Whole-genome copy-number profile analysis as a biomarker of relapse in melanoma

by

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Abstract

Early detection of melanoma relapse is key to maximise benefit from therapies like immune checkpoint inhibitors, and inhibitors of BRAF and MEK. We explored the utility of whole-genome copy-number profiles in circulating cell-free DNA (cfDNA), as a marker of melanoma relapse.

We adapted two published copy-number aberration scoring algorithms, the Copy Number Aberration Score (CNAS) and Extreme Copy Number Aberration Score (eCNAS), exploring their utility as a biomarker of active disease and survival in cfDNA samples from the GEMS study (Genetics and Epidemiology of Melanoma in Sheffield). We found the CNAS to be a good discriminator of active disease (odds ratio, 3.1; 95% CI, 1.5-6.2; $p = 0.002$), and CNAS above or below the 75th percentile remained a significant discriminator in multivariable analysis for active disease ($p = 0.019$, with area under ROC curve of 0.90). Mortality was higher in those with CNASs above the 75th percentile than in those with lower scores (HR, 3.4; 95% CI, 1.5-7.9; $p = 0.005$).

We then set up the MRM study (Markers of Relapse in Melanoma) and obtained serial samples for cfDNA analysis over time. High baseline CNAS scores were associated with significantly poorer survival. We examined the performance of CNAS as a biomarker of relapse, and found this to be significant predictor of relapse status at the time of blood sampling. Sensitivity/specificity analysis of logCNAS showed high specificity (83%) of a low logCNAS in correctly identifying non-relapsed status. However, the low sensitivity (58%) of this approach suggests limited ability of logCNAS to correctly identify relapsed cases. Further exploratory analyses are required to explore scoring algorithms which combine the copy-number score

with other potential biomarkers (e.g. cfDNA mutation burden) to maximise sensitivity, while preserving the high specificity provided by the logCNAS score.

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Publications

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Silva S, Teare MD, Bradford J, Brock I, Connley D, Jarratt E, Cramp H, Taylor F, Cox A, Danson S. Circulating cell-free DNA copy-number profiles as a biomarker in melanoma. NCRI conference, Liverpool, November 2016

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AUC area under curve

BEAMing beads, emulsion, amplification and magnetic

Bp base pair

BRAF proto-oncogene B-Raf, serine/threonine kinase

CAPP-Seq cancer personalised profiling by deep sequencing

CcfDNA circulating cell-free DNA

CCND1 cyclin D1 gene

CDKN2A cyclin-dependent kinase inhibitor 2A gene

CDK4 cyclin-dependent kinase 4 gene

CfDNA cell-free DNA

CGH comparative genomic hybridisation

ChIP-Seq chromatin immunoprecipitation sequencing

CI confidence interval

CNA copy number aberration

CNA kit circulating nucleic acid kit

CNAS copy number aberration score

COSMIC catalogue of somatic mutations in cancer

CT computed tomography

Ct cycle threshold

CTC circulating tumour cell

CtDNA circulating tumour DNA

CTLA4 cytotoxic T-lymphocyte associated protein 4

CV coefficient of variance

ddH_2O double-distilled water

ddPCR digital droplet polymerase chain reaction

DNA deoxyribonucleic acid

dNTPs deoxynucleotides

dsDNA double stranded DNA

dUTP 2’-deoxyuridine, 5’triphosphate

eCNAS extreme copy number aberration score

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

FFPE formalin fixed paraffin embedded

FGFR fibroblast growth factor receptor gene

FISH fluorescence in situ hybridisation

GAPDH glyceraldehyde-3-phosphate dehydrogenase gene

GWAS genome wide association studies

HR hazard ratio

Indel insertion/deletion

KIT KIT proto-oncogene receptor tyrosine kinase gene

LOH loss of heterozygosity

MAF mutant allele fraction

MEK mitogen-activated protein kinase enzyme

MET MET proto-oncogene, receptor tyrosine kinase

MYC MYC proto-oncogene, bHLH transcription factor

MYCN MYCN proto-oncogene, bHLH transcription factor

NGS next generation sequencing

NHS National Health Service

NPV negative predictive value

OR odds ratio

P53 p53 protein

PBS phosphate buffered saline

PCR polymerase chain reaction

PD1 programmed cell death 1

PDGFRA platelet derived growth factor receptor alpha gene

PI3KCA phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene

PGA score plasma genomic abnormality score

PPV positive predictive value

PTEN phosphatase and tensin homolog gene

qPCR quantitative real time polymerase chain reaction

Ratiosn standardised values of copy number ratios

RatiosnZscoreSq standardised copy number ratio Z scores

RB1 retinoblastoma-1 gene

RNA ribonucleic acid

RNA Seq ribonucleic acid sequencing

ROC receiver operating characteristic

RT-qPCR real-time quantitative polymerase chain reaction

SNP single nucleotide polymorphism

SNV single nucleotide variant

SOP standard operational procedure

TAm-Seq tagged amplicon deep sequencing

TCGA The Cancer Genome Atlas

TKI tyrosine kinase inhibitor

TP53 tumour protein p53 gene

WGA whole genome amplification

WT wild type

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Chapter 1: Introduction

1.1 Epidemiology of melanoma:

Melanoma is the most aggressive form of skin cancer, and accounts for just over 2% of all skin cancers. In 2014-2016, the incidence in the UK was 16,000 per year, making it the fifth most common cancer (UK, 2019). Worldwide, the incidence is rising. While some of this increase is explained by improvements in screening with early detection, as well as modifications in diagnostic criteria, there is a real increase attributed to changes in 'risk behaviour' related to sun & sunbed exposure (Corrie, Hategan, Fife, & Parkinson, 2014). Mortality rates in the UK have increased since the 1970s, and this is likely a reflection of the increasing incidence. There were approximately 2,400 deaths per year in 2014-2016 in the UK, which represents a 156% rise in age-standardised mortality in the last 40 years. Over 90% of melanoma lesions occur in the skin, 4% in mucosal membranes, and another 4% in the uvea.

1.2 Risk factors of melanoma:

There is overwhelming evidence that ultraviolet radiation (UVR) is the chief risk factor implicated in cutaneous melanoma. A systematic review carried out nearly twenty years ago established the causal relationship between melanoma risk and sun exposure (particularly intense, intermittent exposure in childhood & adolescence) (Elwood & Jopson, 1997). More recently, a direct mutagenic link has been shown between UVR and the pathogenesis of melanoma (Hodis et al., 2012). The classical phenotype associated with increased melanoma risk is fair/red hair, blue eyes, freckles, and pale white skin that burns without tanning (Gandini et al., 2005).

Other independent risk factors include multiple naevi (typical and atypical)(Chaudru et al., 2004; Tucker et al., 1997), family history of melanoma (Chaudru et al., 2004; Lens & Dawes, 2004), previous history of melanoma

(Bradford, Freedman, Goldstein, & Tucker, 2010; DiFronzo, Wanek, Elashoff, & Morton, 1999), and immunosuppression (Penn, 1996). Familial melanoma accounts for 10% of all cases of melanoma, and is associated with two main high-penetrance germline mutations: CDKN2A mutations (seen in up to 40% of cases of familial melanoma); and CDK4 mutations (seen in 2% of families)(A. M. Goldstein et al., 2006).

1.3 Prognosis & management of melanoma:

Surgical excision is the treatment of choice for non-metastatic disease. 5-year survival for very early stage disease (stage I) is close to 100%. For metastatic disease, however, median survival for untreated disease is 8-12 months (and only 2-3 months, on average, in patients with brain metastases), and treatment is with systemic therapies.

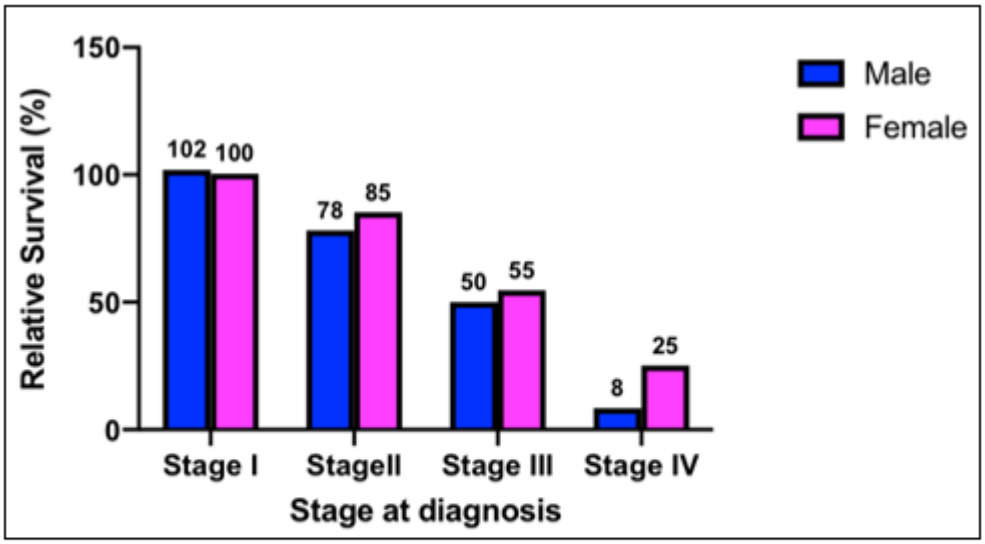


Figure 1.1: Five-year survival by stage of malignant melanoma. Adapted from Cancer Research UK Melanoma skin cancer statistics. Relative survival (shown on the y-axis) for early stage melanoma (I and II) is very good, ranging from nearly 80% in male patients (shown in blue) with stage II disease, to 100% in stage 1. In stage III disease, however, the survival drops to 50%. For stage IV disease, the 5-year survival drops even further (<10% in male patients).

Until recently, standard treatment was with dacarbazine chemotherapy, which offered a very modest response rate of 10-15%, with no proven survival benefit (Hill GJ, 1984). In the last few years, however, a CTLA-4 inhibitor, BRAF-inhibitors and PD-1 inhibitors have been introduced, which are revolutionising the way metastatic melanoma is treated (Chapman et al., 2011; Hauschild et al., 2012; Hodi et al., 2010; Postow et al., 2015; C. Robert, Long, et al., 2015; Weber et al., 2015). The CTLA-4 inhibitor, Ipilimumab, is associated with sustained responses (over three years) in 1 in 5 patients (Schadendorf et al., 2015), while significantly longer five-year survival has been observed with combination immunotherapy with the PD-1 inhibitor Nivolumab plus Ipilimumab, or Nivolumab alone, compared to Ipilimumab alone (James Larkin et al., 2019). But as the immune-mediated responses can take time to take effect, early initiation in those with significant disease-burden seems to be key; hence there is a need for improved methods to detect relapsed/metastatic disease early.

1.4 Hallmarks of cancer

A cancer cell differs fundamentally from a normal cell by virtue of certain characteristic features it possesses. Hanahan and Weinberg described eight “hallmarks of cancer” and two “enabling characteristics” (Hanahan & Weinberg, 2000, 2011). These hallmarks include sustained proliferative signalling, evasion of growth suppressors, replicative immortality, and resistance to cell death. Each of these hallmarks is not a separate unit, but, rather, they form an enormously complex intercalation of mechanisms and pathways, which give the cancer cell its unique ability to survive and proliferate (Cairney et al., 2012). The enabling characteristics (genomic instability and tumour-promoting inflammation), as the term implies, are features, which enable the acquisition of the hallmarks that ensure malignant

proliferation and survival. Figure 1.2 below is adapted from the original figure illustrated by Hanahan and Weinberg, and illustrates the additional hallmarks that have since been proposed (Hanahan, 2022).

Figure 1.2: Hallmarks of cancer. Adapted from Hanahan and Weinberg (Hanahan, 2022). In 2000, Hanahan and Weinburg suggested that cancer cells acquire a set of six distinct and complimentary functional capabilities or hallmarks (outlined in yellow in Figure 1.2), which enable tumour growth and metastatic spread. In 2011, they described two additional hallmarks (outlined in blue in the figure above), as well as two enabling characteristics (outlined in red). In 2022, Hanahan proposed 4 further prospective hallmarks (outlined in pink in the figure).

The transformation from a normal cell to a malignant one possessing these hallmarks is not the result of a single event, but is a process of successive genetic changes ranging from point mutations to chromosomal alterations (Hanahan &

Weinberg, 2000). This genetic instability endows the malignant cell with a gamut of adaptive mutations which ensures its survival (Blagosklonny, 2003). The next section summarises the main genetic and epigenetic changes associated with melanoma.

1.5 Genetic Alterations in Melanoma

Melanoma has a characteristically high rate of somatic mutations, due largely to the damaging effects of ultra-violet radiation (Pleasance et al., 2010). UVB radiation results in distinctive mutations (cytosine-to-thymine substitutions) at the 3' base of pyrimidine nucleotides; while UVA-related oxidative damage typically results in guanine-to-thymine transversions (Lo & Fisher, 2014). The main mechanism behind the development of these mutations is through the formation of photoproducts (cyclobutane pyrimidine dimers, 6-4 pyrimidine-pyrimidones, purine photo-adducts) as a result of DNA damage from UV radiation. If these photoproducts are not cleared by repair mechanisms, the resultant effect is mutation during the DNA replication process (Platz, Egyhazi, Ringborg, & Hansson, 2008).

The most comprehensive characterisation of melanoma genomic alterations to date was carried out by The Cancer Genome Atlas (TCGA), in which various molecular analyses (including whole-exome sequencing, DNA copy-number profiling, mRNA and microRNA sequencing, DNA methylation profiling and protein expression profiling) were carried out on over 300 frozen melanoma specimens (primary and metastatic tumours) in order to map out the landscape of genetic alterations seen in cutaneous melanoma ("Genomic Classification of Cutaneous Melanoma," 2015). Four main mutated genomic subtypes were identified, three of which were defined by the presence of hot-spot mutations in BRAF, NRAS, or NF1.

The fourth sub-type lacked hot-spot mutations in BRAF/NRAS/NF1, but was found to be characterised by structural re-arrangements, including copy-number changes.

Melanomas often evolve from a number of precursor lesions (e.g. melanocytic naevi or melanoma in-situ lesions), and while the sequence of genetic alterations that leads to invasive melanoma is not clearly understood, it has been established that the burden of somatic point mutations increases significantly from benign naevi to melanoma in-situ and invasive melanoma lesions. Also, while copy-number alterations are rarely seen in benign naevi and in-situ lesions, invasive melanomas exhibit an array of copy-number aberrations, affecting whole chromosome-arms or segments, or even focal amplifications of known oncogenes ("Genomic Classification of Cutaneous Melanoma," 2015; Shain et al., 2015).

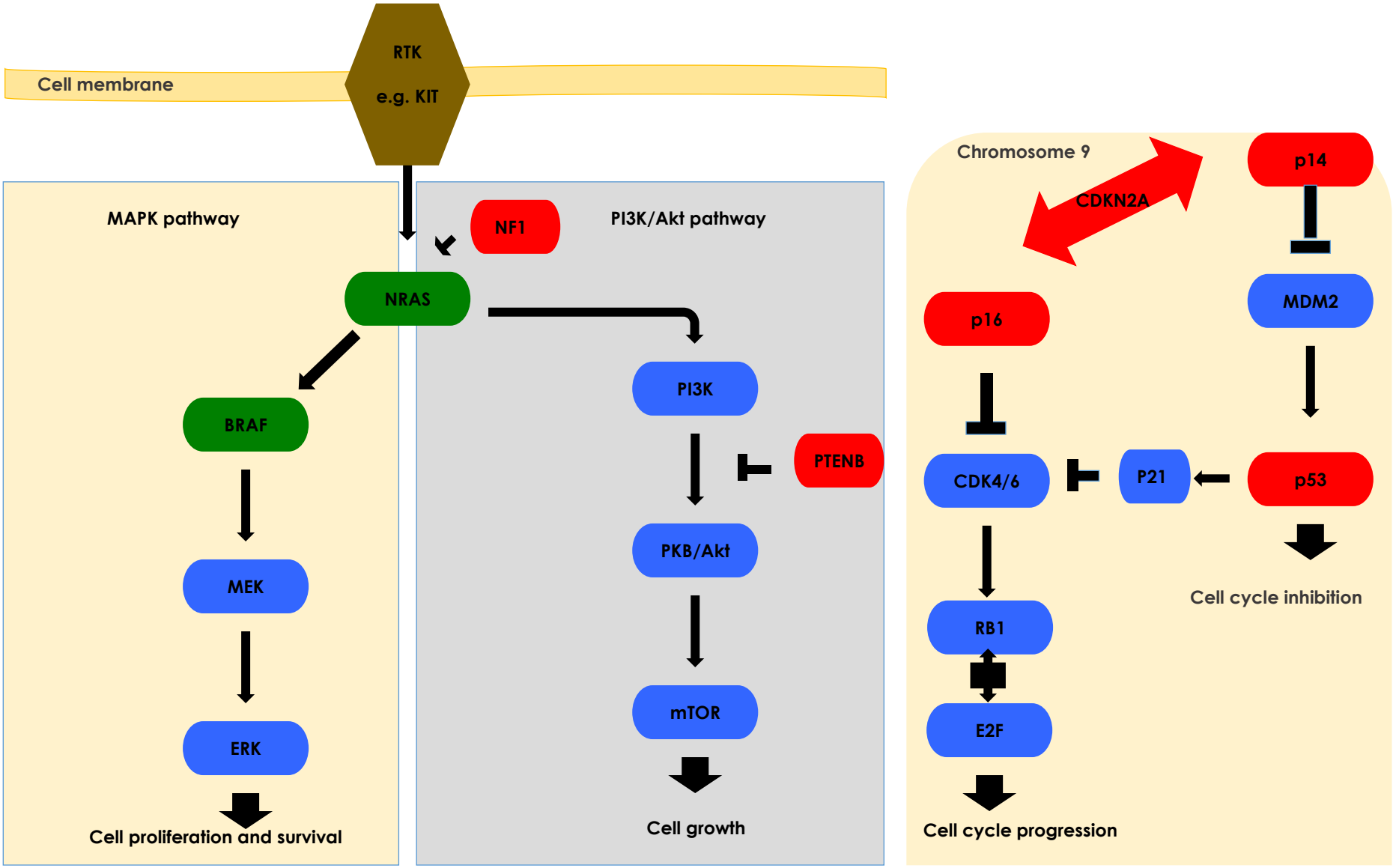


Figure 1.3: Three main molecular pathways that are dysregulated in melanoma

signifies gain of function mutations signifies loss of function mutations

The MAPK and PI3K/Akt pathways are the most common signalling pathways affected in sporadic melanoma; while CDKN2A (on chromosome 9p21) is the most commonly mutated gene in familial melanoma. The MAPK/RAS/Akt, CDKN2A and MDM2/p53 molecular pathways are altered in 91%, 69% and 19% of melanoma cases respectively ("Genomic Classification of Cutaneous Melanoma," 2015).

1.6 Gain of function mutations in melanoma

Oncogenes can be "switched on" by different means including point mutations, amplification, or chromosomal alterations that result in activation and/or increased gene expression. As these changes are dominant, only one allele needs to be affected for the malignant phenotype to be expressed.

The main gain of function mutations seen in melanoma are:

1.6.1 BRAF

This serine/threonine kinase is one of three RAF genes (K Huebner et al., 1986; K. Huebner et al., 1986; Johnson & Lapadat, 2002), which is part of the MAPK pathway (downstream of RAS). When activated, BRAF triggers phosphorylation of MEK, which leads to phosphorylation of ERK, resulting in activation of transcription factors that lead to the expression of genes which regulate cell proliferation and survival (Figure 1.3). BRAF mutations are seen in 40-50% of melanomas (Heinzerling et al., 2013; Long et al., 2011; Saroufim et al., 2014; Yaman, Akalin, & Kandiloglu, 2014). The commonest BRAF mutation (V600E, seen in 92% of BRAF mutated melanoma) occurs as a result of a single-base transversion (T-to-A), which results in the substitution of glutamic acid for valine at codon 600 of exon 15 (Davies et al., 2002). Other BRAF mutations include V600K (12%), V600R (5%) and V600M (5%) (Lovly et al., 2012). Interestingly, the BRAF V600E is seen in over 80% of naevi and primary melanomas, suggesting that this mutation plays an early role in the transformation from normal cell to malignant melanoma (Pollock et al., 2003).

Kumar et al found that the presence of the mutation was associated with longer median disease-free interval. This survival difference was not statistically significant, and the study size was relatively small (Kumar et al., 2003). Other studies

showed that the presence of the BRAF mutation may confer a poorer prognosis (Long et al., 2011; Mann et al., 2013). However, the development of BRAF-inhibitors has significantly improved survival outcomes for BRAF-mutant melanoma patients (Chapman et al., 2011; Hauschild et al., 2012), but resistance to treatment develops, and median duration of response is only 6 months.

1.6.2 N-RAS

This is one of three isoforms of RAS proteins which play a key role in cell growth, proliferation and differentiation (Lowy & Willumsen, 1993). It is mutated in 15-30% of melanomas (Ellerhorst et al., 2011; Jakob et al., 2012; Omholt, Platz, Kanter, Ringborg, & Hansson, 2003). RAS is capable of activating 2 key signalling pathways – MAPK and PI3K/AKT (Figure 1.3).

The NRAS mutation has been shown to be a predictor of shorter median overall survival in metastatic melanoma (Jakob et al., 2012). NRAS and BRAF mutations occur, with few exceptions, in a mutually exclusive manner (Platz et al., 2008).

1.6.3 Other oncogene mutations:

KIT is a transmembrane receptor tyrosine kinase. When it binds with c-KIT ligand, dimerisation of the receptor occurs, resulting in kinase activation, thus promoting cell growth and proliferation (Yarden et al., 1987). Its downstream targets include MAPK and PI3K/AKT pathways. KIT mutations are uncommon in cutaneous melanoma [2% of all skin melanomas (Kunz, 2014)], but occur more frequently in acral and mucosal melanoma (approximately 20%)(Carvajal et al., 2011).

GNAQ/GNAQ 11: these mutations are seen in 80% of uveal melanomas. They cause activation of protein kinase C, which in turn activates the MAPK pathway (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). Thus, MEK inhibitors have a role in the treatment of uveal melanoma, by targeting the MAPK pathway.

1.7 Loss of function alterations in melanoma:

Tumour suppressor genes act by regulating cell proliferation & growth, hence mechanisms that result in their loss of function can cause unchecked cellular proliferation. These mechanisms include mutations, chromosomal alterations or epigenetic alterations.

1.7.1 CDKN2A

This gene, located on 9p21, codes for 2 proteins (p14ARF and p16INK4A), which play inhibitory roles in the progression of the cell cycle (Figure 1.3) (Quelle, Zindy, Ashmun, & Sherr, 1995; Serrano, Hannon, & Beach, 1993; Weinberg, 1995). Mutations in this gene are the most frequently reported aberration (20-40%) in familial melanomas (Alisa M. Goldstein et al., 2006). This gene is also the most frequently affected tumour suppressor gene in sporadic melanoma (ranging from mutations to loci losses to epigenetic changes) (Griewank et al., 2014). A study showed that, in CDKN2A mutation carriers, the lifetime risk of developing melanoma by the age of 80 was between 58-91% (Bishop et al., 2002).

1.7.2 PTEN

This plays a negative regulatory role within the PI3K pathway (Figure 1.3). Deletion or inactivation is seen in up to 60% of melanoma cells lines, altered expression in 40%, and loss of allele in 20% of melanomas (Pollock et al., 2002).

1.7.3 NF1

This negatively regulates RAS function (Martin et al., 1990); thus, when inactivated, downstream effects include activation of MAPK, PI3K and mTOR pathways (Figure 1.3). NF1 loss can lead to resistance to BRAF inhibitors in BRAF-mutant melanomas (Maertens et al., 2013).

Neoplasms that arise from melanocytes are generally thought to be initiated by somatic mutations affecting oncogenes. These mutations are not enough, on their own, to induce the malignant process, but can initiate melanocytic naevi. Subsequently, somatic alterations which result in loss of function of tumour-suppressive mechanisms result in the development of a more malignant phenotype (Bastian, 2014).

1.8 Chromosomal alterations

These occur frequently in cutaneous melanoma, and include gains of CDK4 on chromosome 12q, gains of CCND1 on chromosome 11q, losses of CDKN2A on chromosome 9p, copy number gains in 7, 8, 6p and 1q (Bastian, LeBoit, Hamm, Brocker, & Pinkel, 1998; Gast et al., 2010). Chromothripsis occurs in around 8% of melanoma, and refers to a single catastrophic event, which results in one or more chromosomes or chromosomal arms/sub regions being shattered and then reassembled haphazardly (Hirsch et al., 2013). Chromosomal changes seen in uveal melanoma

include loss of chromosome 3 and 6q, and gains of chromosomes 6p and 8q (Ehlers, Worley, Onken, & Harbour, 2008; Prescher, Bornfeld, Horsthemke, & Becher, 1992).

1.9 Epigenetic alterations

These refer to mechanisms that influence gene expression without actually altering the sequence of DNA (J. J. Lee, Murphy, & Lian, 2014).

DNA methylation aberrations: DNA methylation, catalysed by DNA methyltransferases, results in the addition of a methyl group at the 5' position of the cytosine ring. Methylation of genes associated with cell cycle and cell death, can result in carcinogenesis (Schinke et al., 2010). CDKN2A promoter hypermethylation (which tends to reduce gene expression) was recognized in 19% of cutaneous melanoma patients in one study, and was associated with poor survival (Straume, Smeds, Kumar, Hemminki, & Akslen, 2002). Other tumour suppression genes found to be frequently hypermethylated include retinoic acid receptor – β2, RAS association domain family protein 1A, O6-methylguanine DNA methyltransferase, and apoptosis mediator death-associated protein kinase (J. J. Lee et al., 2014).

Histone modifications: Modifications such as methylation (Bachmann et al., 2006) and hypoacetylation (Ye et al., 2013)), leading to transcriptional silencing, have been described in melanoma.

Alterations in non-coding RNA/microRNA: Several non-coding RNAs (especially microRNA) have been described which regulate a variety of cellular processes (including gene silencing/transcription, and DNA methylation). Thus, through up/down regulation, these ncRNAs have either oncogenic or tumour suppressive potential (J. J. Lee et al., 2014).

1.10 Circulating cell-free DNA

As it is well established that cancers contain numerous genetic alterations (deletions, insertions, chromosomal rearrangements, translocations etc.), which are found in much lower (or zero) frequency in normal cells, these genetic alterations provide a potentially specific means of obtaining personalized information about a patient's cancer (C. Bettegowda et al., 2014).

A means of studying these genetic alterations, which is currently widely being investigated, is to analyse circulating cell-free (cfDNA), which refers to fragments of DNA which can be detected in plasma or serum, as first described in the late 1940s (Mandel & Metais, 1948). Increased concentrations of cfDNA were first demonstrated in the serum of cancer patients with advanced disease nearly forty years ago (Leon, Shapiro, Sklaroff, & Yaros, 1977). Genetic and epigenetic similarities between circulating cell-free DNA and tumour DNA is evidence that at least part of cfDNA originates from tumour (Anker, Mulcahy, Chen, & Stroun, 1999; Gormally, Caboux, Vineis, & Hainaut, 2007; Jahr et al., 2001; Sorenson et al., 1994; Vasioukhin et al., 1994). The means by which tumour-derived cfDNA enters circulation include release from apoptotic and necrotic tumour cells (Choi, Reich, & Pisetsky, 2005; Jahr et al., 2001; H. Schwarzenbach, Hoon, & Pantel, 2011), and active secretion (Stroun, Lyautey, Lederrey, Olson-Sand, & Anker, 2001). Another possibility is that these DNA fragments could be a product of lysis of circulating tumour cells or micro metastases, although this theory has been challenged (Stroun et al., 2000).

However, malignant tumours are not the only source of cfDNA, as there is evidence that other pathological processes [including inflammatory conditions (e.g. inflammatory bowel disease, pancreatitis), infections (e.g. tuberculosis, sepsis), autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosis), stroke,

coronary heart disease, trauma, surgery], can give rise to release of DNA fragments into the circulation (van der Vaart & Pretorius, 2008). cfDNA can also arise from normal cells undergoing programmed cell-death (Ziegler, Zangemeister-Wittke, & Stahel, 2002). In cancer patients, the fraction of circulating DNA which is derived from tumour ranges from as little as 0.01% (Diehl et al., 2005) to 93% (Jahr et al., 2001).

1.11 Methods of detecting cfDNA:

There are two broad stages in the analysis of cfDNA:

The pre-analytical phase, which involves blood sampling and processing, and cfDNA extraction.

The analytical phase, which includes quantification of cfDNA levels, and genomic analysis.

1.11.1 Pre-analytical phase

Although serum samples provide a higher concentration of cfDNA compared to plasma (T. H. Lee, Montalvo, Chrebtow, & Busch, 2001; Taback, O'Day, & Hoon, 2004), a significant proportion of the cfDNA in serum is thought to arise from lysis of normal white blood cells during the clotting process. Thus, plasma has been established as the preferred source of cfDNA in oncology (El Messaoudi, Rolet, Mouliere, & Thierry, 2013).

