they are usually slow growing, but can grow in to the nearby brain tissue. Grade II tumours are more likely to come back after surgery and some can develop in to a malignant tumour. Grade III cells look more abnormal. They can spread to other parts of the brain and spinal cord. Patients are more likely to need post-surgical radiotherapy and chemotherapy. Grade IV cells look very abnormal, resulting in fast growing tumours. They often come back after treatment and can spread to other parts of the brain and sometimes the spinal cord. Surgical treatment is usually accompanied with radiotherapy and chemotherapy.

Astrocytomas are the most common type of brain tumours in both adults and children. There are 4 main astrocytoma types:

**Low grade**

- Pilocytic astrocytoma (grade I)
- Diffuse astrocytoma (grade II)

**High grade**

- Anaplastic astrocytoma (grade III)
- Glioblastomas, also called glioblastoma multiforme (GBM) (grade IV).

Approximately 12,300 new cases of brain tumours are diagnosed each year in the UK, this being 3% of total cancer cases. Astrocytomas represent about 34% of brain tumours diagnosed in England and 80% of astrocytomas are glioblastoma (CRUK, 2021). Approximately 30% of adults with high grade tumours (grades III and IV) survive one year and 13% survive 5 years. The median survival of patients with anaplastic astrocytoma is around 2-3 years and approximately 1 year for patients with glioblastoma (NICE, 2005).

Oligodendrogliomas are rare, representing only 3% of brain tumours diagnosed in England. They are grouped according to their WHO grade, with high grade III described as anaplastic oligodendroglioma. Grade also dictates survival rate, with 66-78% patients with low grade (grade II) surviving 5 years or more after diagnosis, whilst 30-38% patients with high grade anaplastic oligodendroglioma (grade III) surviving 5 years or more after diagnosis. Their treatment depends on grade (see above) but also on the mutation status of key glioma biomarkers.

Ependymomas are a rare gliomal brain tumour derived from ependymal cells that line fluid filled areas of the brain ventricles and spinal cord. They represent approximately 2% of brain tumours but have not been included in this project cohort.

Historically, glioma were histologically classified according to their microscopic similarity to the cells that gave rise to them. However, over the past 15 years evidence has accrued of the association of molecular traits with particular glioma types and their importance in improving diagnostic, prognostic and predictive value. Thus in 2016, a number of defined genetic characteristics were

incorporated in the World Health Organization (WHO) classification of tumours of the central nervous system reporting guidelines, thus redefining the process of the cancer diagnosis (Louis *et al.*, 2016). To define disease entities as precisely and objectively as possible, and to establish biologically and clinically uniform groups, a consensus approach to classification was proposed:

Layer 1: Integrated diagnosis (incorporating all tissue-based information)

Layer 2: Histological classification

Layer 3: WHO grade

Layer 4: Molecular information

This use of integrated phenotypic and genetic parameters of CNS tumour classification was probably the first formalized move away from the century-old principal of diagnosis based entirely on microscopy. The algorithm for classification of glioma generated by the WHO then emphasized that a molecular signature could sometimes outweigh a histological characteristic in achieving an integrated diagnosis (Fig 1.1).

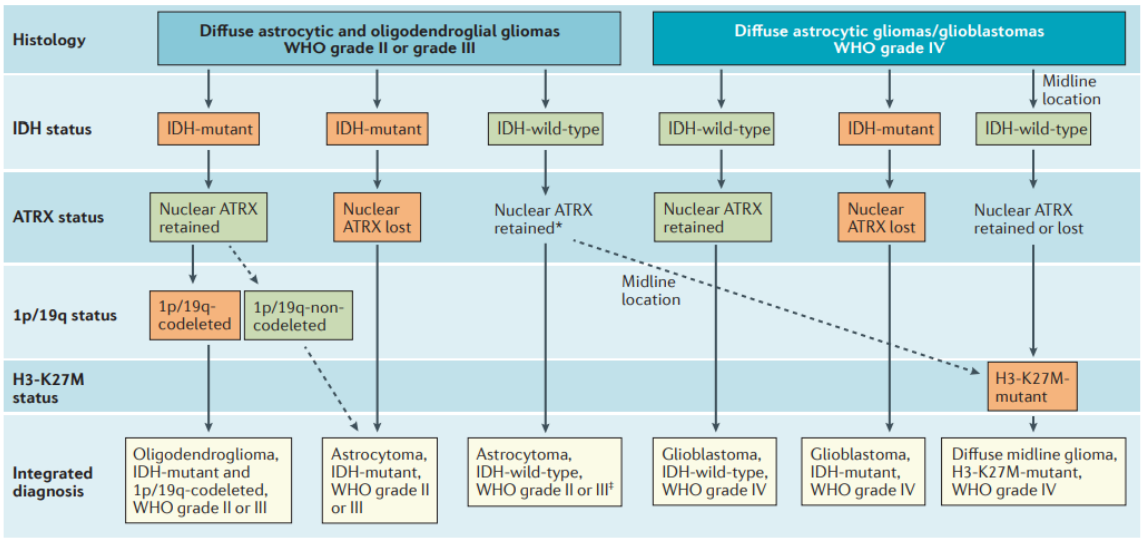



Figure 1.1
**Diagnostic approach for integrated histological and molecular classification of diffuse gliomas according to the 2016 WHO Classification of Tumours of the Central Nervous System**
IDH - Isocitrate dehydrogenase
ATRX - Transcriptional regulator ATRX
H3-K27M - K27M mutation of histone 3
Taken from Reifenberger *et al.*, 2017 (Reifenberger *et al.*, 2017)

Three molecular tests are key in the classification of glioma: Isocitrate dehydrogenase (*IDH*) mutation status, transcriptional regulator ATRX (*ATRX)* loss and 1p/19q chromosome co-deletion. $O^6$-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation status is also of value in the review of adjuvant chemotherapy options.

Early *a priori* investigations in the Cancer Genome Atlas project (TCGA) revealed recurrent missense mutations in *IDH1* in glioblastoma (Parsons *et al.*, 2008). Additional studies revealed that *IDH1* and *IDH2* mutations occurred in a high percentage of WHO grade II and III astrocytomas and oligodendrogliomas (Yan *et al.*, 2009). Isocitrate Dehydrogenase (IDH) enzymes catalyse the oxidative decarboxylation of isocitrate to α-ketoglutarate, during which NADPH is produced. The mutations result in a 'neomorphic change' in IDH enzyme activity, where 2-hydroxyglutarate is generated, rather than α-ketoglutarate. This change in the dynamics of the standard metabolic pathway has 2 major consequences: Firstly, the subsequent reduction in NADPH in the brain reduces oxidative stress protection, a known driver in tumorigenesis. Secondly, 2-hydroxyglutarate is an 'oncometabolite'; it blocks the activity of α-ketoglutarate-dependent dehyrogenases, in particular histone demethylase and TET, a 5'methylcytosine hydrolase, resulting respectively in potential inactivation of differentiation genes and changes in CpG island methylation status. Thus the somatic *IDH* mutations can reprogram the epigenome, transcriptome and metabolome to drive tumour growth (Masui, Cavenee and Mischel, 2016). Mutations in *IDH* are generally heterozygous missense mutations and mutations in *IDH1* and *IDH2* occur at specific arginine residues at the enzymes active site. The most common alteration in *IDH1* is R132H, representing approximately 85% of mutations in this gene. Variants in *IDH2* are much rarer and seen at R172, the most prevalent being R172K, seen at approximately 2.5% in gliomas (Waitkus, Diplas and Yan, 2016). Tumours with *IDH1* and *IDH2* mutations have distinctive and clinical characteristics, and patients with such tumours have better outcomes than those with wild-type *IDH* genes (Yan *et al.*, 2009). In the Laboratory, *IDH1* R132H is detected by immunohistochemistry and other variants are detected by DNA sequencing.

1p/19q co-deletion is defined as the loss of the short arm (p) of chromosomal 1 and the long arm (q) of chromosome 19. This characteristic co-deletion is observed in 50-90% of oligodendrogliomas, particularly those with the classical histologic features of fried egg appearance of tumour cells, with nuclei surrounded by clear cytoplasm, secondary to formalin fixation. Consequently, it is seen in 80-90% of grade II oligodendrogliomas and in 50-70% grade III oligodendrogliomas. Oligodendrogliomas were one of the first gliomas characterized by a distinct genetic alteration. In addition to having high clinical diagnostic value, the loss of 1p/19q has prognostic utility, being associated with prolonged survival. Additionally, it predicts favourable PCV (procarbazine/CCNU/vincristine) and temozolomide chemotherapy and radiation therapy (Aldape, Burger and Perry, 2007). It thus may guide decision to defer radiation therapy. 1p/19q co-deletion is detected by Fluorescent in-situ hybridization (FISH) or Short tandem repeat (STR) dropout analysis.

*ATRX* – α-thalassemia/mental retardation syndrome X-linked - encodes a protein belonging to the H.3.3-ATRX-DAXX chromatin remodelling pathway. Loss of function *ATRX* mutations are widely distributed in glioma and correlate with alternate lengthening of telomeres, but they also affect other cellular functions related to epigenetic regulation (Haase *et al.*, 2018). There is a strong association between *IDH* mutations and *ATRX* mutations, whilst concurrent 1p/19q co-deletion and ATRX loss is very rarely observed. ATRX status has been shown to correlate with patient age, tumour histopathology and prognosis, with ATRX loss conferring a better progression free and overall survival in low grade glioma harbouring *IDH* mutations without 1p/19q co-deletion (Leeper *et al.*, 2015). Hence the triad of *IDH*, ATRX and 1p/19q testing is particular of value in the diagnosis of low-grade glioma. In the Laboratory, loss of ATRX expression is detected by immunohistochemistry, and staining is typically lost in *IDH* mutated astrocytic tumours and glioblastomas derived from them.

Although not a diagnostic requirement, *MGMT* promoter methylation is regularly assessed in high grade glioma molecular pathology investigations, due to its importance in reviewing the likely efficacy of chemotherapeutic agents, most notably temozolomide. Temozolomide is an alkylating agent able to cross the blood brain barrier and so is standard of care for glioblastoma and is also used in other high-grade glioma. Temozolomide modifies the guanine bases of the DNA forming $O^6$-methylguanine adducts, with the consequence methylguanine pairs with thymine rather than cytosine. The fault can be reversed by mismatch repair enzymes, but with continual generation of methylguanine, the futile cycles of mismatch repair eventually leads to cell apoptosis and the required cytotoxic effect of the drug. MGMT ($O^6$-methylguanine-DNA methyltransferase) is a DNA repair enzyme that protects against cytotoxic and mutagenic alkyl groups. It recognizes alkyl adducts and remove them from DNA by transfer of the alkyl group to an internal cytosine acceptor site. MGMT expression is induced by cellular stress indicators such as DNA damage, glucocorticoids, cAMP and protein kinase C. *MGMT* gene structure includes a promoter region with a CpG island composed of 98 CpG sites. Gene expression is modulated by epigenetic modification of the promoter region, through DNA methylation. Methylation at the CpG dinucleotides is followed by recruitment of methylated CpG binding protein, chromosome condensation, a finally a block in transcription. *MGMT* promoter methylation is a predictive biomarker in glioblastoma, with approximately half of cases having *MGMT* promoter methylation. *MGMT* promoter methylation is a predictive biomarker in 2 ways: Firstly, as a prognostic indicator: *MGMT* promoter methylation in glioblastoma confers survival advantage, regardless of therapy *(*Reifenberge*r et al., 2012)*. However, the prognostic evidence of *MGMT* methylation is stronger in the context of treatment, with increased overall survival in patients treated with radiotherapy and temozolomide (Weller *et al.*, 2015). In the study of Hegi *et al*., glioblastoma patients with methylated *MGMT* promoter showed survival rates of 49% and 14% at 2 years and 5 years respectively, when treated with temozolomide and radiotherapy. Patients with unmethylated *MGMT* promoter showed survival rates of 15% and 8% at 2 years and 5 years with the same treatment (Hegi *et al.*, 2005). *MGMT* promoter methylation in glioblastoma in elderly patients is associated with improved overall and progression-free survival (Reifenberger *et al.*, 2014). In lower grade glioma, *MGMT* promoter methylation in anaplastic oligodendroglioma/astrocytoma was associated with longer progression free survival (Minniti *et al.*, 2014a; Minniti *et al.*, 2014b; Esteller, 2000). The second and main clinical utility of *MGMT* promoter methylation status is in its value as a predictive biomarker for response to chemotherapy with alkylating agents, such as temozolomide (Esteller, 2000; Hegi *et al.*, 2005). As temozolomide adds a methyl group to the $0^6$ of the guanine in the DNA, where enzyme MGMT is expressed, it removes methyl groups from the DNA, rendering cancer cells resistant to the chemotherapy. However, where MGMT gene promoter is silenced through CpG island methylation, the alkylating chemotherapy remains active. Consequently, these patients are more responsive to temozolomide compared to patients whose tumours express MGMT.

### 1.2 The brain biopsy sample pathway

As with all cancers, the histological and molecular profile of glioma is essential for accurate diagnosis and evaluation of surgical and therapeutic options. Thus, in spite of the highly invasive nature of a

brain biopsy and its inherent risk (Yong and Lonser, 2013; De la Garza-Ramos *et al.*, 2016), most glioma patients are biopsied, albeit in specialist neurological centres like the Wessex Neurological Centre based at University Hospital Southampton NHS Foundation Trust (UHS). At UHS, biopsy samples are taken at inpatient day surgery, either solely for diagnostic purposes or as part of surgical tumour resection. Generally samples are fixed and embedded to generate a formalin-fixed paraffin embedded (FFPE) samples. From this point slides are generated; for hematoxylin and eosin (H&E) stain for histological assessment and immunohistochemical analysis, or for DNA extraction for downstream genetic testing. Gliomas are diffuse tumours and the high grade tumours often contain significant areas of necrosis. In our service we use paired H&E slides, where the tumour has been marked out by the histologist to allow a microdissection of the matched unstained slide, extracting DNA only from the area of highest tumour density. This ensures our starting material is optimised for tumour enrichment, ensuring maximum sensitivity in the downstream molecular tests.

### 1.3 The promise of 'Liquid biopsy'

Small, labile fragments of DNA are shed from most cells into the plasma through the processes of necrosis, apoptosis and secretion (Diaz and Bardelli, 2014). This circulating cell-free DNA (cfDNA) was first described in 1948 (Mandel and Metais, 1948) and found to be associated with the disease process (Tan *et al.*, 1966) (Leon *et al.*, 1977). Early work showed cfDNA levels were elevated in cancer patients (Leon *et al.*, 1977) and since this time the tumour derived cell free DNA- circulating tumour DNA (ctDNA) - has been intensively researched, particularly with regard to its use as a 'liquid biopsy' (Figure 1.2).

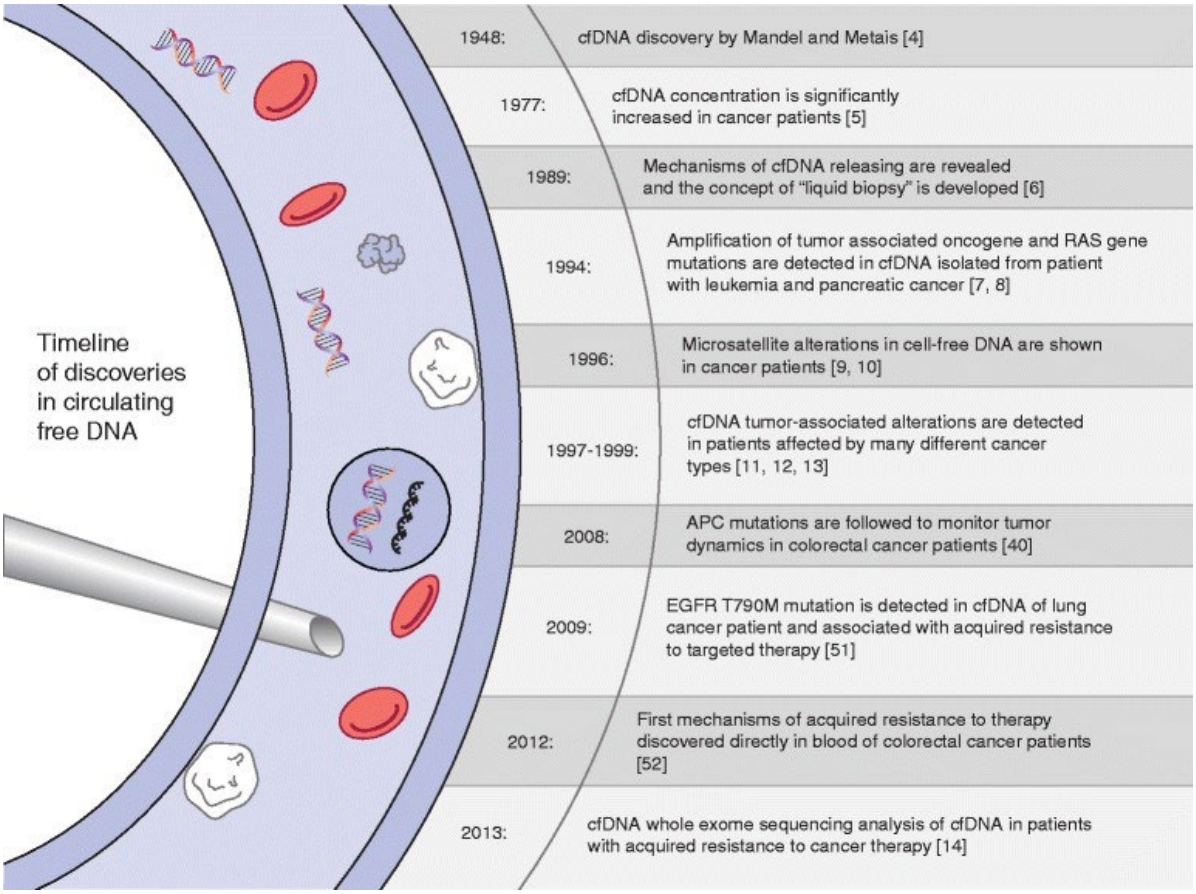



Figure 1.2
**Early discoveries of the utility of cell free DNA in cancer studies**
Taken from Siravegna and Bardelli, 2014, with references cited therein (Siravegna and Bardelli, 2014).

The first direct detection of tumour-derived DNA was demonstrated in a study by Sorensen *et al*. in 1994, when key biomarker single gene *KRAS* mutations were detected in the plasma of patients with pancreatic adenocarcinoma (Sorenson *et al.*, 1994). The discovery of alterations in microsatellite markers in the plasma of small cell lung cancer patients (Chen *et al.*, 1996) and the detection of aberrant methylation profiles illustrated the application cfDNA in the analysis of complex biomarkers. The first clinical studies were done in 2005, with advanced colorectal cancers patients, where mutant adenomatous polyposis coli (*APC*) DNA molecules could be consistently detected in the plasma, detecting allele frequencies down to 0.01% (Diehl *et al.*, 2005). In 2012, the first study using cfDNA as a measure of minimal residual disease (MRD) over time in relation to known prognostic factor was carried out, gaining insight into breast cancer dormancy (Payne *et al.*, 2012). In the same year, this group also showed ctDNA could be used to track cancer progression in breast cancer (Forshew *et al.*, 2012; Dawson *et al.*, 2013). In 2013, cfDNA was used to detect acquired resistance to therapy in range of advanced cancers (breast, ovarian and non-small cell lung cancers) (Murtaza *et al.*, 2013). Here, exome sequencing was performed on circulating DNA from plasma at selected time-points, separated by periods of treatment. Mutations were identified across the plasma samples, and their abundance (allele fraction) at different time-points compared, generating

lists of mutations that showed a significant increase in abundance, indicating underlying selection pressures associated with specific treatments. The seminal paper of Bettegowda *et al.* in 2014, showed the applicability of ctDNA as a method to detect and monitor tumours in most solid cancers types. In a study with 640 patients with various cancer types, ctDNA was detectable in >75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers, but in less than 50% of primary brain, renal, prostate, or thyroid cancers. In patients with localized tumours, ctDNA was detected in 73, 57, 48, and 50% of patients with colorectal cancer, gastroesophageal cancer, pancreatic cancer, and breast adenocarcinoma, respectively (Bettegowda *et al.*, 2014). 2015 showed the utility of ctDNA for monitoring delivery of targeted therapies (Frenel *et al.*, 2015). In early-phase clinical trial of advance cancer patients, this group used next-generation sequencing (NGS) analysis of cfDNA for patient selection and as a tumour clone response biomarker in patients.

Thus by the middle of the last decade, the stage was set for evaluation of cfDNA and specifically ctDNA, as an analyte for molecular monitoring in cancers in mainstream clinical care context.

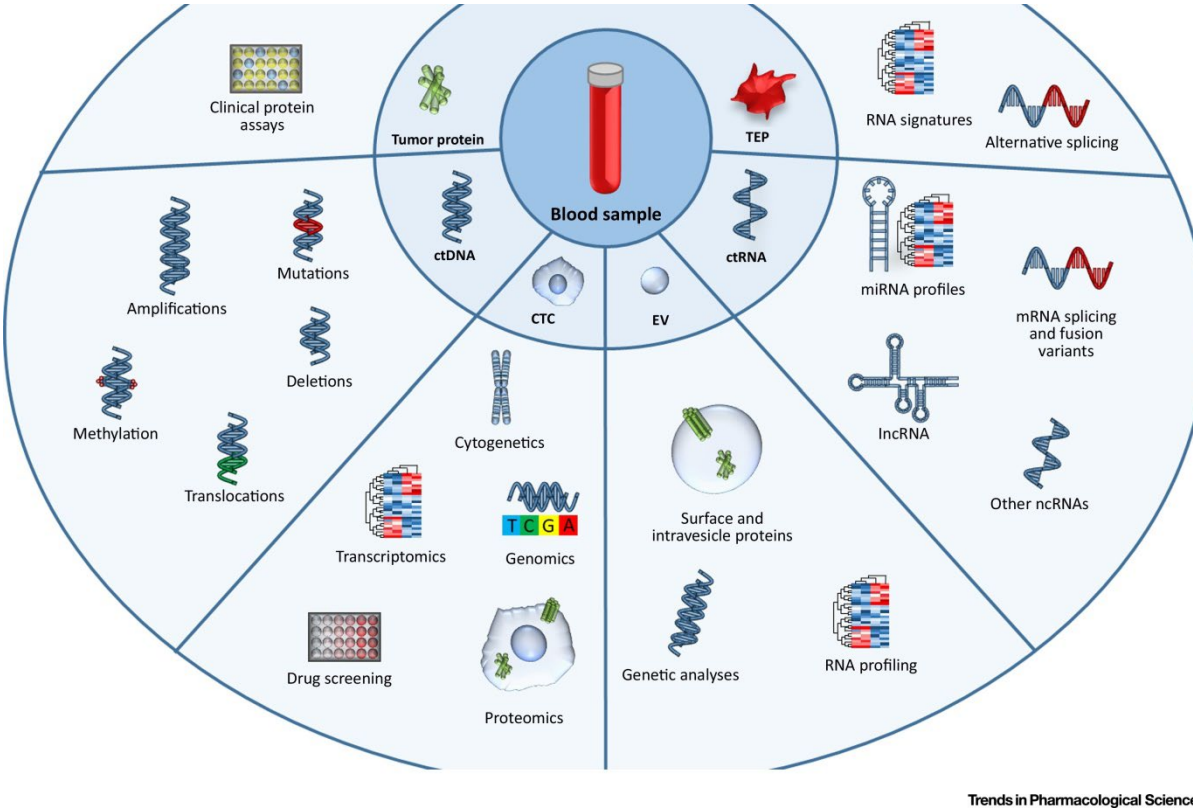



Figure 1.3
**Components of the Tumour Circulome**
The tumour circulome comprises all the tumour-derived elements circulating in the bloodstream that can be used, directly or indirectly, as a source of cancer biomarkers. It includes circulating tumour proteins, circulating tumour (ct)DNA, circulating tumour cells (CTCs), tumour-derived extracellular vesicles (EVs) and their constituents, circulating tumour (ct)RNAs and tumour-educated platelets (TEPs). (Taken from De Rubis *et al*., 2019)

The processes of tumourigenesis results in different types of tumour-derived components being released in to the plasma; DNA (ctDNA), microRNA (miRNA), extracellular vesicles and circulating tumour cells (Figure 1.3) and there is described clinical utility in analysing these components in cancer testing and disease monitoring, including that of brain tumours (Wang and Bettegowda, 2017; Fontanilles, Duran-Pena and Idbaih, 2018; Figueroa and Carter, 2018; Kros *et al.*, 2015; Sareen *et al.*, 2020; Le Rhun *et al.*, 2020). But currently it is cfDNA and ctDNA elements that offer the most clinical utility.

The release of tumour DNA (ctDNA) in to the blood stream (Figure 1.4) represents a huge opportunity for cancer molecular genetics analysis, with a potential replacement for the highly invasive process of physical biopsy.

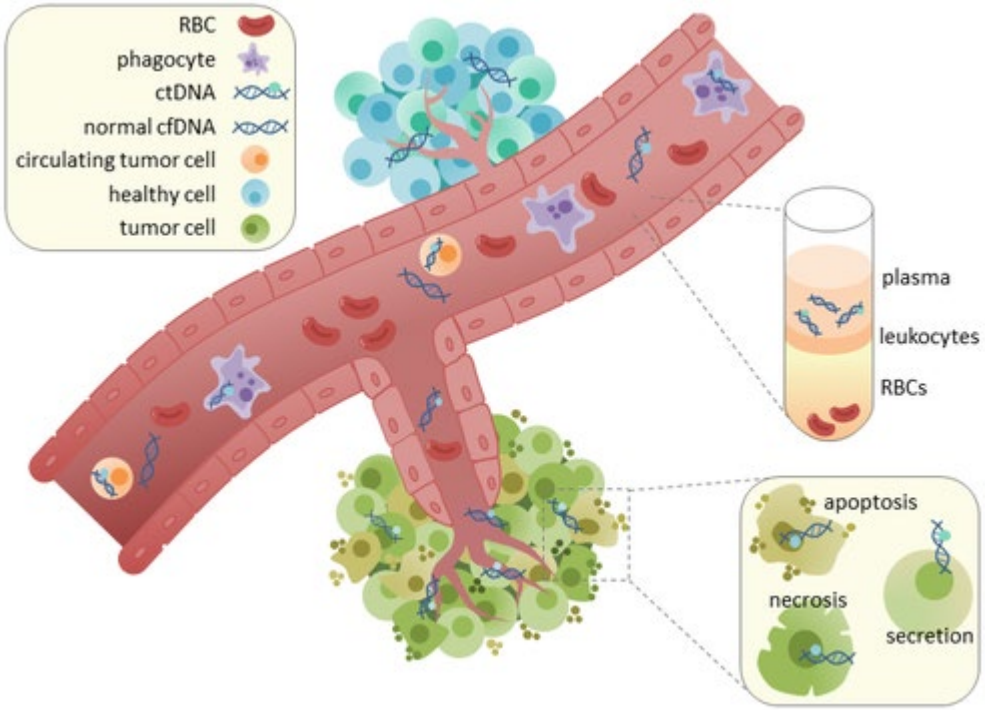



Figure 1.4
**Circulating tumour DNA (ctDNA) release in to the blood system**
Cell free DNA (cfDNA) is released to the plasma through the cellular processes of cell death (necrosis and apoptosis) or by secretion. This small fragment DNA shed specifically from tumour is the circulating tumour DNA – ctDNA.

https://en.wikipedia.org/wiki/Circulating_tumor_DNA#/media/File:CtDNA_in_circulation.png

The potential advantages of a liquid biopsy are many:

Sample collection is less invasive than a conventional tissue biopsy. Most commonly it is a single blood sample, so the process of obtaining the sample is rapid and easy. With the availability of specialist preservative tubes, samples can be collected at a convenient time for the patient and there is no immediate requirement for sample processing. Potentially, ctDNA represents 'fresh' material and can avoid the artifacts that are introduced to the sample through the conventional FFPE (formalin-fixed paraffin embedded) pathway.

The sample can be taken when the primary tissue sample is not available, or is difficult to obtain. A good example of this is with lung tumour biopsies, where sometimes it difficult to obtain a suitable specimen, or in the case of metastatic disease, the patient is too unwell to undergo the highly invasive procedure. As the ctDNA is most often used to genotype the originating tumour, all patients can be considered for mutation-specific therapeutic options, whether or not they were able to have a conventional biopsy.

The homogeneity of ctDNA ensures a representation of a general tumour profile, circumventing the sampling issue in a biopsy/resection analysis. The ctDNA represents both tumour heterogeneity and spatially separated disease foci, facilitating more accurate tumour mutation profiling. Additionally,

potentially, the cfDNA can also be used as a surrogate to measure 'tumour burden'- a measure of tumour bulk/ tumour cell turnover- where the level of ctDNA is prognostic in predicting survival outcomes.

cfDNA has a rapid turnover, with a half-life of less than an hour, and it is rapidly cleared from the circulation. Thus, its testing can provide a snapshot of the current genetic makeup of the tumour and a repeat sample can be readily taken, facilitating longitudinal analysis. This opens up the opportunity of using biomarker profiling in many different ways: to monitor genomic drift and track clonal evolution upon treatment; to understand the mechanisms of primary and especially acquired resistance to therapy resistance and development of secondary cancers; or to detect minimal residual disease following surgery or therapy with curative intent.

It is however important to be mindful of the limitations of using cfDNA as an analytical tumour metric. There is considerable variation in the amount of both cfDNA and ctDNA seen in different cancer types and there is heterogeneity between individual patients - it is apparent not all tumours are 'shedders'. Poor tumour vascularization could hinder ctDNA release or conversely could promote ctDNA release by producing hypoxia and cell death. For example, in the study of Bettegowda *et al*., there is a 100-fold difference between the ctDNA concentration in stage IV disease, with 82 % of patients with stage IV disease and this fell to 47% of patients with stage I disease (Bettegowda *et al.*, 2014). Additionally, there will always be an uncertainty of the source location of the cells producing the ctDNA. This may impact the clinical decision making if the primary tumour is not known. At a practical level, most of the plasma cfDNA is derived from the haematopoitic system and lysis of white blood cells can add genomic DNA to the plasma. Thus contaminating normal DNA may obscure mutant ctDNA within cfDNA. There are considerable challenges in using ctDNA as an investigative tool, particularly in early-stage disease, but the benefits of using ctDNA as a proxy for biopsy testing are clear and over the past few years there had been a burgeoning of studies and applications of the clinical utility of ctDNA as a biomarker analyte (Figure 1.5)

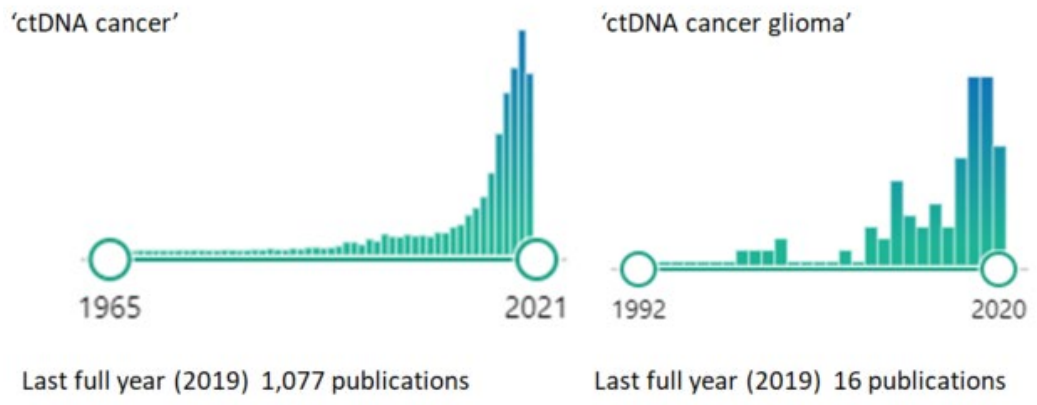



Figure 1.5
**Pubmed literature searched for 'generic' ctDNA cancer and work specifically relating to glioma.** The search for 'all cancer' ctDNA illustrates the rapid increase in publications over the past 6 years, with over 1000 in the last full year of the search, representing the huge interest in ctDNA as clinical analyte and its potential utility. However, the numbers of papers specifically relating to glioma was substantially less, representing only 1.5% of the total for 2019. This does reflect the difficulty of working with this analyte, in this cancer type.

In summary, the use of ctDNA in the management of cancer holds great promise in terms of:

- Diagnosis
- Molecular profiling
- Treatment selection
- Tracking therapeutic response/resistance
- Evaluating tumour heterogeneity
- Monitoring disease burden/ minimal residual disease

(Wan *et al.*, 2017)

ctDNA related cancer management is starting to be used for mutation detection in a number of solid organ cancers, most notably in non-small cell lung cancer. In NHS clinical practice cfDNA samples may be used in lung cancer management:

- **At diagnosis** - in the case of biopsy failure or before tumour tissue testing, so as to inform decisions about prescribing Epidermal Growth Factor (EGFR) tyrosine kinase inhibitor (TKI) therapy.
- **As a first line test** - when patients on 1st or 2nd generation TKI progress. There is ?resistance due to an additional mutation (p.T790M), thus triggering the need for a 3rd generation TKI. (NICE, 2018)

There are also number of UK trials in progress to fully evaluate the clinical utility of ctDNA in a liquid biopsy as an alternative to invasive tissue biopsies in breast cancer (Turner *et al.*, 2020) and a broader range of cancer types (Rothwell *et al.*, 2019).

### 1.4 Methods of analysing ctDNA – the balance of analytical sensitivity *vs* high variant coverage

The type of method used in detecting ctDNA ultimately depends on the clinical question and the ultimate goal of the analysis. As such, the methodological approach follows three lines (reviewed in Wan *et al.,* 2017):

A targeted single-locus or multiplex assay, with high resolution and analytical sensitivity – looking at point mutations or copy number variants in a single gene. This approach enables detection of mutant alleles, even if they are highly underrepresented. Standard approaches include digital PCR, which can reach limit of detection (LOD) sensitivities of 0.001%-0.01%. Conventional laboratory allele specific PCR kits, such as Qiagen Therascreen or COBAS, are slightly less sensitive with a LOD range of 0.05%-12% for different variants. These set ups have high clinical utility for detecting and quantifying recurrent hot-spot mutations and have the advantage of a rapid turnaround time.

A targeted sequencing approach, which can interrogate a much larger locus of 10kb-50Mb. These can be either amplicon-based or hybrid capture and sensitivities range from 0.015-0.5% for specialized panels and 1% for off-the-shelf multiplexed panels. This mid-range analysis is particularly useful as it can be used for mutation profiling and monitoring for *de novo* resistance or clonal evolution in response to therapy. Specialist panels can also be used to look at DNA methylation and limited structural variant analysis.

Genome-wide coverage– either full whole genome sequencing or specialized amplicon-based systems. The value here comes in the ability to identify all structural variants and identify the presence of chromosomal aberrations. It is also additionally possible to stratify patient samples on the basis of overall disease burden. However, the wide coverage inevitably comes a reduced LOD sensitivity of 5-10%. The highly complex nature of the analysis would also be out of the range of most Clinical Laboratories.

### 1.5 Use of ctDNA in detecting glioma biomarkers

In the early literature, a number of publications cited use of serum or plasma derived DNA for successful detection of the triad of single gene or loss of heterozygosity (LOH) tests that are key in the diagnosis and treatment of glioma (Louis *et al.*, 2016; Weller *et al.*, 2012); that of isocitrate dehydrogenase (IDH) mutation status, $O^6$-methylguanine-DNA methyltransferase (MGMT) gene promoter methylation and 1p/19q co-deletion (LOH). Details of studies, including genes, targets and sample source are summarised in Table 1.1.

Table 1.1
**Characteristics of pre-NGS published molecular testing analyses**

| Author | Year | Assay method | Genetic alteration | Target Genes | Sample Type |
|---|---|---|---|---|---|
| Weaver *et al.*, | 2006 | Methylation specific PCR | DNA methylation | *MGMT, p16INK4a, p73,RARβ* | plasma |
| Wakabayashi *et al.*, | 2009 | Methylation specific PCR | DNA methylation | *MGMT, p16INK4a* | serum |
| Lavon *et al.*, | 2010 | LOH PCR | LOH | *1p,19q, 10q* | serum |
| | | Methylation specific PCR | DNA methylation | *MGMT* | |
| Lui et al., | 2010 | MeDIP-qPCR | DNA methylation | *MGMT, p16INK4a, TIMP3,THBS1* | serum |
| Balana *et al.*, | 2011 | Methylation specific PCR | DNA methylation | *MGMT* | serum |
| Boisselier *et al.*, | 2012 | Digital PCR | *IDH1* R132H | SNV | plasma |
| Majchrzak-Celinnska *et al.*, | 2013 | Methylation specific PCR | DNA methylation | *MGMT, RASSF1A, p15INK4B, p14ARF* | serum |
| Fiano *et al.*, | 2014 | Methylation specific PCR | DNA methylation | *MGMT* | plasma |
| Wang *et al.*, | 2015 | Methylation specific PCR | DNA methylation | *MGMT* | Serum, CSF |
| Estival *et al.*, | 2019 | Methylation specific PCR, pyrosequencing | DNA methylation | *MGMT* | Plasma, blood |

Tests highlighted in yellow represent those within the current testing repertoire at UHS.

These early studies reported a wide range of results that are difficult to compare due to a number of factors:

- cf/ctDNA is very labile and conventional collection and extraction of samples affects yield considerably
- cf/ctDNA yield and quality was rarely evaluated
- The methylation specific PCR assay used is not quantitative

However, the diagnostic value and overall performance of ctDNA in glioma assays have very recently been evaluated as part of a meta-analysis (Kang, Lin and Kang, 2020). This meta-analysis evaluated 11 studies, pooling different target genes for the purpose of analysing diagnostic accuracy. Through application of a random-effect statistical model, they were able to determine sensitivity and specificity within the meta-analysis. They concluded pooled specificity was 98% and pooled sensitivity was 69% and that ctDNA had relatively high diagnostic accuracy. Interestingly, the study

showed high heterogeneity in sensitivity that may affect accuracy of results and they identified that sample source could be identified as the source of the heterogeneity seen. Although recognizing the limitations of small sample size, they concluded ctDNA was an effective biomarker for molecular diagnosis in glioma, but also that digital PCR, single nucleotide variant (SNV) detection, high grade glioma and ctDNA derived from cerebral spinal fluid (CSF) yielded better results in the subgroup analysis. They also stated 'the detection methods of circulating mutant DNA in glioma patients need to be improved'.

### 1.6 Glioma ctDNA evaluation as part of pan-cancer genomic ctDNA studies

As the ctDNA technology was applied more systematically across cancer types, it became clear this promising technology gives rise to particular problems in its application to brain cancers (Westphal and Lamszus, 2015; Figueroa and Carter; Wang and Bettegowda, 2017). The seminal study of Bettegowda *et al.*, looked at ctDNA in early and late-stage human malignancy and demonstrated the frequency of detectable ctDNA for glioma was <10%, significantly lower than for most non-CNS malignancies (Bettegowda *et al.*, 2014). Early work by Wang *et al.*, 2015 (Wang *et al.*, 2015) highlighted the use of cerebrospinal fluid (CSF) as a better yield source of ctDNA and subsequent studies have verified the value of CSF in glioma ctDNA studies (Mouliere *et al.*, 2018; Miller *et al.*, 2019). The emerging literature recognizes the best source of ctDNA for glioma patients is the cerebrospinal fluid (reviewed by Simonelli *et al.* (Simonelli *et al.*, 2020)). But obtaining CSF is still a highly invasive process and its collection and processing would be considerably more complex than collecting a blood sample. So if collection methods could be improved and testing strategies made more sensitive, plasma as a ctDNA source would be still be the best practical option in a routine NHS environment.

### 1.7 Focused glioma plasma ctDNA studies

The pan-cancer study of Bettegowda identified ctDNA alterations in less than 10% of 27 glioma patients. (Bettegowda *et al.*, 2014). However, nearly half of the cases were grade II or III, and the plasma cell-free DNA (cfDNA) yield may have been limited by the lower rates of cell proliferation, lack of necrosis, and greater blood-brain barrier integrity of the low grade glioma compared with glioblastoma (GBM) (Volik *et al.*, 2016). There was a lack of data regarding the detection and potential clinical utility of plasma cfDNA when collected from high grade glioma. However, recently, a number of comprehensive cfDNA studies of glioma cohorts have been published (described below) and these now report higher yields of ctDNA, with increased clinical utility for both cfDNA and ctDNA analysis.

The pan-cancer study of Zill *et al.*, (Zill *et al.*, 2018) evaluated 21,807 cell-free circulating tumour DNA samples in advanced cancer patients, of which 107 were glioma/GBM. Their analysis yielded a 51% cfDNA somatic alteration detection rate in advanced primary GBM. Thus, although the glioma ctDNA levels were significantly lower than the other cancer types, they were detectable.

Secondly, a pilot study of 42 patients with newly diagnosed GBM by Bagley *et al.*, (Bagley *et al.*, 2019) indicated preoperative cfDNA concentrations could be linked to inferior progression free survival (PFS). Additionally, ≥1 somatic mutations in the plasma ctDNA occurred in 55% of patients. They concluded cfDNA may be usable as a prognostic tool, as a surrogate to tumour burden and a means of obtaining additional molecular profiling in patients with GBM.

Lastly, in the largest ctDNA primary brain cancer study to date (419 patients), Piccioni *et al*., presented results contrary to the early low ctDNA yield studies (Piccioni *et al.*, 2019). Genomic alterations, eg SNV's and gene amplifications were identified in half of the patients with ctDNA detection rates varying with the histopathology and grade of the cancer. Encouragingly, in this study genomic alterations identified had matched off-label and clinical trials options for almost 50% of patients with detectable ctDNA.

Thus, there is an increasing literature supporting the use of cell free and circulating tumour DNA in molecular diagnosis of glioma. However, it is also clear that a valid testing strategy will depend on assay performance alongside the histopathology and grade of the tumour type being evaluated. In the context of the review of ctDNA testing strategies in section 1.4, the extremely low prevalence of glioma derived ctDNA plasma would necessitate using methodologies of the highest sensitivities. In terms of options in a routine clinical Laboratory there would be two potential options. Firstly, single gene variant detection by allele specific real-time PCR or droplet digital PCR. Secondly, a specialist targeted NGS panel, with a limited coverage of relevant variants and specially adapted to aid detection at low variants allele frequency through unique molecular indices (UMI's).

### 1.8 Practicalities of cfDNA investigations – sample collection and DNA extraction

As with all molecular pathology investigations, pre-analytical variables affect cfDNA yield and quality (Bronkhorst, Aucamp and Pretorius, 2015). There are many compounding factors, for example serum may yield higher levels of cfDNA compared to plasma, but the yield is more variable and the cfDNA quality may be impacted due to lysis of monocytes. The time period a plasma sample is left before the first centrifuge spin of the extraction process heavily influences contamination of genomic DNA from blood cells (El Messaoudi *et al.*, 2013). Where EDTA is used as an anticoagulant, plasma must be separated with double centrifugation within 2h of collection to minimize contamination (Volik *et al.*, 2016); not always practical in the diagnostic setting due to the physical distance between clinical areas and the laboratory which leads to a time delay. It seems unavoidable that the variability in the pre-analytical steps in the early glioma ctDNA biomarker literature has contributed to the difficulties in comparing results. Fortunately, more recently a number of specialized collection tubes formulated to preserve cfDNA have become available. These tubes stabilise cfDNA for up to 7 days at room temperature and are highly suitable for ctDNA stabilisation and subsequent liquid biopsy testing (Alidousty *et al.*, 2017). Even low ctDNA concentrations allow detection of somatic mutations, so use of this tube type would be essential for glioma ctDNA studies where frequency of ctDNA is known to be low. Additionally, there are now a number of commercial DNA extraction kits specifically designed for maximum extraction of cfDNA, and again these would be essential to optimise yield and quality for cfDNA in a glioma study.

## 1.9 Practicalities of cfDNA investigations - Quantification and quality assessment

Comparisons in the literature are difficult due to different approaches in assessing the quantity and quality of extracted cfDNA, and the early studies rarely attempted to evaluate these quality metrics. However, such measurements are highly informative and particularly must be accurate where the cfDNA metrics are in themselves being used as a specific point of investigation. As with all areas of molecular pathology, reviewing quality and quantity is fundamental in analysing cfDNA; to ensure the development of robust and standardized workflows and to facilitate the implementation of ctDNA analysis into clinical laboratory medicine service (Johansson *et al.*, 2019).

The concentrations of extracted cfDNA will nearly always fall below detection range of standard spectrophotometric analysis and a range of more sensitive methods are used.

**Fluorometric measurement** – these methods are generally sensitive enough when using the optimised high sensitivity reagents that are now available.

**Automated Electrophoresis** - this method is of particular value as cfDNA can be visualised at low molecular weight with characteristic trimodal distribution of DNA fragments. Most molecules are approximately 165 base pairs (bp) in length, which matches the length of DNA occupied by the nucleosome. Other fragment lengths then correspond to the two nucleosome units for the 350-bp band and three units for the 565-bp band (see Figure 2.6).

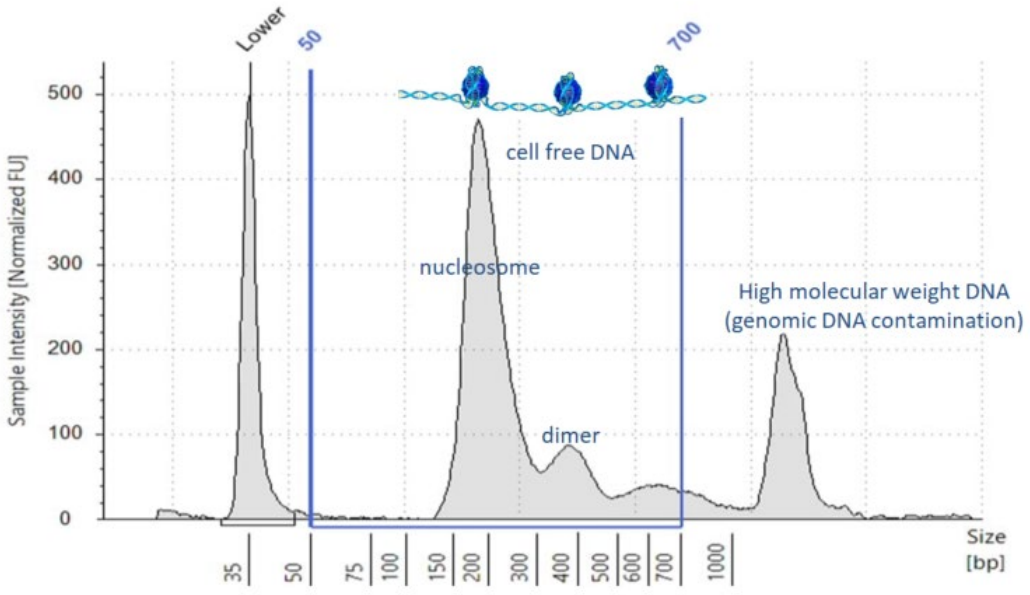



Figure 1.6
**Characteristic profile of cell free DNA analysed by automated electrophoresis**
The electropherogram shows the typical peak of the mono-nucleosome, with smaller peaks representing di- and tri- nucleosomes. Contaminating high molecular weight DNA is also visualised and software can calculate the proportion of cfDNA against total and thus estimate %ctDNA purity.
Adapted from: (Agilent, 2020)

The cfDNA can be quantified and quality evaluated through also assessing genomic DNA contamination

**qPCR to measure cfDNA concentration and evaluate DNA integrity** - qPCR remains an important benchmark method in molecular pathology and cfDNA can be quantified and its integrity evaluated by qPCR, for example using primer sets to amplify consensus ALU sequence (Umetani *et al.*, 2006). Two sets of ALU primers are used, one amplifies both shorter and longer DNA fragments, representing the total amount of cfDNA, whilst a second set is designed to amplify only long DNA fragments, representing the DNA released from non-apoptotic cells. DNA integrity is calculated as the ratio of concentrations in both assays. ALU sequence qPCR has been used in linking cfDNA levels to tumour burden or disease progression and recently has been used in evaluating the utility of cfDNA in newly diagnosed glioblastoma patients (Bagley *et al.*, 2019). An alternate qPCR system of short PDGFRA assay (74 bp) and long FLI1 assay (445 bp) can be used to calculate ctDNA amounts and determine contaminating cellular DNA (Johansson *et al.*, 2019).