EDTA tubes are widely used for blood collection (El Messaoudi et al., 2013), as EDTA has been shown to be superior to heparin or citrate as an anticoagulant (Lam, Rainer, Chiu, & Lo, 2004). In a study of cfDNA in the field of prenatal diagnosis, EDTA collection tubes were shown to be equivalent to specialised cell-free

DNA blood collection tubes if the samples were processed immediately (Hidestrand et al., 2012).

While it is recommended that, for optimal cfDNA concentrations, samples should be processed within two hours of obtaining blood (Jung, Klotzek, Lewandowski, Fleischhacker, & Jung, 2003; Xue, Teare, Holen, Zhu, & Woll, 2009), one study showed no difference in the mean concentrations of cfDNA if samples were processed within twenty-four hours (Board et al., 2008).

Various commercial kits are available for cfDNA extraction, with Qiagen DNA blood mini kits and Qiagen circulating nucleic acid (CNA) kits most commonly used. These two kits have been found to be comparable, although some studies have found the CNA kit to be more efficient (Devonshire et al., 2014; Page et al., 2013; Repiska, Sedlackova, Szemes, Celec, & Minarik, 2013).

The lack of consensus protocols for the pre-analytical phase of cfDNA analysis makes comparison or pooling of available studies difficult (Shaw & Stebbing, 2014).

1.11.2 Analytical phase

There are two main approaches here:

Quantification of circulating cell-free DNA (ccfDNA) levels:

Quantifying total levels does not differentiate between tumour-derived cfDNA and cfDNA from other sources (B. Schmidt, Weickmann, Witt, & Fleischhacker, 2008). However, there is good evidence that ccfDNA levels are higher in those with malignant disease compared to those without, and levels are comparatively higher in advanced cancer compared to early-stage disease (Leon et al., 1977; Pinzani et al., 2011; Sozzi et al., 2003).

In a review of several studies comprising cohorts of patients with various solid tumours versus healthy controls, Van der Vaart et al found that the mean cfDNA level was 15 ng/mL of plasma in healthy patients and 137 ng/mL in cancer patients (albeit with wide discrepancies in the range of cfDNA levels between the various studies)(van der Vaart & Pretorius, 2010); while Jahr et al showed about a hundred fold increase in cfDNA in cancer patients compared with healthy volunteers (mean concentration of 219 ng/mL in cancer patients versus <2 ng/mL in healthy volunteers) (Jahr et al., 2001). The significant differences in cfDNA levels present a potential utility in clinical practice.

Typically, quantification is done following amplification of gene targets (commonly β-globin, Alu sequences, β-actin). Figure 1.4 shows some methods of quantification.

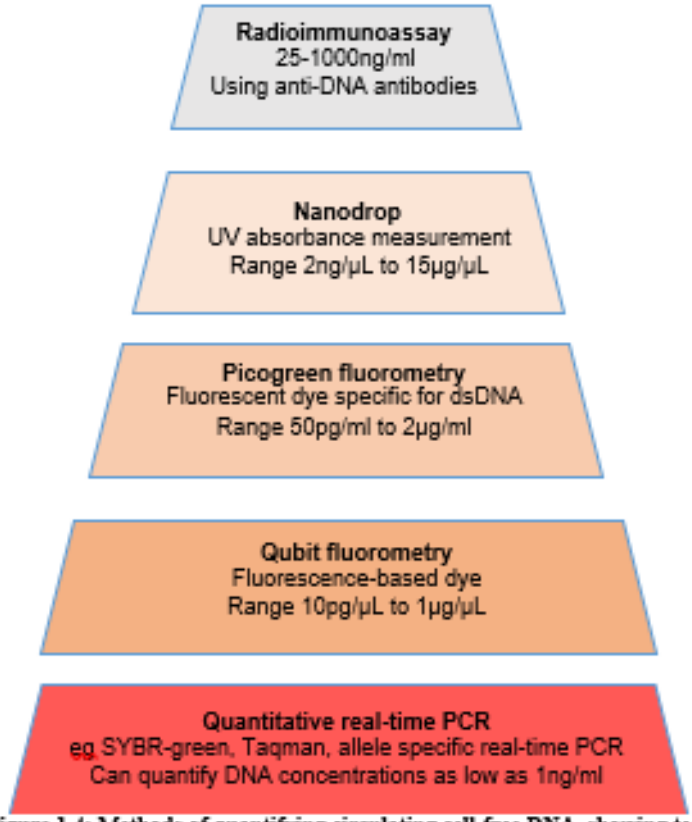


Figure 1.4: Methods of quantifying circulating cell-free DNA, showing technology used, and concentrations reached.
Main methods of quantifying DNA, with increasing accuracy of quantification (from top to bottom).

Qualitative Analysis of cfDNA:

The development of digital genomic technologies has enabled qualitative mutational/structural analysis of ccfDNA. The various technologies utilised in analysing ccfDNA has seen tremendous evolution in the last two decades. The most commonly used methods are based on either single-locus mutational assays (traditional qPCR or droplet digital PCR), or next-generation sequencing (for targeted sequencing panels or whole-genome sequencing. A selection of technologies used in the qualitative analysis of ccfDNA is summarised in Appendix A.

1.12 CcfDNA vs. CTCs

A number of studies have compared ccfDNA with circulating tumour cells in terms of their applicability as biomarker tools.

Dawson et al showed better correlation between tumour burden and ccfDNA, compared with CTCs. They also showed a greater dynamic range in ccfDNA levels (Dawson et al., 2013). Shaw et al agree that ccfDNA is a more useful biomarker, compared to CTCs (Shaw et al., 2012).

Bettegowda et al used whole-genome sequencing to identify somatic rearrangements in tumour DNA. They then used PCR assays to identify these rearrangements in blood pellets (CTCs) and plasma supernatant (ccfDNA) from patients with various malignancies. There was a greater than 50-fold difference in the number of mutant fragments in ccfDNA compared to CTCs. Also, in 81% of cases, ccfDNA was obtained when no CTCs were identified; while there were no cases where CTCs could be identified when no ccfDNA could be detected (Chetan Bettegowda et al., 2014).

1.13 Biomarkers in melanoma:

Biomarkers (in relation to cancer) refer to objective characteristics of the malignant process that can be measured accurately, reliably and reproducibly (Hayes, 2014). They can be utilized for screening, diagnosis, as prognostic/predictive markers, and in monitoring treatment response. The ideal tumour biomarker would be one that can be measured cost-effectively in a minimally invasive manner and provide reliable information (e.g. the presence of early/recurrent disease, the likely clinical course, likelihood of response to treatment) with high specificity and sensitivity. As of yet, an ideal melanoma biomarker has not been identified. The table in Appendix B summarises the main biomarkers that have been explored in melanoma. Till date, the only biomarker with prognostic value in metastatic melanoma remains the lactate dehydrogenase (LDH) enzyme, with elevated levels correlating with poorer outcomes. While other blood-based biomarker proteins, including S100B and melanoma-inhibiting activity (MIA) protein, have diagnostic and/or prognostic utility, their clinical value is limited.

1.14 Why is there a need for more reliable biomarkers?

Standard tissue biopsies can be an uncomfortable procedure for cancer patients, and carry a risk of morbidity that is not insignificant. Biopsies do not always provide the information being sought (due to sampling error, or inadequate tissue, for example), and may not be truly representative of the cancer due to tumour heterogeneity (Gerlinger et al., 2012). Therefore, there is growing recognition of the importance of developing less invasive and more effective means of obtaining information about cancers in order to personalize management. It is well established that mutation status (including BRAF mutation) changes with time, and so the ability

to track mutational status (while minimizing the risks of such interventions) is very important. It is currently difficult to accurately select those who would benefit from adjuvant therapies. If a reliable means of identifying minimally residual disease (following resection of early-stage melanomas) is discovered, it could potentially reduce relapse rates and improve survival outcomes. It is also important to be able to identify responses to therapy early enough, in order to tailor treatment approaches and avoid unnecessary toxicities in those who are not responding. In addition, mutational status as detected in cfDNA could potentially provide a non-invasive means to enrich clinical trials with the right cohort of patients likely to benefit from trial therapies.

CfDNA has the potential to fulfil all these roles, but there are limitations to its utility, including potential technical challenges e.g. in the field of early diagnosis and screening where large panels of genes would be required to be tested, and false-positives and low sensitivity would be a hurdle to be overcome (C. Bettegowda et al., 2014).

1.15 Applications of cfDNA analysis in melanoma

The key published studies investigating cfDNA in melanoma are detailed in the summary below.

Reference	Study type	Study size	Clinical application	Target of interest	Technology	Limit of detection
Tan et al, 2019 (Tan et al., 2019)	Phase III	99 cases	Predicting relapse and survival	Known mutations as identified in tumour specimen	Personalised droplet digital PCR assays	>/=1 copy of mutant DNA per reaction
Lee at al, 2019 (J. H. Lee et al., 2019)	Case series	174 cases	Predicting survival	BRAF/NRAS/KIT mutation	Droplet digital PCR	
Valpione et al, 2018 (Valpione et al., 2018)	Case series	43 cases	Surrogate biomarker for tumour burden, prognostic marker for survival	cfDNA level	TaqMan RNAseP assay	
Hoshimoto et al, 2012 (Hoshimoto et al., 2012)	Case series	56 cases	Methylated AIMI correlated with OS in stage IV patients Methylated LINE-1: no correlation	AIMI LINE-1 cfDNA	Methylation-specific PCR assay	
Hoon et al, 2014 (Hoon et al., 2014)	Case series	18	Detecting metastasis	54 genes of interest	Single-molecule digital sequencing, Guardant 360	0.1%
Santiago-Walker et al, 2015 (Santiago-Walker et al., 2016)	4 phase III studies	732 cases	Predicting response to BRAF-targeted therapy, and predicting survival	BRAF mutation	Beads, Emulsification, Amplification and Magnetics BEAMing	0.01% mutant alleles
Ashida et al, 2015 (Ashida et al., 2015)	Case study	1 case + 5 controls	Diagnosis	BRAF mutation	Competitive Allele-Specific TaqMan PCR castPCR (Life Technologies)	0.1% mutant alleles
Tsao et al, 2015 (Tsao et al., 2015)	Longitudinal case series	6 cases	Monitoring treatment response	BRAFV600E, BRAFV600K, NRASQ61H	Droplet digital PCR (Bio-Rad)	≈ 0.01% mutant alleles
Lipson et al, 2014 (Lipson et al., 2014)	Longitudinal case series	10 cases	Monitoring treatment response	BRAF, NRAS, cKIT, TERT	Beads, Emulsification, Amplification and Magnetics BEAMing (Inostics)	0.01% mutant alleles
Aung et al, 2014 (K. L. Aung et al., 2014)	Case series	208 cases	Diagnosis	BRAFV600E	Allele-specific amplification refractory mutation testing system ARMS PCR (AstraZeneca Genetics)	2% mutant alleles
Ascierto et al, 2013 (P. A. Ascierto et al., 2013)	Single-arm phase II	91 cases	Monitoring treatment response Prognosis	BRAFV600E, BRAFV600K	Beads, Emulsification, Amplification and Magnetics BEAMing (Inostics)	-
Salvianti et al, 2012 (Salvianti et al., 2012)	Case series	76 patients + 63 controls	Diagnosis	Total ccfDNA level, DNA integrity index, RASSF1A promoter methylation, BRAFV600E mutation	qPCR 7900HT Fast Instrument (Applied Biosystems) Allele-specific Taqman-based qPCR	-

Pinzani et al, 2011 (Pinzani et al., 2011)	Case series	79 patients + 34 controls	Diagnosis	DNA integrity index	qPCR 7900HT Fast Instrument (Applied Biosystems)	-
Taback et al, 2004 (B. Taback et al., 2004)	Case series	41 patients	Predicting treatment response	Microsatellite markers for loss of heterozygosity	PCR amplification and gel electrophoresis	-
Fujimoto et al, 2004 (Fujimoto, O'Day, Taback, Elashoff, & Hoon, 2004)	Case series	49 patients	Predicting treatment response	Microsatellite markers for loss of heterozygosity	PCR amplification and capillary array electrophoresis	-
Shinozaki et al, 2007 (Shinozaki et al., 2007)	Case series	103 patients + 18 controls	Prognosis	BRAF mutation	qPCR with iCycler (Bio-Rad Laboratories)	-
Mori et al, 2005 (Takuji Mori et al., 2005)	Case series	50 patients	Predicting treatment response Survival	Methylation markers	Methylation-specific PCR and capillary array electrophoresis	-
Mori et al, 2006 (T. Mori et al., 2006)	Case series	109 patients + 40 controls	Predicting treatment response Prognosis	ER-α methylation	PCR amplification and capillary array electrophoresis	-
Daniotti et al, 2007 (Daniotti et al., 2007)	Case series	41 patients + 15 controls	Diagnosis	BRAFV600E mutation	Allele-specific PCR assays	2 x 10-4 ng DNA
Taback et al, 2004 (Bret Taback et al., 2004)	Case series	10 patients	-	ccfDNA level (plasma vs. serum)	PicoGreen dsDNA quantification kit (Molecular Probes)	

Table 1.1: Key published studies investigating cfDNA in melanoma.

1.16 CfDNA as a marker of relapse/minimally residual disease

In early work, loss of heterozygosity detected in plasma was found to be associated with the presence of metastatic disease in mucosal melanoma studies (Nakamoto et al., 2008; Takagi, Nakamoto, Mizoe, & Tsujii, 2007).

Nigro et al analysed TFPI2 methylation in melanoma cell lines, as well as serum from thirty-five melanoma patients & six healthy volunteers. Using pyrosequencing, they showed that TFPI2 methylated DNA in serum was strongly associated with the presence of metastatic disease ($p < 0.01$). Using ROC curves, and a mean methylation cut-off value of 5%, the diagnostic sensitivity was calculated as 85%, with a specificity of 87%(Lo Nigro et al., 2013). This was a small study, and more prospective work needs to be done to confirm the applicability of TFPI2 methylation as a biomarker.

Tan et al identified somatic mutations in melanoma tumour tissue, and then used ddPCR assays to analyse prospectively collected plasma samples from those patients (Tan et al., 2019). The detection of ctDNA predicted for high risk of relapse at baseline, as well as post-operatively. Similarly, Lee et al found that detectable ctDNA post-operatively (by ddPCR to detect BRAF/NRAS mutations) predicted for worse disease-free interval and survival. (R. J. Lee et al., 2017).

Evidence in other tumour sites:

p53 mutation analysis in breast cancer has potential as a marker of relapse or metastatic disease (Shao, Wu, Shen, & Nguyen, 2001). Other studies have demonstrated similar potential for ccfDNA as a marker of relapse in bowel cancer (Cassinotti et al., 2013; Hao et al., 2014)

Reinert et al analysed patient-specific somatic structural variants in tumour-derived ccfDNA pre- and post-surgical resection of primary bowel tumours, and found this to have 100% specificity and sensitivity in identifying relapses, with an average lead time of 10 months on detection of recurrence compared to conventional methods (Reinert, 2015). A significant limitation of this study was that it was a retrospective study, and prospective analysis is warranted to ascertain if this high level of sensitivity/specificity is reproducible.

1.17 CfDNA as a diagnostic marker in early disease:

In the first pilot study of BRAFV600E as a biomarker for early diagnosis, Daniotti et al compared serum/plasma samples from forty-one melanoma patients with those of fifteen healthy volunteers. They showed good concordance of the BRAFV600E mutation as detected in cfDNA with tumour samples in patients with stage IV disease, but not in those with stage III disease. None of the patients with stage I/II disease tested positive in serum/plasma (although there were only four of such patients) (Daniotti et al., 2007).

Salvianti et al (Salvianti et al., 2012) proposed a “biomarker panel” consisting of cfDNA concentration, cfDNA integrity (i.e. the ratio between the concentration of a 180bp sequence and a 67bp sequence of the APP gene), and RASSF1A promoter methylation, to improve the diagnostic utility of cell-free DNA in the detection of melanoma. This panel showed good predictive capability (with an AUC of 0.945). They excluded BRAF V600E from their panel, as the 95% CI of the AUC for this variable (for stages 0, III-IV disease) included the value 0.5 (indicating a lack of predictive value).

The integrity index of ccfDNA has been studied as a marker of disease, and the 180/67 index was found to be most suitable, while a combination of 3 indexes (180/67, 306/67, 476/67) was even more sensitive in distinguishing between melanoma patients and healthy volunteers (Pinzani et al., 2011).

Evidence in other tumour sites

Shaw et al studied copy number variations (CNVs) in cfDNA of breast cancer patients, comparing pre-operative profiles with those during follow-up, as well as with healthy volunteers. Their analysis differentiated pre-operative patients from healthy volunteers, as well as pre-operative patients from those who had surgical treatment. More interestingly, though, they showed that there was evidence of CNVs, concordant with their original primary tumours, which persisted for over a decade in some patients' samples (in the absence of clinically apparent disease), suggesting minimal residual disease. This study to demonstrates the potential clinical utility of monitoring CNV profiles in ccfDNA during follow-up (Shaw et al., 2012). In another breast cancer study, Beck et al used next-generation sequencing to develop a model of repetitive elements in CNVs which could differentiate stage I patients from healthy controls with a sensitivity of 70% and specificity of 100%(Beck, Urnovitz, Mitchell, & Schutz, 2010).

1.18 CfDNA as a marker of treatment response & outcomes:

This is the area where the most prolific research investigating the utility of cfDNA in melanoma has occurred. Early evidence of correlation between cfDNA and the course of disease on treatment was shown through LOH analysis carried out on serum samples from forty-one patients with metastatic melanoma on therapy (B.

Taback et al., 2004). The presence of loss of heterozygosity was found to be significantly associated with disease progression (p=0.003), poorer response to treatment, and poorer survival outcomes (p<0.001 for PFS and p=0.02 for OS).

Shinokazi et al found that while the presence of BRAF mutation in serum prior to therapy did not correlate with response to therapy; the presence of BRAF mutation after therapy correlated with significantly worse OS (p=0.039)(Shinozaki et al., 2007) (this study predated the use of BRAF-inhibitors in BRAF-mutant patients). Similarly, in a study of BRAF mutational status in a phase II trial of the MEK1/2 inhibitor Selumetinib, in advanced melanoma, the detection of BRAF mutation in serum (taken at enrolment into the study) was not shown to be a prognostic factor for PFS in those patients with BRAF positive tumours (Board et al., 2009).

In a small longitudinal study, BRAF V600E mutations in cfDNA were tracked during BRAF-inhibitor therapy, and shown to correlate with tumour burden ($p < 0.001$ and $R = 0.742$). Additionally, lower basal concentration of the BRAF mutation was associated with longer survival (Sanmamed et al., 2015). Wisell et al found, similarly, that BRAF V600E levels in plasma (as measured using digital PCR) correlated with disease burden, suggesting its potential as a prognostic biomarker (Wisell, Amato, & Robinson, 2014). Exploratory biomarker analysis in the BREAK-2 trial (dabrafenib in metastatic melanoma) looked at BRAF V600E and V600K mutations in ccfDNA. While V600E levels showed strong correlation with tumour burden, overall response rates and PFS, no such correlation was seen with V600K (Paolo A. Ascierto et al., 2013). It is unclear why there was this difference.

Several studies have subsequently shown the utility of baseline cfDNA analysis as a predictor of response, and/or survival, to BRAF-targeted therapy (Santiago-Walker et al., 2016), (Syeda et al., 2021). Longitudinal studies tracking

cfDNA in patients on BRAF targeted therapy have shown that decreasing levels are predictive of response (Haselmann et al., 2018), (Schreuer et al., 2017).

The use of immune checkpoint inhibitors (CTLA-4 inhibitors, PD-1 and PD-L1 inhibitors) can result in improved outcomes for patients with advanced melanoma (compared to conventional chemotherapy). However, assessment of response using standard radiological approaches can be challenging, as in some cases, imaging may show apparent progression initially, and later show regression (pseudo progression). In a study of 12 patients undergoing checkpoint inhibitor treatment, blood was sampled at initiation of treatment, and 2-4 weekly during treatment. CfDNA was analysed using the BEAMing technique (and a customized PCR/NGS approach in one case). Levels were found to correlate with clinical/radiological outcomes, and in one case, cfDNA response preceded clinical response by 3 weeks (Lipson et al., 2014). Although a very small study, this study highlighted the need for larger prospective studies to investigate the utility of cfDNA as a marker of response to this group of therapeutic agents. One such prospective explorative biomarker study demonstrated the ability of cfDNA analysis to differentiate true progression from pseudo progression with 90% sensitivity and 100% specificity (J. H. Lee et al., 2018)). On-treatment analysis of dynamic cfDNA changes accurately predicts for response and survival, as demonstrated in a number of checkpoint inhibitor immunotherapy studies ((R. J. Lee et al., 2017), (Herbreteau et al., 2018), (Seremet et al., 2019).

Bidard et al analysed cfDNA in uveal melanoma, by assessing GNAQ & GNA11 mutations using a bi-PAP technique. Despite a relatively low median ccfDNA level of 4.1 ng/mL (range 0.5 – 512 ng/mL), their analysis demonstrated a prognostic correlation between ccfDNA level and PFS/OS (Bidard et al., 2014).

Hirsch et al showed an interesting distinction in the genetic aberrations between melanomas with good prognosis (i.e. patients alive after at least 10 years of follow-up, with no evidence of local/distant relapse) and those with bad prognosis (melanoma-associated death). Apart from a significant difference in the number of copy number changes in each group ($p = 0.008$), they found chromothripsis and focal copy number changes exclusively in the poor prognosis group; and whole chromosome/chromosomal arm changes in the good prognosis group (Hirsch et al., 2013).

Evidence in other tumour sites

A similar correlation between ccfDNA levels/genetic alterations and tumour burden and/or survival has been demonstrated in other tumour sites, including breast cancer (H. Schwarzenbach et al., 2012; H. Schwarzenbach, Muller, Milde-Langosch, Steinbach, & Pantel, 2011; Shao et al., 2001; J. M. Silva et al., 2002), bowel cancer (Frattini et al., 2008; Spindler, Pallisgaard, Vogelius, & Jakobsen, 2012). One group showed ccfDNA to be superior to the only established serum biomarker in metastatic breast cancer (CA-15 3) in monitoring disease burden (Dawson et al., 2013).

1.19 CfDNA as a surrogate marker for mutation testing:

CfDNA can provide an alternative means of determining BRAF mutational status in order to tailor therapy appropriately. Pinzani et al reported an 80% concordance rate in BRAF status between tumour sample and cfDNA, with a sensitivity of 97% and specificity of 83% in detecting BRAF status in circulating DNA (Pinzani et al., 2010). The concordance rate in earlier studies was lower - 59% ((Yancovitz et al., 2007) and 56%(Board et al., 2009). The differences in mutational

status between tumour samples and cfDNA could represent true molecular differences between the original tumour sample and circulating DNA, or could be explained by the fact that cfDNA is shed in such low amounts that mutations can not be detected by less sensitive assays. Oxnard et al used droplet digital PCR to develop an assay which tested BRAF V600E status, and showed a high AUC (0.94) on the ROC curve (Oxnard, 2014).

Aung et al used a 2-stage ARMS technique to test the robustness of cfDNA as a surrogate marker for BRAF mutation testing. In the first stage, they defined mutation-calling criteria, which were then validated in the second stage. They found that using specific calling criteria improved the sensitivity of BRAF V600 detection (sensitivity 52%, specificity 96%) (Kyaw L. Aung et al., 2014).

Evidence in other tumour sites

CfDNA has been shown to have potential (Oxnard, 2014) as an alternative to tissue biopsies in breast cancer (Garcia-Murillas, Lambros, & Turner, 2013; Page et al., 2011; Rothe et al., 2014); bowel cancer (Kuo, Chen, Fan, Li, & Chan, 2014; Thierry et al., 2014); and lung cancer (Goto et al., 2012; Kimura et al., 2007; Oxnard, 2014).

1.20 Copy number analysis

Copy number aberrations or variants refer to deletions, insertions, or duplications of a segment of DNA of one kilobase or larger, which result in a variable copy number as compared to a reference genome (Feuk, Carson, & Scherer, 2006; Redon et al., 2006). These aberrations are ubiquitous in cancers, and can affect entire chromosomes or segments of chromosomes (Vogelstein et al., 2013).There is an

abundance of somatic copy number alterations in malignant melanoma ("Genomic Classification of Cutaneous Melanoma," 2015; Shain et al., 2015; Wang et al., 2013). Kaufmann et al used array based CGH to study copy number alterations in melanoma cell lines, and found an average of 68 alterations per cell, compared with 8.5 alterations per cell in normal melanocytes. Copy number gains in melanoma have been shown in chromosomes 1q, 6p, 7, 8q, 17q and 20q; while losses occur in 6q, 8p, 9p, 10 and 21q (Blokx, Van Dijk, & Ruiter, 2010; Wang et al., 2013). Focal copy number alterations have also been identified in some key melanoma genes, including CDKN2A, PTEN, BRAF, NRAS, and CCND1(Gast et al., 2010). Thus, copy number analysis in ccfDNA has the great potential to be used as a biomarker tool in melanoma. The table below summaries the key copy-number aberrations seen in melanoma, with estimates of relative frequency.

	Chromosome affected (frequency shown in percentage)			
	Bastion 2003(Bastian, Olshen, LeBoit, & Pinkel, 2003) n=132	Bastion 1998(Bastian et al., 1998) n=32	Curtin 2005(Curtin et al., 2005) n=70	Jonsson 2007(Jönsson et al., 2007) n=47
Gain	1q (33%)	1q (25%)		1q
Gain		2 (13%)		
Gain	6p (37%)	6p (28%)	6p	
Gain	7p (32%)7q (32%)	7 (50%)	7	7
Gain	8q (25%)	8 (commonly q) (34%)	8q	8q
Gain	17q (24%)	17(commonly q) (13%)	17q	17q
Gain	20q (22%)	20 (commonly q) (13%)	20q	20q
Loss				4
Loss	6q (26%)	6q (28%)	6q	6q
Loss		8p (22%)	8p	
Loss	9p (64%) 9q (36%)	9p (81%)	9p	9p
Loss	10q (36%) 10p (30%)	10 (whole or q) (63%)	10	10
Loss	11q (21%)		11q	11q
Loss			13	
Loss			21q	

Table 1.2: Summary of copy-number aberrations in melanoma. Relative frequency of gains and losses (where available in the literature) is shown in brackets.

One approach in the analysis of copy number alterations in ccfDNA is to quantify copy numbers of target regions of the genome. A number of "proof-of-principle" studies have been carried out using this approach, investigating different clinical applications in various tumour sites, including detecting early- stage disease

in breast cancer (Sunami, Vu, Nguyen, Giuliano, & Hoon, 2008); as a molecular biomarker in prostate cancer (Heidi Schwarzenbach, Chun, Isbarn, Huland, & Pantel, 2011); as a prognostic marker in oesophageal cancer (Andolfo et al., 2011); and detecting minimal residual disease in breast cancer (Shaw et al., 2012). The main limitations in these studies were low recovery of ccfDNA, limited sensitivity of the technology used (mainly array-based techniques), and small sample sizes which restricts the conclusions that can be drawn from the results.

The whole-genome approach using massively-parallel next-generation sequencing, on the other hand, offers a means of detecting de-novo copy number alterations across the entire genome, (Leary et al., 2012; Schutz et al., 2015; S. Xia, C. C. Huang, et al., 2015; Xu et al., 2015)

1.21 Thesis aims and overview:

There is a clinical need for reliable biomarkers to detect melanoma relapse early in order to facilitate timely initiation of systemic therapies for improved outcomes. As yet, no blood biomarker has been established that can reliably detect relapsed disease. Whole- genome copy number analysis offers a relatively cheap approach in DNA analysis, without requiring prior knowledge of tumour mutations.

This thesis will test the hypothesis that cfDNA copy-number analysis would be a reliable and cost effective biomarker in detecting melanoma relapse.

The aim of the thesis is to test this hypothesis using copy-number profile analysis in two cohorts of melanoma patients/healthy controls – the Genetics and Epidemiology of Melanoma in Sheffield (GEMS) study and the Markers of Relapse in Melanoma (MRM) study.