### 1.10 Practicalities of ctDNA investigations – hinging on appropriate biomarker assays and their sensitivities

Analyses of ctDNA in liquid biopsies offer considerable biological and technical challenges for cancer testing. Healthy individuals generally have cfDNA plasma concentrations of less than 10 $ngml^{-1}$ with a maximum estimated at 30 $ngml^{-1}$ (Mouliere *et al.*, 2014; Mead *et al.*, 2011) and elevated cfDNA levels can occur for other reasons of disease, trauma and even exercise (Tan *et al.*, 1966; Leon *et al.*, 1977; Pokrywka *et al.*, 2015). The normal concentrations of cfDNA found in plasma fall below the standard DNA ranges of most clinical Laboratory tissue biopsy tests. The fraction of ctDNA in the overall cfDNA has been estimated from less than 0.1% to more than 90% (Diehl *et al.*, 2008; Bettegowda *et al.*, 2014) and so, as of yet, the low concentrations and inherent variability reduce the usefulness of total cfDNA in cancer diagnostics. Levels of circulating tumour DNA depend on many factors and there is substantial variability observed amongst patients with the same tumour type, possibly reflecting biologic differences (for example if the tumour is a 'shedder') or rate of cell death in individual tumours (Bettegowda *et al.*, 2014). Additionally, patients with different tumour types show considerable variation in the frequency of detectable ctDNA. In the seminal study of Bettagowda *et al.*, ctDNA was almost always detected in bladder, colorectal, gastroesophageal and ovarian cancer, but was only seen in less than 10% of the glioma cases tested.

Thus, the key to harnessing clinical utility of cfDNA for cancer testing, is to first specifically pinpoint the proportion that represents cell free DNA specifically from a tumour. This means testing for a 'biomarker' that is unique within the new somatic molecular profile of the tumour and is now different from the patients' germline profile. Then secondly, improve on the sensitivity of the technical analytics platform. Biomarkers have always been the basis of molecular testing, looking for the cancer 'mutation signal' in a biopsy sample against the backdrop of the normal tissue that is also found in the tumour. The difference is that a tissue biopsy sample has been chosen to maximise the proportion of cancer cells within in the sample, whereas in a liquid biopsy sample, there is no opportunity to optimise tumour content; searching for the 'needle in the haystack' becomes largely dependent on the sensitivity of the testing strategy. Given the documented low levels of ctDNA

recorded for glioma, the variable results reported for the early studies and only moderate test sensitivity of 69% in a recent meta-analysis (Kang, Lin and Kang, 2020) this is a particularly acute dilemma for glioma biomarker testing. It is thus of note that the 3 most recent glioma ctDNA studies of the groups of Zill, Piccioni and Bagley (Zill *et al.*, 2018; Piccioni *et al.*, 2019; Bagley *et al.*, 2019) used massively parallel sequencing (Next Generation sequencing) technologies that enable study a suite of cancer-specific biomarkers at enhanced sensitivity. It is of further note, that the studies of Zill and Piccioni have also used the Guardant 360® cfDNA digital sequencing NGS assay, where the sensitivity has been further optimised for the evaluation of cfDNA samples. Use of high sensitivity assays is important in all ctDNA assays but is of exquisite need in glioma related studies where the ctDNA concentration will routinely be very low.

### 1.11 Standalone use of cfDNA metrics in the context of glioma imaging analysis and disease outcomes

Changes in blood brain barrier (BBB) permeability are seen within the different stages of gliomas, as the progression of the disease aggravates its disruption. This disruption is described as 'contrast enhancing' and can be seen on a Magnetic Resonance Imaging (MRI) scan by using a contrast medium such as gadolinium, which does not normally cross the BBB (Dubois *et al.*, 2014) .The early study of Boisselier et al., (Boisselier *et al.*, 2012) described patients with high grade glioma had significantly higher plasma 'small size DNA' – (taken to be cfDNA) concentration than healthy controls and patients with low grade glioma. They suggested the intact blood brain barrier would limit the diffusion of cfDNA in to the plasma. They argued a disrupted BBB, as indicated by the contrast enhancement, could explain the larger amount of cfDNA seen in high grade glioma. So they concluded, larger tumour volumes and higher histological grade were associated with increased plasma cfDNA concentration.

In the more recent pilot study of Bagley et al., (Bagley *et al.*, 2019) cfDNA concentrations were also studied alongside glioma biomarker ctDNA assessment. They concluded prior to surgery, high grade glioblastoma patients had higher plasma cfDNA concentrations than age matched healthy controls. Preoperative cfDNA concentration above a mean value was associated with inferior progression free survival (PFS). In following up patients, they were able to conclude plasma cfDNA may be an effective prognostic tool and a suitable surrogate of tumour burden in newly diagnosed glioblastoma. So, although the BBB limits the contribution of ctDNA to overall plasma cfDNA hampering diagnostic testing, its subsequent 'leakiness' in high grade disease, may have some potential utility, alongside the key imaging tools, in evaluating prognostic outcomes.

In conclusion, the more recent academic work does show some clinical leverage in the evaluation of glioma ctDNA and cfDNA analysis in the hands of a specialist research team.

### 1.11 Project aims and research questions

The values of liquid biopsies are of special interest in brain tumours for several reasons: There remain groups of patients who are not suitable for a brain biopsy; It can be difficult to obtain tissue

for diagnostic purposes, from tumours that are not easily accessible, *eg* in the brain stem; Liquid biopsies offer a non-invasive method to monitor molecular changes in tumours through the evolution of disease, *eg* break down of the BBB; The problems involved in differentiating real tumour from treatment-related process using Magnetic Resonance Imaging (MRI), such as radionecrosis, pseudoprogression and immune-related events (Shankar *et al.*, 2017); The inherent high molecular heterogeneity in glioma may decrease the accuracy and prognostic value of stereotactic biopsy diagnosis.

The benefits of using this analyte in the care of glioma patients are clear and so this pilot study was set up to assess its feasibility in a routine NHS molecular laboratory supporting a UK regional Neurological Centre. The project sought to assess a contemporary method of cell free DNA (cfDNA) extraction and determine if it provide sufficient ctDNA for the routine molecular pathology tests used in the diagnosis and treatment of glioma. The objectives were:

**Pre-analytical phase** - To collect and analyse quantity/quality data for glioma patient cfDNA.

**Analytical phase** – Determine the utility of cfDNA as a ctDNA substrate in existing glioma molecular tests, where possible collecting and analysing data from the relevant laboratory tests (analytical validity).

**Post-analytical phase** – Consider the value of cfDNA and ctDNA analysis as aids in clinical treatment decisions and diagnostic glioma pathology reporting (clinical validity and clinical utility).

# 2 Materials

## 2.1 Specimen collection, cfDNA extraction and analysis

Peripheral blood samples were collected in to Qiagen PAXgene Blood ccfDNA tubes (Qiagen #768165). Low speed centrifugation steps were carried out with a Sorvall Legend T Centrifuge. High speed centrifugation steps were carried out with a Hettich Mikro 22R centrifuge. cfDNA was extracted using Qiagen MinElute ccfDNA Midi kit (Qiagen #55284); this kit includes all reagents required for the processing of this analyte. Magnetic separation steps were carried out with a Qiagen magnet system (Qiagen Adna Mag-L #399921) and end over end roller ambient shaker (Stuart rotator SB2).

cfDNA characteristics were assessed by Agilent 4150 TapeStation system (Agilent #G2992AA), using cell-free DNAScreenTape (Agilent #5067-5630), reagents (Agilent #5067-5631), sample buffer (Agilent #5067-5633) and ladder (Agilent #5067-5632). Samples were vortexed using a IKA MS3 vortexer (IKA #0003319000).

## 2.2 Laboratory Investigations

Droplet digital PCR (ddPCR) was performed using the BioRad QX200 Droplet Digital PCR system: QX200 droplet generator (BioRad #1864002) and QX200 Droplet Digital PCR System (BioRad #1864001). Digital PCR reactions were set up using BioRad designed assay reagents: Droplet generation oil for probes (Bio-Rad #186-3005), Droplet reader oil (Bio-Rad #186-3004), Supermix for Probes (no dUTP) (Bio-Rad #186-3024), sequence-specific primers and TaqMan probes for the *IDH1* p.R132H c.395G>A. hg19|chr2:209113051-209113173 (wild-type BioRad #dHsaCP2000056 and mutant site BioRad #dHsaCP2000055). Specific sequence data is not published for commercially available for BioRad mutation detection kits. ABgene combi thermo plate heat sealer (ABgene #23770) and a Rainin E4 XLS multichannel pipette (StarLab #G9008) were used in set up. PCR reactions were run on a Genetic Research Instruments (GRI) G-Storm GS1 Thermal Cycler.

cfDNA and the EpiTect unconverted unmethylated human DNA control samples were bisulphite modified using a commercially available system, the EpiTect Plus FFPE Bisulfite Kit (Qiagen #59124); this kit includes all reagents required for the processing of this analyte. Temperature-specific incubations were performed on a MJ Research PTC-200 DNA engine thermal cycler or in a Grant QBD4 heating block. Centrifugation steps were performed in a Heraeus biofuge pico microfuge.

*MGMT* promotor methylation PCR reactions were set up with Qiagen EpiTect HRM PCR Kit (Qiagen #59445). Standard of care Laboratory tests were using the primers described by (Wojdacz and Dobrovic, 2007):

**MGMT MS-HRM1 F –** 5’-GCGTTTCGGATATGTTGGGATAGT-3’

**MGMT MS-HRM1 R –** 5’-CCTACAAAACCACTCGAAACTACCA-3’

Primer sequence for the *MGMT* cfDNA developmental assay provided by MethylDetect® were not disclosed. Qiagen EpiTect PCR Control DNA Set (Qiagen #59695) was used for standardized controls and for generation of the semi-quantitative standard curve. PCR reaction and HRM was run on a Corbett Rotor-Gene 6000.

1p19q co-del assessment by STR microsatellite analysis was performed with standard PCR reactions using Qiagen HotStarTaq DNA polymerase Mastermix (Qiagen #2033207). Primer sets used are summarized in Table 2.1. PCR reactions were run on an Applied Biosystems Veriti 96 Well Thermal Cycler and fragment analysis was performed on an Applied Biosystems 3500XL Genetics Analyser with samples denatured with Hi-Di Formamide (ThermoFisher #4440753) and using 400HD Rox (ThermoFisher #402985) dye standard.

Table 2.1
**Primer sequences and size ranges for 1p19q co-deletion STR Microsatellite analysis**

| Microsatellite | Primer Name | Sequence | Size Range bp |
|---|---|---|---|
| **D1S186** | Forward 5' FAM | CCC CTC CTT CCT GCC GCT | |
| | Reverse | TAG CTC ATC CCC CCC TTT CT | 69-110bp |
| **D1S199** | Forward 5' FAM | GGT GAC AGA GTG AGA CCC TG | |
| | Reverse | CAA AGA CCA TGT GCT CCG TA | 85-120bp |
| **D1S226** | Forward 5' HEX | GCT AGT CAG GCA TGA GCG | |
| | Reverse | GGT CAC TTG ACA TTC GTG G | 69-115bp |
| **D1S2734** | Forward 5' FAM | GGT TCA AGG GAT TCT CCT G | |
| | Reverse | TGG CAC TCA GAC CTC AA | 108-134bp |
| **D19S112** | Forward 5' HEX | GCC AGC CAT TCA GTC ATT TGA AG | |
| | Reverse | CTG AAA GAC ACG TCA CAC TGG T | 110-145bp |
| **D19S206** | Forward 5' HEX | AGC CGA AGT CTT TTC ACA AGA G | |
| | Reverse | TTC ATC AAG TCT GTT CCA GCC | 95-155bp |
| **D19S219** | Forward 5' FAM | GTG AGC CAA GAT TGT GCC | |
| | Reverse | GAC TAT TTC TGA GAC AGA TTC CCA | 160-190bp |
| **D19S412** | Forward 5' FAM | TGA GCG ACA GAA TGA GAC T | |
| | Reverse | ACA TCT TAC TGA ATG CTT GC | 89-113bp |

NGS sequencing was performed using Qiagen QIAamp targeted DNA panel (Qiagen #56404); this kit includes all reagents required for the preparation of the NGS library preparation, with the exception of absolute ethanol (Hayman Ltd #122-99-6) and sodium hydroxide (Sigma-Aldrich #333414). Quantitative multiplex reference standard fcDNA (mild) was used as control material (Horizon Discovery #HD798). Temperature-specific incubations were performed on a MJ Research Peltier Thermo Cycler 200 and centrifugation steps on a Heraeus biofuge pico microfuge. PCR steps were on an Applied Biosystems Veriti 96 Well Thermal Cycler and magnetic bead separation steps with a DynaMag 96-side magnet rack (Invitrogen #12331D). Library quality metrics were assessed by Qubit fluorimeter (ThermoFisher #Q33238) with broad range dsDNA assay reagents (Qubit 1× dsDNA BR assay kit ThemoFisher #Q33265) and Tapestation with DNA ScreenTape (Agilent #5067-5582), reagents (Agilent #5067-5583), sample buffer (Agilent #5067-5602) and ladder (Agilent #5067-5586). Sequencing reactions were then performed on an Illumina MiSeq (Illumina #SY-410-1003) using MiSeq V2 reagents (Illumina #MS-102-2002); this kit includes all reagents required for NGS sequencing, with the exception of sodium hydroxide (details above). Sequencing data from the

MiSeq was analysed using a Qiagen CLC Genomics workbench; a bioinformatics analysis package for analysis and visualization of NGS workflows. The pipeline was specifically configured to support analysis of FFPE derived DNA, but should also be appropriate for the small fragment DNA found in cfDNA.

# 3 Methods

## 3.1 Study design and Patient population

This study represented a single time point assessment of adult patients with newly diagnosed high grade glioma. Potential subjects were screened at UHS neurology pre-assessment clinic, by the treating neurosurgical team. They were selected on the basis of review at regional multi-disciplinary team (MDT) meeting where patients had high grade glioma and there was a plan for a diagnostic and/ or therapeutic procedure. 44 patients were consented just prior to surgery and the required blood sample was taken by the nursing team alongside the routine blood test samples. The following patient data was collected:

- Age demographics
- Patient outcomes
    - Patient overall survival - as defined by point of death or the end of the study follow up (June 2020, 16 months).
    - Patient progression free survival, as defined as the point of disease recurrence. This was taken as the point when first mentioned in the UHS electronic patient record (EPR) – CHARTS, either in the letters or as follow up review at MDT meeting.
- Histological /molecular characteristics of the parallel tissue biopsy sample.

Once this data was collected all further analysis was fully anonymized, in line with the ethics approval stipulate.

## 3.2 Ethics approval

Ethics approval for the study was obtained with sponsorship from University Hospital Southampton NHS Foundation Trust Research and Development (IRAS project ID: 234143, protocol number RHM PAT0300, REC reference: 18/SW/0181). Consent forms and Patient information sheets were approved therein (Appendix A1 and A2).

## 3.3 Specimen collection and cell free DNA (cfDNA) extraction

10 ml peripheral blood samples were collected in to Qiagen PAXgene Blood ccfDNA tubes. cfDNA remains stable within these tubes for up to 7 days at room temperature, but samples were routinely processed the same day, occasionally being left for up to 48hr. cfDNA was extracted using Qiagen MinElute ccfDNA Midi kit, according to the manufacturer's instructions:

Samples were centrifuged at low speed for 10 min at 1900 x g @ 4°C. Plasma supernatant was carefully transferred to microcentrifuge tubes for high-speed centrifugation to remove additional cellular nucleic acids attached to cell debris and contamination by genomic DNA and RNA derived from damaged blood cells. Plasma samples were centrifuged at high speed for 10 min at 16,000 x g

@ 4°C. Supernatants were transferred into a 15ml sterile conical tube without disturbing the pellets. Volume of pooled plasma was noted, and additional reagents added according to plasma volume, as per Table 3.1.

Table 3.1
**cfDNA extraction component mix**

| Plasma (ml) | Magnetic Bead Suspension (µl) | Proteinase K (µl) | Bead Binding Buffer (µl) |
|---|---|---|---|
| 4 | 120 | 220 | 600 |
| 5 | 150 | 275 | 750 |
| 6 | 180 | 330 | 900 |
| 7 | 210 | 385 | 1050 |
| 8 | 240 | 440 | 1200 |
| 9 | 270 | 495 | 1350 |
| 10 | 300 | 550 | 1500 |

Components were incubated for 10 min at room temperature (RT), mixing slowly in an end-over-end shaker. Tubes were spun briefly (30 s @ 200 x g) to remove any solution in the cap, then put into the 15ml tube magnetic rack. Tubes were left to stand for at least 1 min, until the solution had cleared, then the supernatant was discarded. Tubes were removed from the magnetic rack and 200µl Bead Elution Buffer added to the bead pellet. Beads were vortex to resuspend, and mixture transferred into a Bead Elution Tube. Mixture was incubated for 5 min on a shaker for microcentrifuge tubes at room temperature and 300 rpm. The Bead Elution Tube containing the bead solution was place the into a magnetic rack for 2 ml tubes. Tubes were left to stand for at least 1 min, until the solution had cleared, then supernatant transferred into a new Bead Elution Tube. 300µl Buffer ACB was added, and vortexed to mix. Tubes were briefly centrifuged to remove drops from inside the lid. The buffer ACB mixture was transferred into a QIAamp UCP MinElute column and centrifuged for 1 min at 6000 x g. The QIAamp UCP MinElute column was placed into a clean 2 ml collection tube, and the flow-through discarded. 500 µl Buffer ACW2 was added to the QIAamp UCP MinElute Column, and centrifuge for 1 min at 6000 x g. The QIAamp UCP MinElute column was placed into a clean 2 ml collection tube, and flow-through discarded. Columns were centrifuge at full speed (20,000 x g) for 3 min, then placed into a clean 1.5 ml elution tube. The lid was opened, and the assembly incubated in a shaker for microcentrifuge tubes at 56°C for 3 min to dry the membrane completely. 50µl of Ultra-clean Water was carefully pipetted into the centre of the membrane, the lid closed and incubate at room temperature for 1 min. Tubes were centrifuge at full speed (20,000 x g) for 1 min to elute the nucleic acids. To maximize yield, elute was then reapply to the column for re-elution. After further incubation of 1 min at RT, tube was centrifuge 1 min at full speed (20,000 x g).

cfDNA was stored at -20°C until later evaluation.

### 3.4 Parallel diagnostic histological, cellular and molecular assessment

cfDNA samples were linked to matched UHS service histology, cellular and molecular results from the paired brain biopsies taken at the same point of surgical intervention. All UHS laboratory

medicine results are from United Kingdom Accreditation Service (UKAS) accredited laboratories and are assessed by appropriately specialized Registrars, Clinical Scientists and Consultants. The matched biopsy results were taken from the UHS Laboratory Information System.

## 3.5 Laboratory investigations

### Evaluation of cfDNA metrics

cfDNA characteristics were assessed by Agilent TapeStation automated electrophoresis system; this is a chip-based system, able to simultaneously analyse size, quality and the integrity of the samples.

Reagents were equilibrated at RT for 30 minutes, then reagents and samples vortexed and spun down before use. 10µl sample Buffer and 1µl Ladder pipetted at position A1 in a tube strip. For each sample, 10µl Sample Buffer and 1µl DNA sample was pipetted in to the tube strip. Caps were applied to tube strips and liquids mixed using a IKA MS3 vortex for 1 min @ 2000 rpm. Samples and ladder were spun down for 1 min. Samples were loaded into the TapeStation with ladder in position A1 on tube strip holder and caps carefully removed of tube strips. After running, TapeStation Analysis software opens automatically and displays results.

### Glioma biomarker analysis

Standard or specially modified laboratory protocols were used to analyse glioma-specific biomarkers.

### Droplet digital PCR for *IDH1* R132H

Three patients were identified from the cohort, where *IDH1* R132H had been identified in the paired FFPE biopsy and could thus be used as a tumour-specific biomarker in the cfDNA.

Droplet digital PCR (ddPCR) was performed using the BioRad QX200 Droplet Digital PCR system, using BioRad designed assay reagents and cfDNA samples and FFPE-derived control material (50ng). Master Mix (1×) was set up with 11µl BIO-RAD Supermix, 1.1µl Primer (mutant), 1.1µl Primer (wildtype), 3.8µl Water. Master Mix was aliquoted into 0.2ml 8-Strip Tubes. 17µl Master Mix was pipetted into each test well (control, NTC or patient sample). 5µl of control @10ngµl$^{-1}$ (50 ng) or cfDNA (DNA input ranging from 1.1-2.6ng) was pipetted into the appropriate wells and 5µl of molecular grade water into the NTC well. Strips were mixed by vortexing and pulsed down in the Sorval centrifuge. Bio-Rad droplet generator DG8 cartridge was loaded in to a holder. A multichannel pipette was used to dispense 20µl of the PCR reaction mix into the sample wells of the cartridge. 70µl of droplet generator oil was pipetted into each oil well of the cartridge and a gasket hooked over the cartridge holder using the holes on both sides. The cartridge holder was loaded in to the QX200 droplet generator and droplet generation initiated; a manifold positions itself over the outlet wells, drawing oil and sample through the micro fluidic channels, where the droplets are created. Droplets flow to the droplet well, where they accumulate. Using the Rainin E4 XLS multichannel pipette, 40µl of the droplets were transferred to an Eppendorf 96 well PCR plate. The plate was

sealed using a pierceable foil plate sealer and the AB gene heat sealer. After droplet generation, PCR reactions were run on a G-Storm PCR engine with the following standard Bio-Rad parameters:

Heated lid set to 105°C

| | | |
|---|---|---|
| 1 cycle | Enzyme activation | 95°C for 10 min* |
| 40 cycles | Denaturation | 94°C for 30sec* |
| | Annealing/extension | 55°C for 1 min* |
| 1 cycle | Enzyme deactivation | 98°C for 10 min* |
| 1 cycle | Hold | 10°C Infinite |

*Ramp @ 2°C/sec

Post PCR read was carried out directly after the PCR has completed. For data analysis, the positive control and NTC control were analysed as single wells. The test samples were analysed grouped together as a triplicate. The results were viewed as 2D amplitude plots with fluorescence recorded through two channels. Channel 1 (CH1) recorded HEX chromophore fluorescence and thus the wildtype signal. Channel 2 (CH2) records FAM chromophore fluorescence and thus the mutant signal.

The 2D amplitude plot showed channel (CH) results as follows:

**A** CH1+ CH2+ represents droplets containing both wildtype (HEX) and mutant (FAM) products.

**B** CH1+ CH2- represents droplets containing only mutant (FAM) products.

**C** CH1- CH2+ represents droplets containing only wildtype (HEX) products.

**D** CH1- CH2- represents negative droplets.

The mutant variant percentage can be calculated from values for **A**, **B** and **C** from above:

Mutant % = ((A+B)/(A+B+C)*100)

### *MGMT* promoter methylation analysis by High Resolution Melt (HRM)

The methylation status of a DNA sequence is most often assessed through bisulphite modification analysis (Frommer *et al.*, 1992). Incubation of the target DNA with sodium bisulphite results in conversion of unmethylated cytosine residues in the dinucleotide sequence, CpG, into uracil, leaving the methylated CpG cytosines unchanged. Therefore, bisulphite treatment gives rise to different DNA sequences for methylated and unmethylated DNA. A reversible 'epigenetic' change has been converted to an irreversible genetic one, for the purposes of detection.

cfDNA and the EpiTect unconverted unmethylated human DNA control sample were bisulphite modified using the EpiTect Plus FFPE Bisulfite Kit. Reactions were set up as follows: 40µl DNA (cfDNA

-DNA input ranging from 11ng to 39ng) and control DNA (100ng), 85µl Sodium Bisulfite Mix, 15µl DNA Protect Buffer, added in this order.

**Bisulphite incubation conditions**

| **Step** | **Time** | **Temperature** |
|---|---|---|
| Denaturation | 5 min | 95°C |
| Incubation | 25 min | 60°C |
| Denaturation | 5 min | 95°C |
| Incubation | 85 min (1 h 25 min) | 60°C |
| Denaturation | 5 min | 95°C |
| Incubation | 175 min (2 h 55 min) | 60°C |
| Hold | Indefinite | 20°C |

After the bisulphite incubation, PCR tubes were briefly centrifuged, and reaction mix transferred to clean 1.5 ml microcentrifuge tubes. A working solution containing BL buffer and RNA carrier was set up for the correct number of samples required. This comprised 310µl BL buffer and 3.1µl carrier RNA per sample. 310µl of this solution was then added to each sample. Solutions were mixed by vortexing and then centrifuge briefly. 250µl absolute ethanol was added to each sample. Solutions were thoroughly mixed by pulse vortexing for 15s, and centrifuge briefly to remove the drops from inside the lid. MinElute DNA spin columns and collection tubes were placed in a rack and entire mixture transferred to the corresponding MinElute DNA spin column. Spin columns were centrifuged at maximum speed for 1 min. Flow-through was discarded and place the spin columns placed into new collection tubes. 500µl Buffer BW (wash buffer) was added to each spin column, and centrifuge at maximum speed for 1 min. Flow-through was discarded and spin columns placed into new collection tubes. 500µl Buffer BD (desulfonation buffer) was added to each spin column and incubated for 15 min at RT. Lids were closed for incubation to avoid acidification from carbon dioxide in air. Spin columns were centrifuged at maximum speed for 1 min, flow-through discarded, and spin columns placed back into new collection tubes. 500µl Buffer BW was added to each spin column and centrifuged at maximum speed for 1 min. Flow-through was discarded and place the spin columns into new collection tubes. The buffer BW wash step was repeated. 250µl absolute ethanol was added to each spin column and centrifuged at maximum speed for 1 min. Spin columns were placed into new 2 ml collection tubes and centrifuged at maximum speed for 1 min to remove any residual liquid. Spin columns were placed with open lids into a clean 1.5 ml microcentrifuge tube and incubated for 5 min at 60°C in a heating block. This step enabled the evaporation of any remaining liquid. Spin columns were placed into clean 1.5 ml microcentrifuge tubes. 18µl Buffer EB (elution buffer) was placed directly onto the centre of each spin-column membrane and the lids gently closed. Spin columns were incubated at room temperature for 1-5 mins. Tubes were centrifuged for 1 min at 15,000 x *g* (13,000 rpm) to elute the DNA.
Bisulphite modified DNA was stored at -20°C until use.

*MGMT* promoter methylation was assessed by methylation-specific High Resolution Melt Analysis (MS-HRM) *MGMT* promoter methylation analysis (Wojdacz and Dobrovic, 2007). In this method, EvaGreen is included in the PCR set up and is used as a reversibly binding intercalating dye which fluoresces brightly when bound to double-stranded DNA. As the PCR reaction progresses, an increase in fluorescence is seen that is proportional to the amount of PCR amplicon being generated. At the end of the PCR, the amplicon is gradually heated in a 'high resolution melt' (HRM) step, until the double stranded molecule melts in to 2 single-stranded molecules. As the dye does not bind to the single-stranded DNA, a change on fluorescence can be used to measure the thermally induced DNA melting by HRM (Figure 3.1a).

a b

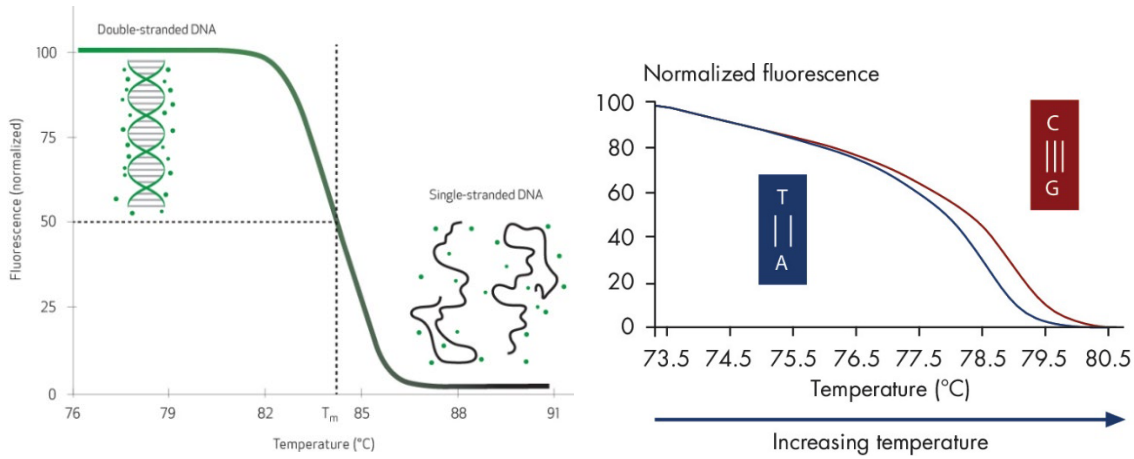



Figure 3.1

a, **Principle of High Resolution Melt (HRM) analysis.**
At the end of the PCR reaction amplicon fluoresces strongly due to the bound intercalated dye. As the temperature increases in the HRM step, the 2 strands of the DNA melt and separate releasing the dye. The consequent reduction in florescence is monitored and data is analysed as normalized plot, where 100% of the DNA is double stranded at the beginning of the melt and it is fully single stranded at the end.
Adapted from Reed *et al.*, 2007 (Reed, Kent and Wittwer, 2007)

b, **Melting curve of amplicon derived from unmethylated DNA and methylated DNA**
In the unmethylated derived amplicon, CpG's are converted to TpG's (blue), whilst amplicon from methylated DNA, where CpG's remain as CpG's (red). There is a higher representation of triple hydrogen bonds in the methylated derived amplicon, so it melts at a higher temperature.
Taken from:
(Qiagen, 2020)

This method uses the bisulphite modified DNA described above. Unmethylated CpG sites are converted to UpG and amplified as TpG in the PCR reaction, whilst methylated CpG sites remain unaltered. Amplicon derived from the methylated sequence has a higher representation of 'C's, hydrogen bond strength is higher and the product 'melts' at a higher temperature. Thus unmethylated and methylated derived sequence can be resolved in a 'high resolution melt' (HRM) analysis (Figure 3.1b).

The routine laboratory assay is based on this methodology, but here was replaced with a developmental assay, designed specifically for this analysis by project collaborators, MethylDetect®. The assay set up was modified to give enhanced sensitivity for the low levels of circulating tumour DNA (ctDNA) signal expected in the cfDNA. 2 µl bisulphite modified cfDNA / 2 or 5 µl of the Epitect control standards (10ngµl$^{-1}$) / 10µl MethylDetect control standards were PCR amplified with MethylDetect MGMT primers and Qiagen EpiTect HRM PCR Kit Mastermix in a total reaction volume of 30 µl. The assay was run on a RotorGene 6000 real-time PCR machine, with built in modifications for accurate and sensitive HRM capacity. The standard SOP/Qiagen Epitect kit PCR conditions were used, but with an increased annealing temperature, designed to increase the sensitivity of the HRM analysis.

**PCR reaction**

| **Step** | **Time** | **Temperature** | |
|---|---|---|---|
| Initial PCR activation step | 5 min | 95°C | |
| 3-step cycling: | | | |
| Denaturation | 10 s | 95°C | |
| Annealing | 30 s | 58°C | |
| Extension | 15 s | 72°C | Fluorescence data acquisition on the 'Green' channel. |
| Number of cycles | 50 | | |
| HRM analysis for: | 2 s | 65–95°C | 0.1 °C increments<br>Fluorescence data acquisition on the 'HRM' channel |

Fluorescence data was collected during the PCR and HRM step of the analysis. Fluorescence levels are initially measured at the end of each PCR extension stage. As the PCR progresses more amplicon is generated. The amount of dye that is able to bind to amplicon is proportional to the amount of double stranded DNA present and so there is real-time monitoring of the amount of amplicon being generated in the PCR. The normalized high resolution melt data used in methylation analysis, and described in Figure 3.1, is automatically generated by the Rotorgene software.

5 samples with the highest concentration cfDNA samples and with a paired biopsy *MGMT* promoter methylation were chosen for a proof of principle analysis.

**1p19q co-del assessment by STR microsatellite analysis**

1p19q co-deletion assessment by STR microsatellite analysis was performed as per the laboratory routine SOP, based on the methods of Hatanpaa *et al*., (Hatanpaa *et al.*, 2003). A mastermix of HotStarTaq and DNA set up for each sample and a single NTC. 4-14 ng of cfDNA was made up to 115µl with nuclease-free water and NTC was composed of 115 µl water only. 2µl of each microsatellite primer mix was added to 25µl of the HotStarTaq/DNA mastermix.

**PCR reaction**

| Step | Time | Temperature | |
|---|---|---|---|
| Initial PCR activation step | 10 mins | 95°C | |
| Denaturation | 1 mins | 95°C | |
| Annealing | 1 mins | 56°C | x 30 cycles |
| Extension | 1 mins | 72°C | |
| Extension | 72°C | 20 mins | |
| Hold | 4°C | Indefinite | |

Sample data was analysed using GeneMapper software. Paired blood and FFPE brain biopsy data was taken from the filed laboratory standard of care analysis. Allelic imbalance factor (AIF) was determined using the peak heights by calculating the ratio of the 2 alleles (1- being the smaller and 2 the larger) for both the constitutional (C) = (PB) and the tumour (T) sample, and then the tumour ratio was divided by constitutional ratio:

$$AIF = \frac{[T_1] / [T_2]}{[C_1] / [C_2]}$$

A reduction of at least 50% (AIF of 0.5) in the peak height of one allele in the tumour, normalised against the retained allele in the healthy control, is used to score LOH for any ambiguous markers.

Three patients were identified from the cohort, where tumour specific 1p19q loss was specifically associated with tumour and could thus be used as a tumour-specific biomarker in the cfDNA.

**Massively Parallel Sequencing (NGS) Analysis**

A single sample where there was an *IDH2* R132K variant in the paired biopsy sample was run through the Laboratory NGS platform. Our Laboratory currently uses the Qiagen QIAseq targeted DNA human actionable solid tumor panel and includes all the *IDH* mutation hot spot regions. The QIAseq Targeted DNA Panels uses integrated unique molecular indices (UMIs) to facilitate ultrasensitive variant detection and so can be used with the compromised DNA templates derived from FFPE samples or very low concentrations of tumour DNA found in the cfDNA fraction of biofluids. The required amount of template for a single QIAseq targeted sequencing reaction ranges from 10 to 40ng for fresh DNA or 40 to 250ng for FFPE DNA. There was only sufficient cfDNA in the sample to load 6 ng. Lower input amounts are possible with this method, however, this leads to fewer sequenced UMI and reduced variant detection sensitivity.

**cfDNA sample fragmentation, end repair and A-tailing:**

DNA was added to 2.5µl ×10 Fragmentation buffer, 0.75µl FERA solution and nuclease-free water up to a volume of 25µl. Reactions were kept on ice during set up, then transferred to pre-chilled thermal cycler.

Incubation steps were then as follows:

| Step | Incubation time | Temperature |
|---|---|---|
| 1 | 1 min | 4°C |
| 2 | 14 min | 32°C |
| 3 | 30 min | 72°C |
| 4 | HOLD | 4°C |

The sample was immediately transferred to ice for the adapter ligation step.

**Ligation at 5' ends of DNA fragments with sequencing platform-specific adapter containing UMIs and sample index:**

The following were added to the 25µl fragmentation reaction: 10µl ×5 ligation buffer, 2.8µl IL-N7## adapter, 5µl DNA Ligase and 7.2µl Ligation Solution (total volume 50µl). Sample was incubated on a thermal cycler for 15 mins @ 20°C. Adapter ligated DNA was cleaned up using magnetic bead technology: 50µl nuclease-free water was added to the 50µl sample and 100µl QIAseq Beads. Reaction was mixed well by pipetting up and down several times then incubated for 5 min @ RT. Tube was placed on a DynaMag 96-side magnet rack for 10 min. Once the solution had cleared, with the beads still attracted to the DynaMag 96-side magnet rack, the supernatant was carefully removed and discarded. With the beads still attracted to the DynaMag 96-side magnet rack, 200µl freshly prepared 80% ethanol was added. The plate was moved from side-to-side between the two column positions of the magnet plate to wash the beads. The wash was carefully removed and discarded the wash. The ethanol wash was repeated, completely removing all traces of the ethanol wash after the second wash. With the beads still attracted the DynaMag 96-side magnet rack, the tube was left to air dry at room temperature for 10 min. The tube was removed from the DynaMag 96-side magnet rack, and the DNA eluted from the beads by adding 52µl nuclease-free water and mixing well by pipetting. The tube was returned to the magnetic rack allowing the solution to clear then the 50µl of the supernatant was transferred to a clean tube. A second 50µl aliquot of QIAseq Beads was added to the sample and mixed well by pipetting up and down several times. It was the incubated for 5 min @ RT, then the ethanol wash process was repeated. The tube was from the DynaMag 96-side magnet rack, and the DNA eluted from the beads by adding 12 µl nuclease-free water, then mixing well by pipetting. The tube was returned to the magnetic rack until the solution has cleared, then 9.4µl of the supernatant to clean tube.

**Target enrichment, post-UMI assignment to ensure that DNA molecules containing UMIs are sufficiently enriched in the sequenced library:**

For enrichment, ligated DNA molecules were subject to several cycles of targeted PCR using one region-specific primer and one universal primer complementary to the adapter. A universal PCR was

then carried out to amplify the library and add platform specific adapter sequences and additional sample indices.

The following were added to the 9.4µl adaptor-ligated DNA: 4µl ×5 TEPCR buffer, 5µl QIAseq Targeted DNA Panel, 0.8µl IL-Forward primer, 0.8µl HotStarTaq DNA Polymerase (total volume 20 µl)

**PCR reaction**

| **Step** | **Time** | **Temperature** |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| 8 cycles | 15 secs | 98°C |
| | 10 mins | 96°C |
| 1 cycle | 5 min | 72°C |
| HOLD | 5 min | 4°C |
| HOLD | indefinite | 4°C |

Target enriched sample was cleaned up by addition of 80µl nuclease-free water and 100 µl QIAseq Beads, then mixed well and incubated for 5 min @ RT. The tube was placed on a magnetic rack for 5 min. After the solution had cleared, with the beads still attracted to the DynaMag 96-side magnet rack, the supernatant was carefully removed and discarded. With the beads still on the DynaMag 96-side magnet rack, 200µl 80% ethanol was added. The plate was moved side-to-side between the two column positions of the magnet to wash the beads. The wash was carefully removed and discarded. The ethanol wash was repeated ensuring all traces of the ethanol wash were removed. With the beads still attracted to the DynaMag 96-side magnet rack, they were left to air dry for 10 min @RT. The tube was removed from the DynaMag 96-side magnet rack and DNA eluted from the beads by adding 16µl nuclease-free water. The tube was returned to the magnetic rack until the solution had cleared. 13.4µl of the supernatant was transferred to clean tubes.

Universal PCR was set up as follows: 13.4µl Target-enriched product from previous step, 4µl ×5 UPCR Buffer, 0.8µl IL-Universal Primer, 0.8µl IL-S50 Index Primer and 1µl HotStarTaq DNA Polymerase (Total Volume 20 µl)

**PCR reaction**

| **Step** | **Time** | **Temperature** |
|---|---|---|
| Initial denaturation | 13 min | 95°C |
| | 2 min | 98°C |
| 21 cycles | 15 secs | 98°C |
| | 2 mins | 60°C |

| | | |
|---|---|---|
| 1 cycle | 5 min | 72°C |
| HOLD | 5 min | 4°C |
| HOLD | indefinite | 4°C |

Universal PCR reaction was cleaned up by addition of 80µl nuclease-free water and 100µl QIAseq Beads, then ethanol washed as described above. 28µl of the final supernatant was transferred to a clean tube for Qubit quantification and Tapestation analysis prior to normalisation and pooling. The library was quantified to obtain the nanograms of library prep needed for sequencing then diluted to 4nM using nuclease free water. 5µl 4nM library was mixed with 5µl freshly made 0.2N NaOH in a microcentrifuge tube, vortexed briefly, pulsed down in a centrifuge and then incubated for 5 minutes @ RT. Immediately after this incubation had finished, 990µl of prechilled HT1 buffer was added to the tube containing denatured library to produce 1ml of a 20pM denatured library. Denatured 20pM Library was further diluted depending on the input concentration:

| Concentration | 6pM | 8pM | 10pM | 12pM | 15pM | 20pM |
|---|---|---|---|---|---|---|
| 20pM library | 180µl | 240µl | 300µl | 360µl | 450µl | 600µl |
| Prechilled HT1 | 420µl | 360µl | 300µl | 240µl | 150µl | 0µl |

The tube was inverted to mix and then pulse centrifuge, ready to load onto the MiSeq cartridge. The Custom read 1 primer was also diluted out to a final concentration of 0.5µM by adding 3µl QIAseq A Read 1 Custom Primer to 597µl pre-chilled HT1 buffer. 600µl pre-diluted library and 600µl of diluted custom read 1 primer were loaded onto the MiSeq V2 cartridge. MiSeq V2 reagents (Buffer bottle and flow cell) were loaded on to the sequencer then set up and run performed according to the machine prompts.

**Data analysis with Qiagen CLC Genomics workbench:**

The raw fastq files generated from the sequencing run were aligned and annotated using the Qiagen CLC Genomics workbench bioinformatics pipeline. The final variants were also filtered to remove poor quality variants which did not meet stringent quality control requirements and fell below a clinically useful threshold.

Sequence data was transferred as a fastq file from the MiSeq instrument to the Hospital intranet. The files were imported in to desktop PC-based CLC Genomics Workbench and processed with the bespoke bioinformatics '5% Frequency cut off of Qiaseq DHS-101Z somatic Gx12 v55.3' pipeline. This generated a table of final filtered variants. Run QC metrics were checked with parallel 'Horizon Discovery' control samples to accept the run as valid. Sample QC analysis checked final target coverage, to ensure the target genes had appropriate coverage: read count >100, reported variants are > 5% Frequency, Forward/Reverse balance approximately 50% (0.5) and not close to 0% or 100%. For UMI group report: Average reads per group is between 2 and 6, Check that the Groups with size ≤ 1 (% of groups) (% of reads) is between 10-20% and increase in Q scores between 'Average Q score for Input reads' and 'Average Q score for UMI reads'.

### 3.6 Statistical analysis

Comparisons between subject survival times of 2 groups ‘high cfDNA vs low cfDNA’ defined as a cfDNA concentration above vs. below the mean value. Comparisons were performed with a test of normality (Shapiro-Wilk) and nonparametric (Mann-Whitney U) tests. The Kaplan-Meier (KM) method was used to estimate median overall survival (OS). Log-rank (Mantel-Cox), Breslow (Generalized Wilcoxon) and Tarone-Ware were used to test for equality of survival distributions for the 2 groups. OS was defined as the number of months from the date of surgery /biopsy until the date of death, or the date of last follow up, if progression had not occurred. The date of last follow up was set at 16 months, when the last death was recorded in the cohort. Progression Free Survival (PFS) was defined as the number of months from the date of surgery /biopsy until the date of tumour progression, or the date of last follow up, if progression had not occurred. Statistical analyses were performed using SSPS statistics package v26.

# 4 Results

## 4.1 Study Cohort composition

The study followed up a cohort of 44 glioma patients collected over 6 month period.

Age and gender demographics and the relevant histopathology and molecular pathology data of tumour type, tumour grade and molecular characteristic (ATRX status, Ki67 proliferation, *IDH* mutation status, 1p19q co-deletion, *MGMT* promoter methylation) are described in appendix A3.

cfDNA was obtained for 39 patients with the remaining 5 cases not processed. The 39-case sub-set was followed up in the cfDNA metrics and/or biomarker analysis. The breakdown of analysis is summarized in Figure 4.1.

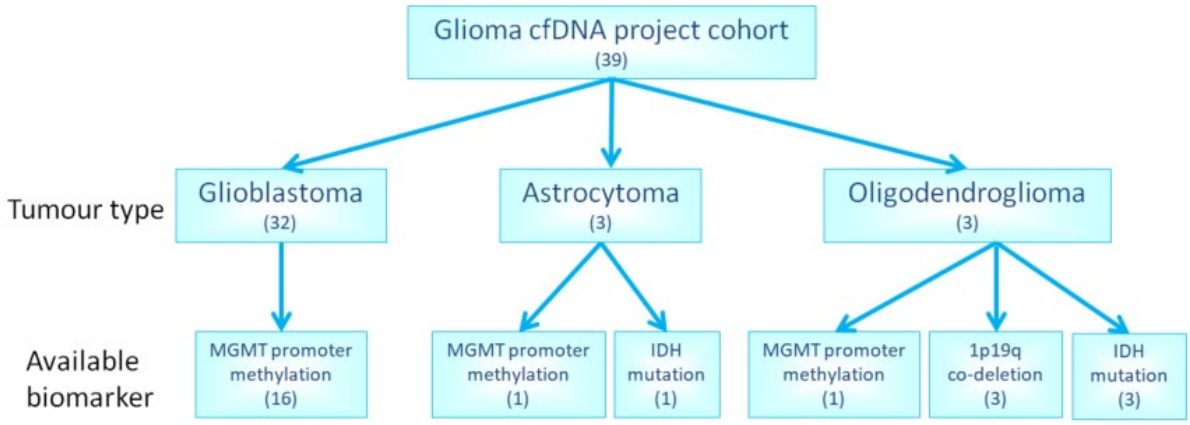



Figure 4.1
**cfDNA project cohort specimen breakdown**
The cohort was predominantly made up of glioblastoma (GBM) patients, with a smaller number of high grade anaplastic astrocytoma, oligodendroglioma and a single case of diffuse midline glioma. The biomarker tests available for each glioma types are indicated. Number of patients/samples are indicated accordingly. No biomarker tests were available for the diffuse midline glioma case and so it is not represented in this figure.

Additionally three lung cancer samples were also obtained and assessed for EGFR biomarker, to evaluate the analytical validity of the cfDNA extraction pathway.

## 4.2 Cohort Age Demographics

The cohort was not diverse enough to do a detailed evaluation of age demographics with reference different glioma types, but the glioblastoma (GBM) group is illustrated in Figure 4.2.

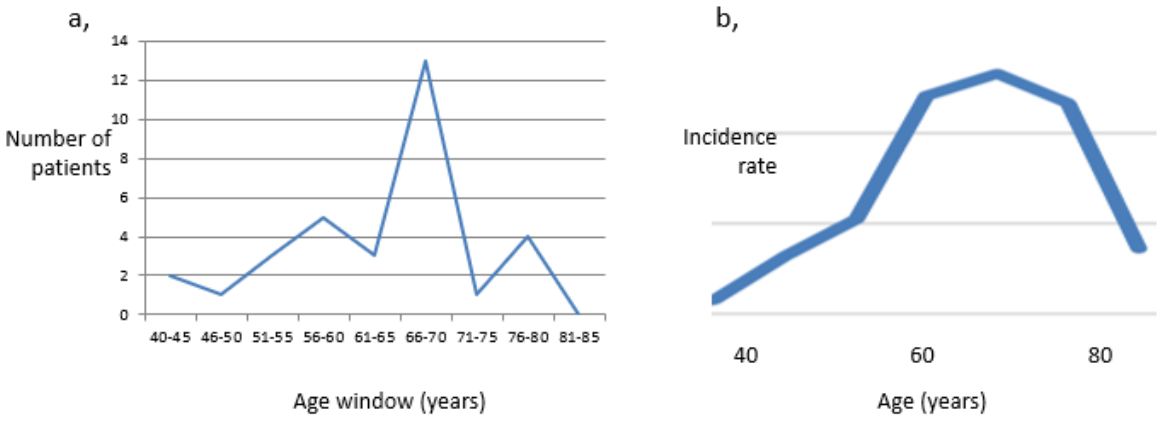



Figure 4.2
**Age at diagnosis for glioblastoma (GBM) cases**
(a) in this cfDNA cohort – the number of GBM patients in concurrent 5-year age windows was tallied and plotted in ascending age
(b) the incidence rate seen nationally. Taken from: (UCL, 2020)

GBM is primarily diagnosed at older ages with a median age of diagnosis of 64 years. The incidence continues to rise with increasing age, peaks at 84 years of age and drops after 85 years (Thakkar *et al.*, 2014). In this cohort, the median age of diagnosis was 66 years (Figure 4.2a) and the age range of initial diagnosis reflected the national incidence rate (Figure 4.2b). Thus, although the cohort was small, age demographics were broadly in line with what might be expected.

### 4.3 Recommendations for plasma separation and storage

The kit protocol followed that of the literature, in recommending a double centrifugation step to separate the plasma (Volik *et al.*, 2016). A second high g-force spin removes cellular debris and thereby reduces the amount of cellular or genomic DNA and RNA in the sample. This was not easy to implement in the laboratory as we only have microfuges that we are able to refrigerate, but was done by sub-aliquoting the samples. Figure 4.3 illustrates the need for this additional spin to ensure minimum carry over of genomic DNA and therefore optimal extraction of cfDNA.