The thesis is composed of three results chapters, which address the following objectives:

i. Pre-analytical testing to optimise the cfDNA extraction process; and investigation of the utility of copy-number aberration scoring algorithms as a biomarker of active disease and survival in cfDNA samples from the GEMS study (Chapter 3).

ii. Investigation of the copy-number aberration scoring algorithm as a marker of melanoma relapse in the MRM study (Chapter 4).

iii. Identification, from published literature, of chromosomes associated with copy number aberrations in cutaneous melanoma, to assess if these chromosomes of interest are similarly aberrant in cfDNA samples from the MRM study (Chapter 5)

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General equipment and consumables

Laboratory equipment	Supplier
AB104-S Balance	Mettler, Toledo
ABI 7900 Genotyping Platform	Applied biosystems
Benchtop Micro Centrifuge Heraeus Pico 17	Thermo Fisher Scientific
Benchtop Rotamixer	HATI
Benchtop Temperature Controlled Centrifuge	MSE Sanyo
Class II Microbiological Safety Cabinet	Envair
CO_2 Incubator MCO175	Sanyo
Covaris® S220 Focused-ultrasonicator	Covaris
Heating Block	Grant Boekel BBA
Ice machine	Scotsman Ice Machine
Magnet stand-96	Thermo Fisher Scientific
Nanodrop Spectrophotometer ND-1000	Labtech International
P2, P10, P20, P100, P200, P1000 Gilson Pipettes	Fisher Scientific
Power pack	Bio-Rad
QBD4 Incubator for Eppendorfs	Grant Boekel BBA
Thermal cycler: GeneAmp PCR system 96 well	Applied Biosystems
UV Sterilisation Cabinet	Bignet
Vortex Genie 2	Scientific Industries
Water Purification Unit	Lab Technologies
Water bath	Grant Instruments
Western Gel Mini Protean II Cell	Bio-Rad

Laboratory consumables	Supplier
0.2ml Microcentrifuge Tubes	Starlab
0.5ml, 1.5ml, 2ml Microcentrifuge Tubes	Fisher Scientific
1.5 ml DNA Lo-bind Microcentrifuge Tubes	Eppendorf
1.5 ml Cryovials	Scientific laboratory suppliers (SLS)
15 ml, 50ml Sterile Conical Tubes	BD Falcon
96 Well PCR Plates	Applied Biosystems
384 Well PCR Plates	Starlab
6 ml EDTA Blood Phlebotomy Tubes	BD
GIBCO Distilled DNase/Rnase Free Water	Life Technologies
Graduated 10µl Microfilter Tips	Starlab
Nitrile Powder Free Gloves	Fisher Scientific
Pipette Tips	Starlab
Plate Seals	Biorad

2.1.2 Laboratory solutions

All laboratory solutions were made up with ddH_2O, purchased from Sigma-Aldrich Co, and were of molecular biology grade.

TAE buffer (10x, pH8.0): 0.4 M Tris-base, 200mM glacial acetic acid, 10 mM EDTA (pH adjusted to 8.0).

Phosphate Buffered Saline (PBS) (Dulbeccos A, 1x): Sodium chloride 0.137M, Potassium Chloride 0.003M, Disodium Hydrogen Phosphate 0.008M, Potassium Dihydrogen Phosphate 0.0015M.

6X Sample loading buffer: Glycerol 60%, Tris-HCL pH 7.6 10mM, EDTA 60mM, Bromophenol blue 0.03%, Xylene Cyanol FF 0.03%.

2.1.3 Buffers and reagents for molecular biology techniques

DNA processing

Xylene	Fisher Scientific
Absolute Ethanol	Fisher Chemical
Isopropanol 99.5% extra pure	ACROS organics
Nuclease free distilled water	Gibco by Life Technologies
Ready to use TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) X1 buffer	Thermo Fisher Scientific
NaOH 2M	Illumina

Commercial kits purchased for DNA extraction:

QIAamp® DNA FFPE Kit	Qiagen
QIAamp® Blood Mini Kit	Qiagen
QIAamp® Circulating Nucleic Acid Kit	Qiagen
FlexiGene DNA kit	Qiagen

Commercial kits purchased for Illumina whole genome sequencing:

NEBNext® Ultra II DNA library Prep Kit	NEW ENGLAND BioLabs®Inc.
NEBNext® multiplex oligonucleotides	NEW ENGLAND BioLabs®Inc.
Dual lane HiSeq Rapid PE Flow Cell	Illumina

PCR

PCR water	
Nuclease free distilled water	Gibco by Life Technologies

2.2 Recruitment of Patients to the Markers of Relapse in Melanoma Study

The Markers of Relapse in Melanoma (MRM) study was an academic, single-centre study set up to recruit melanoma patients and healthy controls for the primary objective of obtaining longitudinal blood samples for analysis of ccfDNA. Participants consented to providing blood samples when they attended their routine melanoma follow-up clinic appointments. In addition, participants completed a questionnaire, which provided detailed epidemiological information about their lifestyle (including "sun behaviour"), medical history and family history. This study received ethical approval from Yorkshire and Humber Research Ethics committee (see Appendix C & E). The Sheffield Teaching Hospitals (STH) Research and Development department authorised the recruitment of participants from oncology, plastic surgery and dermatology clinics, as did the Cancer Clinical Trials Executive (see Appendix C).

Eligible patients included those with a diagnosis of cutaneous melanoma of any stage (either with a new diagnosis or prevalent cases). Patients were excluded if they had received chemotherapy within 4 weeks of recruitment, had a concurrent second malignancy, or were unable to give informed consent. Partners and/or siblings of patients were invited to participate as controls.

Potential participants were invited to participate in the study through a letter posted out prior to their follow-up appointment, and were provided with information leaflets. Participants were required to provide written informed consent for participation in the study and for use of their donated blood specimens and any surplus pathology tissue specimens (see Appendix C – for information leaflets, letter

of invitation, and consent forms). Each participant provided a blood sample at each follow-up appointment.

2.3 Use of blood and tumour tissue samples from the GEMS study

Blood and tumour samples, which had been collected from patients recruited to the Genetics and Epidemiology of Melanoma in Sheffield (GEMS) study (Chief Investigator Professor Sarah Danson), were utilised in this project. Patients were recruited to this study between January 2008 and June 2009, from Dermatology and Oncology outpatient clinics in Sheffield Teaching Hospitals (STH) and Barnsley District General Hospital. Eligible patients had a confirmed diagnosis of cutaneous melanoma of any stage, and no chemotherapy within the previous four weeks. They either had active non-resected disease, or had had their primary tumour and/or metastatic disease excised, with clear margins, within four weeks of recruitment. Controls were co-habiting partners of study patients or siblings of study patients. Patients could have any number of controls. All patients and controls gave informed consent to participate. The study was approved by the local research ethics committee (REC 10/H1003/72) and received STH Research Governance Approval. Plasma and buffy coats had been processed from whole blood, and stored at -80 °C. Tumour DNA had been extracted from available FFPE tumour specimens and stored -80 °C. (Page et al., 2013)

2.4 Processing MRM participants' blood samples:

From each MRM participant, up to 48 mls of blood was collected in 6 ml EDTA vacutainers, and processed within two hours of collection. At baseline, one EDTA sample of whole blood was stored at -20 °C. The remaining EDTA samples

were centrifuged at 800g for 10 minutes at 4 °C, and the resulting supernatant plasma transferred into 15 ml conical tubes. These plasma samples were centrifuged at 1600g for 10 minutes at 4 °C to pellet any cell debris. The clear plasma was then pipetted in 1ml aliquots into 1.8 ml cryovials which were stored at -80 °C. The double-spin procedure has been shown to reduce contamination from lymphocyte DNA and enzymes (Page et al., 2013). In addition, the buffy coat layer remaining from the first centrifugation was transferred from 3 vacutainers into separate 1.8 ml cryo-vials. The cells in one of these vials were re-suspended in 1ml growth medium containing 90% foetal calf serum and 10% DMSO and frozen gradually at -80 °C for at least 24 hours before being transferred to liquid-nitrogen storage. The other 2 buffy coat cryo-vials were also stored at -80°C.

2.5 CfDNA extraction from plasma samples

CfDNA was extracted from plasma using the Qiagen QIAMP circulating nucleic acid kit. Taking 2 ml* aliquots of thawed plasma, 200 µl of QIAGEN proteinase K was added, followed by 1.6 ml of ACL buffer, before vortexing. This step ensured that any proteins, lipids or vesicles in the plasma were inactivated. Where required, the plasma volume was adjusted to 2 ml by adding Phosphate Buffered Saline (PBS). The samples were then incubated in a water bath at 60 °C for 30 minutes, before adding 3.6 ml of ACB buffer. This buffer allows optimal binding of circulating nucleic acids to the silicone membrane in the subsequent vacuum extraction step. A further incubation step was carried out, in ice, for 5 minutes.

The QIAamp mini-columns provided in the kit were attached to the vacuum manifold, and each sample was loaded into a separate column before applying a vacuum at a pressure of 17 in Hg. Through this process, the extracted DNA was

bound to the silica-based membrane within each column. Three separate washes were then carried out (using ACW1 & ACW2 buffers, followed by ethanol) to eliminate peptides and other contaminants. Each column was then placed in a separate collection tube, and centrifuged at 13000g for 3 minutes, and any remnant liquid was discarded. Finally, DNA was eluted from each column by adding 200µl** AVE buffer, incubating for 3 minutes at room temperature, before centrifuging at 13000g for 1 minute. The eluted DNA was stored at 4 °C for further downstream processing.

* for GEMS samples, cfDNA was extracted from 1 or 2 ml of blood, based on availability

** see section 2.8 on optimising yield of cfDNA extraction

2.6 Lymphocyte DNA extraction from buffy coat samples:

Extraction of lymphocyte DNA from 500 µl buffy coat samples was carried out using the QIAGEN Flexigene DNA kit. The previously frozen buffy coats were thawed quickly in a water bath at 37 °C with gentle agitation, then stored on ice prior to extraction. To a 1.5 ml centrifuge tube containing each buffy coat sample, 1250 µl of Buffer FG1 was added, mixed by inverting the tube 5 times, and the tube centrifuged at 10,000 g for 20 seconds. The supernatant was discarded, and the tube gently inverted over a clean absorbent paper for 2 minutes, leaving behind the pellet of DNA at the base of the tube. A mixture of 500µl Buffer FG2 and 5 µl Protease was then added to the tube containing the pellet, and the tube vortexed thoroughly, using 3-4 pulses of vortexing at high-speed for 5 seconds each, until the pellet was completely homogenised. The tube was then centrifuged briefly for 5 seconds. After incubating at 65 °C for 10 minutes, the DNA was precipitated in 500 µl of 100% isopropranolol. After centrifuging at 10,000 g for 3 minutes, the supernatant was

again discarded by inverting the tube onto a clean sheet of absorbent paper, ensuring that the DNA pellet remained in the tube. The DNA pellet was washed with 500 µl of 70% ethanol before air-drying for at least 5 minutes, until all liquid had evaporated. Finally, the pellet was dissolved in 200 µl of Buffer FG3 by incubating for 30 minutes in a water bath at 65 °C. If needed, incubation was continued to ensure the DNA was dissolved. The DNA was then stored at 4 °C for further downstream processing.

2.7 Lymphocyte DNA quantification using Qubit dsDNA broad range assay kit

This fluorometric assay uses a proprietary dye that is highly specific for dsDNA. The amount of fluorescence generated when the dye binds to nucleic acids is measured by the Qubit fluorometer, as this is proportional to the concentration of DNA in the sample. This assay is technically simple compared to other fluorometry-based assays (e.g. PicoGreen or SYBR green assays), as the kit comes with a set of standards, which is utilised to prepare a standard curve. Another advantage is that the assay is robust to common contaminants like free nucleotides, proteins, salts or detergents. The range of quantification is from 10pg/µl to 100ng/µl for the high sensitivity (HS) kit, and 100pg/µl to 1000ng/µl for the broad range (BR) kit. In order to quantify the lymphocyte DNA samples, a working solution was first prepared by diluting the Qubit reagent 200-fold using the buffer provided in the kit. 190 µl of working solution was placed in two 0.5 ml tubes. 10 µl of Standard #1 (which is a blank solution) was added to the first tube, and the same volume of Standard #2 (which contains 10ng per µl of dsDNA) added to the second tube, and both tubes vortexed briefly (both standards are provided in the kit). For each DNA sample to be quantified, 198 µl of working solution was mixed (by vortexing) with 2 µl of DNA.

All tubes were incubated at room temperature for 2 minutes. Each standard was read on the Qubit 2.0 fluorometer, before the DNA samples were run. The fluorometer was set to display the concentrations of each DNA sample, based on the volume of sample used, by measuring against the known standards provided in the kit.

2.8 Plasma DNA quantification by SYBR green real-time PCR (qPCR)

SYBR green is a dye that binds specifically to double-stranded DNA, emitting fluorescence that can be measured by a detector. qPCR using SYBR green dye is a molecular technique used to quantify DNA by amplifying specific DNA fragments, and measuring, in real time, the fluorescence generated during the PCR reaction. Standards of known DNA concentration are prepared, and the number of cycles (C_t) at which the fluorescence generated exceeds a threshold value (set to account for background fluorescence) is determined for each standard (see Figure 2.1), and plotted on a standard curve (see Figure 2.2). The concentration of DNA samples to be quantified is then determined by reference to this curve. SYBR green master mix contains SYBR Green I Dye, AmpliTaq Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components. SYBR green dye binds to double-stranded DNA releasing fluorescence that can be measured. The GAPDH (glyceraldehyde -3- phosphate dehydrogenase) primer pair (Sigma Genosys) was used for the PCR amplification. This 87 base-pair amplicon is present at a single copy in the genome, and the small size of this amplicon made it ideal for this quantification due to the fragmented nature of ccfDNA. The primer sequences were as follows:

forward 5’ AACAGCGACACCCATCCTC

reverse 5’ CATACCAGGAAATGAGCTTGACA.

The primer was re-suspended in sterile nuclease-free water at a concentration of 100pmol/µl.

5 standards were prepared using known concentration human genomic DNA (Promega), ranging in concentration from 1 pg/µl to 1 ng/µl. A negative control (using DNAse free distilled water) was also used. The eluted DNA aliquots to be quantified were thawed on ice, and vortexed and centrifuged prior to use. The mastermix for the PCR reaction was prepared as shown in Table 2.1 below (quantities shown are in µl per well).

	Volume
SYBR green dye	5 µl
GAPDH primer (forward)	0.3 µl
GAPDH primer mastermix	0.3 µl
DNAse free distilled water	1.4 µl

Table 2.1: Components of the SYBR green qPCR mastermix reaction.
The mastermix comprised SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components dNTPs deoxynucleotides; dUTP 2'-deoxyuridine, 5'triphosphate; GAPDH glyceraldehyde -3- phosphate dehydrogenase.

Using a 384-well plate, 7 µl of this master mix and 3 µl of DNA (standard or unknown) were mixed by pipetting repeatedly into each well. This was done in triplicate for each plasma DNA sample, and in 5 replicates for each of the standards. The plate was sealed, and centrifuged briefly (800 g) before the qPCR process was carried out using the Applied Biosystems ht7900 machine. PCR cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles of 95 °C for 15 seconds and 60 seconds for 1 minute, followed by a final cycle of 95 °C for 15 seconds, 60 °C for 15 seconds and 95 °C for 15 seconds. To ensure accuracy of the standard curves, the following quality criteria were used:

a. the mean slope (m) had to be between -3.6 to -3.0;

b. the mean correlation coefficient ($R^{2)}$ greater than 0.99;

c. the efficiency of the PCR reaction [calculated using the equation $10^{(-1/slope)}$ -1 x 100%], had to be between 90-105%; and

d. a low standard deviation (less than 0.167) of the threshold cycle values for the triplicates was ideal.

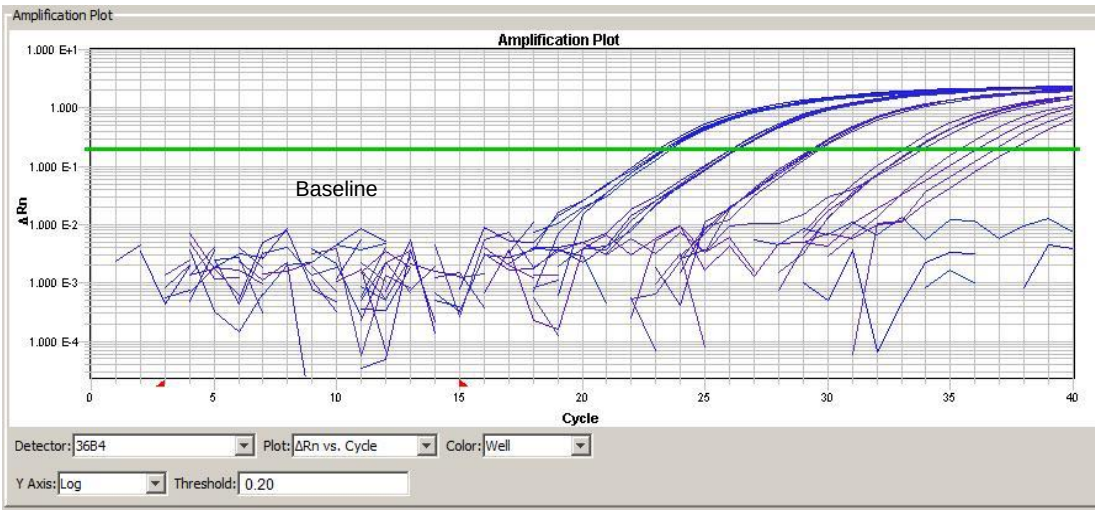


Figure 2.1: Example of amplification plots for a dilution series of 5 standards (5 replicates each), over the duration of a real-time PCR reaction, representing relative fluorescence (y-axis) plotted against cycle number (x-axis).The baseline refers to low-level signal at the start of the PCR reaction with minimal change in fluorescent signal. The green horizontal line represents the threshold, set at the exponential phase of the PCR reaction, which marks a significant increase in fluorescent signal over the baseline. The threshold cycle (C_t) for each sample is the cycle number at which the fluorescent signal crosses the threshold. This is inversely related to the starting amount of DNA target, such that as the target amount decreases, the number of cycles, at which significant amplification is seen, increases.

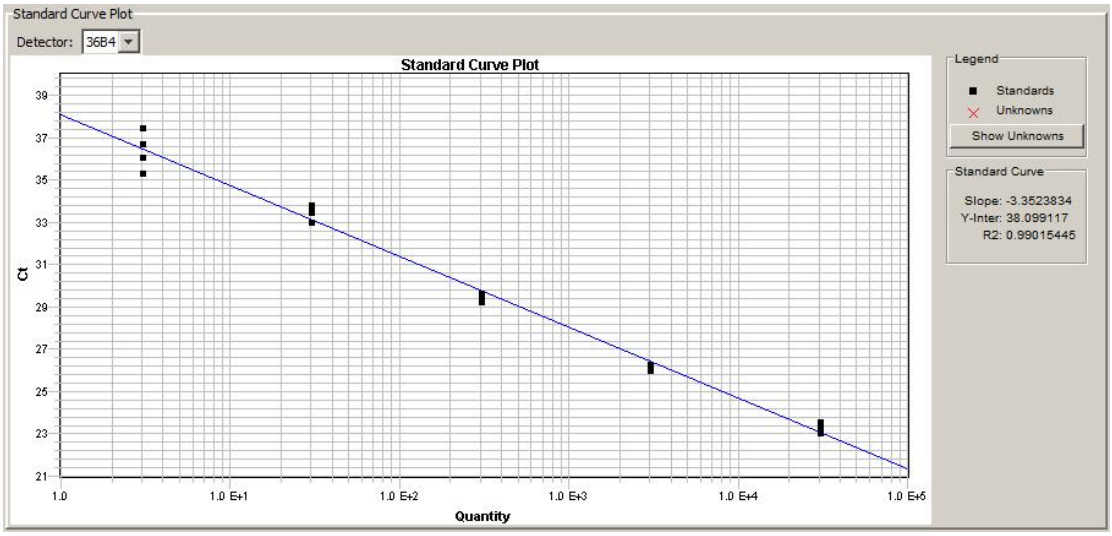


Figure 2.2: Example of a standard curve of real-time PCR data. Five replicates for each DNA standard (ranging in concentration from 1 pg/µl to 1 ng/µl) were analysed. In this quantification, one of the replicates for the 1 pg/µl standard was omitted from the analysis as it failed to amplify during the PCR reaction. Threshold cycle C_t (y-axis) was plotted against starting quantity of DNA (x-axis) for each replicate. Following creation of the standard curve, unknown DNA samples could then be quantified by comparison with this curve (absolute quantification).

2.9 Optimising cfDNA extraction

In order to optimise the process of extraction of ccfDNA from plasma, we compared elution volumes to see the effect on DNA yield, after spiking plasma with DNA extracted from tumour FFPE samples, and measuring the yield of DNA extracted. The methods for this experiment are described below.

2.9.1 Tumour DNA extraction using QIAamp DNA FFPE kit

Six colon cancer FFPE samples were selected (four slides from each sample), and, using a scalpel, tumour tissue was scraped off each slide, with each tumour sample collected in a separate 2 ml tube. 1 ml Xylene was added and each tube vortexed and centrifuged at 13,000g for 2 minutes. The resulting supernatant was discarded using a pipette, leaving a pellet behind. 1 ml ethanol (100%) was added to this pellet and each tube vortexed and centrifuged at 13,000g for 2 minutes. Again, the supernatant was discarded, making sure the pellet was not disturbed. The tubes

were then left open to incubate at room temperature until all residual ethanol had evaporated (at least 10 minutes).

To each pellet, 180 µl Buffer ATL was added followed by 20 µl proteinase K, and then vortexed. Each tube was incubated at 56 °C for 1 hour, and then left overnight at -20 °C. The overnight incubation step is a modification on the original QIAamp protocol, and was introduced to ensure complete cell lysis. Each tube was then incubated at 90 °C for 1 hour, and centrifuged. Two hundred microliters of Buffer AL was added, each tube vortexed, followed by the addition of 200 µl ethanol (100%), and further voxtexing. Each tube was again briefly centrifuged.

The contents of each tube were then transferred to a separate QIAamp MinElute column (placed within a 2 ml collection tube). Each column was centrifuged at 6,000g for 1 minute, and then transferred to a clean 2 ml collection tube, after ensuring that all the lysate had passed through the column. When required, the centrifuge step was repeated to ensure that the column was empty. 500 µl Buffer AW1 was added to each column and centrifuged at 6,000g for 1 minute, and each column transferred to another clean 2ml collection tube. 500 µl Buffer AW2 was added and each tube again centrifuged at 6,000g for 1 minute, before transferring the column to a fresh 2 ml collection tube. A further centrifuge at 20,000g for 3 minutes was done to ensure the membrane was completely dry. Each column was then placed in a clean 1.5 ml tube and the DNA was eluted by applying 50 µl Buffer ATE to the centre of the membrane, and incubating at room temperature for 5 minutes, followed by a final centrifuge at 20,000g for 1 minute to collect the eluate.

2.9.2 Tumour DNA quality check using gel electrophoresis

In order to assess the quality of the six extracted tumour FFPE DNA samples, they were subjected to a gel electrophoresis after PCR amplification using a short BRAF amplicon (Sigma-Aldrich Co.). Forward primer: 5'-TGTTTTCCTTTACTTACTACACC, Reverse primer: 5'-AGCCTCAATTCTTACCATCCA.

For each DNA sample, a mastermix was prepared as shown in Table 2.2 below.

	Volume
MyTaq HS red mix (Bioline)	12.5 µl
BRAF forward primer	0.5 µl
BRAF reverse primer	0.5 µl
DNAse free distilled water	10.5 µl

Table 2.2: Components of PCR mastermix for tumour DNA gel electrophoresis.

23 µl of the mastermix was then mixed with 2 µl of each DNA sample. A positive control was prepared using 23 µl of mastermix, 1 µl of human genomic DNA (Promega), and 1 µl of DNAse free distilled water. In addition, a negative control, using 23 µl of mastermix and 2 µl of DNAse-free distilled water was prepared. The DNA samples, along with the positive and negative controls, were PCR amplified in the Geneamp PCR thermal cycling system 2700, using the conditions outlined in Table 2.3 below.

Temperature	Time	Number of cycles
95 °C	1 minute	1 cycle
95 °C	20 seconds	40 cycles
60 °C	30 seconds	
72 °C	20 seconds	
72 °C	2 minutes	1 cycle

Table 2.3: PCR cycling conditions for gel electrophoresis

For the gel electrophoresis, a 1.5% agarose gel was prepared by mixing 2.25 g of agarose powder (Sigma) with 150 ml of 1x TAE (40 mM Tris/20 mM acetate/ 1mM EDTA). The mixture was heated, then cooled, before adding 6 µl of ethidium bromide (10 mg/µl) and allowed to set to a gel in a tray fitted with a comb to create channels for sample loading. Each DNA sample, as well as the negative and positive controls, was then loaded into separate channels in the gel (3 µl each sample, along with 2 µl of loading buffer. A Hyperladder (Bioline, IV) was also loaded to provide size standards. 1x TAE was used as a running buffer, and the gel was run at 60 volts for 1½ hours.

2.9.3 Tumour DNA quantity and quality check using Nanodrop spectrophotometry

This technique is based on the principle that DNA and RNA absorb UV radiation at specific frequencies. Therefore, nucleic acids can be quantified by passing UV light through a sample, and measuring the frequencies absorbed using a spectrophotometer. An optical density of 1.0 corresponds to 50 ng/µl of dsDNA. The Nanodrop (Thermo Scientific) uses specialised technology to quantify nucleic acids ranging from 2 ng/µl to 3700 ng/µl in concentration.

The quantity and quality of the tumour DNA samples were assessed using the Nanodrop ND-1000 instrument, following manufacturer's instructions. To summarise, the optical surfaces of the receiving and source fibres of the instrument were cleaned using compatible wipes. 2 µl of DNAse-free water was loaded onto the instrument as a further cleaning step. 1 µl of Buffer ATE was loaded onto the instrument as a blank prior to 1 µl of each DNA sample.

The concentration (in ng/ µl), as well as the A_{260}/A_{280} and $A_{260}/_{230}$ ratios were determined by the Nanodrop software. An A_{260}/A_{280} ratio of around 1.8 and $A_{260}/_{230}$

range of 2.0-2.2 were considered acceptable in terms of purity of the extracted DNA. Nucleic acids absorb at 260 nm, proteins at 280 nm, and other contaminants at 230 nm.

As Nanodrop is recognised to consistently overestimate dsDNA quantities, the tumour DNA samples were re-quantified using Qubit (as outlined in Section 2.6).

2.9.4 Spiking plasma samples with tumour DNA:

Plasma samples from healthy volunteers (recruited to the "Optimisation of plasma nucleic acids" study at University of Sheffield) were thawed and pooled (to get two pools of 6 ml of plasma each) and then centrifuged at 1600g for 5 minutes to separate any contaminants. 1 pool of plasma was spiked with tumour DNA (extracted from colon cancer FFPE samples) of known concentration, while the second pool was left un-spiked. FFPE tumour DNA was used to spike the plasma as it is fragmented, and would mimic ccfDNA. The two plasma pools were then stored as 1 ml aliquots at -80 °C prior to extraction.

Three separate spiking experiments were carried out to compare different elution volumes during the DNA extraction process, in order to see what effect varying the elution volume had on the yield of DNA extracted.

2.9.5 DNA extraction using different volumes of elution buffer

The two pools of plasma (spiked and un-spiked samples) were thawed before extracting the tumour DNA, following the protocol described in Section 2.4. As we were comparing the effect of varying the volume of elution buffer on DNA yield, three different volumes of buffer (50 µl, 100 µl, and 200 µl) were used in the final elution step (as shown in Figure 2.3 below). The eluted DNA was quantified using

SYBR-green qPCR (as described in Section 2.7). Once the DNA yield of the spiked and un-spiked plasma samples were determined, the percentage yield of DNA extracted was calculated using the following formula:

$$\frac{\text{Spiked plasma DNA yield (ng)} - \text{Un-spiked plasma DNA yield (ng)}}{\text{Tumour DNA spike quantity (ng)}} \times 100\%$$

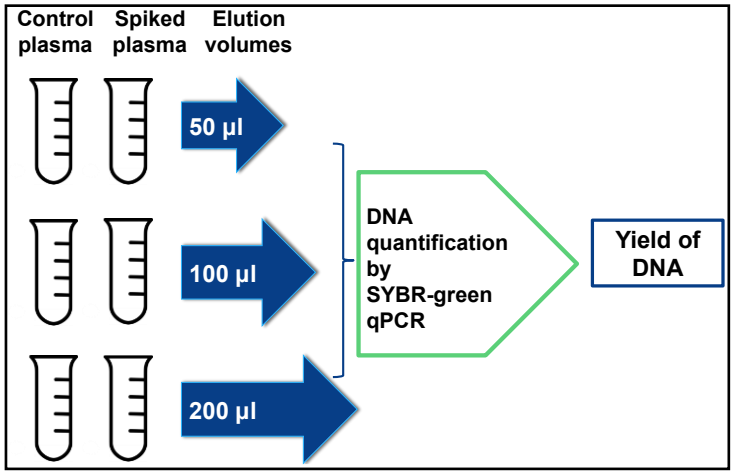


Figure 2.3: Outline of DNA-spiking experiment to compare DNA yield in varying volumes of elution buffer. Three sets of paired plasma samples (controls and DNA-spiked samples, each containing I ml of plasma) were compared to evaluate the effect of elution volume on DNA yield. The spiked samples were spiked with tumour DNA to achieve a concentration of 50ng of DNA/ml of plasma.