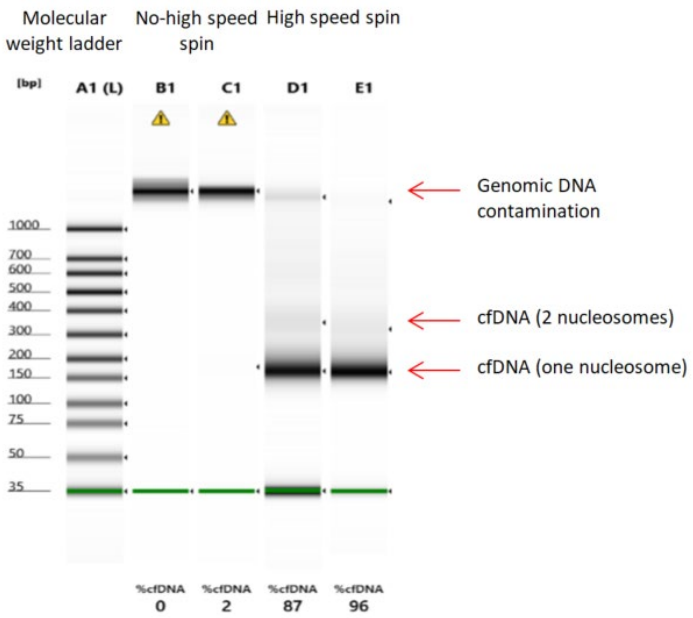



Figure 4.3

**Tapestation analysis of extracted cfDNA with and without additional high speed spin**

The Tapestation system is a chip-based automated electrophoresis system used to evaluate sample quality of nucleic acids. The data can be presented as a 'virtual' electrophoresis gel to aid interpretation. Track A1 is the DNA ladder, that is used to calibrate and calculate sizes of components. Tracks B1 and C1 are two samples that only received a low speed centrifuge spin (1900 x g). The sample consists entirely of high molecular weight genomic DNA. Samples in tracks D1 and E1 had an additional high speed (16,000 x g) and this almost eliminated the genomic DNA contamination. cfDNA is clearly visualized with a strong band at approx. 150bp, which corresponds to the DNA wrapped around one nucleosome. A second fainter band can be seen at approx. 350bp, which corresponds to the DNA wrapped around two nucleosomes. The purity of the cfDNA as a percentage of all the nucleic acid present is calculated for each sample and is indicated at the bottom of the figure.

## 4.4 Plasma cfDNA quality and quantity evaluation

cfDNA characteristics were evaluated by running samples through the Agilent Tapestation automated electrophoresis system. As well as providing a 'virtual' electropherogram, this system can calculate cfDNA metrics defining cfDNA concentration and purity. Full data is summarized in Appendix A4. For the full data set the mean extracted DNA concentration was 1067± 946 (SD) pg $\mu l^{-1}$ and the %cfDNA 91% ±5.5 (SD). For the glioblastoma subset the mean extracted DNA concentration was 1186 ± 1001 (SD) pg $\mu l^{-1}$.

## 4.5 Parallel analysis of cfDNA Epidermal Growth Factor (*EGFR*) companion testing for lung cancer patients

Due to the expected difficulty in obtaining sufficient glioma DNA within the cfDNA fraction, there would be an initial unknown as to whether the extraction process *per se* would generate suitable material for standard laboratory PCR assays. During the course of the project an opportunity arose to extract cfDNA in parallel with plasma lung cancer samples being sent to an external laboratory for cfDNA *EGFR* mutation analysis. Affirmation of results with those of another Laboratory would indicate the extraction process itself could produce DNA that was viable in a downstream application.

Two of the samples were run on the Tapestation and had cfDNA concentrations of 271 and 618 pg $\mu l^{-1}$, thus within the range of the glioma cohort. To test the analytical utility of this cfDNA, all three samples were run in the laboratory *EGFR* mutation assay, Qiagen Therascreen RGQ-PCR version 2 (Qiagen #874111). Results are summarized in Table 4.1.

Table 4.1
**cfDNA *EGFR* mutation analysis for liquid biopsy lung cancer testing**

| | **Standard of care result** | **In-house analysis** |
|---|---|---|
| cfDNA extraction method | Roche cobas® cfDNA sample preparation kit | Qiagen MinElute ccfDNA kit |
| *EGFR* mutation detection assay | Roche cobas® *EGFR* mutation kit | Qiagen Therascreen *EGFR* RGQ PCR version 2kit |
| Sample | mutation detected | |
| 30789477A | Exon 21 c.2573T>G p.(Leu858Arg) **L858R**<br><br>Exon 20 (c.2369C>T p.(Thr790Met) **T790M** | L858R positive<br><br>T790M positive |
| 30761630U | Exon 21 c.2573T>G p.(Leu858Arg) **L858R**<br><br>Exon 20 (c.2369C>T p.(Thr790Met) **T790M** | L858R variant amplicon detected, but out of QC range for variant calling.<br><br>T790M variant amplicon detected, but out of QC range for variant calling. |
| 30788331F | No mutation detected | No mutation detected |

cfDNA extracted from a duplicate sample of one sent to external Laboratory for *EGFR* cfDNA testing. cfDNA was run though the in-house assay, Qiagen Therascreen *EGFR* mutation RGQ-PCR assay.

Two samples were fully concordant for results returned from the referral laboratory. In the remaining sample, amplification could be seen for both variants detected in the referral result, but the software did not call the variants, with them falling out of the Quality Control (QC) threshold

cycle (Ct) acceptance range. The referral laboratory uses the cobas® *EGFR* test than has been optimized for both FFPE and cfDNA samples whereas the Qiagen Therascreen assay used here is for use with FFPE samples, a separate kit is available for plasma analysis. So the lack of full calling of the cfDNA variants is likely due to the lesser sensitivity in our assay system. However, there is overall concordance of results with the parallel testing here and that of the referral laboratory. The ability to successfully execute companion *EGFR* mutation testing in cfDNA from lung cancer liquid biopsy supports a conclusion that the collection and extraction process, *per se*, produced a viable DNA template for standard molecular pathology diagnostic tests.

## 4.6 Glioma biomarker analysis

### Droplet digital PCR for *IDH1* R132H

*IDH* mutations represent powerful glioma specific biomarkers, being of strong diagnostic and prognostic value and detected in 40% of gliomas (Yan *et al.*, 2009). The *IDH1* R132H substitution represents 88% of the variants seen in these genes (Brandner and Jaunmuktane, 2018). The mutation is usually screened for by immunohistochemistry, but this method is subjective, and the droplet digital PCR (ddPCR) method gives a highly accurate and sensitive alternative. The *IDH* mutations occur in two classes of gliomas – astrocytomas and oligodendrogliomas (Louis *et al.*, 2016; Brandner and Jaunmuktane, 2018) and there were 3 samples within the cfDNA cohort where an *IDH1* R132H) was recorded as part of the paired biopsy histology. An example plot is illustrated in Figure 4.4.

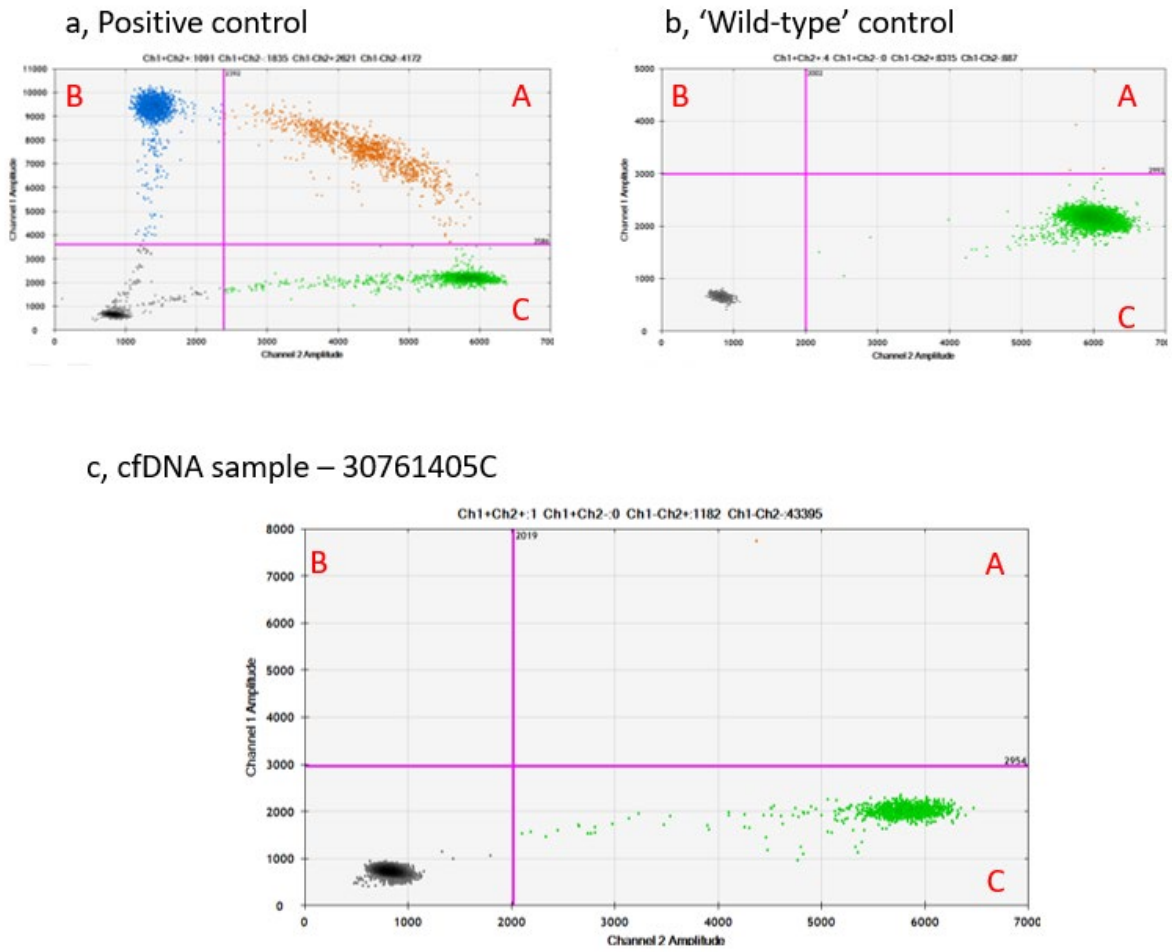



Figure 4.4

***IDH1* R132H droplet digital PCR 2-D amplitude graphical representation**

2-D plot shows the droplets that carry the *IDH1* R132H amplicon (FAM-Blue), *IDH1* Wild-type amplicon (HEX-green) or both (FAM/HEX-orange)

a, positive control – FFPE DNA from a known *IDH1* R132H positive sample – positive droplets in all 3 quadrants

b, 'wild-type control' – The Laboratory wild type (Wt) FFPE DNA standard - positive droplets in the wild type quadrant (green).

c, glioma cfDNA sample – wild type signal with single dual signal droplet.

Letters coloured red (A, B, C) represent the quadrants used in the mutation percentage calculations (see below)

A single pilot run was performed for this assay and the results are summarized in Table 4.2

Table 4.2
**Summary of cfDNA *IDH1* R132H droplet digital PCR analysis**

| **Sample** | **DNA input (ng)** | **Combined droplet numbers** | | | | ***IDH1* R132H %** |
|---|---|---|---|---|---|---|
| | | A (CH1+CH2+) | B (CH1+ CH2-) | C (CH1- CH2+) | Total events | |
| 30761405C | 6.9 | 1 | 0 | 1182 | 1183 | 0.08 |
| 30761407U | 4.4 | 0 | 0 | 598 | 598 | 0.00 |
| 30788615U | 3.3 | 0 | 0 | 341 | 341 | 0.00 |
| Positive control | 50 | 1091 | 1835 | 2621 | 5547 | 52.75 |
| Wild type control | 50 | 0 | 0 | 8319 | 8319 | 0.00 |

Channel 1 (CH1) records HEX chromophore fluorescence and thus the wildtype signal.
Channel 2 (CH2) records FAM chromophore fluorescence and thus the mutant signal.

Mutation percentage, calculated as follows:
*IDH1* R132H % = ((A+B)/(A+B+C)*100)

**A** CH1+ CH2+ represents droplets containing both wildtype (HEX) and mutant (FAM) products.
**B** CH1+ CH2- represents droplets containing only mutant (FAM) products.
**C** CH1- CH2+ represents droplets containing only wildtype (HEX) products.

A range of 3.3-6.9 ng glioma cfDNAs, corresponding to a fixed volume of varying concentration, were used in the assay. These small amounts were added out of no choice, because of the limited amount of material available. They were considerably less than the 50ng of both samples and controls used in the routine diagnostic test. This was then reflected in the low template-containing droplet numbers, with a correlate between the input DNA amount and the observed droplet numbers. In the diagnostic test we aim to get 10,000 droplets to ensure a limit of detection of >0.1%. So the cfDNA analysis here did not generate enough droplets to give an accurate result at the current stated level of sensitivity. Given the expectation of low percentage glioma ctDNA fraction within the cfDNA, the low droplet numbers here would compromise the detection of expected very low levels of *IDH1* R132H DNA. Unfortunately, these samples had also been used in other biomarker tests and there was insufficient material remaining to expand the analysis; to increase input DNA, ensure 10,000 droplets and guarantee a limit of detection of 0.1%.

It was therefore not possible to draw conclusions about whether the ddPCR assay could be used to pick up the ctDNA *IDH1* R132H biomarker, within the plasma derived cfDNA. However, our experiences have shown that even if droplet numbers are low, when a mutation is present, the 3-quadrant pattern can often be seen even though the sectors are sparsely populated. Although a single *IDH1* R132H droplet was seen in one sample (30761405C), there was no observed mutation pattern in the 2-D plots (Figure 4.4c), that might have hinted at this method having potential to detect glioma-specific biomarker.

**MGMT promoter methylation analysis by High Resolution Melt (HRM)**

The method used in the MGMT promoter methylation was carried out using a kit and protocol designed by the project collaborators MethylDetect®. The in-house assay and the kit are based on the original assay design of Wojdacz and Dobrovic (Wojdacz and Dobrovic, 2007). The key design point of this assay is to use CpG's in the primer sequence to introduce a PCR bias towards methylated template; this being the clinical utility in the assay. An added feature of the assay design was that, by manipulating the annealing temperature of the PCR amplification, the assay could be made more sensitive to methylation detection. However, there is a trade off with this, as at higher annealing temperature the overall stringency of the PCR is higher and yield of amplicon is lower. So in assay design there needs to be consideration of where the annealing temperature should sit; to provide sufficient amplicon in the PCR for an accurate result, whilst still generating a melt profile that is of analytical utility for the assay. As for all of the assays, the expectation is that ctDNA will only be a small fraction of the cfDNA. Thus existing assays must be further optimised to ensure they are sensitive as possible. The company had carried out initial optimisation studies to improve the sensitivity of their commercially available product using control material (see Appendix A5).

**Analytical Validity of the new test protocol within the in-house assay protocol**

The Methyldetect protocol used a different Mastermix than that used in our laboratory – Roche®LightCycler®480 High-Resolution Melting Master PCR reagents - so the assay was first run to confirm the new assay reagents worked within the in-house set up. The standard HRM Mastermix reagents (EpiTect HRM PCR Kit) were set up with bisulphite modified DNA control material; the in house method - EpiTect PCR Control DNA Set- and those from the new, developmental assay- Methyldetect.

The PCR amplification results are illustrated in Figure 4.5

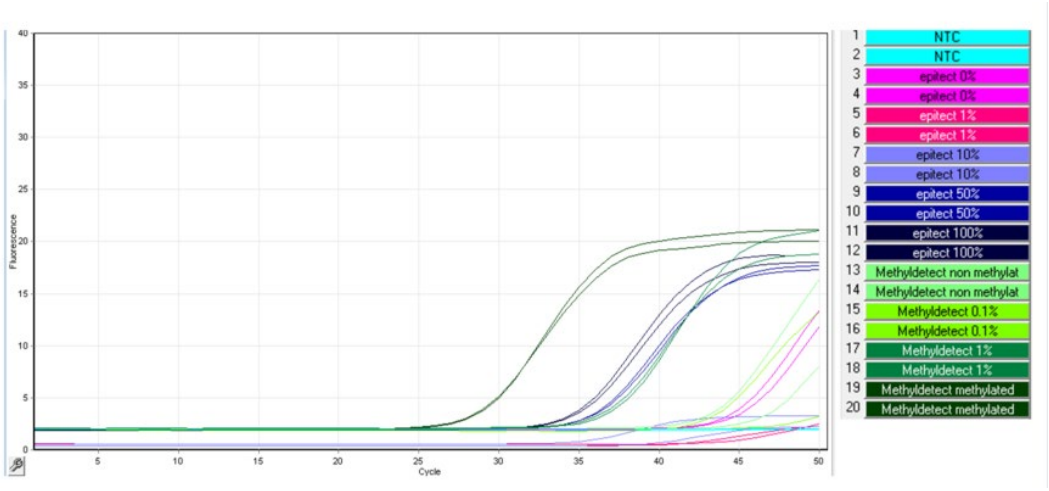



Figure 4.5

**Controls PCR for assay adapted protocol**

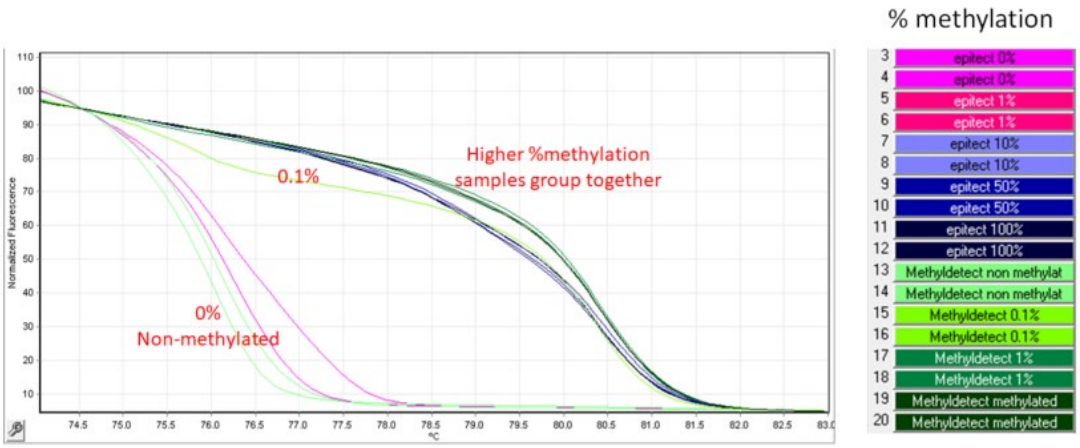
Figure shows level fluorescence tracked over increasing PCR cycle number. As the PCR proceeds, more amplicon is generated, more intercalating dye binds and the level of fluorescence increases. Of note, no amplification seen in the 'no template control' (NTC). Due to high number of cycles used in the PCR, it is particularly important to monitor the NTC fluorescence. Increased NTC fluorescence may be due to external contamination, but is more often seen resulting from primer dimers. Unlike separating PCR products out on a gel, it is not possible to discriminate true product from primer dimers by monitoring the fluorescence generated from double stranded template alone.

Although all reactions are loaded with the same amount of DNA more amplification is seen in the higher %methylated samples due to the bias in the design of the PCR reaction.

Due to covid lockdown constraints, this assay was only performed once.

The results showed the standard in-house PCR reagent and conditions generated amplicon similarly to that of the MethylDetect protocol and there was no amplification in the non-template control (NTC). With the high cycle number of 50 it is essential to check there was no non-template related amplification from the primers *eg* amplification of primer dimers. When using intercalating dyes to monitor product amplification, it is not possible be completely sure fluorescence signal is specifically related to the required amplicon (this can be readily evaluated by electrophoresis).

a,




b,

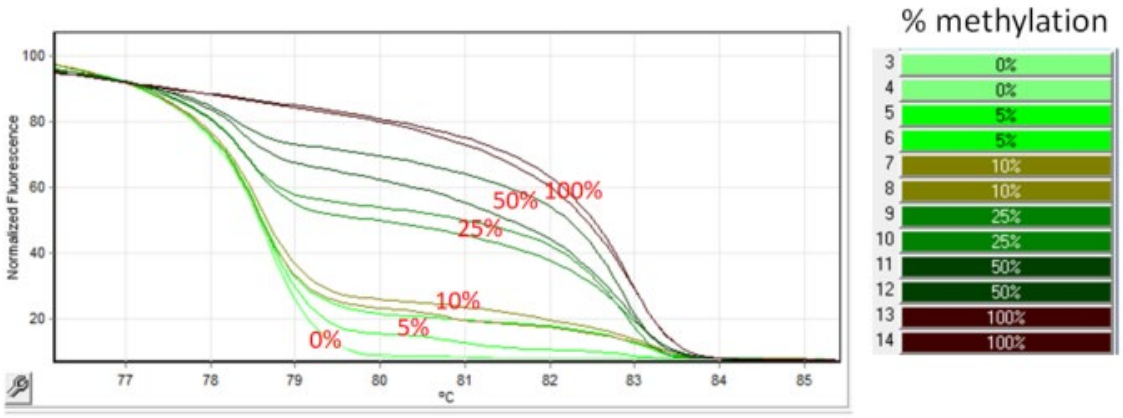



Figure 4.6

**Controls HRM analysis for assay adapted protocol**

HRM plots are presented as 'normalised plots' by the Rotorgene software. That is, starting at the lowest temperature where all the DNA is double stranded, fluorescence is at a maximum and set as the 100% value. As the temperature is increased, the double strands melt and separate, dye is lost and fluorescence declines (see Figure 3.1). When all the DNA is single stranded, no dye is left bound and the normalized fluorescence is set at 0%.

a, Illustration of HRM of the control standards with maximum resolution of %methylation at <0.1% - The high annealing temperature enables maximum resolution of the very low percentage standards, anticipating the low levels of tumour derived methylated *MGMT* promoter DNA expected in the cfDNA.

b, Example control calibration curves in the routine Laboratory assay – Here the annealing temperature is lower, to get resolution of the standards to better fit the methylation ranges seen in routine analysis of the *MGMT* promoter methylation in glioma FFPE biopsy samples. Under these conditions, this assay has a limit of detection of 5%. The assay as it stands would not be sufficiently sensitive for detection of ctDNA methylation signal in cfDNA.

Due to covid lockdown constraints, this assay was only performed once.

The HRM analysis also mirrored MethylDetect protocol with an optimised resolution of the methylated DNA, favouring the discrimination of the very low %methylated samples (see Figure 4.6a). The current in-house assay has a limit of detection of 5% (illustrated in Figure 4.6b), so would

be insufficiently sensitive to pick up the very low levels of glioma ctDNA expected as a sub-fraction of the cfDNA. Concordance of the in-house adapted protocol with that of the MethylDetect preliminary work up, showed the assay to have appropriate analytical utility.

**Application of the new MGMT promoter methylation test protocol to a pilot set of glioma cfDNA samples**

The new protocol was used to test the 5 glioma cases with the highest cfDNA where the diagnostic biopsy sample had shown MGMT promoter methylation. The EpiTect unconverted unmethylated human DNA control sample was included for independent evaluation of the bisulphite conversion and subsequent melt assay, given the sub-optimal input of cfDNA in both steps. DNA input to the assay was estimated to be ranging from 2-6ng for the cfDNA and approximately 15ng for the control DNA, so both considerably below the recommended 50ng used in the diagnostic service assay set up.