2.10 Workflow for DNA preparation prior to whole-genome sequencing

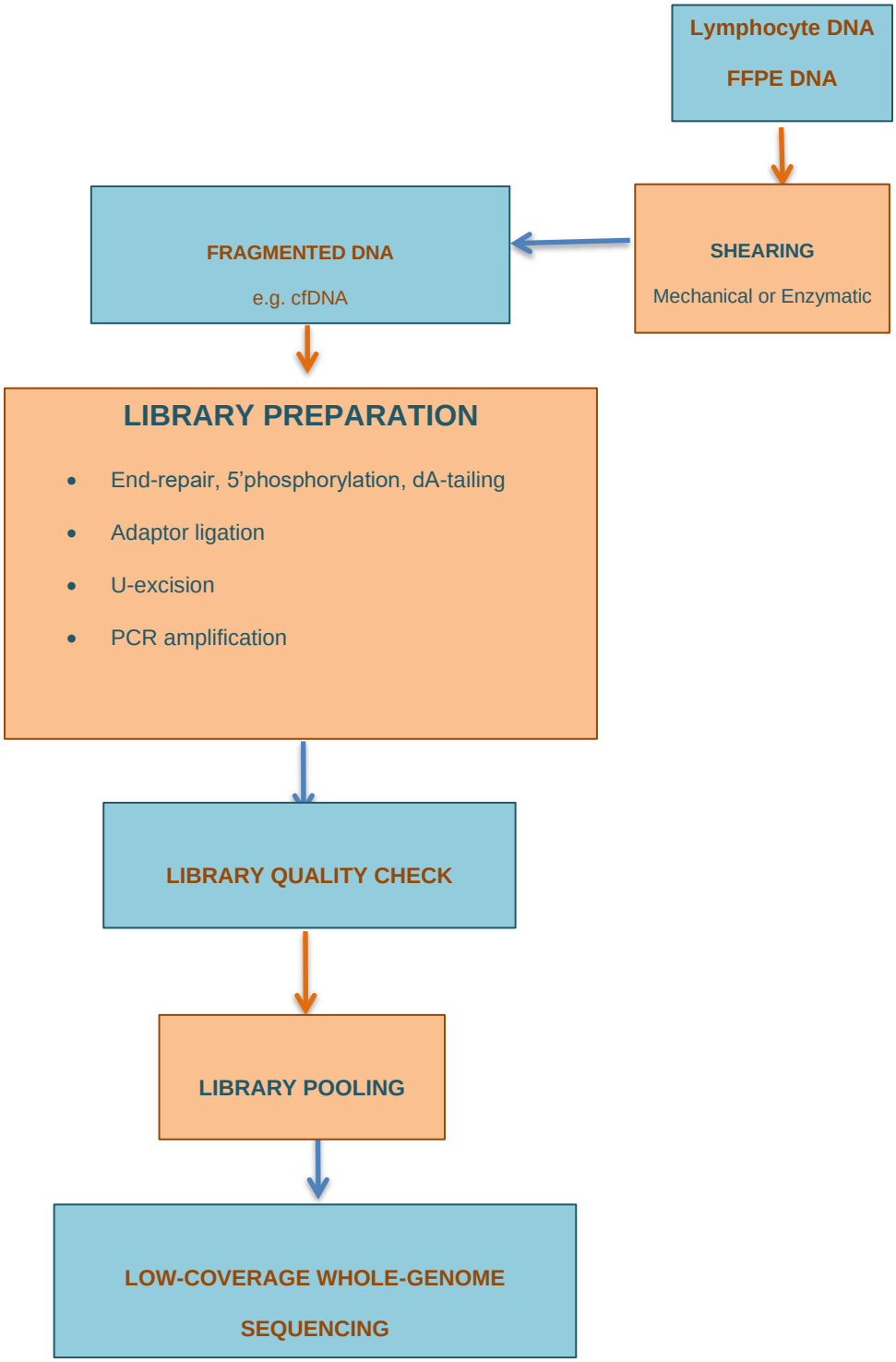


Figure 2.4: Steps involved in DNA preparation prior to sequencing.

2.11 Shearing lymphocyte and FFPE DNA using Covaris:

Prior to library preparation, FFPE and lymphocyte DNA samples were mechanically sheared using the Covaris E220 Focused-ultrasonicator, to achieve a target size of 200 base pairs. This step was necessary to enable optimal reading of DNA fragments during downstream paired-end sequencing. Care was taken when loading the DNA samples into the microtubes for the Covaris, to avoid introducing air-bubbles, which could interfere with the shearing process. For each sample, 1000 ng of DNA in 130 µl solution (using nuclease-free distilled water) was sheared. If less than this quantity was available for any of the samples, the entire quantity available was sheared. The sonicator settings utilised are shown in Table 2.4 below.

Target BP (Peak)	200
Peak Incident Power (W)	175
Duty Factor	10%
Cycles per Burst	200
Treatment Time (s)	180
Temperature (°C)	7
Water Level	6
Sample volume (µl)	130
E220 Intensifier	Yes

Table 2.4: Settings for the Covaris E220 focused-ultrasonicator.
BP: base pairs

2.12 Library preparation using NEBNext kit

The Ultra II DNA library Prep kit for Illumina® (New England Biolabs®) was used to generate libraries with appropriate adaptors and barcodes for sequencing. This kit allows DNA inputs as low as 500pg, making it an ideal choice given the very low quantities of cfDNA available from the majority of the samples. A starting DNA volume of 50 µl was used, as recommended by the manufacturer, and where less than this volume was available, the deficit was made up using 1x TE. We aimed for 100ng of input DNA for the FFPE/lymphocyte DNA samples. Where this quantity was not

available, the available amount per 50 µl was used. For the cfDNA samples, the available quantity per 50 µl was used. The process of library preparation followed five main steps which are detailed in the following sections:

2.12.1 End Repair, 5´ Phosphorylation and dA-Tailing:

Fragmented DNA contains a variety of 3' and 5' ends, some recessed and others overhanging. In this step, any 3' and 5' recesses or overhangs were filled in, and removed, respectively. The 5' ends were then phosphorylated, and a deoxyadenylate (A) residue added to each 3' end. This was achieved by adding 3 µl of the End Prep Enzyme Mix and 7 µl of the End Prep Reaction Buffer to each DNA sample in a 200 µl, after which, the mixture was gently mixed by pipetting, and incubated in a 54hermos-cycler for 30 minutes at 20 °C, followed by 30 minutes at 65 °C.

2.12.2 Adapter Ligation and U-excision:

In this step, adaptors with a single T-overhang were ligated to the end-repaired dA-tailed DNA fragments. These adaptors contained sequences complimentary to the Illumina flow -cell oligonucleotides used downstream during sequencing.

30 µl of NEBNext Ultra II Ligation Master Mix was added to the End Prep Reaction Mixture, followed by 1µl of NEBNext Ligation Enhancer and 2.5 µl of NEBNext Adaptor for Illumina was added. Where less than 100 ng of input DNA was available for library preparation, the adaptor was diluted (see Table 2.5 below) to reduce the risk of adapter-dimer formation

DNA input (ng)	Adaptor dilution
> 100 ng	No dilution
5 - 100 ng	1:10
< 5 ng	1:25

Table 2.5: Ratio of adaptor dilution based on quantity of input DNA

This mixture was mixed and then incubated for 15 minutes in a thermocycler at 20 °C, with the heated lid turned off. 3 µl of USER Enzyme was added, and the mixture incubated for a further 15 minutes at 37 °C, with the heated lid set at a minimum of 47 °C. This cleaves the adaptors, making the adaptor-ligated DNA fragments available for PCR amplification.

2.12.3 Clean-up of Adapter-ligated DNA without size-selection:

The size-selection step was omitted in order to conserve the amount of DNA available for PCR amplification (size-selection is not recommended for DNA inputs less than 50 ng, which was the case for most of the cfDNA samples).

Agencourt AMPure XP beads (Beckman Coulter Life Sciences®) were used for this step. They were allowed to stand at room temperature for at least 30 minutes, and vortexed thoroughly, prior to use. Each adaptor-ligated DNA sample was placed in a separate well in a 96-well plate, and 87 µl of beads was then added to each well, mixing by pipetting up and down at least 10 times. The samples were incubated at room temperature for 5 minutes, before placing the plate on a magnet stand for about 5 minutes, until the solution was clear, with the beads magnetised to the base of each well. The clear supernatant was discarded by pipetting out, taking care not to disturb the beads, which contained the DNA targets. The AMPure XP beads® solution

contains an optimised buffer which selectively binds DNA fragments (of 100bp or more) to paramagnetic beads, so that unwanted PCR products (salts, enzymes, primers etc.) can be removed by washing, resulting in more purified DNA targets. Two sequential washes were carried out by incubating with 200 µl of freshly prepared 80% ethanol in each well, before air-drying the samples for about 5 minutes. Care was taken not to over-dry the samples (as evidenced by the samples turning a lighter brown and cracking up), as this would result in DNA-loss. The plate was then removed from the magnetic stand, each DNA sample eluted in 17 µl of 0.1X TE, mixed well and incubated for 2 minutes at room temperature. The plate was then returned to the magnetic stand, and after the solution had cleared again (about 5 minutes), 15 µl of clear supernatant (containing the target DNA) was transferred to a new PCR tube. This was stored at -20 °C until the PCR-enrichment step was carried out.

2.12.4 PCR-enrichment and Indexing of adapter-ligated DNA:

The PCR step enriches for adaptor-ligated fragments, while increasing the amount of library available for sequencing. Distinct barcodes (NEBNext® Multiplex Oligos for Illumina®) were also incorporated into each sample to enable downstream multiplexing.

To each adaptor-ligated sample, 25 µl of NEBNext Ultra II Q5 Master Mix and 10 µl of Index/Universal Primer (supplied pre-mixed) was added, such that each amplified sample would include a unique index sequence. The mixture was mixed by gently pipetting up and down at least 10 times. PCR amplification was then carried out on a thermocycler, using the cycling conditions outlined in Table 2.6.

PCR enrichment step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1
Denaturation	98 °C	10 seconds	7 - 15*
Annealing/Extension	65 °C	75 seconds	
Final Extension	65 °C	5 minutes	1
Hold	4 °C	∞	

Table 2.6: NEBNext Ultra DNA library prep PCR cycling conditions.

The number of PCR cycles increased with reducing DNA input (starting DNA), and was based on recommendations in the instruction manual. Table 2.7 shows the number of PCR cycles based on the input DNA amount.

Input DNA (ng)	Number of PCR cycles
60 - 100	7
30 - 59.9	8
20 - 29.9	9
10 - 19.9	10
5 - 9.9	11
3 - 4.9	12
2 - 2.9	13
1 - 1.9	14
0.5 - 0.9	15

Table 2.7: Number of PCR cycles during DNA library preparation, based on quantity of DNA input.

2.12.5 Clean-up of PCR-amplification:

A further clean-up step was carried out, following the same process outlined in Step 10.3, but using 45 µl of beads. The DNA was then eluted in 33 µl of 0.1X TE, and 30 µl of clear supernatant eluted into a fresh PCR tube, and stored at -20 °C.

2.13 TapeStation analysis of libraries:

The 2200 TapeStation System (Agilent®) was used to check the size distribution of the libraries, and to quantify them, prior to pooling. This system provides a quick, automated tape-based electrophoresis of the libraries. The High Sensitivity D1000 Screen Tape assay was used because the size range (35-1000 bp) and quantity range (10 – 1000 pg/µl) offered by this assay were ideal for the libraries.

To summarise, the High Sensitivity D1000 Ladder and High Sensitivity D1000 Sample Buffer were allowed to equilibrate at room temperature for 30 minutes, and vortexed before use. Using a 96-well plate, 2 µl of Sample Buffer was pipetted into separate wells (depending on the number of DNA libraries to be run).

A 1:5 dilution of all DNA libraries was carried out, and 2 µl of each diluted library was added to a separate well containing the buffer. In addition, 2 µl of Sample Buffer and 2 µl of the Ladder were added to one separate well. The plate was then vortexed at 2000 rpm for 1 minute, and briefly centrifuged to ensure the contents were at the base of the wells. The plate was then loaded on to the 2200 TapeStation, along with the ScreenTape and loading tips.

2.14 Library Quality Control:

The Tapestation reports of the libraries were examined to ensure that they fulfilled two essential conditions: (a) presence of a single predominant peak representing the library; and (b) the peak was of the expected molecular weight of around 300 bp, which represents the size of the ccfDNA fragment (180 bp) plus the adaptor/index primer added during library preparation (120 bp). Importantly, if any significantly smaller peaks were seen (e.g. 80 bp peaks representing primer dimers, or 120 bp peaks representing adapter dimers), the library would be regarded as having

failed the QC step. Much larger molecular-weight peaks would indicate over-amplification at the PCR step during library preparation.

2.15 Quantification and Pooling of Libraries:

The high throughput of the Illumina platform, as with most current sequencers, means that its capacity for generating sequence reads far exceeds the requirements of a single library. Thus, it was possible to pool together several libraries during a single sequencing run to maximise the sequencing capability of the platform, while keeping the process cost-effective.

In order to carry out such multiplexed sequencing, up to forty-eight samples were pooled together, prior to loading on to both lanes on the Illumina HiSeq flow cell. Samples were pooled in equimolar amounts. It was also ensured that the absolute total molarity of the pool did not exceed the manufacturer's recommended molarity (4 nM for Illumina HiSeq). The volume of each library added to the pool was calculated using the following formula:

$$\text{Volume of library (µl)} = \frac{\text{Volume of final pool (µl)} \times \text{Concentration of pool (nM)}}{\text{Number of libraries} \times \text{Concentration of library (nM)}}$$

The libraries were pooled into a 1.5ml eppendorf tube, and the total volume was made up to 1500µl by adding QIAGEN buffer EB + 0.1% tween.

Other important considerations taken during pooling included:

- samples being pooled together share a similar fragment size distribution (shorter fragments amplify more efficiently than longer fragments, so it was important to avoid mixing very differently sized-libraries, to ensure relatively equal reads across samples)

- any libraries with very low concentrations, were reconstituted (using either a 1:2 dilution factor, or using the stock library, instead of a 1:5 dilution as outlined in section 1.11) to ensure more even concentrations (and so molarity) across samples.

2.16 Sequencing on Illumina platform:

The HiSeq platform, which utilises the Sequencing by Synthesis technology for high throughput sequencing, was utilised to carry out paired-end sequencing of the pooled libraries. Paired-end sequencing, as the name suggests, involves the reading of a DNA fragment from each end, providing twice as many reads, and through the alignment of paired reads, enables more accurate read alignment and, thus, more reliable identification of structural variants. The "Rapid Run Mode" was used, which is capable of generating up to 600 million reads, at a read length of 2 x 100bp across the 2 lanes of the flowcell. Using the formula shown below, this would achieve a coverage of approximately 0.41, if 48 samples were multiplexed in a single run.

$$\text{Coverage} = \frac{\text{Number of reads (600 million)} \times \text{Average read length (100)}}{\text{Number of samples multiplexed (48)} \times \text{Length of original genome (3 billion)}}$$

2.16.1 Loading the libraries on the HiSeq:

Along with the DNA libraries, a positive control (the PhiX Control v.3) was loaded on the HiSeq during each sequencing run. The PhiX is a ready-to-use adaptor-ligated library, which, given its relatively small size, can be rapidly aligned to provide relevant sequencing metrics for quality control. The DNA libraries and Phi X control were prepared as follows:

A pair of 1.5ml eppendorf tubes was labelled 'DNA 20' and 'PhiX 20' respectively. 5µl of the pooled DNA was added to the 'DNA 20' tube, while to the

‘PhiX 20’ tube, was added 2µl of PhiX control and 3µl Qiagen Buffer EB with 0.1% tween.

5µl of a 0.2N NAOH solution was added to each of the tubes, and these were vortexed, and pulse-centrifuged, before incubating for 5 minutes. 990 µl of pre-chilled buffer HT1 was added to each tube and these tubes placed in a 0 °C cold block.

Another pair of 1.5ml eppendorf tubes was labelled ‘DNA 11’ and ‘PhiX 11’ and 270 µl pre-chilled buffer HT1 added to each before placing on ice. 330 µl of ‘DNA 20’ and ‘PhiX 20’ was then added to ‘DNA 11’ and ‘PhiX 11’ respectively, and both tubes placed on ice.

Another 1.5ml eppendorf tube was labelled ‘Final’ and to this was added 495 µl of ‘DNA 11’ and 5 µl of ‘PhiX 11’. This tube was placed in a heating block at 95 °C for 2 minutes, and then kept on ice until ready to load on the HiSeq machine.

The Illumina HiSeq workflow consists of 3 main processes, described below:

2.16.2 Cluster generation:

During this process, the adaptor-ligated DNA fragments are allowed to flow across the lanes of the flow cell, which is coated on its surface with oligonucleotides which are complementary to the adaptors at the ends of the fragments. Each fragment is then isothermally amplified through the process of bridge amplification, resulting in clonal clusters.

2.16.3 Sequencing:

Fluorescently labelled nucleotides are allowed to wash over the hybridised clusters, and each tethered DNA fragment serves as a template for complementary strand synthesis. The synthesised strand is excited by a light source, and from the

wavelength of the fluorescence emitted from each nucleotide, as well as its signal intensity, the base is called. This cycle is repeated continually, with the number of cycles determining the length of the read. With each cycle, the presence of a blocking 3' hydroxyl group prevents more than one base being added at a time. Cleavage of this hydroxyl-nucleotide group after calling, enables the next sequencing cycle to begin. This process occurs in a massively parallel manner across the flowcell, resulting in millions of reads being generated within one sequencing run.

2.16.4 Data analysis:

The reads are separated based on the distinct barcodes incorporated into each DNA sample during library preparation. Forward and reverse reads are paired, and alignment algorithms map contiguous reads to a reference genome.

2.17 Creating a pool of genomic copy-number ratios from healthy controls

Twenty healthy controls were randomly selected from the Markers of Relapse in Melanoma study, ensuring a balance between male and female controls. The read counts for each one Mb window derived from the reads obtained from sequencing their lymphocyte DNA were pooled bioinformatically to obtain a pooled reference to which each cfDNA sample (from patients and controls) could be normalised when creating copy-number profiles. This approach provided an alternative to sequencing the lymphocyte DNA from each participant, thus reducing sequencing costs significantly.

For each of the cfDNA samples from the twenty controls, the mean of the copy-number ratios from all 1Mb windows was obtained as above. The mean and

standard deviation of these twenty means were used to standardise copy number ratios when calculating the copy-number scores described in section 2.21.

2.18 Copy-number ratios calculated using CNAnorm:

As shown in the workflow in Figure 2.5, sequence reads were mapped to the reference genome (GCh 38), following which any reads that were duplicated or of poor mapping quality were removed using an in-house bioinformatics pipeline developed by Dr James Bradford.

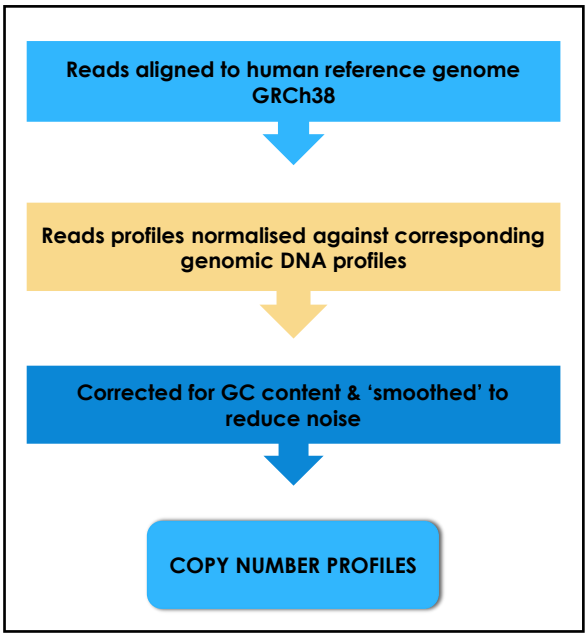


Figure 2.5: Workflow for creation of copy-number ratios

The resulting BAM files (for each cfDNA, lymphocyte DNA and FFPE tumour DNA sample) were inputted to the software CNANORM. Blacklisted regions of the genome were removed by this software, which also corrected for GC content. Copy number ratios were generated for each 1 Megabase window across the genome by normalizing each sample to either matched lymphocyte DNA (for the GEMS cohort), or pooled lymphocyte genomic DNA from a cohort of healthy controls (described in section 2.15).

2.19 Copy-number scores:

In order to numerically summarise the aberrations identified in each participants' copy-number profile across the whole genome, two scoring algorithms were applied, which are described below:

2.19.1 Copy-number Aberration Score (CNAS)

This score was described by Heitzer (Heitzer et al., 2013). For each cfDNA sample, Z-scores were calculated for each 1Mb window of sequencing reads by standardising the copy-number ratio to the mean copy-number ratio from a cohort of healthy controls (see Section 2.16), i.e. the mean and standard deviation of copy number ratios of each 1Mb window across the genome of each healthy control were calculated. The average of these means and the standard deviation was then used to calculate z-scores. The square of z-scores was then summed across the whole genome.

2.19.2 Extreme Copy-number Aberration Score eCNAS

This was adapted from the score described by Xia et al (Shu Xia et al., 2015). For each cfDNA sample, Z-scores for each 1Mb window were calculated by

standardising its copy-number ratio to the mean copy-number ratio of all windows for that sample.

The log $_{10}$-transformed squared z-scores were ranked, and the sum of the 95-99th percentiles of these was obtained.

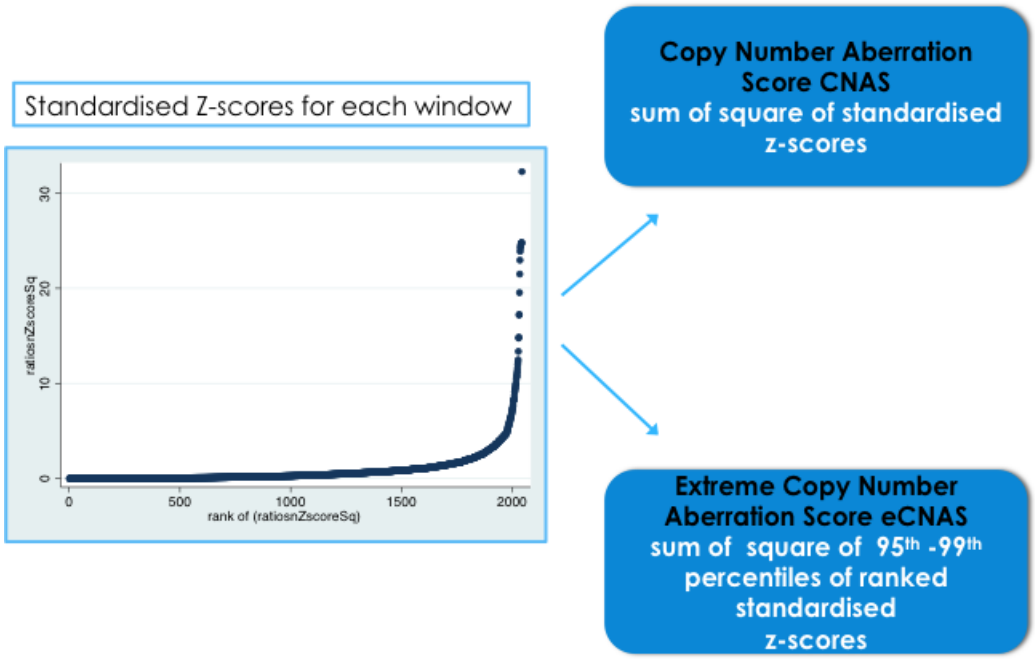


Figure 2.6: Summary of the two copy-number scores described in sections *2.17.1 and 2.17.2*

2.20 Melanoma cell-line culture (BRAF mutant and wild-type)

Three human melanoma cell lines (A375, MDA-MB-435 and WM1361) were obtained from the American Type Culture Collection (ATCC), (Bethesda, USA). Cell lines were cultured to obtain DNA for use in quality control analysis (specifically to investigate reproducibility of the low-coverage copy-number approach).

2.20.1 Cell-line sub-cultures

Two BRAF mutant melanoma cell-lines (A375, MDA-MB-435), and one BRAF wild-type cell line (WM1361) were identified and thawed (from liquid-

nitrogen storage). Media mixes appropriate for each cell-line were prepared (as shown in Table 2.8) and warmed to 37 °C prior to use.

	Medium/FBS mix	Volumes
A375	DMEM	27 ml
	10% FBS	3 ml
MD-MB-435	DMEM	28.5 ml
	5% FBS	1.5 ml
WM-1361	RPMI	27 ml
	10% FBS	3 ml

DMEM	Dulbecco's Modified Eagle Media
FBS	Fetal Bovine Serum
RPMI	Roswell Park Memorial Institute medium

Table 2.8: Media mixes for each of the three cell-lines cultured to obtain DNA for quality-control analyses

Using a T_{25} flask for each cell-line, 1 ml of re-suspended cells was mixed with 9 ml of the appropriate media mix. The flasks were placed in an incubator, checked daily, and media changed regularly to encourage cell growth. When cells were approximately 90% confluent, they were sub-cultured. Cells were rinsed twice with PBS, 1 ml of Trypsin EDTA was added, and the excess decanted. The flask was allowed to stand for about 2 minutes, and then viewed under a microscope to look for dissociation of cells from the base of the flask. The cells were then re-suspended in 5 ml of medium/FBS mix, before 2 ml of these cells was transferred to two T_{75} flasks, each containing 20 ml of medium/FBS mix. The flasks were stored in the incubator, and the process repeated until sufficient cells had grown for DNA extraction could be carried out.

2.20.2 Cell-line DNA extraction

The QIAGEN Flexigen kit was used to extract DNA from the three cell-line cultures. Prior to extraction, the cells in each culture were counted. To do this, the medium from the flasks were removed, and each flask was rinsed twice with PBS, before adding 2 ml of Trypsin EDTA. When cells were dissociated, 5 ml of medium/FBS mix was added, and the cells collected in a 15 ml centrifuge tube. Each sample was then loaded onto the chambers beneath the coverslip of a haemocytometer. After mounting on a microscope set at 10 X objective, the cells within each of the four sets of 16 squares of the haematocytometer grid were counted manually, using a hand tally counter. Only cells within a square, or on the right/bottom rows of each square were included in the count. The average of the cell counts from the four sets was calculated, and multiplied by 10^4 to achieve a measure of the number of cells per ml of solution. The recommended number of cells required for extraction using the Flexigen kit was between 1-2 x 10^6 cells. If the count revealed an inadequate number of cells, the cell-line was sub-cultured, and allowed to grow until there was adequate growth for extraction.

The required number of cells was centrifuged in a 1.5 ml microcentrifuge tube for 5 minutes at 300 x g. The supernatant was discarded, taking care not to disturb the pellet. The cells in the pellet were re-suspended by adding 300 µl of Buffer FG1 and pipetting up and down repeatedly. 300 µl of pre-mixed Buffer FG2/QIAGEN protease mix (300 µl Buffer FG2 with 3 µl of reconstituted QIAGEN protease) was added, and the closed micro-centrifuge tube inverted three times. The tube was then placed in a water bath and incubated for 10 minutes at 65 °C. 600 µl of 100% isopropanol was added, and mixed thoroughly, until the precipitated DNA became visible (as threads or as a clump). The tube was then centrifuged for 3 minutes at 10,000 x g. The

supernatant was discarded, and the tube inverted gently onto a clean paper towel to ensure the supernatant was completely discarded. Then, 600 µl of 70% ethanol was added and vortexed for 5 seconds, after which it was centrifuged for 3 minutes at 10,000 x g. Again, the supernatant was discarded, and the tube left inverted on a paper towel for about 5 minutes. The pellet was air-dried for at least 5 minutes, after which 200 µl of Buffer FG3 was added, followed by a gentle vortex for 5 seconds, before the DNA was dissolved by incubating for 30 minutes at 65 °C. If the DNA was not completely dissolved at this stage, the incubation was prolonged until the DNA was completely dissolved.

The extracted DNA was then quantified using QUBIT, and a quality check was carried out on the Nanodrop (as previously described in Sections 2.6 and *2.8.3*)

2.21 Investigating the reproducibility of the copy-number approach

To investigate the reproducibility of the low-coverage copy-number approach, DNA extracted from the melanoma cell line MDA-MB-435 was analysed on ten separate next-generation sequencing runs. 1000 ng of the extracted DNA was sheared on the Covaris (see Table 2.9 for settings) to achieve 200 bp fragments. 10 ng of this fragmented DNA was then made into libraries using the NEBNext Ultra II Library Prep kit (as described in Section 2.11). For each of the ten sequencing runs carried out (as described in Section 2.15), a cell-line DNA library was pooled along with the cfDNA/genomic DNA libraries being sequenced. The copy-number profiles, as well as the CNAS and eCNAS, for these cell-line DNA samples were compared across the runs. The coefficient of variation for CNAS and eCNAS across the 10 runs was calculated (using Stata) to assess the reproducibility of the copy-number analysis.

2.22 Determining the technical sensitivity of the copy-number approach

In order to determine the technical sensitivity of the copy-number approach, we carried out serial dilutions of DNA extracted from the MDA-MB-435 melanoma cell-line DNA and examined their copy-number profiles, as well as the CNAS and eCNAS scores. Dilutions of the cell-line DNA were carried out using lymphocyte DNA extracted from a healthy control. The dilutions ranged from 50% to 0.01%, and were all done in duplicate. To reflect the average concentration of typical cfDNA samples, each dilution contained a total of 12 ng of fragmented DNA (cell line plus healthy control DNA).

Similarly, we carried out serial dilutions of a sarcoma cell line for which the copy-number profile produced by array-CGH was available, and sequenced these using the low-coverage sequencing approach, in order to compare the profiles obtained by both platforms.

2.23 Statistical analysis

All analyses were carried out using Stata (version 12) and GraphPad Prism (version 7). The two-tailed t- test was used in analyses comparing continuous variables. Patient deaths were determined from hospital records, up to a last date of follow-up of 17.08.17, and survival times were plotted using the Kaplan Meier method. Hazard ratios were derived in a Cox regression framework with adjustment for time from diagnosis to recruitment. Tumour-related factors significant at the $p<0.05$ level were included in a multivariable survival analysis. Univariable logistic regression analyses comparing relapsed and non-relapsed melanoma included log CNAS, log cfDNA level (ng/ml plasma), stage at recruitment (coded as binary I/II *vs.* III/IV), age at recruitment, gender, and BRAF status. Significant variables ($p<0.05$)

were included in a multivariable logistic regression, and predicted probabilities based on the model were used to generate Receiver-Operator Characteristic curves.

The random coefficients mixed-effects model was utilised in the longitudinal analyses, taking into account “within-patient” as well as “between-patient” variations in continuous variables. This model was necessary as the logistic regression analysis does not adjust for repeated measures. This model was recommended by Dr Dawn Teare (co-supervisor and statistician).

Chapter 3: Results: Genetics and Epidemiology of Melanoma in Sheffield study (GEMS)

3.1 Introduction

While the incidence of melanoma in the UK has increased steadily since the 1970s (Cancer Research UK, 2018), the overall survival is improving, especially for early stage disease. Tumours in early stage disease are usually amenable to surgical excision, but, for those patients with high-risk disease, relapse rates can be up to 50%, and relapses are often metastatic. The prognosis for patients with unresectable metastatic disease is not very good. However, the emergence, within the last decade, of BRAF-targeted agents and immune checkpoint inhibitors is changing the prognosis for these patients dramatically(Hodi et al., 2010; J. Larkin et al., 2014; James Larkin et al., 2015; Caroline Robert et al., 2014; Wolchok et al., 2013). Early detection of metastatic relapsed disease could enable initiation of systemic therapies sooner, potentially improving clinical outcomes.

There is currently a reliance on radiological imaging and physical examination to detect relapses in patients. There is, as yet, no blood test that can be used to indicate melanoma relapses reliably. While the serum protein lactate dehydrogenase (LDH) has some use as a prognostic biomarker in melanoma (patients with markedly elevated serum levels have been shown to have a shorter median survival, and poorer response rates to systemic therapy (Balch et al., 2009; Martens et al., 2016)), its value as a marker of relapse is limited, given its low sensitivity (Finck, Giuliano, & Morton, 1983).

As cfDNA shares genetic and epigenetic similarities with its parent tumour, there has been considerable interest in cfDNA analysis as a biomarker in various tumour sub-types, including melanoma. Salvianti et al developed a model (incorporating cfDNA concentration, cfDNA integrity, and RASSF1A promoter methylation in cfDNA) which showed good predictive ability to discriminate healthy

controls from melanoma patients (Salvianti et al., 2012). A number of other groups have utilised cfDNA to assess treatment response by tracking mutations in cfDNA during therapy (R. J. Lee et al., 2017; Lipson et al., 2014; Tsao et al., 2015). As yet, copy number aberrations have not been examined, either as a marker of relapse, or to assess treatment response.

As a step towards the possible use of genome-wide copy number aberration analysis as a biomarker for relapse, this chapter introduces the concept of copy-number aberration analysis in cfDNA as an indicator of active disease in melanoma patients. Copy-number aberrations are large structural alterations in part, or the whole, of a chromosome, which result in an abnormal number of copies of sections of DNA as compared to a reference genome. There is an abundance of somatic copy number alterations in melanoma, as shown rather nicely by Curtin et al (Curtin et al., 2005), who showed low level copy number gains, losses, amplifications and deletions, in various sub-groups of melanoma. Coupled with the fact that copy-number aberrations are detectable by next-generation sequencing with shallow coverage, in either FFPE samples or cfDNA, (Camacho et al., 2017; Chen et al., 2017; Kader et al., 2016) unlike point mutations which require considerable depth of sequencing, the copy number approach in detecting relapse in melanoma is a potentially attractive, and cost-effective, option.

Heitzer et al (Heitzer et al., 2013) describe an elegant method of capturing the breadth of copy-number aberrations detected by whole-genome sequencing in cfDNA from prostate cancer patients, by summing the square of standardised copy-number ratios calculated for 1Mb windows of sequencing reads. This algorithm provides a score that can serve as a surrogate marker of tumour burden. An alternative method of estimating the fraction cfDNA that is tumour-derived is provided by Xia et al in their

analysis of cfDNA in prostate cancer patients, in which copy-number ratios in 1Mb windows were calculated by comparing to lymphocyte-derived DNA for each sample, before subjecting to segmentation using the CNAM algorithm. The ratios were then ranked, before summing the square of the 95^{th} – 99^{th} percentile of ratios. They assumed the copy-number aberrations within these percentiles to be the most significant ones, and excluded the highest (100^{th}) percentile aberrations, as they found the most extreme aberrations to be associated with centromere and telomere regions, representing high sequence homologs and poor quality libraries.

The aims of this chapter are:

- In the first part of the chapter, optimisation of the cfDNA extraction process is explained, followed by an examination of cfDNA levels and average sequencing library size as possible factors to be controlled for.
- Following this, in order to provide proof of concept that the copy-number approach has potential as a biomarker of relapse, we explore the two copy-number scoring algorithms (described above) to explore their ability to discriminate active melanoma from relapsed disease, and survival, in subjects from the GEMS study.
- We end by providing data about the sensitivity and technical reproducibility of the copy-number approach.

3.2 Optimisation of cfDNA extraction

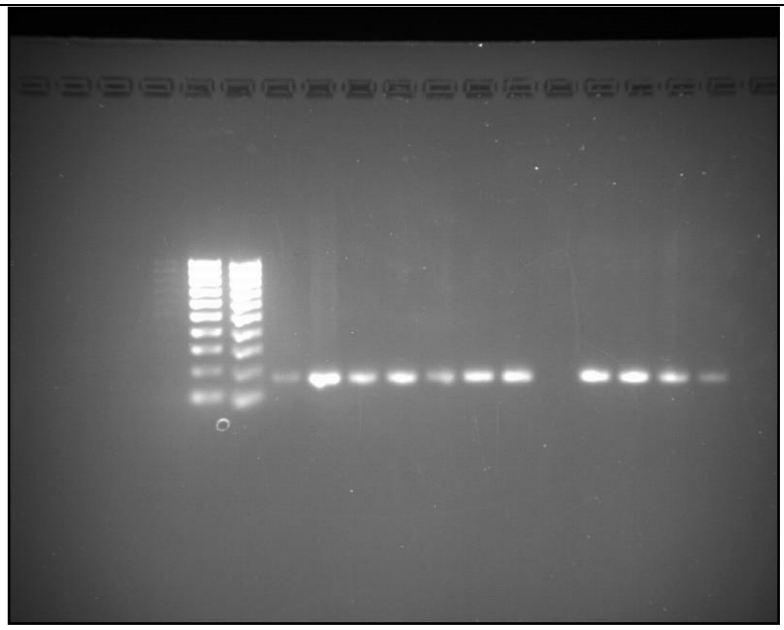
In order to optimise the process of extraction of ccfDNA from plasma, I compared different elution volumes to investigate the effect on DNA yield. This was done by spiking plasma from healthy volunteers with DNA extracted from tumour FFPE samples, and measuring the yield of DNA extracted when three different

elution volumes were applied to the extraction column. The FFPE DNA used in the spiking experiments was first subjected to quality checks prior to being used in spiking, as described below.

3.2.1 Tumour FFPE DNA quality check using gel electrophoresis

FFPE DNA was selected for the spiking experiment, as this type of DNA, by virtue of its fragmented nature, mimics cfDNA in some respects. To assess the quality of the tumour DNA samples extracted from the FFPE specimens, they were subjected to a gel electrophoresis after PCR amplification using short-amplicon BRAF primers (as described in Section 2.8).

All six tumour DNA PCR bands corresponded to a molecular weight of between 100-200 bp, the expected size of the BRAF amplicon, as shown in figure 3.1. Thus, although fragmented, the FFPE DNA is of sufficient size and quality to generate PCR product with these BRAF primers.



Lane 1&2:	Hyperladder IV
Band 3 - 8:	Tumour DNA samples (T1, T2, T3, T4, T5, T6)
Band 9:	Positive control
Band 10:	Negative control, water
Bands 11-14:	Repeat tumour DNA samples (T3, T4, T5, T1)

Figure 3.1: Electrophoresis readout for DNA quality check

3.2.2 Tumour FFPE DNA quantification (using Qubit fluorometry) and quality check (using Nanodrop spectrophotometry)

A further quality check on the tumour FFPE DNA was carried out using Nanodrop spectrophotometry. The A_{260}/A_{280} and $A_{260/230}$ ratios provided information about the quality (purity) of the samples, as shown in table 3.1 below.

FFPE sample	Concentration ng/µl	$A_{260/A280}$	$A_{260/230}$
T1	3.6	1.17	-3.7
T2	80.98	1.94	2.42
T3	9.16	1.57	1.77
T4	14.52	1.53	1.83
T5	59.47	2.15	2.57
T6	19.36	1.88	2.53

Table 3.1: DNA concentration and purity as determined by Nanodrop

An A_{260}/A_{280} ratio of between 1.8 and 2.0, and an $A_{260}/_{230}$ ratio above 1.9 are considered acceptable for good quality DNA samples, indicating the absence of protein and organic contaminants respectively. Significantly lower ratios indicate the presence of contaminants like protein and phenol. The A_{260}/A_{280} and $A_{260}/_{230}$ ratios of all the tumour samples (except Sample T1) reflected that, although variable, these samples did not appear to have significant contamination.

Quantification of double-stranded DNA by Nanodrop spectrophotometry is not wholly accurate and tends to over-estimate DNA concentration. There are a number of reasons for this (Gallagher, 2011). The UV absorbance method is not selective for DNA, RNA or protein. Secondly, this method has low accuracy at lower concentrations. In addition, absolute quantifications vary significantly in the presence of contaminants. In order to improve accuracy, a further quantification of the FFPE DNA samples was carried out by Qubit fluorometry (using the method described in Section 2.6). The DNA concentrations are shown in the table 3.2 below.

Tumour sample	DNA concentration ng/µl
T1	1.81
T2	31.7
T3	4.8
T4	6.92
T5	17.2
T6	6.52

Table 3.2: DNA concentrations of tumour FFPE samples, as determined by Qubit fluorometry

3.2.3 Percentage DNA yield from spiking experiments

Two separate pools of plasma (6 mls each) were made, once of which was spiked with FFPE DNA to achieve a concentration of 50 ng/ml of plasma. Each pool was separated into three 2ml aliquots, and the DNA was extracted from each aliquot using the QIAamp circulating nucleic acid kit, as described in Section 2.4). At the final elution step, one of three different elution volumes of buffer AVE (50 µl, 100 µl and 200 µl) was applied to each aliquot. The yield of DNA was then calculated for each aliquot.

Comparison of the DNA yields from each aliquot of the spiking experiment showed higher yields from the bigger elution volumes (Figure 3.2). Therefore, for all subsequent DNA extractions using the QIAamp kit, 200 µl was used for DNA extractions from 2ml plasma aliquots.

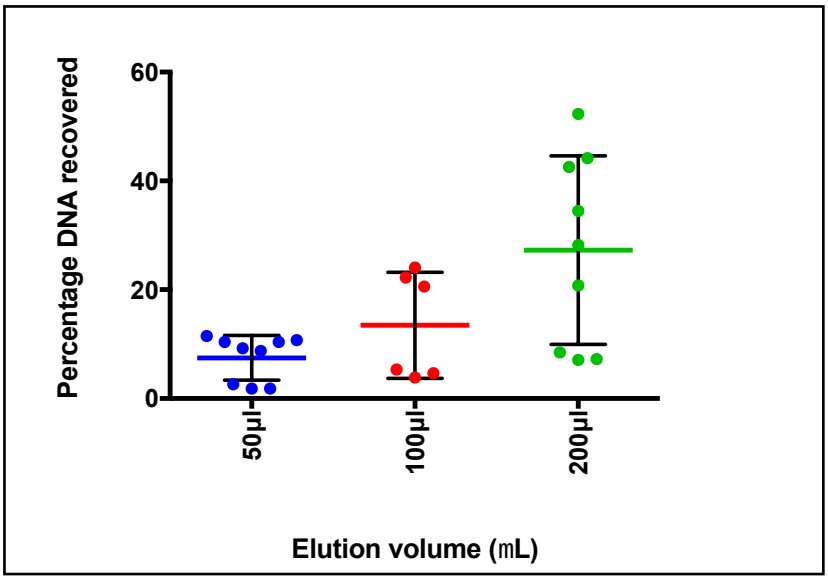


Figure 3.2: Comparing DNA yields using three different elution volumes. Three different elution volumes of AVE buffer were compared (50 µl shown in blue, 100 µl in red and 200 µl in green). 3 aliquots were used for each volume, and the experiment was conducted thrice, giving 9 datapoints for each of the volumes compared. Note that in one of the experiments, the 100ml aliquot quantification failed (hence there are only six datapoints for that volume).

3.3 CfDNA concentration in active and resected disease

The GEMS study and its participants are described in Section 2.1. We hypothesised that cfDNA levels would be greater in those with active and more severe disease, and for comparison purposes we first examined cfDNA levels as a biomarker of active disease and survival. CfDNA was extracted from the plasma of participants recruited to the GEMS study, and the cfDNA concentrations determined through quantification by SYBR-green qPCR (as described in Section 2.7).

Significantly higher levels of cfDNA were observed in those with active disease (n=44; median (range) 7.63 (1.28-54.01) ng/ml) compared to those with resected disease (n=39; median (range) 3.78 (1.47-10.39) ng/ml; p=0.0001) (Figure 3.3).

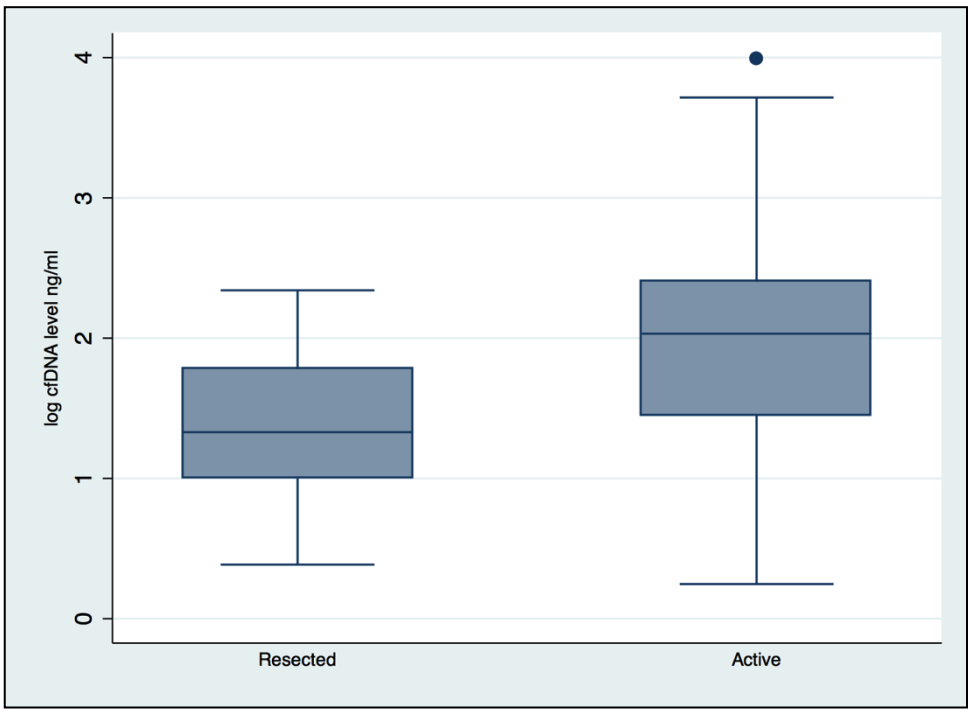


Figure 3.3: Comparison of cfDNA levels between active and resected cases. Box and whisker plot of the cfDNA levels in cases with active melanoma (n=44) compared to those with resected disease (n=39)

3.4 Survival analysis based on cfDNA levels in GEMS

Since a higher level of cfDNA was associated with active disease, the association between cfDNA level (as a continuous variable in ng/ml) and patient survival was investigated. Using Cox regression there was a significant association between cfDNA level and patients' survival, with a hazard ratio (95% CI) of 5.52 (3.44 – 8.86), p< 0.0001, as shown in Table 3.3. In multivariable analysis this association remained significant after correcting for stage of disease, status of disease (active or resected), and BRAF status (HR (95% CI) 2.78 (1.68 – 4.61); p <0.0001).

	HR	p-value	95% CI
Univariable analysis			
log cfDNA level	5.52	<0.0001	3.44 - 8.86
Multivariable analysis			
log cfDNA level	2.28	<0.0001	1.68 - 4.61
Stage	2.76	0.032	1.09 - 7.0
BRAF status	2.74	0.002	1.44 - 5.21
Active/Resected	5.26	<0.0001	2.44 - 11.36

Table 3.3: Cox regression analysis for log cfDNA level

The melanoma cases were then grouped based on log cfDNA quartiles. As shown in Table 3.4, there was a trend for increasing mortality, with the 3rd and 4th quartiles (log cfDNA levels between the 50th & 75th percentiles, and levels above the 75th percentile, respectively) having significantly higher hazard ratios for death, compared to the reference quartile (log cfDNA level below the 25th percentile). Thus, the data demonstrate that higher cfDNA levels in plasma are associated with active disease and poor survival.

Log cfDNA level	HR	p-value	95% CI
< 25th percentile	reference		
25th - 50th percentile	1.87	0.189	0.73 - 4.75
50th - 75th percentile	3.24	0.011	1.31 - 8.02
> 75th percentile	14.4	<0.0001	5.81 - 35.68

Table 3.4: Cox regression analysis for cfDNA quartiles

3.5 Stability of cfDNA levels over time

Plasma samples collected from melanoma patients in the GEMS study between January 2008 and June 2009, were stored at -80 C. An initial cfDNA extraction was carried out from the plasma of all 83 cases in November 2009 using the QIAamp circulating nucleic acid kit. A second cfDNA extraction was carried out

in August 2016 from the remaining plasma samples from the same 83 cases. The yields of cfDNA extracted from the stored plasma at these two time points seven years apart was compared. This showed good agreement (Spearman's correlation for log cfDNA level = 0.75, p<0.0001), with a median drop in yield of 2.8 ng/ml plasma over that time period (interquartile range 0.59 - 6.2 ng/ml of plasma).

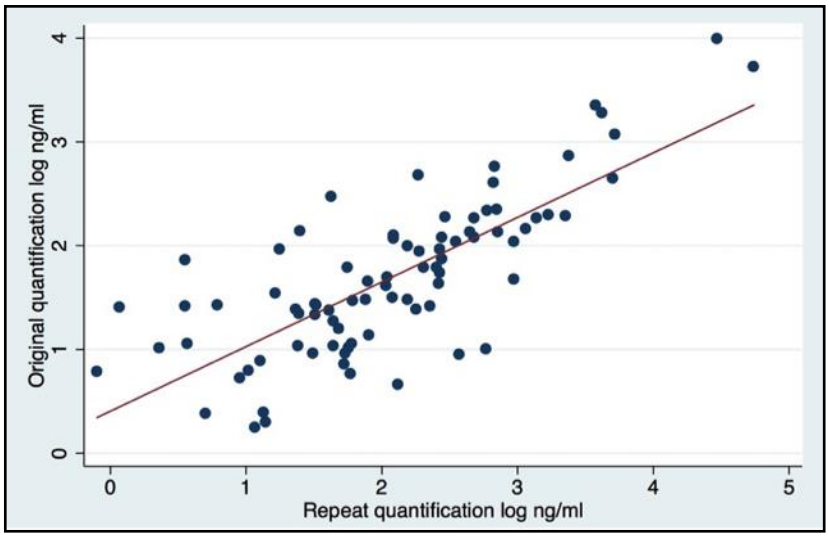


Figure 3.4: Scatter plot of DNA yields from extractions carried out over 2 separate time-points, 7 years apart, showing good correlation.
Scatter plot of cfDNA yields from the same plasma samples (n=83) carried out in 2009 (y axis) and 2016 (x axis). Spearman's correlation for log cfDNA level was 0.75, p<0.0001.

3.6 Library size analysis

Sequencing libraries were generated from the GEMS cfDNA samples as described in Section 2.15. A tape-based electrophoresis of the DNA libraries was carried out using the 2200 TapeStation System (Agilent®), to check the size distribution of the libraries prior to pooling. This system used capillary electrophoresis to provide an accurate measurement of DNA fragment size distribution in each library. We hypothesised that mean library size in bp might be greater in samples from patients with active or more severe disease due to the presence of higher levels of cfDNA as demonstrated above.

A comparison was made to examine if there were differences in library sizes between cases with active and those with recently excised disease. Patients with active disease tended to have larger fragment sizes than those with resected cases (p= 0.0124), as shown in the table below.

Cases	Mean (bp)	Standard deviation	95% CI
Resected	290	10.4	287-293
Active	297	15.8	292-301

Table 3.5: T-test comparison of library sizes between active and resected cases

Cases were grouped according to library size quartile, and those cases in the highest quartile (library size >300 bp) were found to have significantly increased risk of active disease (OR (95% CI) 5.82 (1.53–22.17), p=0.010, as shown in Table 3.6.

Library Size quartile	Odds Ratio	*p*-value	95% CI
< 285 bp	Base		
285 – 289 bp	1.29	0.681	0.38 - 4.39
289 – 300 bp	1.31	0.656	0.40 - 4.27
>300 bp	5.82	0.010	1.53 - 22.17

Table 3.6: Logistic regression analysis of library size quartiles for active disease

Univariable logistic regression analysis comparing cases with active melanoma to those with resected disease showed that library size above or below 300 bp (i.e. above or below the 75th percentile) was a significant predictor of the presence of active disease (OR (95% CI) 4.99 (1.50 – 16.65), p= 0.009). Adjusting for cfDNA level and stage of disease, multivariable logistic regression analysis showed the library size category (above or below 300 bp) to be a significant predictor of active disease as shown in the regression analysis in table 3.7.

Univariable analysis			
	OR	***p*-value**	**95% CI**
Library size category*	4.99	0.009	1.50 - 16.65
Multivariable analysis			
	OR	***p*-value**	**95% CI**
Library size category*	14.11	0.022	1.48 - 134.79
Log cfDNA level	2.12	0.110	0.84 - 5.31
Stage	61.41	<0.0001	6.85 - 550.86

Table 3.7: Logistic regression analysis of library size as a predictor of active disease
*Library size above and below 300 bp

To examine if library size was a predictor of overall survival, a univariable Cox regression analysis was carried out (Figure 3.5). This showed mortality to be higher among cases with library size above 300 bp (HR (95% CI) 2.65 (1.44 – 4.87), $p = 0.002$).

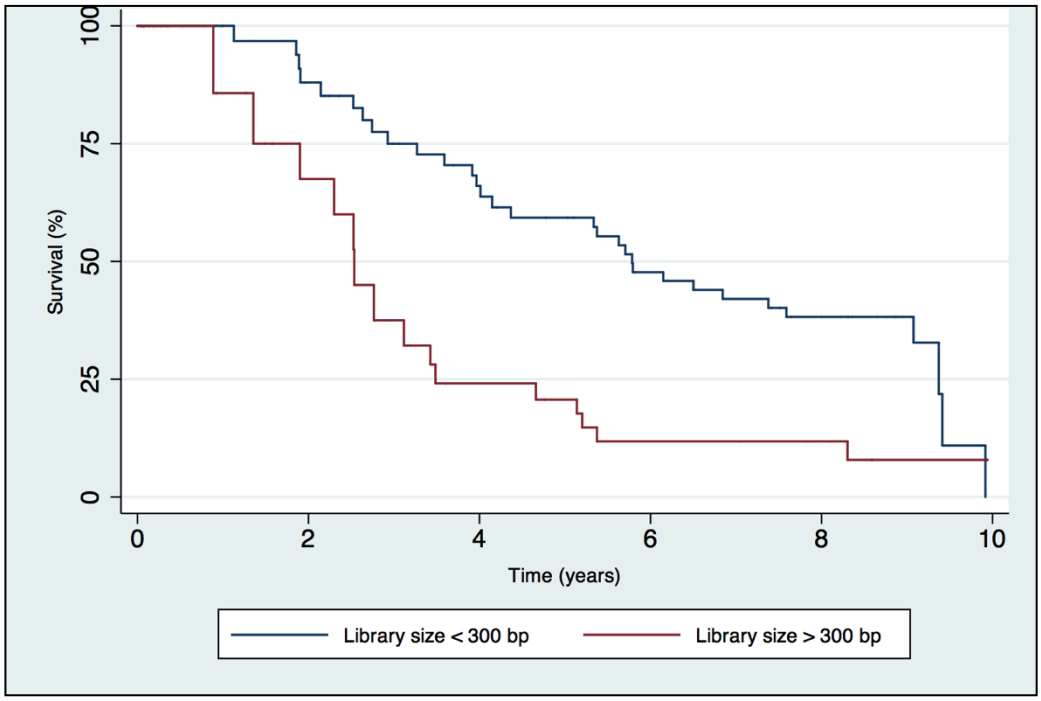


Figure 3.5: Kaplan-Meier survival estimates for overall survival, showing higher mortality among case with library size > 300bp, compared to cases with library size <300bp
Kaplan–Meier survival graph comparing survival for patients with library size above 300bp (red, n=23) and below 300bp (blue, n=60). Mortality was higher among cases with library size above 300 bp (HR (95% CI) 2.65 (1.44 – 4.87), $p = 0.002$).

However, after adjusting for other significant predictors of survival, such as stage of disease and BRAF status, as shown in the multivariable Cox regression table below, this association was no longer significant.

	HR	*p*-value	95% CI
Univariable analysis			
Library size category*	2.65	0.002	1.44 – 4.87
Multivariable analysis			
Library size category*	1.23	0.581	0.59 - 2.56
cfDNA level	2.84	<0.0001	1.69 - 4.74
BRAF status	2.79	0.002	1.46 - 5.35
Active/Resected	4.78	<0.0001	2.05 - 11.15
Stage	2.82	0.029	1.11 - 7.16

Table 3.8: Cox regression table of library size category* as a predictor of overall survival
*Library size above and below 300 bp

3.7 Low coverage sequencing of GEMS DNA samples

Following extraction of cfDNA and quantification by qPCR, DNA libraries were prepared using the NEBnext library prep kit for Illumina. These libraries were quantified using the 2200 TapeStation System (Agilent®) system, then pooled and sequenced on the Illumina HiSeq platform. These methods are described in Sections 2.12 and 2.14. The libraries were prepared and quantified by Dr. Shobha Silva, and staff in Sheffield Diagnostic Genetics Service carried out pooling and sequencing.