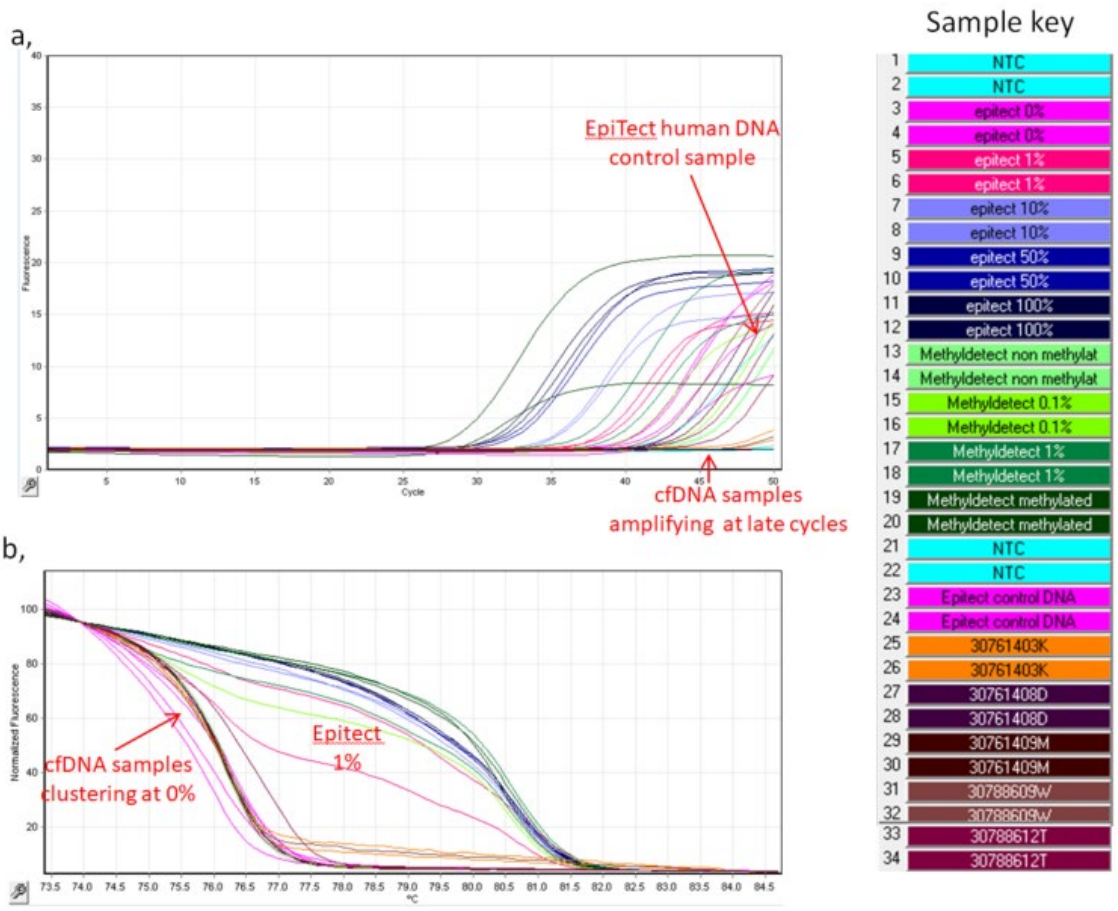



Figure 4.7

**PCR amplification and HRM of glioma cfDNA samples with 58°C annealing temperature**

5 glioma samples were used as a trial – those that were the highest cfDNA concentration and where the mirrored FFPE sample showed *MGMT* promoter methylation. The *MGMT* promoter methylation could be used as a positive glioma-specific ctDNA biomarker, standing out against the unmethylated profile that would be seen as the bulk of the normal cell derived cfDNA.

a, PCR amplification plots showing very late cycle amplification for the cfDNA samples due to the stringent PCR conditions.

b, Normalised HRM plot showing clustering of the tumour derived cfDNA samples around the 0%, unmethylated standards.

Due to covid lockdown constraints, this assay was only performed once.

Figure 4.7 (a) shows the PCR amplification plot for tumour derived cfDNA samples, the EpiTect human control DNA and the calibration standard from the two kits. The cfDNA samples and low %methylation calibration standards are amplifying very late. Rotorgene software can be used to calculate Ct values for the amplification PCR and it is usually recommended that HRM data is only reliable when Ct <30 (Rotorgene manufacturers manual recommendation). All of the samples except the 100% and 50% calibration standards failed to meet our usual acceptance criteria. The EpiTect human control DNA has a mean Ct of 39.0 and the cfDNA samples ranged from 38.4- 47.2.

This late amplification would likely be due to a combination of reasons:

- Low input DNA concentration (well below the recommended 50ng per sample)
- The fragmented nature of the cfDNA

- The stringent annealing temperature of 58°C

Under these circumstances we would normally advise caution in interpreting the HRM analysis. But the example data provided by MethylDetect also exhibited this late cycle amplification (see Appendix A5), so given there was no clear amplification in the NTC samples, the HRM plots might still be considered for evaluation. Figure 4.7 (b) shows the 58°C annealing temperature derived HRM plot. Under these settings the >1%methylation calibration standards predominantly sit together at the right hand side of the plot. The %methylation calibration standards start to separate approximately <1%. The glioma derived samples (30761403K, 30761408D, 30761409M, 30788609W, 30788612T) all sit with the 0%/unmethylated calibration standards.

**Lowering of annealing temperature to improve amplicon yield in the PCR**

The high annealing temperature and stringent amplification conditions would be one aspect of the poor yield in the PCR reaction. Therefore, the assay was repeated, reducing the annealing temperature to 55°C, which is recommended with the EpiTect kit HRM reagents protocol. The results are illustrated in Figure 4.8.

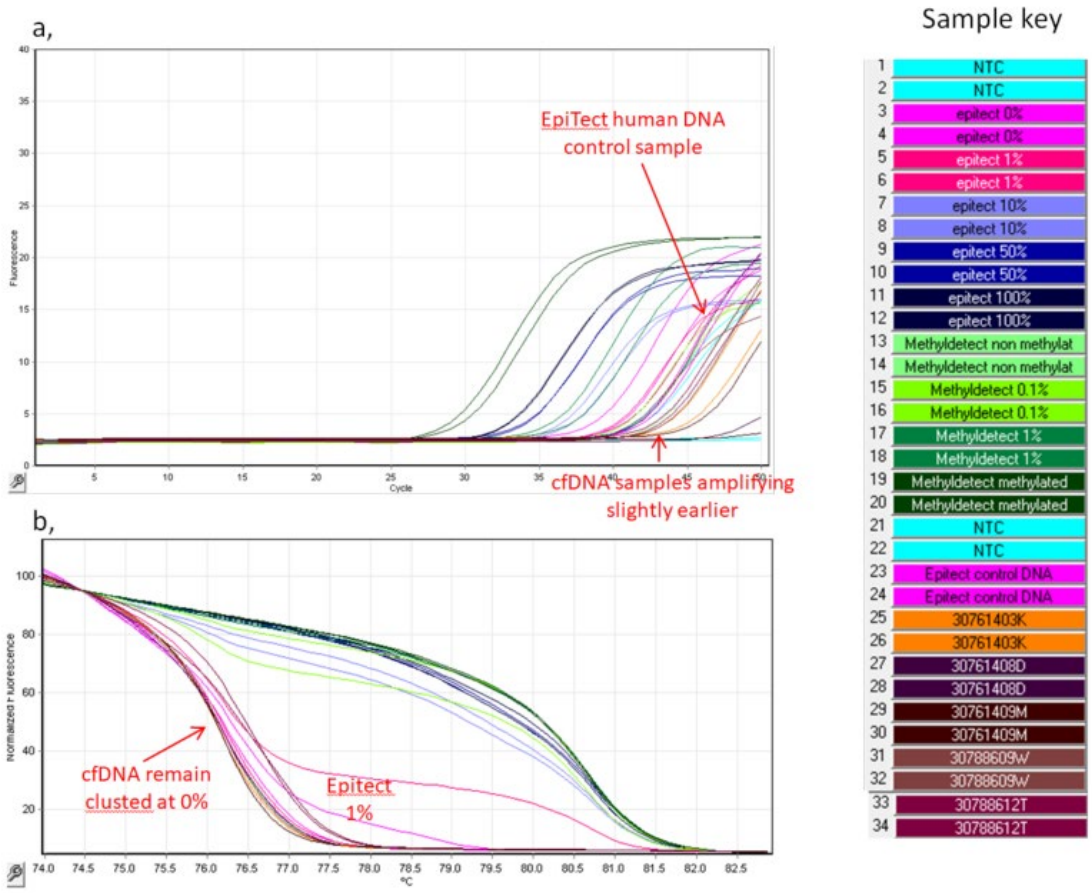



Figure 4.8
**PCR amplification and HRM of glioma cfDNA samples with 55°C annealing temperature**
a, PCR amplification plots showing earlier cycle amplification for the cfDNA samples.
b, Normalised HRM plot showing the tumour derived cfDNA samples remain clustered around the 0%, unmethylated standards.
Due to covid lockdown constraints, this assay was only performed once.

Figure 4.8 (a) shows cfDNA samples and low %methylation calibration standards did amplify slightly earlier than for the 58°C. However only the 100% and 50% calibration met the usual Ct >30 standards QC cut off. The EpiTect human control DNA has a mean Ct of 38.2 and the cfDNA samples ranged from 37.6- 42.2. Thus, reducing the annealing temperature did not substantially improve the yield of the PCR amplicon. Figure 4.8 (b) shows the 55°C annealing temperature derived HRM plot. Under these settings the >1%methylation calibration standards track lower and to the left of the plot, illustrating the reduced resolution of the lower annealing temperature for the very low %methylation samples. The glioma derived cfDNA samples remain with the 0%/unmethylated calibration standards, replicating the HRM results at the 58°C annealing temperature.

The results of the pilot cfDNA MGMT promotor methylation run showed that with amounts of template available it was not possible to generate sufficient amplicon to ensure an accurate result, in terms of current QC acceptance criteria. However, our experience is that even at low amplicon yield with a Ct >30, the HRM plot will match that of that where the Ct <30. Therefore the pilot HRM analysis may still have some validity. The glioma derived samples (30761403K, 30761408D, 30761409M, 30788609W, 30788612T) all sat with the 0%/unmethylated calibration standards. For

the paired diagnostic samples, the %methylation ranged from 10-25% to 50-100%, so the cfDNA samples did not reflect the diagnostic counterpart. The assay did not have the sensitivity to pick up the methylated signal even if there was glioma derived ctDNA in the cfDNA fraction. Tumour signal is getting 'masked' by cfDNA derived from 'normal' tissue genomic sources. However, also of note is that should there have been significant genomic contamination from lysed lymphocytes this would have amplified in the assay (Dr Tomasz Wojdacz, personal communication) so the assay results do support the cfDNA metric data, indicating good purity of cfDNA with the isolation and extraction protocol.

**1p19q co-del assessment by STR microsatellite analysis**

There were three cfDNA samples from patients diagnosed with an oligodendroglioma. Chromosomal deletion in 1p and 19q (Loss of heterozygosity) defines this tumour type and so could be used as a tumour-specific biomarker. Chromosome deletion is detected by loss of one STR allele and can be visualized by capillary electrophoresis of a multiplex PCR containing fluorescently labelled STR microsatellite markers. Two representative electropherogram plots are illustrated in Figure 4.9

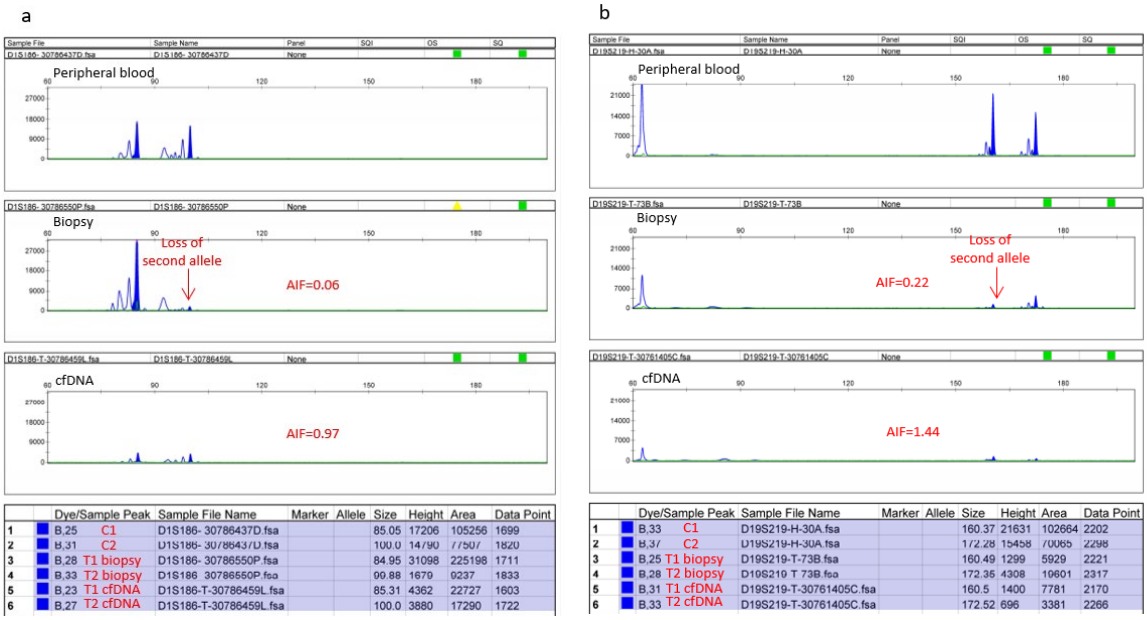



Figure 4.9

**Example electropherograms of 1p19q co-deletion analysis for paired blood, biopsy and cfDNA samples**

**(a) low bp size range (D1S186) and (b) high bp size range (D19S219)**

LOH interpretation is aided by calculation of an allelic imbalance factor (AIF), calculating the ratio of alleles for both the constitutional, germline (C) = Peripheral blood (PB) and the tumour (T) sample, with the tumour ratio divided by constitutional ratio:

$$AIF = \frac{[T_1] / [T_2]}{[C_1] / [C_2]} = \frac{[\text{peak height of smaller tumour allele}] / [\text{peak height of larger tumour allele}]}{[\text{peak height of smaller PB allele}] / [\text{peak height of larger PB allele}]}$$

A reduction of at least 50% (AIF of 0.5) in the peak height (or area) of one allele in the tumour, normalised against the retained allele in the healthy control, is used to score LOH. The peak height (or area) is a semi-quantitative proxy for the amount of each specific STR marker.
Biopsy samples show loss of a second allele (Loss of Heterozygosity).
There is no loss of second allele in the cfDNA sample, with the plot showing a similar profile to the peripheral blood (germline) sample. No loss is confirmed with the AIF >0.5.

PCR signal, as indicated by scan plot height, was reduced in the cfDNA samples compared to biopsy. The routine diagnostic assay uses 400ng FFPE-derived DNA whereas a maximum of 15ng could be used for the pilot cfDNA evaluation. PCR amplification would be expected to be suboptimal due to very low input of DNA in to the assay. However, the STR PCR reaction is not quantitative and despite the low DNA input, amplification could still be seen and electropherogram peak height was still sufficient to calculate the AIF used to confirm LOH. Also, as expected, amplification of the larger sized STR marker products (Figure 4.9b) was poorer than from a smaller STR marker reaction (Figure 4.9a). This is due to lower representation of large fragments in the cfDNA (Figure 2.6) compared to genomic DNA, from which PCR in more efficient. Figure 4.9b also illustrates a reduced amplification in the larger STR fragment biopsy analysis. This reduced efficiency is also often seen in DNA derived from FFPE material, due to the damaging nature of the fixation process, causing both chemical modification and fragmentation (Do and Dobrovic, 2015). The cfDNA analysis suggests there does

not appear to be significant contamination of high molecular weight genomic DNA that would preferentially amplify in this assay, if present in excess. So as for the MGMT analysis, the STR analysis also suggests the extracted DNA is substantially pure cfDNA.

Table 4.3
**Summary of the STR analysis for the cfDNA samples and paired biopsies with 1p19q co-deletion**

| | | 30761405C | | 30788615U | | 30786459L | |
|---|---|---|---|---|---|---|---|
| STR marker | **Allele range (bp)** | **biopsy** | **cfDNA** | **biopsy** | **cfDNA** | **biopsy** | **cfDNA** |
| D1S186 | 69-110 | no loss | germline pattern | LOH | germline pattern | LOH (plot 4.8a) | germline pattern (plot 4.8a) |
| D1S199 | 85-120 | LOH | germline pattern | NI | germline pattern | LOH | germline pattern |
| D1S266 | 69-115 | no loss | germline pattern | NI | germline pattern | NI | germline pattern |
| D1S2734 | 108-134 | LOH | germline pattern | LOH | germline pattern | NI | germline pattern |
| D19S112 | 110-145 | LOH | germline pattern | NI | germline pattern | LOH | germline pattern |
| D19S206 | 95-155 | no loss | germline pattern | LOH | germline pattern | NI | germline pattern |
| D19S219 | 160-190 | LOH (plot 4.8.b) | germline pattern (plot 4.8b) | NI | germline pattern | LOH | Minimal amplification |
| D19S412 | 89-113 | no loss | germline pattern | LOH | germline pattern | LOH | germline pattern |

NI – not informative
LOH – loss of heterozygosity

Table 4.3 summarizes the 1p19q biomarker analysis results. Bar one, all of the STR marker products were picked up in all 3 patient samples. This indicated cfDNA fragments, up to approximately 170 bp, were present and of sufficient integrity for the successful PCR and STR microsatellite analysis.

However all cfDNA profiles match those of the germline rather than pick up the profile of the tumour, showing locus specific loss of heterozygosity (LOH). Hence for these 3 samples the method was insufficiently sensitive to pick up tumour specific profile (ctDNA) within the cfDNA.

**Massively Parallel Sequencing (NGS) Analysis**

Running the single sample NGS analysis through Qiagen CLC Genomics workbench bioinformatics pipeline yielded no variant detection. The sample loaded for sequencing was under the recommended minimum DNA concentration, and the glioma ctDNA fraction within the cfDNA would be expected to be even lower, Thus, as a pilot run, in spite of the use of UMI's to facilitate

ultrasensitive variant detection, the NGS method routinely used in the laboratory was not sufficiently sensitive to pick up the variant seen in the diagnostic biopsy sample.

### 4.7 Analysis of the Glioblastoma cohort clinical outcomes data in the context of the cfDNA concentration

As a number of publications had indicated potential value in using cfDNA concentration metrics as an aid to complement imaging analysis and disease outcomes assessment (Boisselier *et al.*, 2012; Dubois *et al.*, 2014; Bagley *et al.*, 2019), the cfDNA concentration data in this study was analysed in this context. The glioma cohort was predominantly made up of patients with a diagnosis of Glioblastoma and this sub-cohort (n=32) was further analysed to investigate the relationship of cfDNA concentration to clinical outcome (Data summary in Appendix A6). Data was taken from the UHS electronic patient record (EPR) where available, or from NHS digital Spine database where not. For the purposes of statistical analysis an 'end of follow up date' was set at 16 months, the last month of a documented death within the cohort. Progression free survival (PFS) was estimated as the interval between the date of initial surgery or biopsy and the date of progression, as ascertained from letters and MDT notes in the EPR. With the difficulties of the Covid pandemic I was unable to get support with evaluating accurate timelines of PFS for UHS patients. Additionally, UHS hosts the Wessex Neurological centre and patients come from across the region for their neurosurgery. Once surgery is completed, patients are discharged to their local hospital. So, although the regional Nurse Specialists were contacted it was not possible to obtain follow up data for many discharged patients (see Appendix A6, marked yellow). Given the unreliability of the data, it was not possible to proceed with statistical analysis of the cfDNA concentration and PFS clinical outcome.

**Statistical Analysis of cfDNA concentration data and Overall Survival outcomes**

To examine a potential relationship of cfDNA concentration at the time of surgery with Overall Survival outcome, the cohort was divided in to 2 groups – high cfDNA vs. low cfDNA - based on whether a subject's cfDNA concentration fell above or below the mean. cfDNA concentrations were determined by TapeStation automated electrophoresis (Data summarised in Appendix A6). The

mean cfDNA concentration of the extracted DNA solution was 1186 pg $\mu l^{-1}$ ±1001 (SD). Mean and standard error of the mean (SEM) are illustrated in Figure 4.10

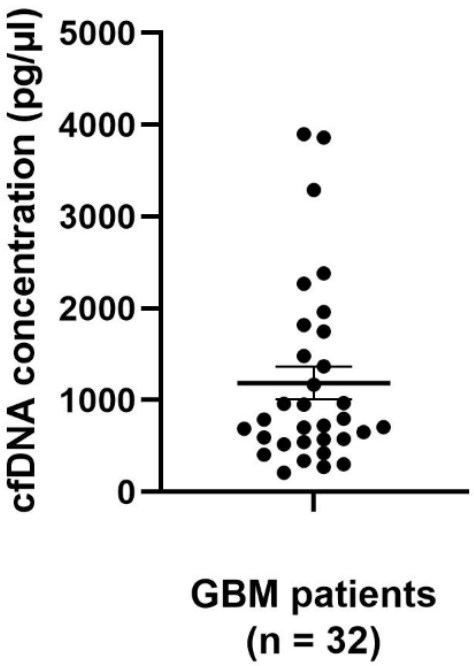



Figure 4.10
**Plasma cfDNA concentration in the glioblastoma cohort**

The mean rather than the median value was chosen as the cut off, in line with the publication of Bagley *et al*. In their study, the mean value of cfDNA was regarded to better capture the cut off point for identifying cfDNA concentration that were truly elevated, *i.e.* beyond what would be expected in a healthy population.

Comparison of OS in months, between subjects with high vs. low cfDNA was first checked for normal distribution by Shapiro-Wilk test for normality. The high cfDNA group follows a normal distribution (p=0.882; Shapiro-Wilk), the low cfDNA group significantly deviates from a normal distribution (p=0.007; Shapiro-Wilk).

As the data was not normally distributed, it was further analysed by a non-parametric test, which additionally, is robust against small sample sizes present in the current study. Data was analysed with an independent samples Mann-Whitney U test comparing survival time between groups. Comparison of survival times between low cfDNA and high cfDNA is statistically non-significant (p=0.052; 2-sided Mann-Whitney U). Despite analyses not reaching statistical significance, comparison of survival times between the two groups was on the cusp of significance (p=0.052). The lack of statistical significance may be a consequence of small sample sizes and deviation from a normal distribution. Review of the histograms of the data, to access whether the data approximates

a normal distribution (illustrated Figure 4.11) show the low cfDNA group distribution is skewed by the inclusion of all the 'alive' patients that are included in the 'end of study' numbers at 16 months.

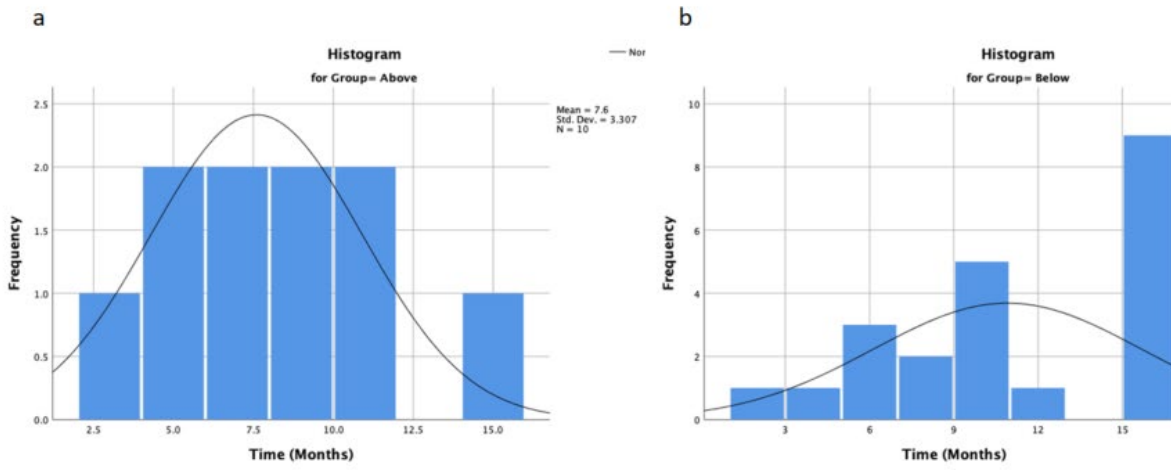



Figure 4.11
**Histograms for test of normality analysis with Overall Survival Data**
Frequency of subject data *vs.* time (months)
a, patient samples above the mean
b, patient samples below the mean

It may be that for longer period of analysis, for example up to the 20 months at which data collection was stopped, the data would follow a normal distribution and the comparison of overall survival time between groups would reach the significance threshold of p=0.05.

The test of equity of survival distributions for the high cfDNA *vs.* low cfDNA was then analysed as a Kaplan-Meier curve. This non-parametric test method can take account of some types of censored

occurrence at follow up and was the main statistical analysis used for the similar dataset of Bagley *et al.* (Bagley *et al.*, 2019). Results are illustrated in Figure 4.12.