Sample ID	Total number of reads	Number of mapped reads	Percentage of mapped reads	Number of paired reads	Coverage	Percentage duplicate reads
C0001	18531250	16564138	89.38	8282069	0.47	0.0113
C0002	22958166	20616322	89.80	10308161	0.57	0.0101
C0003	20731302	18562336	89.54	9281168	0.53	0.0115
C0004	19831050	17811890	89.82	8905945	0.49	0.0064
C0005	23255198	20857458	89.69	10428729	0.58	0.0093
C0006	31954072	28651572	89.66	14325786	0.79	0.0097
C0007	31193286	27847822	89.28	13923911	0.77	0.0124
C0008	17250950	15572026	90.27	7786013	0.43	0.0063
C0009	17724582	15644474	88.26	7822237	0.44	0.009
C0010	22147474	19657176	88.76	9828588	0.47	0.0105
C0011	20680930	18181590	87.91	9090795	0.48	0.0123
C0012	16812808	15174990	90.26	7587495	0.42	0.0059
C0013	17238482	14815642	85.95	7407821	0.43	0.0106
C0014	20074210	18064074	89.99	9032037	0.47	0.0081
C0015	21390562	18968538	88.68	9484269	0.54	0.0099
C0016	18541274	16392856	88.41	8196428	0.48	0.0127
C0017	26044926	23433046	89.97	11716523	0.65	0.011
C0018	16671062	14875580	89.23	7437790	0.41	0.0092
C0019	31642696	28367128	89.65	14183564	0.78	0.0098
C0020	36099034	32418440	89.80	16209220	0.88	0.0074
C0021	18511878	16587612	89.61	8293806	0.46	0.0089
C0022	14694214	13215044	89.93	6607522	0.38	0.0081
C0023	14806006	13301624	89.84	6650812	0.37	0.008
C0024	19927852	17466078	87.65	8733039	0.52	0.013
C0025	12687594	11408242	89.92	5704121	0.32	0.0077
C0026	11296600	10066960	89.11	5033480	0.29	0.0102
C0027	16053452	14197832	88.44	7098916	0.41	0.01
C0028	8079976	6913292	85.56	3456646	0.20	0.0098

C0029	16528932	14960764	90.51	7480382	0.42	0.0068
C0030	15163060	13583598	89.58	6791799	0.39	0.0103
C0031	8461776	7334506	86.68	3667253	0.21	0.0099
C0032	16676062	14878180	89.22	7439090	0.43	0.0084
C0033	25797610	22862648	88.62	11431324	0.65	0.0106
C0034	13559550	12216000	90.09	6108000	0.35	0.0082
C0035	20648348	18306552	88.66	9153276	0.52	0.0092
C0036	15294234	13578904	88.78	6789452	0.39	0.0108
C0037	13720912	12306098	89.69	6153049	0.35	0.0098
C0038	14022436	12562388	89.59	6281194	0.35	0.0086
C0039	24344912	21881516	89.88	10940758	0.61	0.0085
C0040	12493660	11246788	90.02	5623394	0.31	0.0095
C0041	11001320	9883016	89.83	4941508	0.28	0.0094
C0042	16382262	14642974	89.38	7321487	0.42	0.0111
C0043	14489886	12990960	89.66	6495480	0.38	0.01
C0044	31193134	27896938	89.43	13948469	0.77	0.0081
C0045	11319802	10159234	89.75	5079617	0.28	0.0069
C0046	6572132	5723590	87.09	2861795	0.17	0.0101
C0047	8884378	7806376	87.87	3903188	0.23	0.011
C0048	7458012	6658056	89.27	3329028	0.19	0.0098
C0049	7215946	6446680	89.34	3223340	0.18	0.0097
C0050	18342314	16145968	88.03	8072984	0.48	0.0105
C0051	10705928	9511872	88.85	4755936	0.27	0.0123
C0052	14567470	13041802	89.53	6520901	0.38	0.0099
C0053	7699302	6769752	87.93	3384876	0.19	0.012
C0054	7327456	6531404	89.14	3265702	0.19	0.0097
C0055	8284660	7341582	88.62	3670791	0.21	0.0098
C0056	14083856	12581922	89.34	6290961	0.36	0.0106
C0057	14162254	12612736	89.06	6306368	0.36	0.0093
C0058	9129178	8248586	90.35	4124293	0.24	0.0111
C0059	10752752	9423258	87.64	4711629	0.27	0.0157
C0060	6709336	5824060	86.81	2912030	0.17	0.0106

C0061	20610174	18389864	89.23	9194932	0.53	0.0102
C0062	15708910	13894880	88.45	6947440	0.41	0.0104
C0063	14830294	12943424	87.28	6471712	0.37	0.0086
C0065	18975366	16984694	89.51	8492347	0.48	0.009
C0066	15342232	13809974	90.01	6904987	0.40	0.0097
C0067	16736516	15035364	89.84	7517682	0.42	0.0091
C0068	18302520	16229220	88.67	8114610	0.48	0.012
C0069	15186536	13417684	88.35	6708842	0.38	0.0102
C0070	11920860	10630044	89.17	5315022	0.31	0.0099
C0071	16108868	14426532	89.56	7213266	0.41	0.0097
C0072	17114970	15246910	89.09	7623455	0.44	0.0126
C0073	15626596	13979730	89.46	6989865	0.41	0.0096
C0074	17001942	15260964	89.76	7630482	0.43	0.0099
C0075	11693546	10250548	87.66	5125274	0.29	0.0101
C0076	14085442	12418656	88.17	6209328	0.36	0.0128
C0077	15596640	13947540	89.43	6973770	0.40	0.0107
C0078	13888632	12432206	89.51	6216103	0.33	0.0082
C0079	15157268	13578540	89.58	6789270	0.39	0.0099
C0080	14661366	12725028	86.79	6362514	0.37	0.0415
C0081	13920148	12384608	88.97	6192304	0.36	0.0084
C0082	14063838	12453022	88.55	6226511	0.35	0.0103
C0083	13465032	12077710	89.70	6038855	0.34	0.0104
C0084	14446972	13046552	90.31	6523276	0.36	0.01

Table 3.9: Metrics summary from sequencing runs of 85 cfDNA samples from the GEMS study.

The 83 cfDNA samples were sequenced on 5 separate runs, the individual metrics for each sample are shown in Table 3.9 above, and the metrics are summarised below. The median number of reads across the samples was 15.3 million. On average, 89% of the reads mapped to the human reference-genome GRCh38, with a median coverage of 0.4 X the genome.

	Median	Range
Number of reads	15,342,232	6,572,132 - 36,099,034
Number of mapped reads	13,809,974	5,723,590 - 32,418,440
Percentage mapped reads	89.34	85.56 - 90.51
Number of paired reads	6,904,987	2,861,795 - 16,209,220
Coverage	0.4	0.17 - 0.88
Percentage duplicate reads	0.0099	0.0059 - 0.0415

Table 3.10: Summary of metrics from sequencing runs

3.8 Copy-number aberration scores

To investigate if whole-genome copy-number profiles could be used to differentiate active melanoma cases from those with recently excised disease, we explored two different scoring algorithms, both of which sought to numerically summarise the copy-number profiles for each case, to enable comparisons. Copy number ratios for 1Mb windows were calculated based on numbers of sequencing reads as described in Section 2.17.

Both of these algorithms were based on z-scores (standardised copy number ratios). Briefly, for the CNAS score, Z-scores for each 1Mb window were summed across the entire genome; while for the eCNAS, Z-scores across the genome were, ranked, and the Z-scores in the 95-99th percentiles were then summed. The derivation of the scores is described in detail in Section 2.18.

3.9 Normalising tumour DNA copy-number ratios to corresponding lymphocyte DNA *vs.* normalising to pooled genomic DNA from healthy controls

The reads obtained from sequencing lymphocyte DNA from twenty randomly selected healthy controls from the Markers of Relapse in Melanoma Study were pooled bioinformatically, to obtain a reference to which each cfDNA or tumour DNA sample could be normalised.

Sequenced tumour DNA samples extracted from FFPE biopsy or surgical resection samples from forty-nine patients (from the GEMS study) were normalised to their corresponding genomic DNA, to create copy-number ratios from which eCNAS and CNAS scores were calculated (as previously described). In a separate analysis, the sequenced tumour DNA was normalised to the genomic pool of reads from healthy controls, before calculating eCNAS and CNAS scores.

We compared the copy number scores (CNAS and eCNAS) for each tumour DNA sample, when normalised to the corresponding lymphocyte DNA sample and when normalised to the pooled genomic DNA, in order to see how well these scores correlated.

Both the eCNAS and CNAS scores showed an excellent correlation when normalised either to corresponding genomic DNA or to the genomic pool, as shown in Figures 3.6 and 3.7. The Spearman coefficient of correlation for the eCNAS was 0.9295 ($p<0.0001$), and 0.9957 ($p<0.0001$) for the CNAS score.

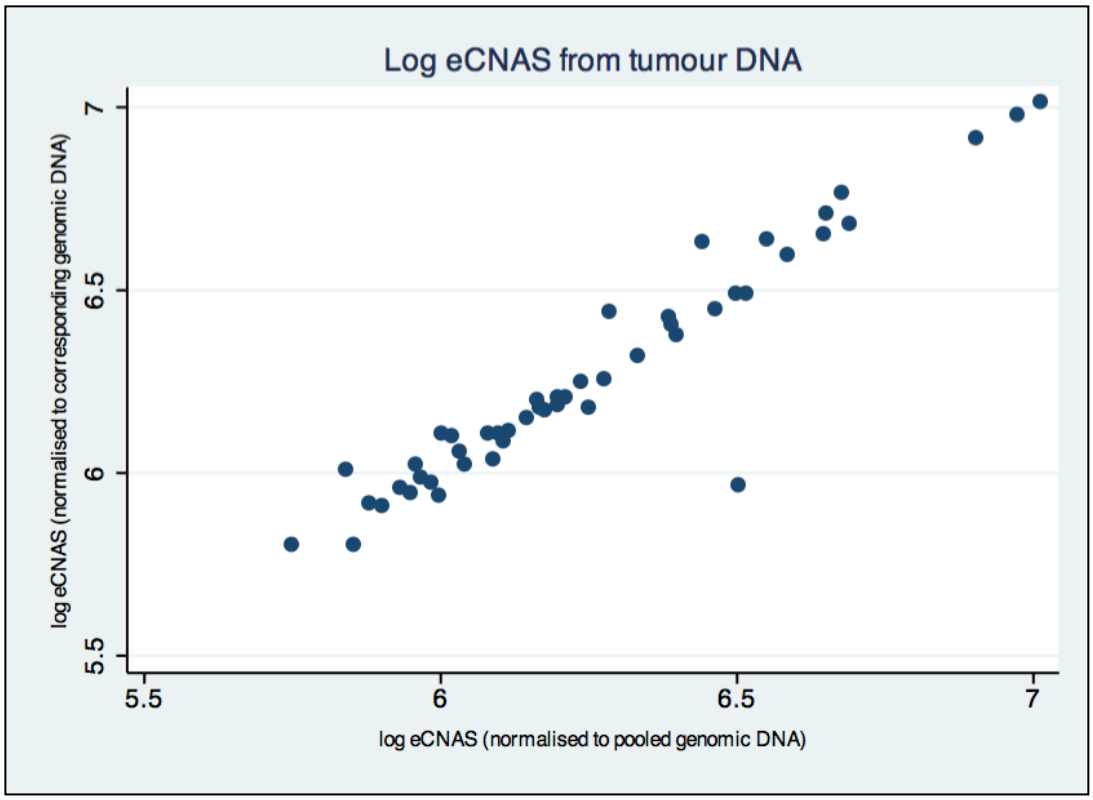


Figure 3.6: Scatter plot showing correlation of log eCNAS normalised to pooled genomic DNA (x-axis, n=20) and log eCNAS normalised to corresponding genomic DNA (y-axis, n=20). The Spearman coefficient of correlation was 0.9295 (p<0.0001)

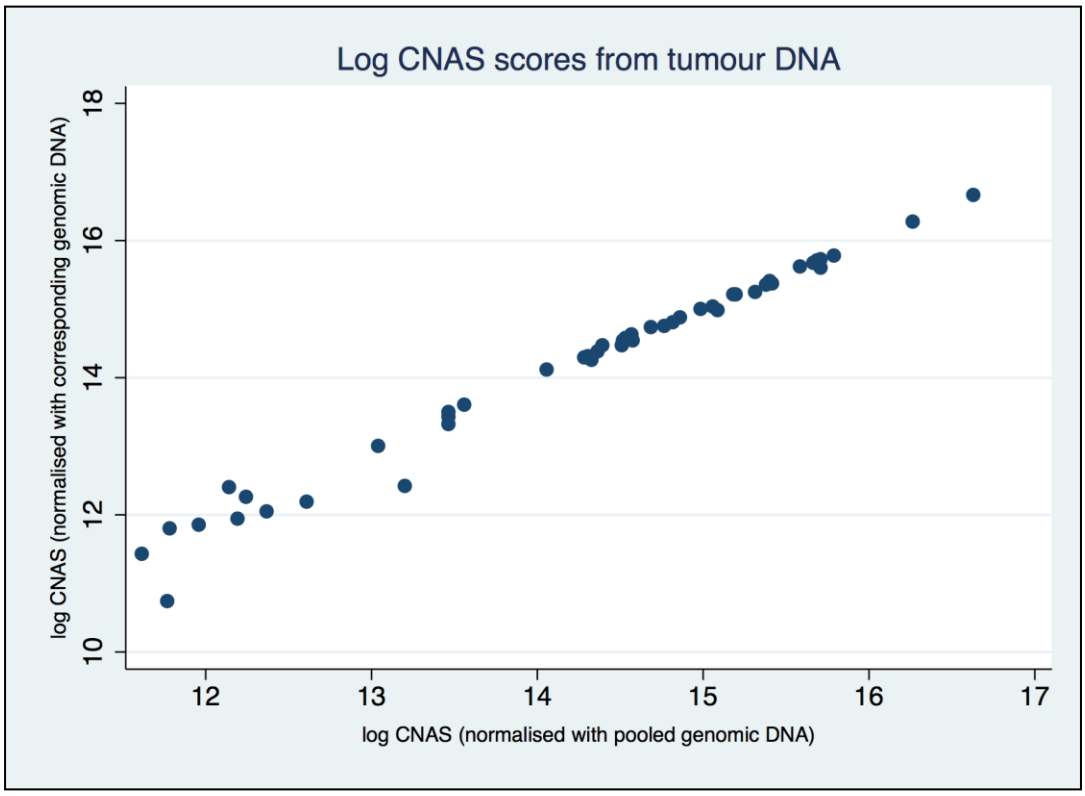


Figure 3.7: Scatter plot showing correlation of log CNAS normalised to pooled genomic DNA (x-axis, n=20) and log CNAS normalised to corresponding genomic DNA (y-axis, n=20). The Spearman coefficient of correlation was 0.9957 (p<0.0001)

3.10 No correlation of tumour DNA copy-number scores with cfDNA copy-number scores

We compared the copy number scores (eCNAS and CNAS) for forty-nine tumour DNA samples (including active melanoma cases and recently-excised cases) with the scores of the corresponding cfDNA samples, and found no significant correlation between these scores (Table 3.11).

When we analysed those cases for which there was less than a 180-day interval between the tumour DNA sample and cfDNA sample, there remained no significant correlation. Similarly, samples with less than a 30-day interval between samples did not show any significant correlation.

All cases (n=49)	**Spearman coefficient**	***p*-value**
eCNAS	0.0671	0.6467
CNAS	0.0376	0.7978
Resected cases (n=22)		
eCNAS	0.2298	0.3035
CNAS	0.0175	0.9384
Active cases (n=27)		
eCNAS	0.0140	0.9446
CNAS	-0.0452	0.8229
< 180 days between tumour and cfDNA samples (n=30)		
eCNAS	0.0701	0.7129
CNAS	-0.0656	0.7304
> 180 days between tumour and cfDNA samples (n=19)		
eCNAS	0.0316	0.8979
CNAS	0.1877	0.4415

Table 3.11: Comparison of copy-number scores in 49 tumour DNA samples, compared with corresponding cfDNA samples, showing no significant correlation.

3.11 Optimisation of number of PCR cycles for enrichment of adaptor-ligated DNA with the NEBNext Ultra II kit

The NEBNext Ultra II kit utilises a PCR amplification step during DNA library preparation process to enrich for adaptor-ligated DNA, while increasing the amount of library available for sequencing. The protocol recommends a specific range of PCR cycles based on the amount of input DNA. In order to optimise this PCR-amplification step, we conducted an experiment comparing three different number of PCR cycles (8,10 and 12 cycles), using two different input DNA amounts (2ng and 10ng), in order to compare the quality of libraries obtained.

We subjected three aliquots each of 2ng and 10ng of cfDNA (extracted from the plasma of a patient with advanced lung cancer) to the same library preparation conditions, except for varying the number of PCR amplification-cycles. The prepared libraries were then analysed on the 2200 TapeStation System (Agilent®) to check the quality of the libraries.

For the low-volume (2ng) DNA libraries, the Tapestation report showing the library peaks are shown in figure 3.8 below, with the corresponding library concentrations shown in Table 3.12. The electropherogram shows that 8 PCR cycles did not produce significant library peaks for 2ng of input DNA; while the table shows that 10 PCR cycles produced a limited amount of library for sequencing, therefore making 12 PCR cycles the preferred option for this low level of input DNA.

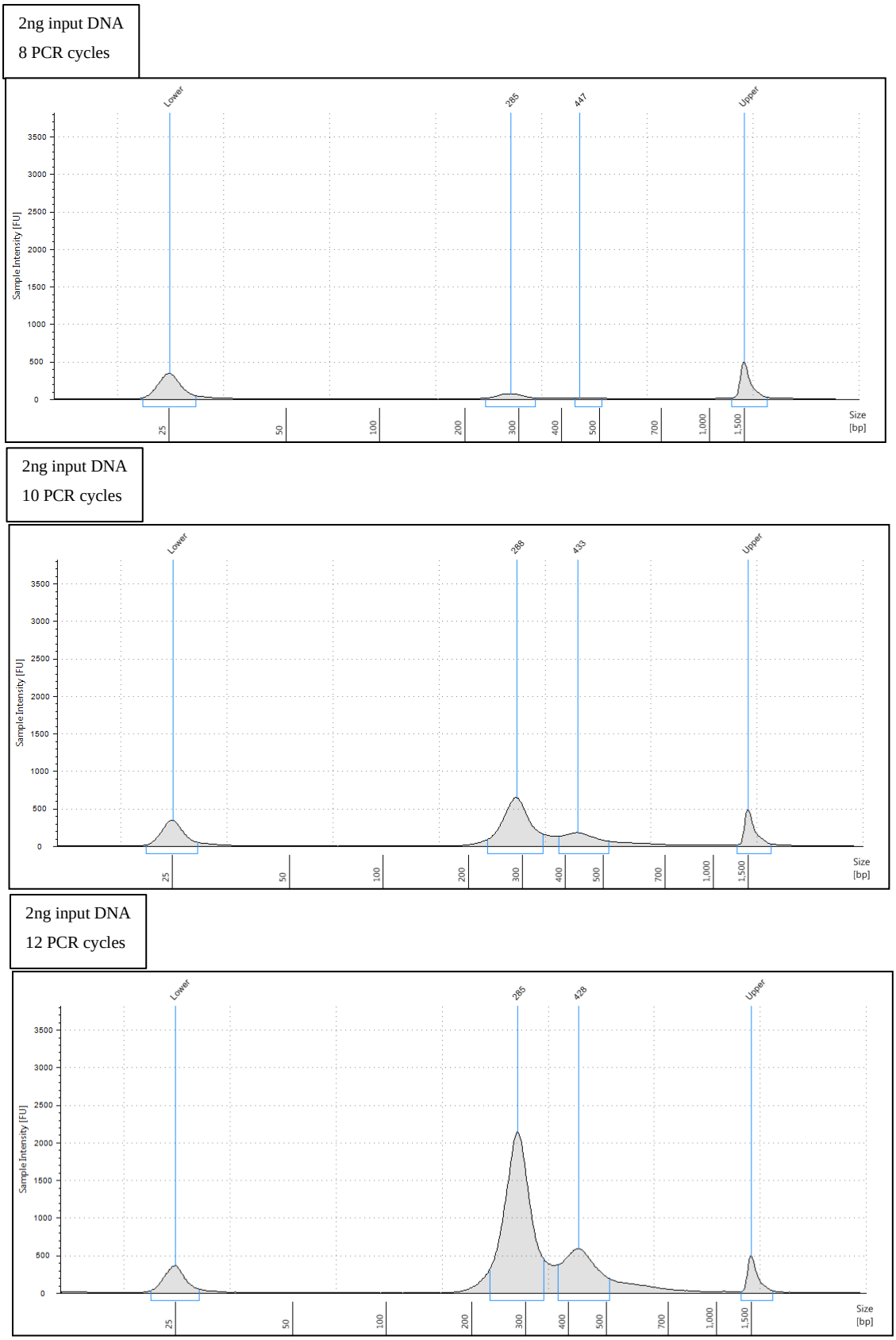
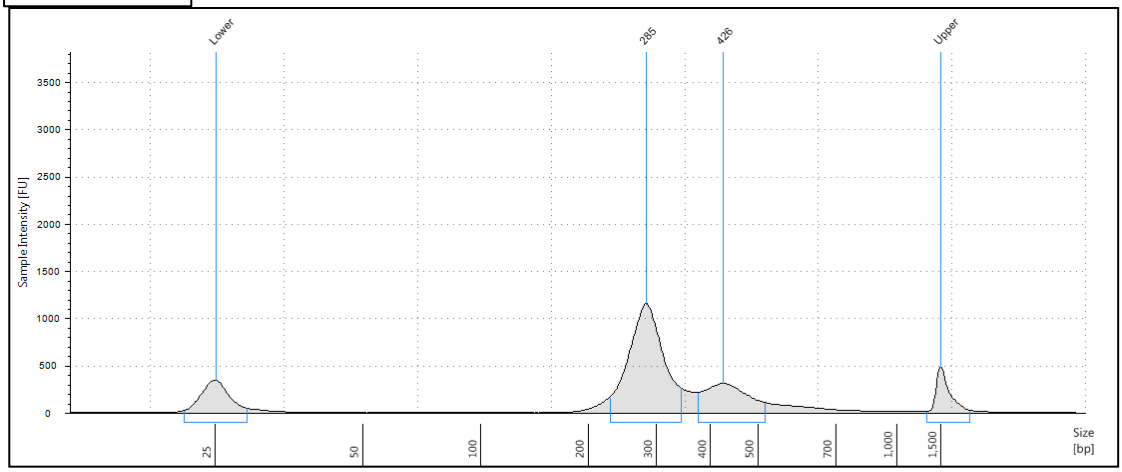
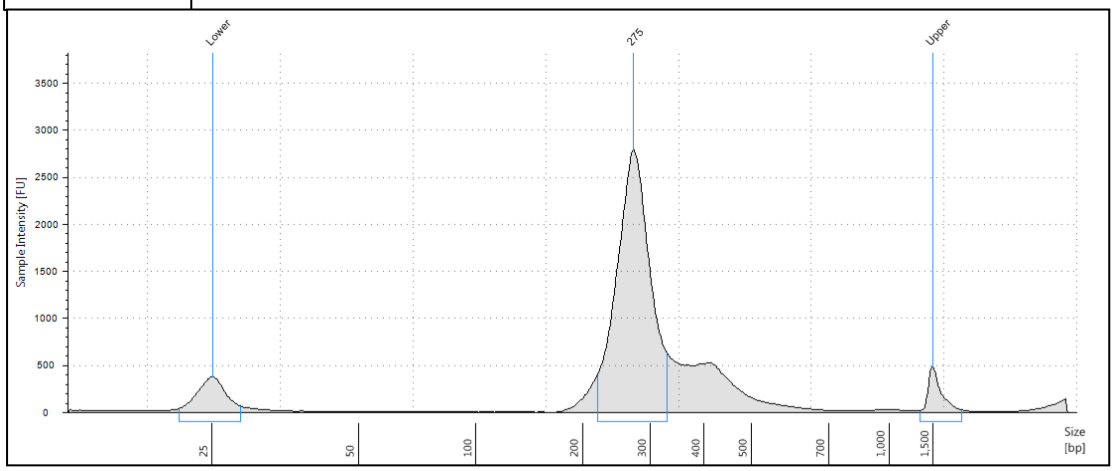


Figure 3.8: Tapestation reports for low-volume (2ng) DNA libraries, comparing three different PCR cycles (8, 10, and 12 cycles)

10 ng input DNA
8 PCR cycles



10 ng input DNA
10 PCR cycles



10 ng input DNA
12 PCR cycles

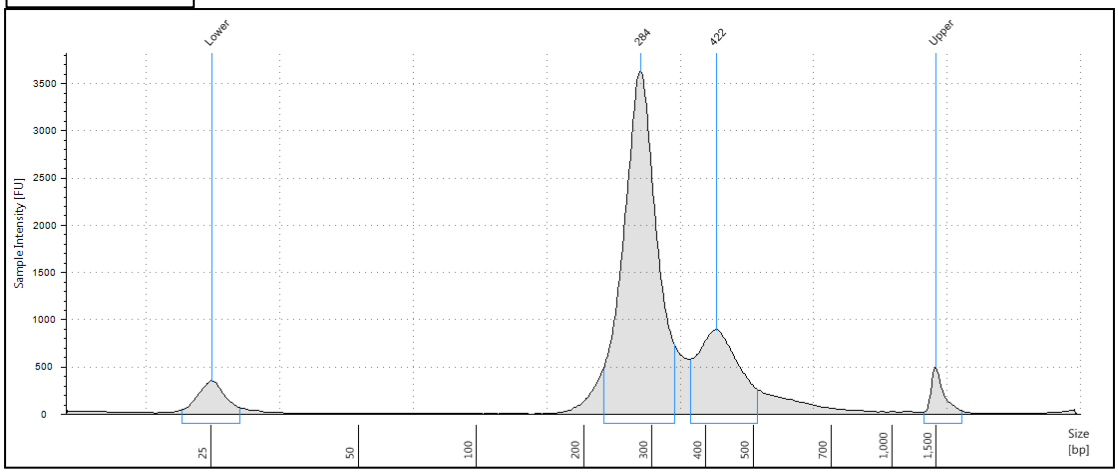


Figure 3.9: Tapestation reports for 10ng DNA libraries, comparing three different PCR cycles (8, 10, and 12 cycles)

DNA input	Number of PCR cycles	DNA library concentration (pmol/L)
10ng	8	11,400
10ng	10	36,000
10ng	12	27,500

DNA input	Number of PCR cycles	DNA library concentration (pmol/L)
2ng	8	994
2ng	10	6450
2ng	12	20,600

Table 3.12: Summary of DNA library concentration (pmol/L) comparing three different PCR cycles, with low (2ng) and high (10ng) DNA input

The electropherograms for the 10ng input DNA libraries are shown in Figure 3.9. 10 PCR cycles produced an ideal library concentration for the 10ng DNA input aliquot. 12 cycles resulted in concatenation of the DNA library.

3.12 Good reproducibility of copy-number approach

In order to investigate the reproducibility of the low-coverage copy-number approach, DNA libraries prepared from the MDA-MB-435 cell line were sequenced on nine separate runs. The copy-number profiles, as well as the CNAS and eCNAS, for these cell-line DNA samples were compared across the runs, to assess the reproducibility of the copy-number analysis.

The copy-number profiles for the cell-line control from each of the nine sequencing runs are shown in figure 3.10 below. Comparison of the profiles shows

the same patterns of major copy-number gains and losses across all 9 runs, giving an indication of high reproducibility of the approach.