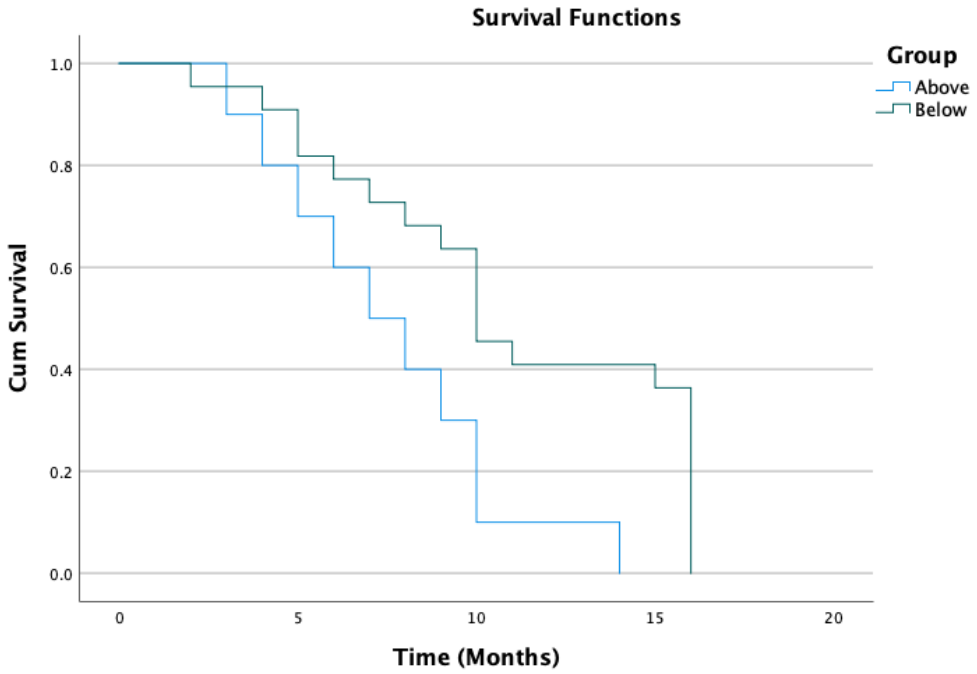



Figure 4.12
**Kaplan Meier Curve for Overall Survival after initial surgery according to the cfDNA concentration**
Curves represent:
Cases with high cfDNA, above the mean (blue)
Cases with low cfDNA, below the mean (green)
Log Rank p=0.014

SSPS Kaplan Meier analysis gives three outputs for probability significance:

| | |
|---|---|
| Log Rank (Mantel-Cox) | p=0.014 |
| Breslow (Generalized Wilcoxon) | p=0.04 |
| Tarone-Ware | p=0.024 |

All three methods show the survival distribution of the high cfDNA (above mean) concentrations and the low cfDNA (below mean) concentrations is of statistical difference. The data suggests that the high cfDNA concentration is independently associated with inferior outcome in terms of OS. The analysis here results in a higher significance for the difference in OS between high cfDNA *vs.* low cfDNA compared to the study of Bagley *et al*., where p=0.122. There were a number of points to consider in why the results were different:

- In this published study Cox proportional hazards regression was applied to adjust for relevant prognostic variables including age (<65 *vs.* >65), IDH1/2 mutational status (positive *vs.* negative, MGMT promoter methylation status (methylated *vs.* unmethylated), extent of surgical resection (gross / near total resection *vs*. biopsy only) and Karnofsky performance

status (≤60 *vs.* ≥70). It was not possible to obtain detail of for all of these correction factors in this pilot study, so there is a limitation in the result of the analysis as these correction factors may well reduce the significance of the difference found here.

- The study of Bagley *et al.* also used a quantitative PCR method to calculate the cfDNA concentration. As our method used an electrophoresis trace to calculate cfDNA concentration, there is also a possibility of differences in results was due to difference in the methods of quantification.
- In an effort to maximise extraction of ctDNA all of the plasma in the peripheral blood sample rather than a standard volume used in the Bagley study. There was a slight variation from sample to sample, in the amount of plasma used for the cfDNA extraction (estimated at ± 10%). There was thus an element of error in working with the mean cfDNA concentration of the extracted DNA for this analysis, as it was sourced from marginally different volumes of plasma and did not wholly reflect the actual plasma cfDNA concentration.

This study has replicated other studies in that the combination of specialist collection tubes and bespoke extraction kits can be used to generate analyte of high quality and yield for glioma cfDNA studies. The investigation of using an automated electrophoresis method measuring cfDNA concentration looks a promising, simple alternative to a qPCR based method. If a standardised volume of plasma is ensured, this pilot study does give grounds for a potential further full study, looking at cfDNA concentration, as a prognostic indicator for newly diagnosed glioblastoma.

# 5 Discussion

## 5.1 Pre-analytical phase review - Collecting glioma patient cfDNA and analysing quantity/quality data

Collection of plasma samples from UHS glioma patients was highly successful. Patients of interest were identified by the neurosurgical team and the surgeons and nurse specialists briefed the patients at pre-assessment clinic, giving out patient information sheets (see Appendix A2). At consent, only one patient declined and two were not approached due to impaired cognition. In spite of the short timeline in which patients were coming to terms with their diagnosis and treatment plan they were very willing to participate in the study. It had been observed by the neuro-oncologist that this group of patients and their families were remarkably amenable to partaking in research work – the dire consequences of their diseases more often than not, left a wish to be able to help in any way to understand their disease and work towards better treatments. Taking a single extra blood sample with the others needed at assessment was straightforward and thus patients did not need to take complex decisions about providing samples for the study. In total 44 samples were collected over a 6 month period. Plasma was processed either immediately, or within no more than 2 days, but then the cfDNA samples were stored frozen at -20°C. Unfortunately cfDNA assessment was not performed until approximately a year later due to conflicting commitments and then the covid pandemic. There are documented problems with long term storage of plasma (reviewed in Johansson et al., 2019) and although purified DNA is generally stable, there is a possibility DNA quality was impaired due to a period of long storage, particularly as the DNA fragments are small and the solutions were dilute.

The cfDNA concentrations for the extracted DNA solution for the cohort ranged from 223-3900 pg $\mu l^{-1}$ and the 2 lung cancer samples also fell within that range (271 and 618 pg $\mu l^{-1}$). Twelve of the first extracted cfDNA sample concentrations were also estimated by fluorometric measurements using the Qubit dsHS assay and these measurements tended to run a little higher than the Tapestation electrophoresis analysis (data not shown). Given the added purity data of the Tapestation and the described limitations of the fluorometric analysis (Ullius, 2019), cfDNA concentration assessment was confined to the electrophoretic analysis. However, the broad concordance was supportive of the electrophoretic assessment of the cfDNA as a reliable and accurate method of measuring DNA concentration.

It is not easy to directly compare this yield with the literature, as many groups dispense with this metric and go straight to biomarker linked ctDNA analysis (Zill *et al.*, 2018; Piccioni *et al.*, 2019; Bagley *et al.*, 2019). In an early glioma *IDH1* mutation study of Boisselier *et al.* (Boisselier *et al.*, 2012), plasma DNA size and concentration was measured by a similar electrophoresis system, the Agilent Bioanalyser. They reported similar results for healthy controls and a glioma cohort. The DNA ranged in size from 150 base pairs (bp) to 250 bp and the median DNA concentration in the plasma of the patients with glioma was 1.2 ng $ml^{-1}$ (with a range of 0.1- 50.3). This was not significantly different from the median DNA concentration in the plasma from healthy controls (1.2 ng $ml^{-1}$, with a range of 0.1- 6.6). Our study had cfDNA size ranges of 150 -650 bp, reflecting the DNA derived from

nucleosomes, but also from their dimers/trimers, suggesting a better integrity of cfDNA compared to the Boisselier study. Our glioma cohort extracted median cfDNA concentration was 704 pg $\mu l^{-1}$ (Appendix A4). The isolated cfDNA was eluted DNA 50 µl, so assuming an average 5 ml plasma was obtained from each sample this would represent a concentration of 7 ng $ml^{-1}$ in the originating plasma, so within the range described by Boisselier.

The glioblastoma only cohort of Bagley *et al*. used a qPCR-based method of assessment and determined a mean cfDNA concentration of 13.4 ng $ml^{-1}$ in the originating plasma (with a control group mean concentration of 6.7 ng $ml^{-1}$). Our glioblastoma only cohort would approximate to 12 ng $ml^{-1}$ in the originating plasma, so in good agreement with that of the Bagley group.

The Tapestation analysis was able to calculate the purity of the cfDNA and the method used for the study gave a calculated purity of a mean of 91% ±5.5 (SD) with a range of 77%-99%. This high percentage cfDNA purity therefore would be expected to equate to low % genomic contamination. Low genomic contamination was then also inferred from two of the biomarker tests, *MGMT* promoter methylation and 1p19q co-deletion, where leaching of DNA from lymphocytes would have been picked up as a strong amplification signal in the former and higher molecular weight amplification in the latter. The workflow for cfDNA produced high quality cfDNA of appropriate utility in downstream ctDNA biomarker studies.

## 5.2 Evaluating the utility of cfDNA as a ctDNA substrate in existing glioma molecular tests

The key requirements of an effective molecular biomarker test are that it has clinical and analytical validity and delivers clinical utility (Figure 5.1).

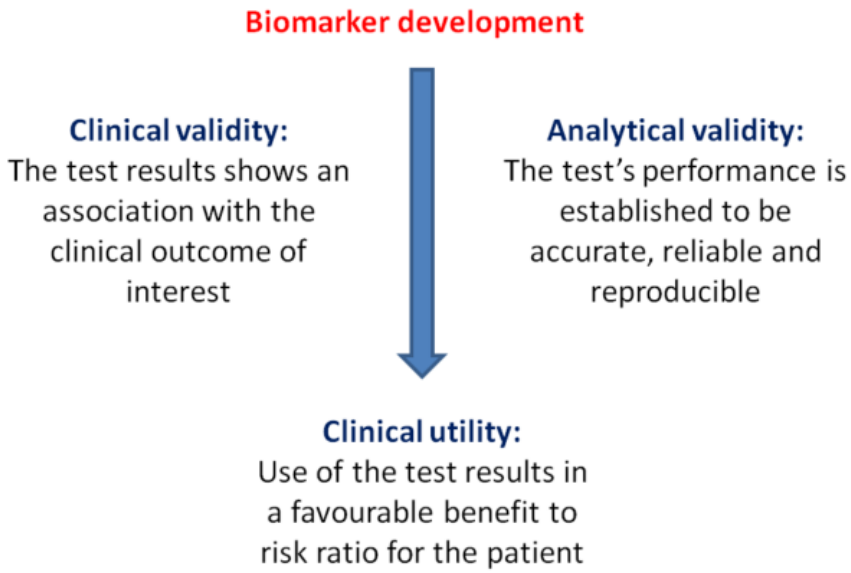



Figure 5.1
**The requirements of molecular biomarker test**

A limited lung cancer/cfDNA analysis showed that the cfDNA collection and extraction process could deliver appropriate substrate for the EGFR mutation test (Table 4.1). So the process of extraction of ctDNA *per se* could deliver template of analytical utility.

The current FFPE biopsy laboratory tests deliver clinical utility for diagnosis (*IDH1* R132H and 1p19q co-deletion) and appropriate use of chemotherapy (*MGMT* promoter methylation). For the ctDNA analysis of glioma, these results would need to be replicated from the plasma extracted cfDNA, to be a valid proxy for the biopsy test. However at this pilot test level stage, the amount of cfDNA extracted from the glioma patient plasma was consistently too low to add in cfDNA equivalent to that used in the diagnostic biopsy test. The assays all delivered PCR product, so it could be concluded there was viable cfDNA. However, for the tests that were quantifiable (*IDH1* R132H ddPCR) or semi-quantifiable (*MGMT* promoter methylation HRM), results fell below the ascribed limit of detection (LOD) for the diagnostic biopsy assay. So, although the cfDNA tests were unable to replicate the biopsy assays, it was not possible to say of this was because of an inability to detect the glioma ctDNA fraction within the cfDNA. As the analysis had failed to pass the assay LOD there was insufficient sensitivity in the assays, as they stood, to detect an expected ctDNA low signal. In a further study, it would be essential to investigate all factors that may affect the quality and quantity of the cfDNA in delivering analyte that was suitable for the diagnostic assays. In the case here, due to the Covid pandemic, it was 6-9 months before the assays could be run. Although the samples had been stored at the recommended temperature of -20°C, it was unknown if this delay would have affected the quality and utility of the ctDNA within the cfDNA fraction. In this study, the use of collection tubes and extraction protocols specifically designed for cfDNA would have ensured good assay input material. However, given the difficulties of working with glioma, if the study was continued, the pre-analytical process would need to be fully optimized to ensure maximizing amount of cfDNA extraction, through consideration of the impact of sample collection volume, times to processing and storage conditions of purified cfDNA *etc.*

The review of the existing literature (Table 2.1) showed that a number of the early studies had successfully identified glioma-specific biomarkers in the cfDNA. However, the very recent meta-analysis of Kang *et al.* (Kang, Lin and Kang, 2020) concluded that although ctDNA analysis was an effective method to detect genetic mutation status with high specificity, sensitivity was relatively moderate. The collected plasma sample for this study was similar to that of the published ones, so there might have been an expectation that we would have picked up the biomarkers. The discrepancy is likely due to the differences in methodology. The work of Boisselier *et al.* (Boisselier *et al.*, 2012) used a method that is not conventionally used in routine diagnostic laboratories. This group used a COLD PCR (co-amplification at lower denaturation temperature-PCR) which preferentially amplifies mutant DNA, and digital qPCR which is a highly sensitive method for *IDH1* R132H mutation detection. Although the ddPCR is also a highly sensitive method and should lend itself well to ctDNA analysis (Olmedillas-Lopez, Garcia-Arranz and Garcia-Olmo, 2017), we were unable to meet the LOD requirements of our in-house assay, so the ddPCR *IDH1* R132H assay may not have had the sensitivity of the COLD PCR/digital qPCR assay. Of note, the COLD PCR/digital qPCR assay has not been used since this publication, so it has not been possible to review the merits of this assay in the hands of other groups.

The MethylDetect *MGMT* promoter methylation test had been adapted to give a higher sensitivity and LOD of 0.1%. However, the amount of cfDNA extracted from the plasma sample was insufficient

for the test requirements, so in the pilot study it was only possible to use approximately 2-6ng, when 50ng was required. It was not possible to properly analyse cfDNA methylation, as amplification was sub optimal. However, our previous experience is that often the methylation-specific melt pattern can be seen at sub-optimal template loading. For example, in our diagnostic assay we load 10ng rather than 50 ng for the calibration curve, and the methylation differences can still be easily visualized (Figure 4.6b). The HRM analysis was suggestive that the methylation profile of the original biopsy could not be seen in the paired cfDNA analysis (Figures 4.7b and 4.8b). A number of the early studies had detected *MGMT* promoter methylation in cfDNA samples and we did not replicate this finding in our study. The majority of the early *MGMT* promoter methylation studies utilised a methylation specific PCR assay. This assay utilises a bisulphite modified DNA substrate, but then consists of a paired PCR analysis, with 2 PCR reactions designed to amplify from either unmethylated or methylated sequence. It is qualitative endpoint PCR, with products run out on a gel and interpretation of the bands on the gel is based on subjective judgement. A particular problem with this analysis is that of false positives; where there is no clear process for defining a cut off for negative results (Tomasz Wojdacz, personal communication). A recent review of this field by Simonelli *et al.* (Simonelli *et al.*, 2020) concluded the MS-PCR results to be very high specificity (close to 100%), but of unsatisfactory sensitivity, ranging from 11%-50%. An additional problem with the method is that cannot be used to analyse heterogeneously methylated samples (Yamashita 2019), which would be expected as glioblastoma are often diffuse. Recently, the need for a more standardised and objective method for MGMT promoter methylation has been addressed in a MS-PCR *vs* pyrosequencing analysis (Estival *et al.*, 2019). As it stands it is difficult to compare the semi-quantitative HRM derived *MGMT* results here, with the body of results in the literature where MS-PCR qualitative analysis has been used.

The over-riding problem of the biomarker investigations was that there was insufficient cfDNA obtained from the plasma samples to run our in-house assays. This was such that it was not then possible to evaluate test sensitivity, in terms of detection of an expected small ctDNA fraction within the total cfDNA. We used specialised tubes and extraction kits for isolation of cfDNA and running a parallel lung cancer patient derived samples through to EGFR testing showed the process could generate cfDNA of clinical utility. It would be difficult to expect to take more than one tube of blood from patients, so to move this glioma ctDNA testing forward it would be necessary to find a better way of using the samples as they exist. This is easier to consider with genomic variant analysis, as use of unique molecular identifiers (UMI's) can barcode to improve sensitivity, so that input amount is not so limiting (Kinde *et al.*, 2011; Schmitt *et al.*, 2012; Stahlberg *et al.*, 2017). Although UMI's are a component of the new combined genomic / transcriptomic NGS panels (eg Illumina Trusight 500), the SNV and structural variant targets do not extend to the needs of neuropathology, where generally there are few genetic biomarkers, compared to other types. Improving the sensitivity of the *MGMT* promotor gene test is particularly problematic and potentially a SNP array, such as the Illumina EPIC methylation arrays would be more sensitive. However, it remains particularly difficult to circumvent the contribution of unmethylated signal for the 'normal' tissue. This is a considerable component in a liquid biopsy, as there is thought to be little glioma ctDNA in the peripheral circulation. However it is also an issue in tissue biopsy samples, due to the heterogeneous nature of this tumour type.

The developing literature now concludes that after an initial optimism for blood based approaches to liquid biopsies biomarker levels are usually low and detectable in only a few cases and that CSF is

the best source of ctDNA for glioma cases (Miller *et al.*, 2019; Mouliere *et al.*, 2018; Simonelli *et al.*, 2020). However, obtaining CSF is also a highly invasive process and does not seem a particularly valuable alternative, given the now almost routine integration of burr hole biopsy in to the neuropathology investigations. The recent successful research work using glioma cfDNA work using highly sensitive NGS strategies to mine the small glioma ctDNA fraction of the cfDNA (Zill *et al.*, 2018; Piccioni *et al.*, 2019; Bagley *et al.*, 2019) along with developing methods to enhance the detection of circulating tumour DNA, *eg* by fragment size analysis (Underhill *et al.*, 2016; Mouliere *et al.*, 2018; Ivanov *et al.*, 2019) suggest pursuit of plasma as source of glioma ctDNA should remain a goal.

### 5.3 Consideration of the value of cfDNA and ctDNA analysis as aids in clinical treatment decisions and diagnostic glioma pathology reporting

The last phase of the projects was to investigate clinical validity and clinical utility of the ctDNA and cfDNA as a proxy for patient laboratory and clinical investigations.

As described above, the study conclusively showed that cfDNA yield was too low to meet the requirements of the existing laboratory tests and so under these conditions had no clinical utility as a proxy for tissue biopsy testing.

However, we had planned to investigate clinical validity and clinical utility of the cfDNA in the context of the other clinical investigation's glioma patients receive:

#### 1 As an aid in evaluating pseudoprogression

Follow up of glioma patients during and after standard treatment can be confounded by treatment related effects that can mimic tumour progression. Pseudoprogression, or radionecrosis, is a treatment related effect that typically occurs 3-6 months after chemoradiotherapy. It is thus difficult to diagnose tumour progression in the first 12 weeks after chemoradiation completion, unless there is an indication of new tumour (measured as enhancement) outside the radiation field or with repeat tissue diagnosis (reviewed by Raza *et al.* (Raza *et al.*, 2020)). So as image-based monitoring of disease burden in glioma is frequently confounded by treatment effects, review of circulating tumour biomarkers could theoretically augment image-based response monitoring. The meta-analysis of Raza et al. concluded whilst extensive data on biomarker dysregulation in various response categories were reported, no biomarkers ready for clinical application were identified. (Raza *et al.*, 2020). However, the metric of overall cfDNA concentration, as an aid to imaging, would be a promising candidate in this role.

#### 2 As a proxy for assessing the leakiness of the blood brain barrier

The Neuro-oncologist raised the observation that some patients seemed to respond better than others to chemotherapy and that this might be related to 'leakiness' of the BBB. We then hypothesised that measuring the ctDNA or cfDNA concentration could be a metric for quantifying this leakiness. It might also be an interesting metric for correlation with tumour enhancement signal, which is regarded as measure the breakdown of the blood brain barrier.

#### 3 As a correlate for radiographic tumour burden

Radiographic tumour burden is calculated by the neuro-oncologists for chemotherapy dosing calculations. We were interested in if cfDNA concentrations reflected the calculated size of the tumour. There is very little literature in this area with the study of Bagley et al 2019 stating that plasma cfDNA concentration did not correlate with radiographic tumour burden. Inevitably there will be a variety of factors that contribute to cfDNA release in to the circulation and volumetric tumour measurements cannot adequately address the interplay between tumour biology and cfDNA concentration. But in consideration, higher cfDNA concentrations may relate to tumour cellularity, perfusion, BBB disruption and vessel size (Bagley *et al.*, 2021).

**4 Correlate of cfDNA concentration and patient survival**

Towards the end the study, the group of Bagley *et al.* (Bagley *et al.*, 2019) published a pilot study linking cfDNA concentrations with patient overall survival and progression free survival. Some of this published work could be replicated using the data generated in this study.

Unfortunately, due to the Covid-19 pandemic, I was unable to get the clinical support I needed to accurately address points 1-3. However, I was able to carry out some pilot investigations for point 4 – looking at a correlate of the cfDNA concentration of with the glioblastoma cohort subset, in line with the published study. The cohort of 32 glioblastoma patients was split in to 2 groups for above and below the mean cfDNA concentration of the extracted sample. This analysis showed a significant difference in OS between the above and the below average result, this being of higher significance than for the similar study of Bagley study. The Bagley study had used a standardized plasma volume for the cfDNA extraction, however, in an effort to maximise the cfDNA yield for the biomarker analysis, all of the plasma was used, and this did vary from sample to sample. Some variability in plasma volume, once spun, might marginally affect final concentration of extracted cfDNA concentration and would not fully reflective of the concentrating originating in the circulating plasma. The variation was approximately ±10% and would affect the numerical statistical difference between the above and below average groups. But with a greater than 3-fold difference in DNA concentrations above and below this mean, the small difference in plasma extraction volume should not have reduced the outcomes considerably. The provisional analysis of the data here suggests that the high cfDNA concentration was independently associated with inferior outcome in terms of OS. However this pilot study would need to be replicated in a cohort of at least similar size, and with a standardized volume of originating plasma, for this finding to be fully validated.

Subsequent to the submission of this thesis, a number of other papers were published where studies concluded measurements of glioma cfDNA concentrations were of potential clinical utility. In a follow up paper to their pilot study, the group of Bagley *et al.* showed that in an *IDH*-wildtype glioblastoma cohort, a post-hoc analysis of change in cfDNA post-chemoradiotherapy compared to pre-surgery showed increasing cfDNA concentration was associated with worse progression free survival and overall survival (Bagley *et al.*, 2021). This consolidates the finding here, the potential value of cfDNA concentrations as a promising biomarker, enabling more accurate estimates of survival and effective clinical stratification. From the same group, Nabavizadeh *et al.* showed imaging and histopathologic correlates of cfDNA concentration with newly diagnosed glioblastoma. In patients with treatment-naïve glioblastoma, tumour volume with elevated metrics of BBB disruption was independently associated with higher cfDNA concentration (Nabavizadeh *et al.*, 2020). The work of Fontanilles *et al*. further confirmed patients with newly diagnosed glioblastoma

had elevated mean baseline cfDNA concentration, compared to a control group (19.4 ng $ml^{-1}$ *vs* 5.6 ng $ml^{-1}$). Additionally, patients with progressive disease had a significant increase in cfDNA concentration from pre-radiotherapy/temozolomide treatment to point of disease progression, compared to patients where there was no progression (Fontanilles *et al.*, 2020). As mentioned above, the diagnosis of disease recurrence is often challenging due to the confounding pseudoprogression. They reiterate the point that a reliable definition of patients with progressive disease and non-progressive disease is required for clinical decision making and evaluating response within clinical trials; and that cfDNA represents a promising tool in management of these patients.

There is continual recognition of the technical and biological problems with cell-free DNA technologies for the analysis of brain cancers. However, with the combined use of alternative bio-fluids, tumour-guided sequencing, epigenomic and fragmentomic methods, it will hopefully be possible to reap the gains of using liquid biopsy in glioma testing and treatment (Mair and Mouliere, 2021).

# 6 Conclusions

This study showed that a new protocol for cfDNA extraction could be successfully introduced to a diagnostic NHS laboratory, generating high quality cell free DNA that had analytical validity, clinical validity and clinical utility. The work has set the department up to move forward with working with this exciting new analyte, with its prospects for improving patient care; with less invasive means of diagnoses and avenues for post treatment monitoring of disease.