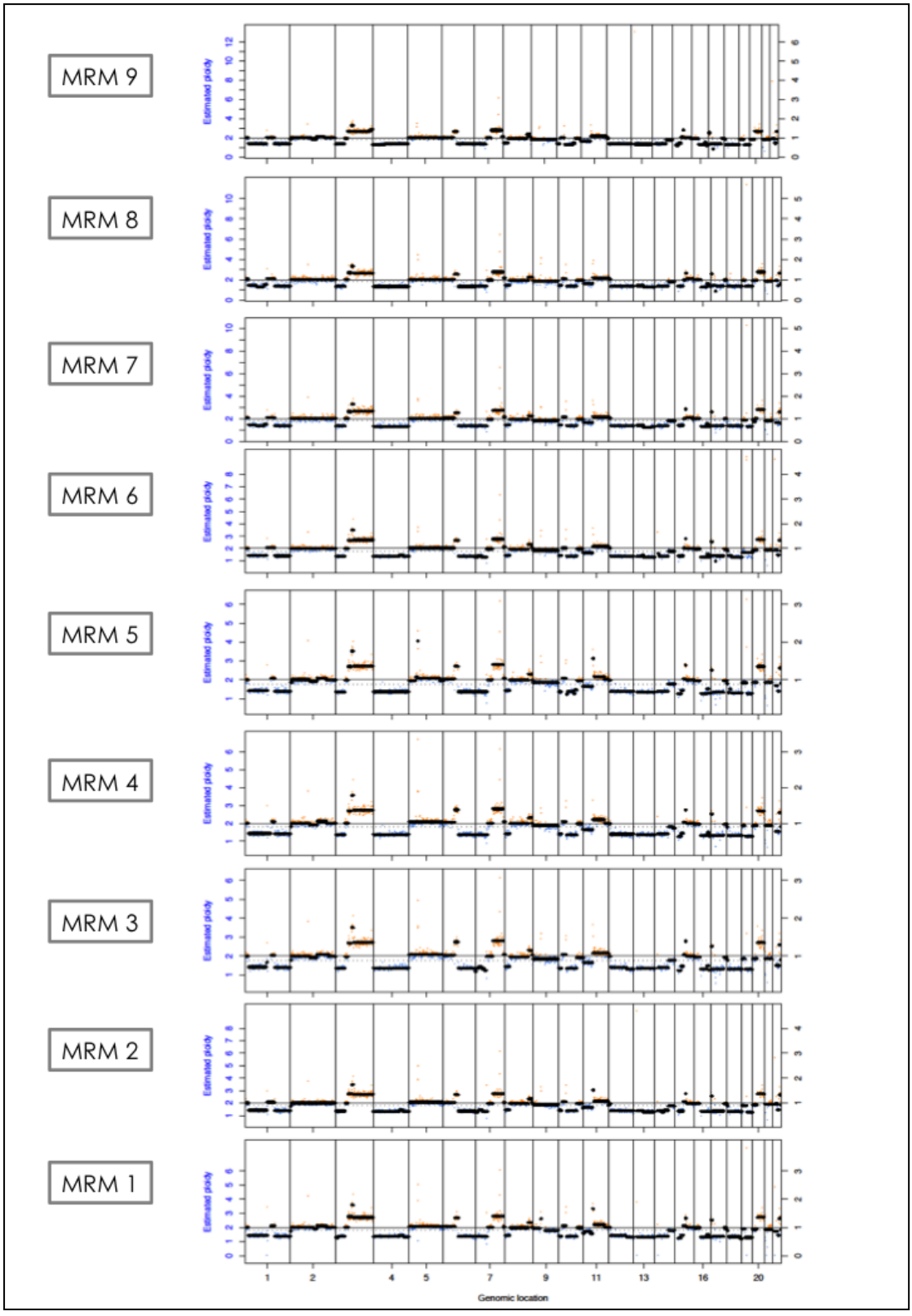


Figure 3.10: Whole-genome copy-number profiles for the cell-line control from 9 separate sequencing runs showing same patterns of copy-number gains and losses. Copy number profiles of MDA-MB-435 cell line DNA produced from 9 sequencing runs on IIumina Hi-Seq. x- axis: genomic coordinate, y- axis: estimated copy-number ratio

In order to objectively compare the copy-number changes, the CNAS and eCNAS were calculated for each profile, and the coefficient of variation of each score was calculated to estimate the degree of dispersion of the scores across the runs. While both scores showed very little dispersion across the runs, the eCNAS was more variable.

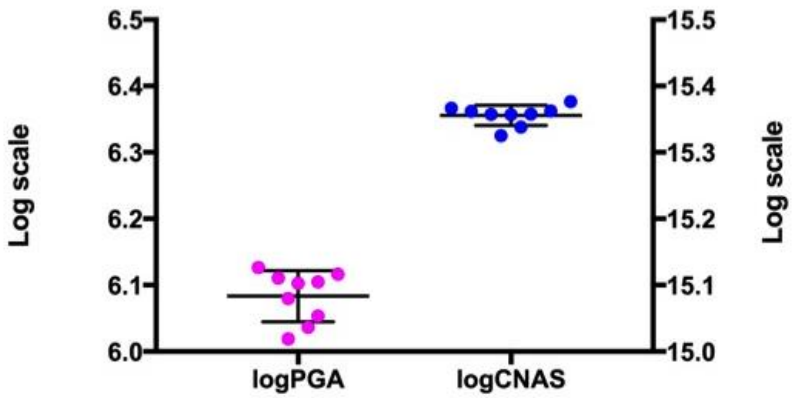


Figure 3.11: The CNAS shows highly reproducible yields across sequencing runs. Copy number aberration scores of MDA-MB-435 cell line DNA determined on 9 occasions. Left-hand axis: log eCNAS; right-hand axis: log CNAS.

3.13 Technical sensitivity of copy-number approach

To estimate the technical sensitivity of this approach, serial dilutions (ranging from 50% to 0.01%) of MDA-MB-435 DNA were sequenced. Their copy-number profiles, as well as the CNAS and eCNAS scores, were compared with that of lymphocyte DNA extracted from a healthy control.

As shown in the figure below, copy-number aberrations could be detected as a raised CNAS above baseline down to a dilution of 6.25% cell-line DNA.

For the eCNAS, however, no increase above the baseline was detected in any of the dilutions (including the 100% cell line DNA).

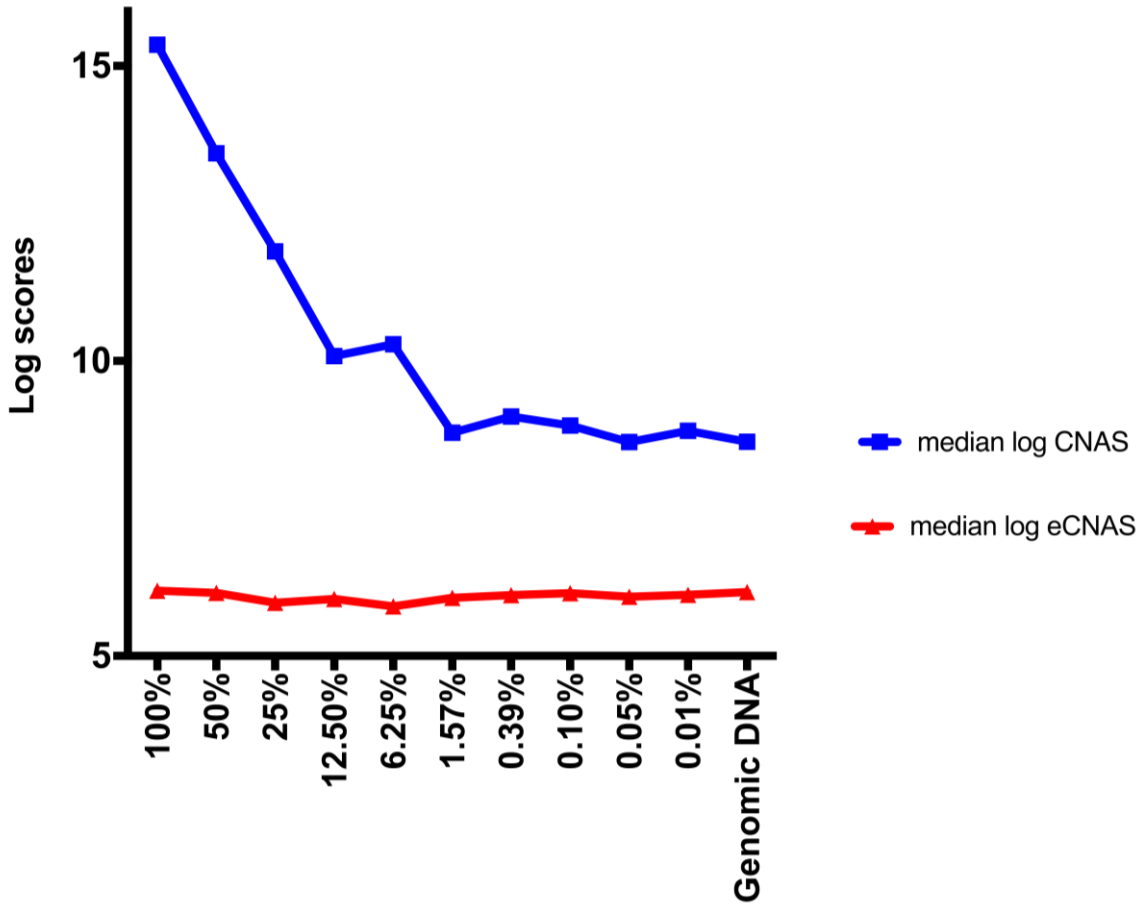


Figure 3.12: Copy-number aberrations are detected in serial dilutions of cell-line DNA, with a limit of detection of 6.25%. Copy number aberration score determined for serial dilutions of MDA-MB-435 cell line DNA in control lymphocyte DNA. Median of duplicate determinations is shown.

3.14 Comparison of CNAS and eCNAS scores between active and resected cases

When I examined the CNAS for all 83 cases, I found that those with active melanoma had higher scores compared to those with recently excised disease (p=0.0011). On the other hand, for the eCNAS scores, no significant difference was found between the two groups (p=0.14).

	Mean	SD	95% CI
log eCNAS			
Active (n=44)	6.16	0.327	6.06 – 6.26
Resected (n=39)	6.03	0.360	5.91 – 6.14
log CNAS			
Active (n=44)	11.59	1.450	11.15 – 12.03
Resected (n=39)	10.64	0.448	10.49 – 10.78

Table 3.13: Summary of log CNAS and eCNAS scores for active and resected cases

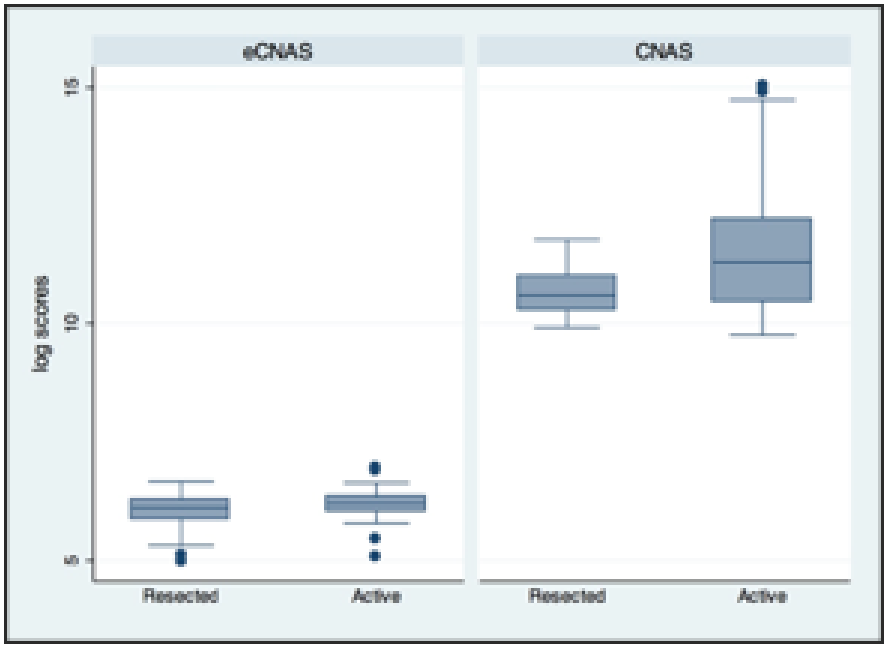


Figure 3.13: CNAS was higher for cases with active disease than those with resected disease; while eCNAS showed no difference between the 2 groups. Box and whisker plot of the log scores (log eCNAS on the left, log CNAS on the right) in cases with active melanoma compared to those with recently excised disease.

3.15 CNAS and eCNAS as predictors of the presence of active disease

To identify significant predictors of active disease, we carried out univariable logistic regression analysis comparing patients with active melanoma to those with recently resected disease. In addition to cfDNA level and library size, various variables were explored as outlined in the table below.

	OR	*p*-value	95% CI	AUC
Univariable analysis				
cfDNA category	12.81	**0.001**	2.73-60.02	0.68
Library size category	4.99	**0.009**	1.50 – 16.65	0.63
Stage	42	**<0.0001**	8.76 – 201.27	0.81
Age	0.98	0.206	0.96 – 1.01	0.59
Gender	1.19	0.685	0.50 – 2.86	0.52
BRAF status	1.28	0.595	0.51 – 3.23	0.53
log CNAS	3.06	**0.002**	1.50 – 6.22	0.71
log eCNAS	3.14	0.099	0.81 – 12.18	0.59
CNAS category	31.67	**0.001**	3.99 – 251.55	0.71
Multivariable analysis				
CNAS category	21.70	**0.043**	1.10 – 426.61	-
cfDNA category	3.66	0.150	0.63 – 21.31	-
Stage	51.29	**0.001**	5.05 – 521.12	-
Library size category	17.69	**0.026**	1.41 – 222.58	-

Table 3.14: Logistic regression analysis of predictors of the presence of active disease

Univariable logistic regression analysis identified logCNAS, cfDNA category, library size category and disease stage as significant predictors of the presence of active disease. The log eCNAS variable, on the other hand, was not a significant predictor of active disease.

To further explore the relationship between CNAS and the presence of active disease, patients were ranked and grouped into four quartiles, based on their log CNAS. Those in the highest quartile of CNAS had a significantly higher risk of active disease (OR (95% CI) 47.5 (5.15 – 438.49), p=0.001.

	OR	*p*-value	95% CI
< 25th percentile	reference		
25 – 50th percentile	3.33	0.066	0.93 – 12.01
50th – 75th percentile	1.25	0.739	0.34 – 4.64
> 75th percentile	47.50	0.001	5.15 – 438.49

Table 3.15: Logistic regression analysis of CNAS quartiles as a predictor of active disease

A multivariable regression analysis, adjusting for cfDNA category, stage and library size category, showed that CNAS category was a significant predictor of active disease (OR (95% CI) 21.70 (1.10 – 426.61), p= 0.043. The area under the receiver operating characteristic curve (AUC = 0.93) showed this model to be a good predictor of the presence of active disease. We went on to examine log CNAS as a predictor of patient survival.

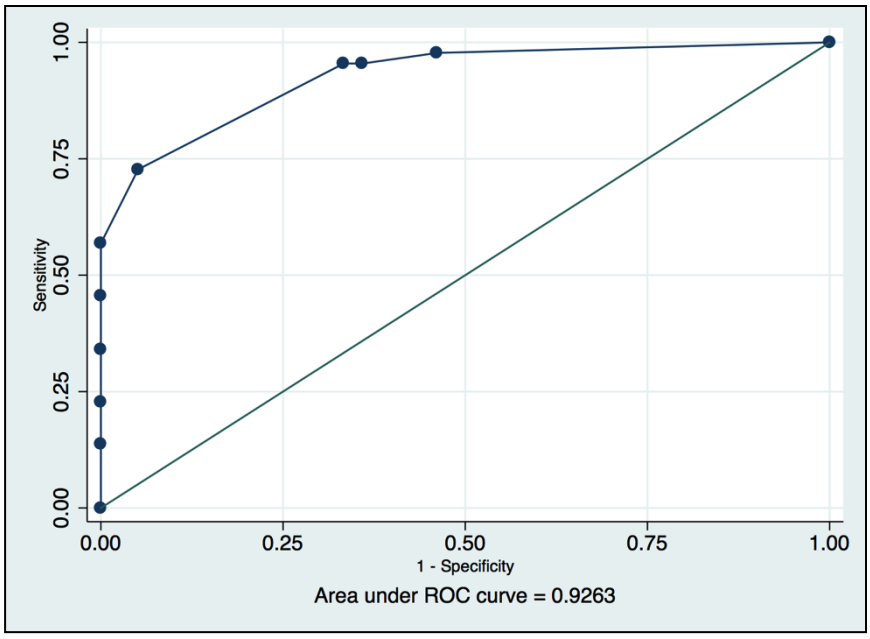


Figure 3.14: ROC analysis of multivariable model as a predictor of the presence of active disease. ROC analysis shows CNAS above or below the 75th percentile is a significant discriminator of the presence of active disease. CNAS above or below the 75th percentile remained a significant discriminator in multivariable analysis for active disease (p = 0.043, with area under ROC curve of 0.93)

3.16 CNAS as a predictor of survival

Univariable Cox regression analysis showed CNAS, as well as CNAS category, to be significant predictors of overall survival. Other variables identified as significant predictors of survival included cfDNA category, disease stage, BRAF status, and the presence of active disease. For patients with log CNAS above the 75th percentile, mortality was significantly higher (HR (95% CI) 7.68 (4.15 – 14.22), p<0.0001). After adjusting for the other significant variables, CNAS category remained a significant predictor of survival (HR (95% CI) 3.62 (1.47 – 8.90), *p*=0.005. eCNAS was not found to be a significant predictor of survival (HR (95% CI) 1.35 (0.58 – 3.18), *p*= 0.486.

	HR	p-value	95% CI
Univariable Cox regression			
log CNAS	2.09	<0.0001	1.59 - 2.74
log eCNAS	2.11	0.119	0.82 - 5.43
log cfDNA level	5.52	<0.0001	3.44 - 8.86
Stage	9.35	<0.0001	4.16 - 21.00
BRAF status	2.55	0.001	1.44 - 4.49
Gender	0.7	0.229	0.39 - 1.25
Active/Resected	9.26	<0.0001	4.94 - 17.34
Library size category	2.65	0.002	1.44 – 4.87
Multivariable Cox regression			
CNAS category*	3.62	0.005	1.47 – 8.90
cfDNA category**	2.23	0.037	1.05 - 4.74
Stage	3.17	0.017	1.23 - 8.17
BRAF status	3.16	0.001	1.65 - 6.03
Active/Resected	5.04	<0.0001	2.15 - 11.82
Library size category	0.83	0.646	0.38 – 1.81

Table 3.16: Cox regression analysis of predictors of overall survival.
* log CNAS above and below the 75th percentile
** log cfDNA level above and below the 75th percentile

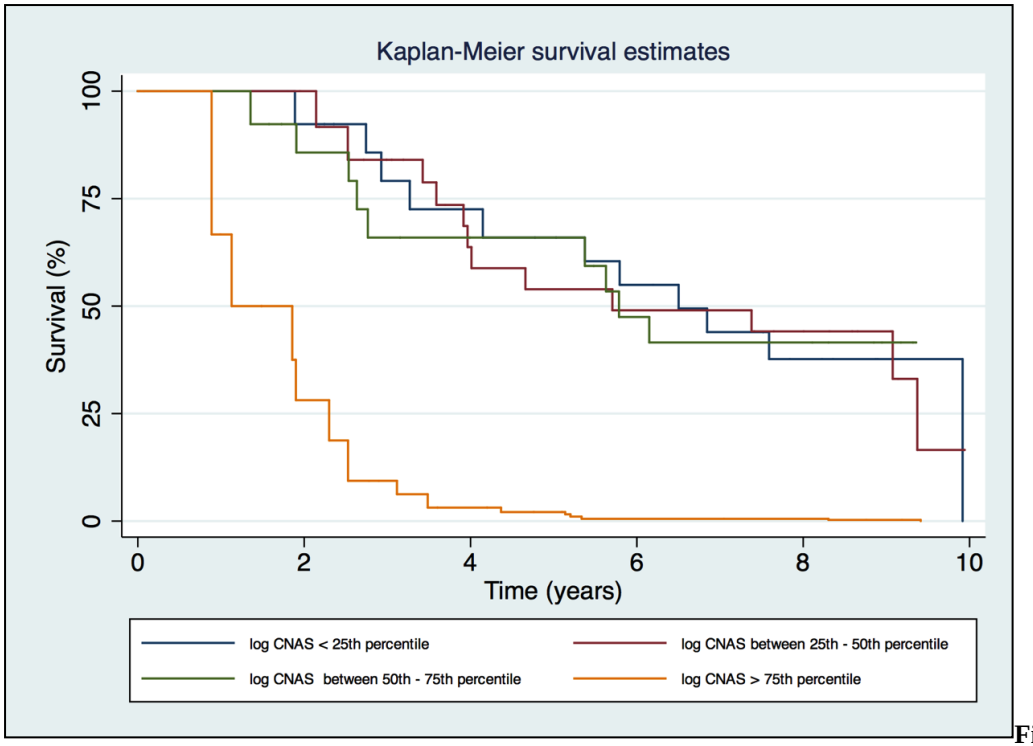


Figure 3.15: Kaplan-Meier survival estimates of CNAS quartiles. This compares survival for patients with log CNAS above the 75th percentile (yellow, n=21), between 50th -75th percentile (green, n=17, between 25th -50th percentile (red=24), and below 25th percentile (blue, n=21)

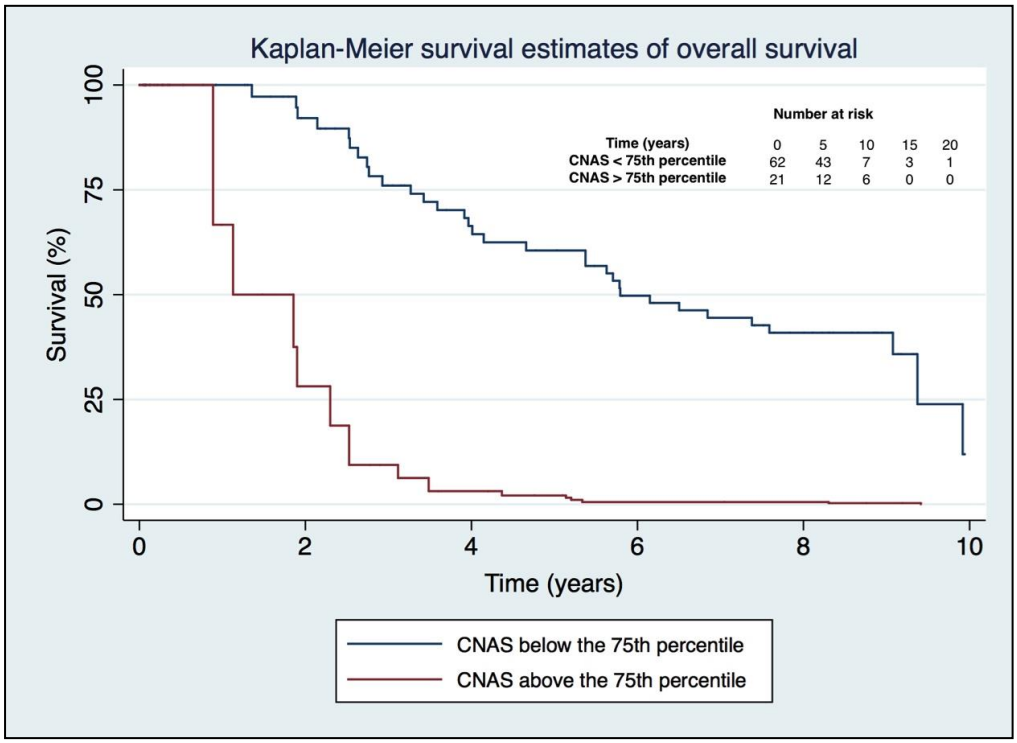


Figure 3.16: Kaplan-Meier survival estimates of CNAS above and below the 75th percentile. This shows mortality was higher in patients with log CNAS above the 75th percentile. Kaplan–Meier survival graph comparing survival for patients with CNAS above (red, n=21) and below (blue, n=62) the 75th percentile. Inset shows the number of patients at risk at each time point.

Median survival for patients with CNAS above the 75th percentile was 13.5 months, compared to 73.8 months for those with scores less than the 75th percentile (S. Silva et al., 2018) see Appendix F for published paper.

3.17 Discussion

The more starting DNA that is available for incorporation into libraries for sequencing, the less PCR amplification is required, thus enhancing the complexity of these libraries. Various aspects of pre-analytical DNA processing methods have been examined in an attempt to enhance DNA recovery, including comparisons between extraction kits, plasma collection tubes and centrifugation speeds during plasma processing (Devonshire et al., 2014; Sherwood et al., 2016). Both Sherwood et al and Devonshire et al found the QIAamp circulating nucleic acid kit to generate the highest DNA yield compared to other kits. The final step in the QIAamp extraction protocol is the application of an elution buffer to release the extracted DNA from the column. I compared different elution volumes using this QIAamp CNA kit to investigate the effect on DNA yield, and have shown that the higher elution volumes resulted in greater DNA yields, which is similar to what Xue et al and Graziano et al have reported (Xue et al., 2009).

The high volume of DNA obtained however, makes it necessary to concentrate the DNA samples prior to library preparation, as the commercially available library kits require smaller input volumes of DNA. While this concentrating step potentially makes the library processing more laborious, this needs to be weighed up against the benefit of achieving higher DNA input for the libraries, especially when dealing with plasma samples with potentially very low amounts of tumour-derived DNA. Options for concentrating the DNA samples include drying in a

laboratory fume hood or using a vacuum concentrator (e.g. SpeedVac). I opted for the former, due to the higher risk of drying down samples completely (and hence losing DNA) with the latter.

Given the relatively small amounts of cfDNA expected in our plasma samples, the choice of qPCR as a method of DNA quantification was made, as fluorometric methods of quantification (such as QUBIT and PicoGreen), although specific for dsDNA are less accurate at lower concentrations of DNA (Nakayama, Yamaguchi, Einaga, & Esumi, 2016; Szpechcinski et al., 2015; Szpechcinski et al., 2008). The fluorometric methods were sufficient, however, for the more concentrated DNA samples (genomic DNA and FFPE-derived DNA samples). Nanodrop spectrophotometry was utilised solely as a quality check for the DNA.

There is good evidence that cfDNA levels are higher in those with malignant disease compared to those without, and levels are comparatively higher in advanced cancer compared to early-stage disease (C. Bettegowda et al., 2014; Daniotti et al., 2007; Leon et al., 1977; Pinzani et al., 2011; Sozzi et al., 2003; Valpione et al., 2018). This was mirrored in our analysis, where we found higher concentration of cfDNA in melanoma cases with active disease compared to those with resected disease. As those with active disease tended to have higher stage melanoma (III-IV), our findings reflect that cfDNA level gives an indication of the burden of disease.

This concept that cfDNA can serve as surrogate marker of melanoma tumour burden was nicely demonstrated by Valpione et al (Valpione et al., 2018), who, like us, showed that there was a clear association between cfDNA and survival, with those with higher cfDNA (and so greater burden of disease) having a worse survival. This association between cfDNA and survival has been demonstrated in lung cancer cohorts as well (Cargnin, Canonico, Genazzani, & Terrazzino, 2017; Sirera et al.,

2011; Yi, Liu, Guan, & Ma, 2017). In our study, this association was significant even after adjusting for other factors known to influence survival in melanoma, including stage of disease and BRAF status. We did not have LDH levels available for this cohort of patients, and so were unable to adjust for this factor. Interestingly, Valpione et al showed a significant association with survival even after adjusting for LDH level, which is known to be a standard prognostic factor of survival in melanoma.

We demonstrated that cfDNA is relatively stable in plasma that is appropriately processed and stored at -80°C, for up to 7 years, with a low median decrease in yield over that time period. It is important to point out that two different DNA extraction methods were used at the different time points. The earlier extraction was carried out using a phenol-chloroform based extraction method, which has actually been shown to produce higher cfDNA yields compared to kit-based extractions (Xue et al., 2009). Therefore, some of the drop in DNA yields may be accounted for simply by the different extraction processes utilised, suggesting that the median drop in DNA yield could be an over-estimate of the effects of storage time.

We found cfDNA library size greater than 300 bp, as determined by Tapestation, to be a significant predictor of active disease. This contradicts the findings of keys studies (Mouliere & Rosenfeld, 2015; Underhill et al., 2016), which found that shorter cfDNA fragments were more likely to be tumour-derived, and so indicative of active disease. Library size was not a significant predictor of survival, though, after adjusting for other factors which are known to influence survival. A possible explanation for the incongruence in our finding would be in the method used in determining fragment size (Tapestation). This provides an estimate of the average fragment size which may not be an accurate representation of the true fragment size distribution within the library. A more accurate sizing can be achieved by calculating

the absolute distances between the outermost bases for each paired forward and backward read from the paired-end sequencing data.

While qPCR is recognised as the technique of choice in quantifying DNA libraries prior to pooling for multiplexed sequencing as it is specific for adaptor-ligated (i.e. sequenceable) fragments, it is very time-consuming and labour-intensive. Tapestation is an acceptable method for library quantification, provided the user is experienced, and the technique has been validated in-house to demonstrate consistency, hence our choice for this method of library quantification.

To objectively summarise the aberrations detected across the genome for each cfDNA sample, we explored two scoring algorithms the Copy Number Aberration Score (CNAS) and Extreme Copy Number Aberration Score(eCNAS). The CNAS is a genome-wide score (Heitzer et al., 2013), which captures the whole spectrum of copy-number aberrations regardless of amplitude or length of aberration, therefore providing a comprehensive summary of the copy-number profile for each sample. In contrast, the eCNAS score (similar to the PGA score (S. Xia, C. C. Huang, et al., 2015; S. Xia, M. Kohli, et al., 2015) focuses on the 95-99th percentile of copy-number ratios, the objective being that the biggest, and theoretically most important, aberrations are captured. The Illumina HiSeq platform provided the means to carry out low-coverage whole-genome paired-end sequencing for copy-number analysis in a cost-effective manner (through multiplexing several samples in each run), while successfully detecting copy-number aberrations with a sensitivity down to 6.25% as demonstrated by a CNAS score above the baseline (control genomic DNA). This is compatible to what others have achieved (between 5-10%). Interestingly, the eCNAS did not show any aberrations above that of the baseline control DNA. It is likely that the eCNAS scoring algorithm, by focusing on the 95-99th percentile of aberrations,

ignores the potentially significant contribution made by smaller aberrations, which may be quite numerous. This score proved to be a poor reflection of the copy-number profile for our melanoma samples.