However, the yields for cfDNA were too low to meet the requirements of the standard laboratory molecular tests for *IDH* mutation and 1p19q co-deletion used for diagnosis, or the *MGMT* promotor methylation testing used for chemotherapy treatment decisions. Input DNA was insufficient to reach the limit of detection for the tests and so it was not possible to define assay sensitivity, and whether very low levels of circulating tumour DNA could be picked up as a component of the cfDNA. To have the required analytical and clinical utility, a testing strategy that significantly improved sensitivity (e.g. molecular barcoding) and could off-set the very low levels of glioma ctDNA expected in the plasma would be required for this type of analysis. So in spite of the considerable advantages to glioma plasma molecular testing, this is currently not possible in the routine diagnostic environment.

However, within the limitations of the testing strategy, the work did find an interesting correlate where high cfDNA concentration was independently associated with inferior outcome in terms of OS. This work would need to be repeated in a second cohort for verification. But given the simplicity of obtaining this quantifiable metric, there are grounds for further analysis, not only with survival outcomes, but also for correlation with the clinical assessment of tumour burden, BBB integrity and disease pseudoprogression. The pursuit of cfDNA as an analyte for neuropathological investigations and to augment image-based treatment response monitoring, remains a worthy and valuable goal for improved patient care.

# Appendices

A1 Patient Consent Form

# Research Study Participant Consent Form

**Title of Project:** Use of Circulating Tumour DNA for Genetic Tests in Brain Tumours
**Contact Telephone number:** 02381-206638
**Patient name:** ……
**Patient date of birth:** ……..

**To confirm agreement with each of the statements below, please initial the box**

| | | |
|---|---|---|
| 1 | I have read and understood the participant information sheet 'Can we use a blood sample for the genetic testing required in brain tumour diagnosis ?' Version: ..... Date: ……..) and have had the opportunity to ask questions. | |
| 2 | **Sample collection**<br>I agree to give a sample of blood for this project. | |
| 3 | **Use of samples**<br>I agree that my donated sample can be used to collect circulating tumour DNA (ctDNA) and that this ctDNA can be used in standard and developing genetic tests used in brain tumour diagnostics. | |
| 4 | **Cross checking ctDNA results with existing biopsy findings**<br>I agree to allow the comparison of ctDNA results with those in my existing records, understanding that this analysis will be anonymised, so my personal identification remains protected. | |
| 5 | **Data storage**<br>I understand, and agree to the anonymised data relating to my donated sample being stored electronically, within the hospital computer systems. | |
| 6 | I understand my participation is completely voluntary and I may withdraw at any time by contacting the research team. | |

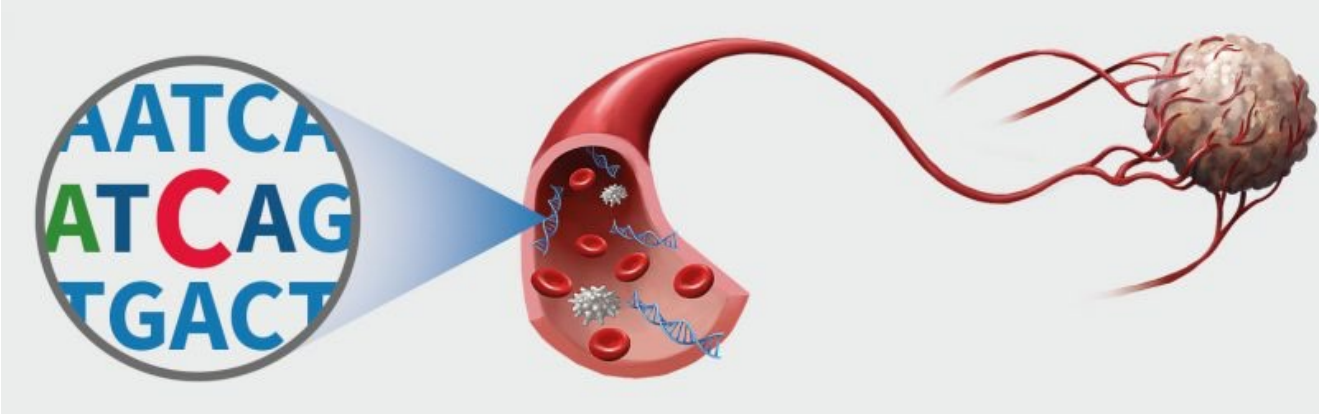
Name of volunteer……
Date…… Signature……
Name of person taking consent……
Date…… Signature……

**When completed:**
1 (original) to be kept in the research project records.
1 copy for the participant

University Hospital Southampton NHS
NHS Foundation Trust

**Appendix A2**

# Can we use a Blood Sample for the Genetic Testing required in Brain Cancer Diagnosis ?
# Research Study Patient Information Sheet




## Introduction to our study

Genetic tests are an important part of helping patients with brain tumours. They help with diagnosing the disease accurately, understanding what the outcome might be and working out the best treatment for the patient.
Tests are normally carried out on the brain biopsy sample, taken at the time of surgery.
In some other cancers it has been found that tiny pieces of DNA (circulating tumour DNA) are shed from the tumour into the bloodstream and these can be used to carry out genetic tests, instead of always requiring a biopsy sample. This means we may be able to use less invasive tests, and sometimes offer the chance to follow up in disease monitoring, without the need for further biopsy.
In a brand new pilot study, we would like to see if we can pick up this circulating tumour DNA (ctDNA) in the blood of our brain tumour patients. But it will be difficult. The blood-brain barrier stops substances passing between the brain and the rest of the body. So far, scientists have found different amounts of ctDNA are detected in brain tumour patients.
University Hospital Southampton (UHS) Molecular Pathology carries out a number of genetic tests for our brain tumour patients and we have some potential new ones that may be even more accurate. So we are in a good place to work out if brain tumour ctDNA is present in the blood.

## What would I be asked to do ?

Whilst you are having your routine blood tests, before surgery, we will ask you to give one extra blood sample. This will be collected in to a special tube that helps preserve ctDNA (taken during the same blood test)
You would only be required to give one blood sample and there would be no further follow up.

## What will we do ?

We will use standard laboratory tests to see if we can isolate the brain tumour ctDNA.
If we find any ctDNA, we will run it through two of the tests we often do as part of the routine work up, but also a new test we are trying, which is particularly accurate. This test uses droplet digital PCR (ddPCR) and is the method often successfully used for detecting ctDNA in other tumour types.
At the end of the research project all samples will be disposed.

## Do I benefit in any way ?

This is a pilot project – a proof of principle study.
There would be no personal benefit from the study, but we hope to add to the evidence of whether the idea of using ctDNA 'liquid biopsies' is possible in brain tumour testing. So it may help many other patients in the future.

## Are there any risks to me ?

There are no risks to you.
The study just requires one additional tube of blood, that will be taken at the same time as your other bloods by your day of surgery care nurse.

## This is genetic information – is my data safe ?

Our tests do use genetic material, but only DNA specifically from the tumour. We will **not** analyse your genomic DNA – that which you inherited from your parents and that you would pass on to your children.
To determine if the ctDNA test results are accurate, we do need to compare with the results that have been obtained from your brain biopsy, as part of your routine care. The data will be anonymised, so that we can see the biopsy test results, but cannot see your full patient details. We will arrange for a state registered UHS scientist, unconnected to the project, to access to participants' medical records and generate the anonymised brain biopsy results.

## We will comply with General Data Protection Regulations (GDPR)

University Hospital Southampton (UHS) NHS Foundation Trust is the sponsor for this study based in the United Kingdom. We will be using information from your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. UHS NHS Foundation Trust will keep identifiable information about you for 3 years after the study has finished.
Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
UHS NHS Foundation Trust will use your name, and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from UHS NHS Foundation Trust Research and Development (R&D) and regulatory organisations may look at your medical and research records to check the accuracy of the research study. UHS NHS Foundation Trust will pass these details to UHS NHS Foundation Trust R&D, along with the information collected from your medical records. The only people in UHS NHS Foundation Trust R&D who will have access to information that identifies you will be people who need to contact you to audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name or contact details.
UHS NHS Foundation Trust will keep identifiable information about you from this study for 3 years after the study has finished.
You can find out more about how we use your information by contacting Dr Ros Ganderton, UHS Molecular Pathology, at the below contact information.

## Do I have to say yes ?

No – it is entirely up to you whether you agree to allow your blood sample to be used for this study.

## What happens if I change my mind ?

You do not have to give a reason if you want to withdraw; it is completely your choice.
You can change you mind at any time by contacting Dr Ros Ganderton, UHS Molecular Pathology, at the below contact information

## What else do I need to do now ?

If you have received enough information and have decided that you would like to agree, then you will be asked to sign a consent from. This would be done on your day of surgery, when you are having your other blood tests done.
If you do not agree, then you need to do nothing,

## Contact information

If you require any further information or have any questions, please contact Dr Ros Ganderton.
Email: UHS.MolecularPathology@nhs.net
Phone: 02381-206638
Address: UHS Molecular Pathology, MP 225
University Hospital Southampton, NHS Foundation Trust
Southampton, SO16 6YD

## Appendix A3

## Demographics, Histopathology and Molecular Pathology Results for the full glioma cohort

| ID no. | Diagnosis | Grade | gender | age at diagnosis | ATRX | Ki67 | IDH | 1p19q codel | MGMT |
|---|---|---|---|---|---|---|---|---|---|
| 30761400J | Glioblastoma | IV | M | 56 | retained | moderate, focally high | IDH (R132H) non-mutated | NT | Yes, 10-25% |
| 30761401S | Glioblastoma | IV | M | 45 | retained | moderate | IDH (R132H) non-mutated | NT | unmethylated |
| 30761402B | Glioblastoma | IV | F | 68 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30761403K | Glioblastoma | IV | F | 68 | retained | low | IDH (R132H) non-mutated | NT | Yes, 10-25% |
| 30761404T | infiltrating high grade glioma | IV | F | 76 | NT | low | IDH (R132H) non-mutated | NT | NT |
| 30761405C | anaplastic oligodendroglioma | III | M | 57 | retained | focally high | IDH (R132H) +ve | 1p19q codel | Yes, 50% |
| 30761406L | glioblastoma (epitheloid subtype) | | M | 76 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30761407U | anaplastic astrocytoma | III | M | 57 | retained | low-moderate | IDH (R132H) +ve | no loss | Yes, 10-25% |
| 30761408D | glioblastoma | IV | F | 66 | retained | high | IDH (R132H) non-mutated | NT | Yes 25-50% |
| 30761409M | glioblastoma | IV | F | 70 | retained | moderate | IDH (R132H) non-mutated | NT | Yes, 10-25% |
| 30761410R | glioblastoma | IV | M | 64 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30761411A | likely glioblastoma | IV | M | 64 | no stain | no significant labelling | no stain | NT | NT (necrotic) |
| 30761412J | glioblastoma | IV | M | 77 | retained | moderate | IDH (R132H) non-mutated | NT | Yes, 25-50% |
| 30761413S | glioblastoma | IV | M | 66 | retained | moderate | IDH (R132H) non-mutated | NT | unmethylated |
| 30761414B | glioblastoma | IV | M | 58 | retained | moderate | IDH (R132H) non-mutated | NT | unmethylated |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 30761415K | glioblastoma | IV | M | 66 | retained | moderate-high | IDH (R132H) non-mutated | NT | unmethylated |
| 30761419U | glioblastoma | IV | M | 62 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30761420Y | glioblastoma | IV | M | 55 | retained | moderate | IDH (R132H) non-mutated | NT | unmethylated |
| 30788600T | glioblastoma | IV | F | 50 | retained | high | IDH1/ IDH2 -ve | NT | unmethylated |
| 30788601C | glioblastoma | IV | M | 71 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30788602L | glioblastoma | IV | M | 55 | retained | high | IDH (R132H) non-mutated (equivocal) | NT | unmethylated |
| 30788603U | glioblastoma | IV | F | 53 | retained | moderate | IDH (R132H) non-mutated | NT | Yes - 10% |
| 30788604D | anaplastic astrocytoma | III | M | 79 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30788605M | glioblastoma | IV | M | 57 | retained | moderate | IDH (R132H) non-mutated | NT | unmethylated |
| 30788606V | glioblastoma | IV | M | 80 | NT | high | IDH (R132H) non-mutated | NT | NT (too little tissue) |
| 30788607E | anaplastic astrocytoma | III | M | 50 | retained | moderate | IDH (R132H) non-mutated | no loss | unmethylated |
| 30788608N | glioblastoma | IV | F | 44 | retained | high | no IDH mutations (UCL sequencing) | NT | Yes, 25% |
| 30788609W | glioblastoma | IV | F | 67 | retained | high | IDH (R132H) non-mutated | NT | Yes, 50-100% |
| 30788610B | glioblastoma | IV | M | 66 | retained | high | IDH (R132H) non-mutated | NT | unmethylated |
| 30788611K | diffuse midline glioma | IV | F | 48 | loss | moderate-high | IDH (R132H) non-mutated | NT | unmethylated |
| 30788612T | glioblastoma | IV | M | 58 | retained | moderate-high | IDH (R132H) non-mutated | NT | Yes, 25% |
| 30788613C | glioblastoma | IV | F | 69 | retained | moderate-high | IDH (R132H) non-mutated | NT | Yes, 25-50% |
| 30788614L | glioblastoma | IV | M | 68 | retained | moderate - high | IDH (R132H) non-mutated | NT | NT |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 30788615U | Oligodendroglioma | II | M | 43 | retained | low | IDH (R132H) +ve | 1p19q codel | NT |
| 30788616D | glioblastoma | IV | F | 45 | retained | high | IDH 1/ IDH2 no mutation | NT | Yes, 25% |
| 30788617M | glioblastoma | IV | M | 58 | retained | high | IDH (R132H) non-mutated | NT | Yes. 5-10% |
| 30788618V | glioblastoma | IV | M | 58 | retained | moderate | IDH (R132H) non-mutated | | unmethylated |
| 30788619E | glioblastoma | IV | M | 70 | retained | moderate | IDH (R132H) non-mutated | | unmethylated |
| 30786459L | anaplastic oligodendroglioma | III | M | 38 | retained | focally high | IDH2 (R172K) +ve | 1p19q codel | NT |
| 30786460Q | glioblastoma | IV | M | 70 | retained | moderate-high | IDH (R132H) non-mutated equivocal | NT | Yes, 25-50% |
| 30786461Y | glioblastoma | IV | M | 66 | retained | focally high | IDH (R132H) non-mutated | | Yes, 25-50% |

## Appendix A4

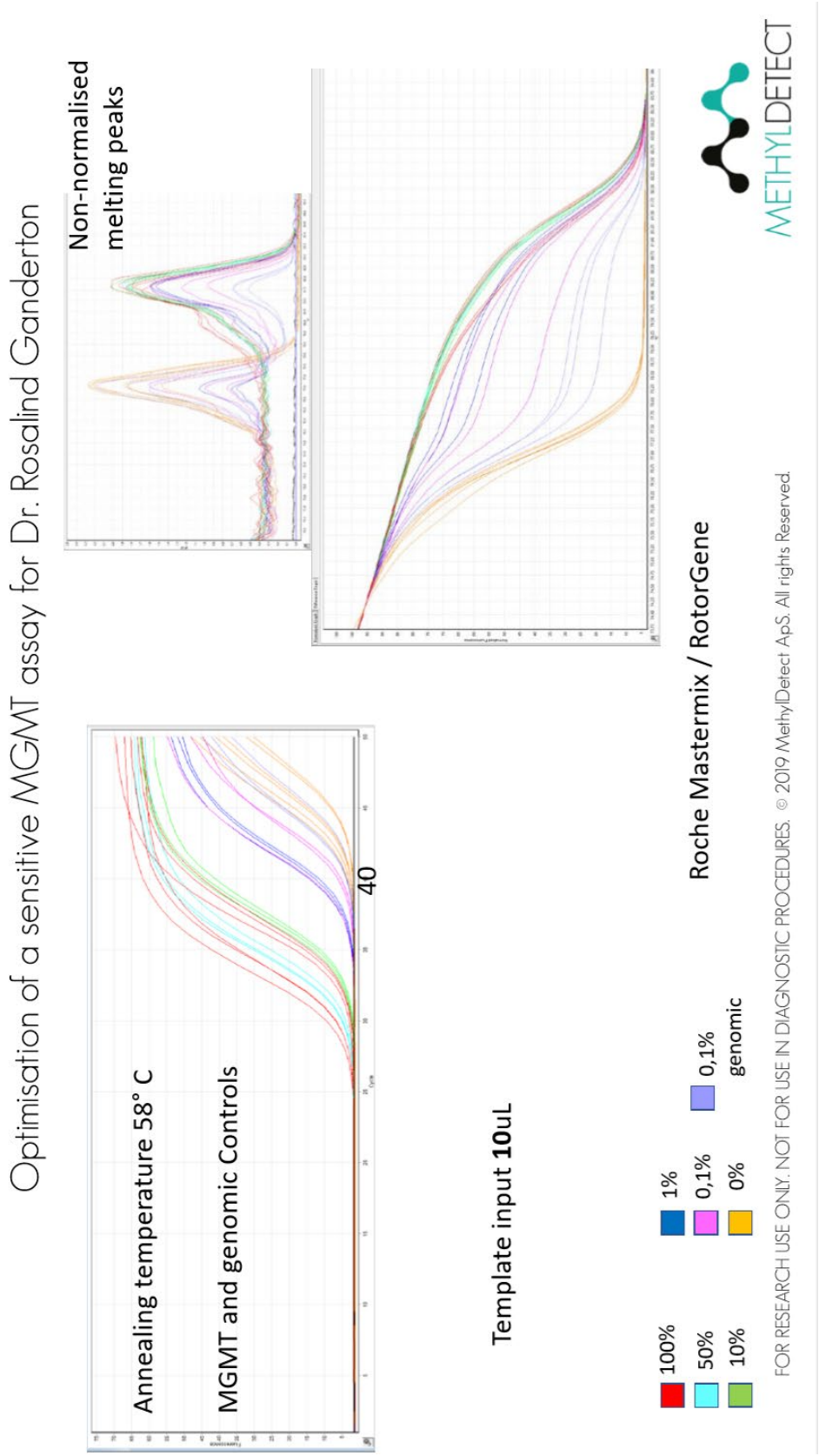
## cf DNA metrics for the Glioma Cohort

| Lab no. | Diagnosis | Grade | tapestation metrics | |
|---|---|---|---|---|
| | | | pg/ul | %cfDNA purity |
| 30761400J | Glioblastoma | IV | overload | 0 |
| 30761401S | Glioblastoma | IV | overload | 2 |
| 30761402B | Glioblastoma | IV | 790 | 87 |
| 30761403K | Glioblastoma | IV | 3860 | 96 |
| 30761404T | infiltrating high grade glioma | IV | 545 | 90 |
| 30761405C | anaplastic oligodendroglioma | III | 458 | 81 |
| 30761406L | glioblastoma (epitheloid subtype) | | 968 | 93 |
| 30761407U | anaplastic astrocytoma | III | 294 | 87 |
| 30761408D | glioblastoma | IV | 3290 | 92 |
| 30761409M | glioblastoma | IV | 1820 | 92 |
| 30761410R | glioblastoma | IV | 423 | 90 |
| 30761411A | likely glioblastoma | IV | 651 | 77 |
| 30761412J | glioblastoma | IV | 592 | 93 |
| 30761413S | glioblastoma | IV | 573 | 75 |
| 30761414B | glioblastoma | IV | 691 | 93 |
| 30761415K | glioblastoma | IV | 1960 | 92 |
| 30761419U | glioblastoma | IV | 575 | 84 |
| 30761420Y | glioblastoma | IV | 339 | 90 |
| 30788600T | glioblastoma | IV | 408 | 91 |
| 30788601C | glioblastoma | IV | 2270 | 93 |

| | | | | |
|---|---|---|---|---|
| 30788602L | glioblastoma | IV | 960 | 95 |
| 30788603U | glioblastoma | IV | 274 | 99 |
| 30788604D | anaplastic astrocytoma | III | 614 | 88 |
| 30788605M | glioblastoma | IV | 520 | 98 |
| 30788606V | glioblastoma | IV | 799 | 93 |
| 30788607E | anaplastic astrocytoma | III | 418 | 88 |
| 30788608N | glioblastoma | IV | 210 | 90 |
| 30788609W | glioblastoma | IV | 1170 | 96 |
| 30788610B | glioblastoma | IV | 302 | 85 |
| 30788611K | diffuse midline glioma | IV | 992 | 88 |
| 30788612T | glioblastoma | IV | 3900 | 96 |
| 30788613C | glioblastoma | IV | 704 | 92 |
| 30788614L | glioblastoma | IV | 2380 | 97 |
| 30788615U | Oligodendroglioma | II | 223 | 85 |
| 30788616D | glioblastoma | IV | 1750 | 98 |
| 30788617M | glioblastoma | IV | 705 | 87 |
| 30788618V | glioblastoma | IV | 724 | 84 |
| 30788619E | glioblastoma | IV | 1480 | 93 |
| 30786459L | anaplastic oligodendroglioma | III | 663 | 96 |
| 30786460Q | glioblastoma | IV | 1370 | 96 |
| 30786461Y | glioblastoma | IV | 954 | 91 |
| | | mean | 1067 | 91 |
| | | SD | 946.0 | 5.5 |
| | | median | 704 | 92 |

**Appendix A5**

MethylDetect assay optimization



## Appendix A5

Overall and Progression free survival data for the Glioblastoma cohort

| Lab no. | Diagnosis | Grade | Overall survival (months) | progression free survival (months) | extracted cfDNA conc (pg/ul) | Above or below mean |
|---|---|---|---|---|---|---|
| 30761402B | Glioblastoma | IV | 4 | | 790 | Below |
| 30761403K | Glioblastoma | IV | 8 | | 3860 | Above |
| 30761404T | infiltrating high grade glioma | IV | 8 | ?4 | 545 | Below |
| 30761406L | glioblastoma (epitheloid subtype) | | 5 | | 968 | Below |
| 30761408D | glioblastoma | IV | 14 | | 3290 | Above |
| 30761409M | glioblastoma | IV | 4 | | 1820 | Above |
| 30761410R | glioblastoma | IV | 10 | 7 | 423 | Below |
| 30761411A | likely glioblastoma | IV | | 20 | 651 | Below |
| 30761412J | glioblastoma | IV | 5 | 2 | 592 | Below |
| 30761413S | glioblastoma | IV | 7 | 3 | 573 | Below |
| 30761414B | glioblastoma | IV | 15 | 14 | 691 | Below |
| 30761415K | glioblastoma | IV | 10 | 8 | 1960 | Above |
| 30761419U | glioblastoma | IV | | 13 | 575 | Below |
| 30761420Y | glioblastoma | IV | 10 | | 339 | Below |
| 30788600T | glioblastoma | IV | | 3 | 408 | Below |
| 30788601C | glioblastoma | IV | 3 | n/a | 2270 | Above |
| 30788602L | glioblastoma | IV | | 10 | 960 | Below |
| 30788603U | glioblastoma | IV | 11 | 6 | 274 | Below |
| 30788605M | glioblastoma | IV | 16 | | 520 | Below |
| 30788606V | glioblastoma | IV | 10 | | 799 | Below |
| 30788608N | glioblastoma | IV | 9 | n/a | 210 | Below |
| 30788609W | glioblastoma | IV | 2 | | 1170 | Below |
| 30788610B | glioblastoma | IV | 10 | 7 | 302 | Below |
| 30788612T | glioblastoma | IV | 9 | 5 | 3900 | Above |
| 30788613C | glioblastoma | IV | | 5 | 704 | Below |
| 30788614L | glioblastoma | IV | 6 | ?6 | 2380 | Above |
| 30788616D | glioblastoma | IV | 10 | ?6 | 1750 | Above |
| 30788617M | glioblastoma | IV | | 13 | 705 | Below |
| 30788618V | glioblastoma | IV | 6 | ?4 | 724 | Below |
| 30788619E | glioblastoma | IV | 7 | | 1480 | Above |
| 30786460Q | glioblastoma | IV | 5 | | 1370 | Above |
| 30786461Y | glioblastoma | IV | | 7 | 954 | Below |
| | | | | mean= | **1186** | |
| | | | | median= | 790 | |
| | | | | ±SD | 1001 | |

Yellow boxes indicate where it was not possible to find the relevant data

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