Analysis showed that the CNAS for cases with active disease were significantly higher than those with resected disease (p=0.0011). There was, however, a wide variation in the CNAS, with some overlap between the scores for active and resected cases. It is possible that the presence of microscopic residual disease in some patients with resected disease may be contributing to higher scores in those patients, hence the overlap. We also established a multivariable model (based on the CNAS, cfDNA level and disease stage) which is a good discriminator of active disease, with area under ROC of 0.90. There have been a number of recent publications which have utilised cfDNA to differentiate melanoma cases from controls [9] or to assess treatment response in melanoma [21,22]. These studies utilised either cfDNA levels, mutation analysis (predominantly BRAF) or cfDNA integrity indices (or a combination of these approaches) in their analyses. We further demonstrated, using a multivariable Cox regression analysis correcting for other factors known to affect survival, that high CNAS scores (>75th percentile) were associated with significantly poorer survival. Although preliminary, these results are consistent with those of Lee et al (R. J. Lee et al., 2017) who observed that ctDNA predicts relapse and survival in stage II/III patients.

We assessed how CNAS quartiles were associated with overall survival, and found that there was a trend for worsening survival with the higher quartiles, with those in the highest quartile (above the 75th percentile) having the worst survival. Interestingly, in their analysis of chromosomal instability based on a gene expression signature in breast cancer tumour specimens, Swanton et al, showed a worsening

survival with increasing chromosomal instability score except for the highest quartile, which actually had an improved survival, suggesting that extreme genomic instability beyond a certain threshold renders tumour cells unviable, hence the better prognosis in this quartile. Our results did not support this paradox, but were more in keeping with earlier studies that had established chromosomal instability to be generally associated with worsening prognosis (Carter, Eklund, Kohane, Harris, & Szallasi, 2006; Walther, Houlston, & Tomlinson, 2008)

The excellent correlation we found in the copy number aberration scores when cfDNA copy number reads in each megabase window were normalised to the corresponding reads from the pooled DNA from healthy controls, compared to the normalisation using each patient's corresponding lymphocyte DNA supports the pooled option (Samman et al., 2015) as a cost-effective means of achieving copy-number ratios, without having to run each patient's lymphocyte DNA along with their cfDNA sample.

Chapter 4: Markers of Relapse in Melanoma Study

4.1 Introduction

Genomic instability, resulting in DNA copy number aberrations, is one of the main mechanisms by which cells acquire the hallmarks of cancer (Hanahan & Weinberg, 2011). The number and magnitude of genetic aberrations increase as tumours progress, and the more genetically unstable melanomas are associated with poorer outcome for patients (Hirsch et al., 2013). A copy number profile of the whole genome taken at regular time intervals could reveal new and changing genomic aberrations as they arise during tumour relapse and progression. Thus, longitudinal copy number analysis in cfDNA has great potential as a source of biomarkers of relapse in oncology, as it provides a minimally invasive means of surveillance that could easily be incorporated as part of standard follow-up. Not only does this approach not require *a priori* knowledge of mutations that may be present in a tumour, it can be successfully carried out using low-coverage whole-genome sequencing with low-input DNA, and can be applied in a variety of cancer subtypes (Azad et al., 2015; Schutz et al., 2015; Weiss et al., 2017). Additionally, the copy-number approach can be combined with mutational analysis, for a more comprehensive analysis.

The previous chapter outlined the establishment of the approach utilising low-coverage copy-number profiling of cfDNA in a single sample per subject as a biomarker of active disease (and survival) in melanoma (Chapter 3) (S. Silva et al., 2018)). Higher levels of cfDNA were found in those with active disease, compared with those with resected disease (chapter 3.3). cfDNA concentration correlated with survival, even after correcting for factors known to correlate with melanoma survival, including BRAF status and stage of disease (see chapter 3.3.1). The copy number aberration score CNAS, which is based on the sum of squared Z-scores of copy

number ratios of 1Mb windows across the genome, was found to be a good discriminator of active disease, as well as a good predictor of survival. The CNAS remained a good predictor of survival, after accounting for cfDNA concentration. This chapter will explore a longitudinal analysis using this low-coverage copy-number profile approach, in order to investigate the relationship between changes in these profiles, and the clinical course of melanoma, specifically with relation to relapse.

The hypothesis to be tested is that individual measures and/or changes in CNAS over time are potential markers of relapse in melanoma.

The aims of the work described in this chapter were:

1. To set up a study and recruit melanoma patients and healthy controls, in order to obtain longitudinal blood samples for genomic analysis and outcome data.
2. To explore cfDNA levels (plasma concentration) longitudinally over time.
3. To track changes in CNAS over time, and explore this score as a marker of relapse in melanoma.

The objectives of this chapter were:

1. To design and set-up a study to recruit melanoma patients, and healthy controls, from whom serial blood samples were to be obtained for cfDNA analysis.
2. To measure cfDNA concentrations over time in both cases and controls recruited to the study. In the GEMS analysis (refer to chapter 3) (S. Silva et al., 2018), higher concentrations of cfDNA were found in melanoma cases with active disease compared to those with resected disease. As those with active disease tended to have higher stage melanoma (III-IV), these findings reflect that cfDNA level gives an

indication of the burden of disease, hence the potential utility of cfDNA concentration as a biomarker of relapse.

3. To compare copy number aberration scores between cases that relapse and those which do not experience a relapse, in order to assess the ability of the CNAS to reliably detect melanoma relapses in this study cohort, and assess whether it is a better predictor than cfDNA concentration in the longitudinal setting. A random coefficients mixed-effects statistical model will be utilised to investigate the performance of CNAS as a biomarker of relapse. This statistical model is required to take into consideration intra-patient variation in CNAS over time.

Circulating tumour DNA levels have been shown to correlate with melanoma tumour burden (McEvoy et al., 2018; Wong et al., 2017). We demonstrated (in section 3.3) that cfDNA concentration was higher among melanoma cases with active disease, compared with those who had had their melanomas surgically resected. We explored the changes over time, of cfDNA concentrations, to see how these changed with the occurrence of radiological/clinical relapse within our cohort of melanoma patients, and compared these trends with those of the healthy controls in the MRM study, in order to see how this compared with CNAS over time.

4.2 Methods

4.2.1 Study design

The Markers of Relapse in Melanoma (MRM) study was an academic, single-centre study set up in Sheffield Teaching Hospitals NHS Trust to recruit melanoma patients and healthy controls to obtain longitudinal blood samples cfDNA analysis. The aim of the study was to identify whether a genome instability score derived from a weighted sum of the number and magnitude of copy-number aberrations in cfDNA,

and/or circulating levels of cfDNA, could act as early markers of relapse in melanoma.

The primary objectives of the study were to recruit 120 patients with melanoma and 120 healthy controls (cohabiting partners or siblings) over 2 years, and obtain blood samples every 3 months (and at any clinically relevant additional hospital appointments); to determine plasma cfDNA levels, and quantify genomic copy-number aberrations; to assess if there was a temporal relationship between these and the onset of melanoma relapse. The secondary objective was to confirm the stability of cfDNA levels, and genomic profiles, in control subjects over time.

4.2.2 Funding

The study, and subsequent cfDNA analysis including next-generation sequencing, was funded by Weston Park Cancer Charity (grant number R/141934).

4.2.3 Ethical approval

The protocol for the study was written to include details of recruitment, blood sample collection, clinical data collection, as well as regulatory and ethical issues (see Appendix C and D for trial protocol). Dr Shobha Silva led on the ethics application, and was the named chief investigator on the study. The protocol was reviewed by Yorkshire & The Humber (Sheffield) research ethics committee, and approval was given on 23rd January 2015 (REC reference 14/YH/1275). See approval letter in Appendix C.

4.2.4 Study population

Patients who had undergone surgical resection of their primary (and/or metastatic) melanoma lesion(s), and who were undergoing surveillance at Oncology/Dermatology/Plastic surgery departments, consented to providing blood samples when they attended their routine melanoma follow-up clinic appointments. Partners and/or siblings of these patients were invited to participate in the study as controls. Dr Shobha Silva led the recruitment of patients and controls for the study.

4.2.5 Inclusion and exclusion criteria

Eligible patients included those with a diagnosis of cutaneous melanoma of any stage (both incident and prevalent cases). Patients were excluded if they had received chemotherapy within 4 weeks of recruitment (as cfDNA levels are significantly affected by chemotherapy (Sanz-Garcia, Zhao, Bratman, & Siu, 2022)), had a known concurrent second malignancy, or were unable to give informed consent.

4.2.6 Consent process

Participants were invited to participate in the study through a letter posted out to their home address prior to their follow-up appointment, and were provided with information leaflets following discussion of the study. Participants were required to provide written informed consent for participation in the study and for use of donated blood specimens and any surplus pathology tissue specimens (Appendix C). Written consent was obtained by Dr Shobha Silva, Sister Jo Bird (a senior Research Nurse), and Professor Sarah Danson, (Consultant Medical Oncologist). Each participant provided a blood sample at each 3-monthly follow-up appointment.

All participants completed a questionnaire, which provided detailed epidemiological information about their lifestyle (including “sun behaviour”), medical history and family history.

4.2.7 Processing and storage of blood samples

All blood samples were taken by venepuncture, using EDTA tubes, at the relevant outpatient clinic departments in Sheffield Teaching Hospital, either by Shobha Silva or Andrew Newsome (phlebotomist). Forty-eight millilitres (48ml) of blood was collected at each sampling. Samples were anonymised and promptly taken to the research laboratory based in the Medical School in University of Sheffield, where they were processed to plasma and buffy coat within two hours (as outlined in Chapter 2). The processing was carried out by Ian Brock (lab technician) and Dan Connelly (data manager trained in processing protocol). All plasma samples were stored in 1ml aliquots at -80 °C, while for each participant, a baseline whole blood sample was stored at -20 °C.

4.2.8 Analysis of cfDNA

All the cfDNA extractions (from case and control plasma samples), and subsequent quantification, DNA library preparation and pooling for sequencing, were carried out by Dr Silva (as outlined in Chapter 2). Loading of pooled libraries on to the Illumina HiSeq was carried out Emilie Jarratt (technician at Sheffield Diagnostic Genetics Service). Bioinformatics support was provided initially by Dr James Bradford (University of Sheffield) and subsequently by Matt Parker (Sheffield Diagnostic Genetics Service). Copy number ratios were generated (using the pipeline

outlined in Chapter 2) by Matt Parker, and copy-number aberration scores were calculated (using STATA) by Dr Shobha Silva, as outlined in Chapter 2.

4.2.9 Data collection

Relevant clinical details were obtained from clinic letters as well as from the electronic database for clinical investigations (refer to section 1.11 for details of data collected). All participant information was stored in a password-protected database (excel workbook), which was only accessible by Dr Shobha Silva. Anonymised information was stored in a separate database (excel workbook), which was accessible to my supervisors (Professor Angela Cox and Dr Dawn Teare).

4.2.10 Statistical analysis

All analyses were carried out using Stata (version 12) and GraphPad Prism (version 7). See section 2.22 for details.

4.3 Results

4.3.1 Patient and control summary

The trial opened to recruitment in February 2015, and recruitment was completed in October 2016. 140 melanoma patients in total were identified as potential subjects for recruitment. 31 declined with no specific reason given, 3 patients were deemed unsuitable due to significant cognitive impairment making them unable to give informed consent, 1 declined due to a needle-phobia, 1 patient withdrew due to poor venous access, 1 patient relapsed prior to entry into the study, and another patient had a concurrent malignancy which made them ineligible for the study, 2 patients requested follow-up local to them making them ineligible.

In total, 100 melanoma patients and 47 healthy control subjects were recruited. Of the 100 patients recruited, 4 withdrew (2 due to poor venous access resulting in difficulty in obtaining blood samples, 1 due to a concurrent malignancy, 1 did not give a reason for withdrawal). Their samples were excluded from subsequent analyses, leaving 96 case subjects for analysis.

Of the 47 controls recruited, 10 were not analysed further. Two of these controls were spouses of patients who withdrew from the study, and samples from 8 controls, for whom it was only possible to collect a single blood sample, were excluded due to time and funding constraints.

Patient/control characteristics are summarised in Table 4.1. There was a slightly higher proportion of male patients recruited (59.4%), compared to females (40.6%). As the controls recruited were predominantly spouses of the patients, there was thus a higher proportion of female controls recruited (56.8%), compared to male controls (43.2%), but the difference was not statistically significant (p=0.095). The median age among cases was 64 years; and 68 years among controls, with no significant difference between both groups (p=0.62). 59.4% of patients had stage III/IV tumours (AJCC 7th edition). Of the 54 patients for whom BRAF status was available, 19 (35.2%) had BRAF mutant tumours.

Thirty six out of the 96 case subjects had a relapse event during the study. Of those who relapsed, 14 patients (38.9%) had surgical excision as the first treatment of their relapsed disease (2 of these patients relapsed again during the study). Of the remaining 22 patients who relapsed, 17 (47.2%) received systemic therapies as their first-line treatment for relapsed disease (7 patients had BRAF-targeted therapy, 9 immunotherapy, and 1 patient had chemotherapy), 1 patient had palliative radiotherapy, and 4 patients received best supportive care.

	Patients (*n*=96)	Controls (*n*=37)
Male	57 (59.4%)	16 (43.2%)
Female	39 (40.6%)	21 (56.8%)
Median age in years at diagnosis (range)	64 (23-90)	68 (35-85)
Stage at recruitment		
I	2 (2.1%)	
II	37 (38.5%)	
III	47 (49.0%)	
IV	10 (10.4%)	
BRAF status		
Mutant	19 (19.8%)	
Wildtype	35 (36.5%)	
Unknown	42 (43.8%)	
Relationship of control to patient		
Spouse		29
Sibling		4

Table 4.1: Baseline characteristics for patients and controls recruited to the Markers of Relapse in Melanoma study

4.3.2 Distribution of longitudinal samples in the Markers of Relapse in Melanoma study

All patients (and control participants), who had provided consent to take part in the MRM study, were invited to provide a blood sample when they attended for their regular outpatient Oncology/Plastic Surgery/Dermatology clinic appointments. Figure 4.1 and Table 4.2 summarize the numbers of samples collected. Over a period of two years (March 2015 – April 2017), a total of three hundred and seventy-one longitudinal samples were processed, with eighty-one per cent of all participants providing two or more longitudinal samples, and over half of participants providing at least three samples. Control samples accounted for a quarter of all samples obtained.

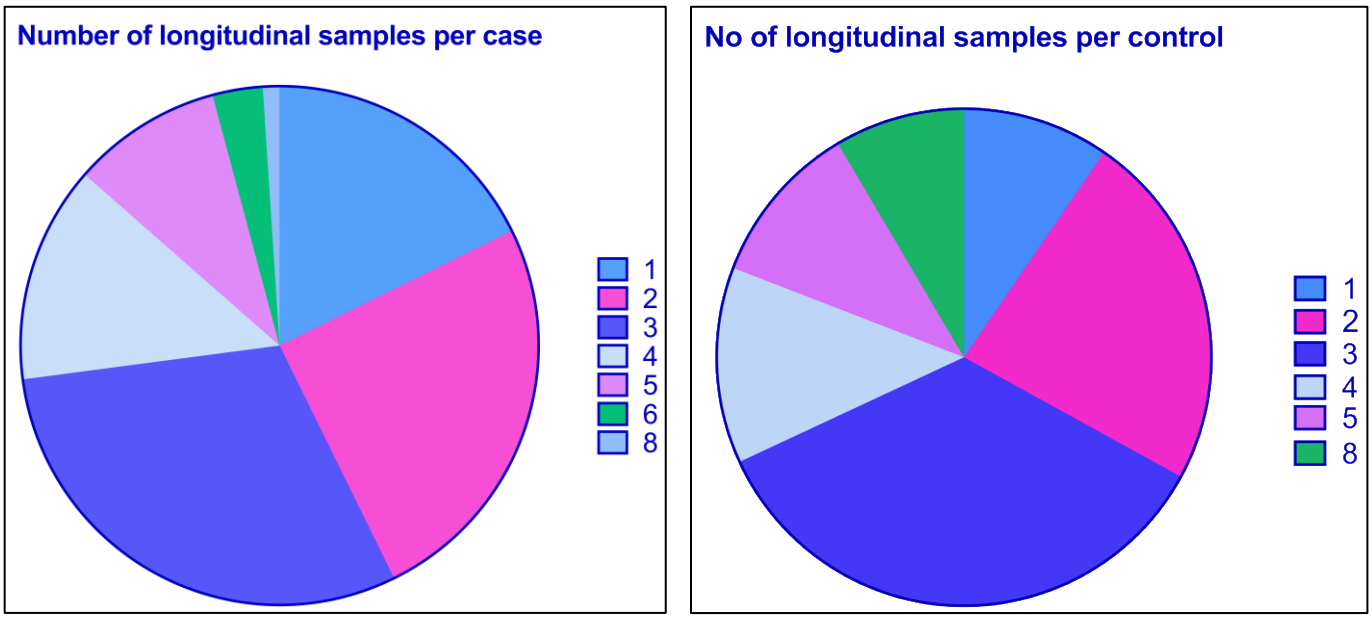


Figure 4.1: Pie charts showing distribution of samples obtained from cases and controls. Cases shown on left, and controls shown on right, as in the Markers of Melanoma study.

Number of samples per subject	Number of subjects		Total number of samples from cases	Total number of samples from controls	Total number of samples from cases and controls	
	Cases (*n*=96)	Controls (*n*=37)			(*n*)	(%)
1	17	8	17	8	25	6.7%
2	24	11	48	22	70	18.9%
3	29	12	87	36	123	33.2%
4	13	3	52	12	64	17.3%
5	9	2	45	10	55	14.8%
6	3	0	18	0	18	4.9%
7	0	0	0	0	0	0.0%
8	1	1	8	8	16	4.3%
		Total (%)	**275 (74%)**	**96 (26%)**	**371**	**100**

Table 4.2: Summary of samples obtained from cases and controls.

4.3.3 cfDNA concentration in MRM samples

Having demonstrated, in Chapter 3, that cfDNA concentration reflects burden of disease, we explored the potential utility of cfDNA concentration in serial samples as a biomarker of relapse.

Comparison of cfDNA concentration in relapsed samples, non-relapsed samples and controls

Firstly, each longitudinal sample was classified as "relapsed "or "not relapsed", based on whether or not the sample was associated with a relapse event (clinical and/or radiological relapse). A sample was said to be associated with a relapse event if, at the time of blood sampling, a clinical, or radiological, diagnosis of relapse was/had been made (see Figure 4.2 below).

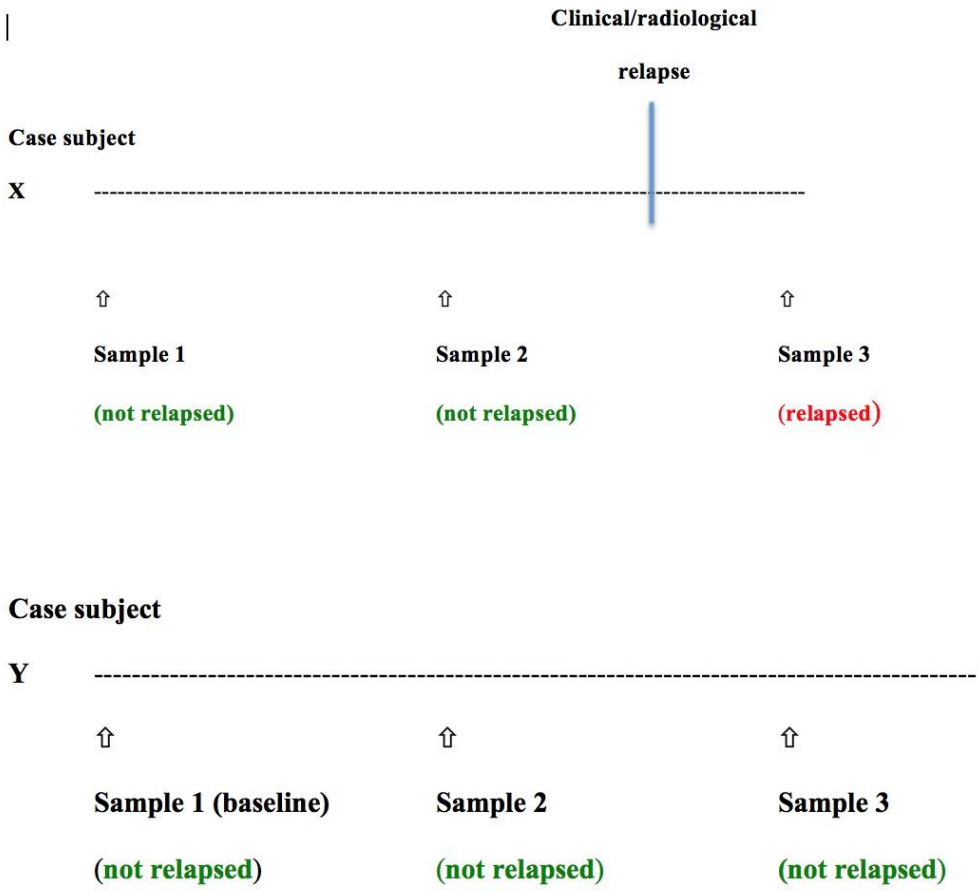


Figure 4.2 – A sample was attributed a "relapsed" status if, at the time of blood sampling, a clinical, or radiological, diagnosis of relapse was/had been made

The mean log cfDNA concentrations in samples associated with a relapse was compared, both with the mean of those samples not associated with a relapse, and

control samples. Samples associated with a relapse had higher mean log cfDNA concentrations (*n*=38, from 36 cases, mean 1.95, SD 0.79), compared to those samples not associated with relapse (n=237, from 60 cases, mean 1.65, SD 0.55), (*p*= 0.029), see figure 4.3.

Interestingly, there was a significant difference in the cfDNA concentration in longitudinal samples not associated with relapse, and those of healthy controls, with the samples not associated with a relapse having a higher mean log concentration compared to controls (*n*=96, samples from 37 subjects, mean 1.41, SD 0.47), *p*= 0.0001).

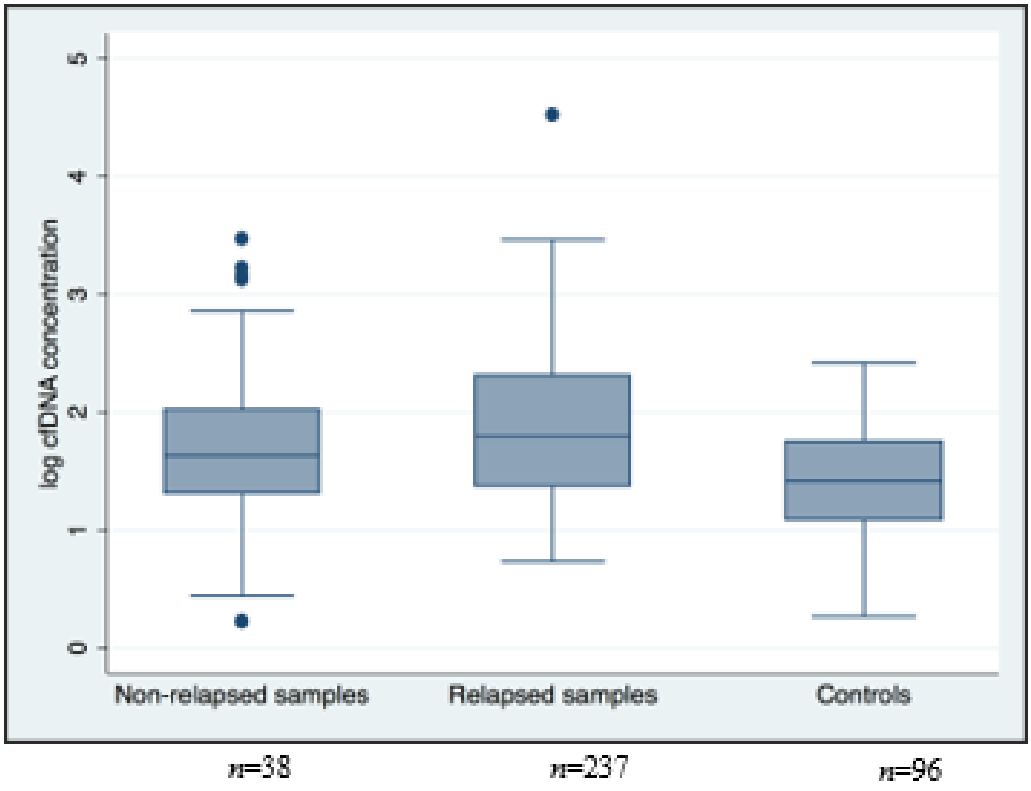


Figure 4.3: Box and whisker plot of the log cfDNA concentration (ng/ml of plasma) in relapsed samples vs. non-relapsed samples vs. controls. Log cfDNA concentrations (ng/ml of plasma) in samples associated with a relapse were higher than non-relapsed samples (p=0.029), and the cfDNA concentrations in non-relapsed samples were higher than control samples (p=0.0001)

Comparison of cfDNA concentration in relapsed cases, non-relapsed cases and controls

In a second analysis, each case was attributed a status as relapsed or not, based on whether or not the case had had a relapse event (clinical and/or radiological relapse) at any point during the study, (see Figure 4. below).

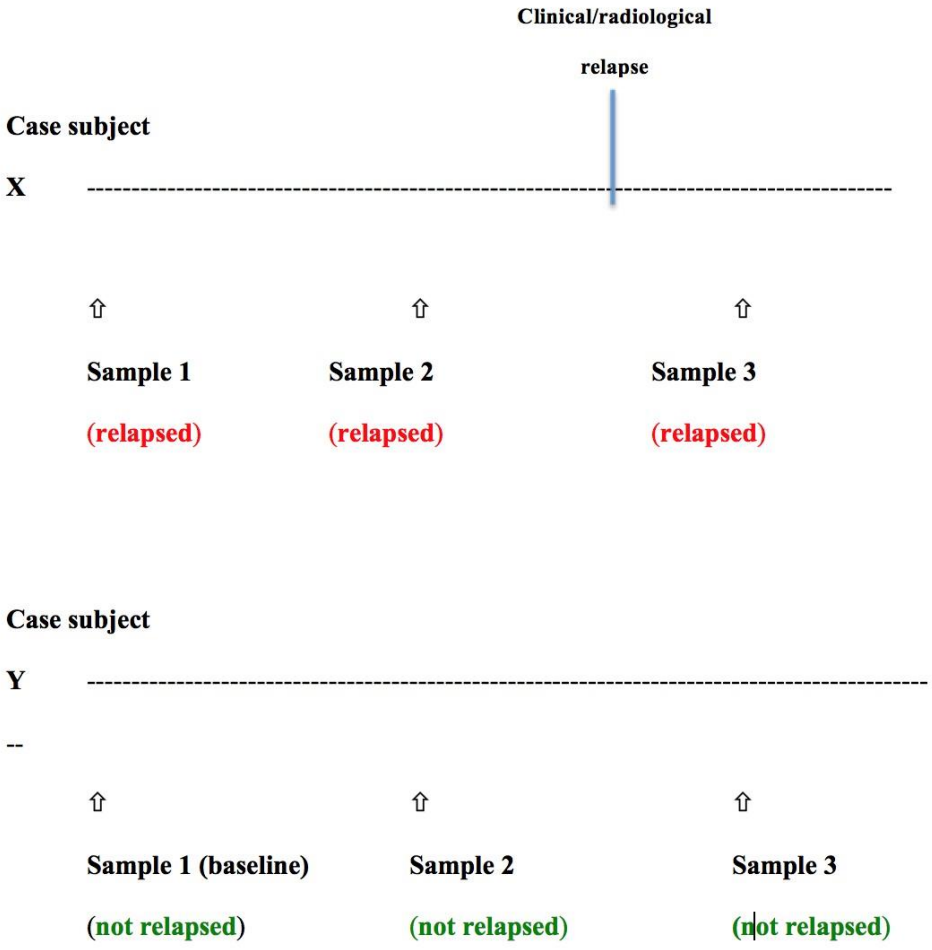


Figure 4.4 – A case was attributed a "relapsed" status if, at any point during the study, a clinical, or radiological, diagnosis of relapse was made.

This analysis was done to explore if cases in this cohort which relapsed had higher cfDNA concentrations overall. The log cfDNA concentration in all longitudinal samples obtained from cases who had relapsed (n=97, samples from 36

cases, mean 1.74, SD 0.66) was compared with all samples obtained from all cases who had not relapsed (n=178, samples from 60 subjects, mean 1.66, SD 0.56), and control samples (*n*=96, from 37 subjects, mean 1.41, SD 0.47), see figure 4.5. In this analysis, there was no significant difference in the mean concentration between relapsed cases and non-relapsed cases. (*p*= 0.34). There was, however, a significant difference in log cfDNA concentration in non-relapsed samples compared to controls (*p*=0.0001).

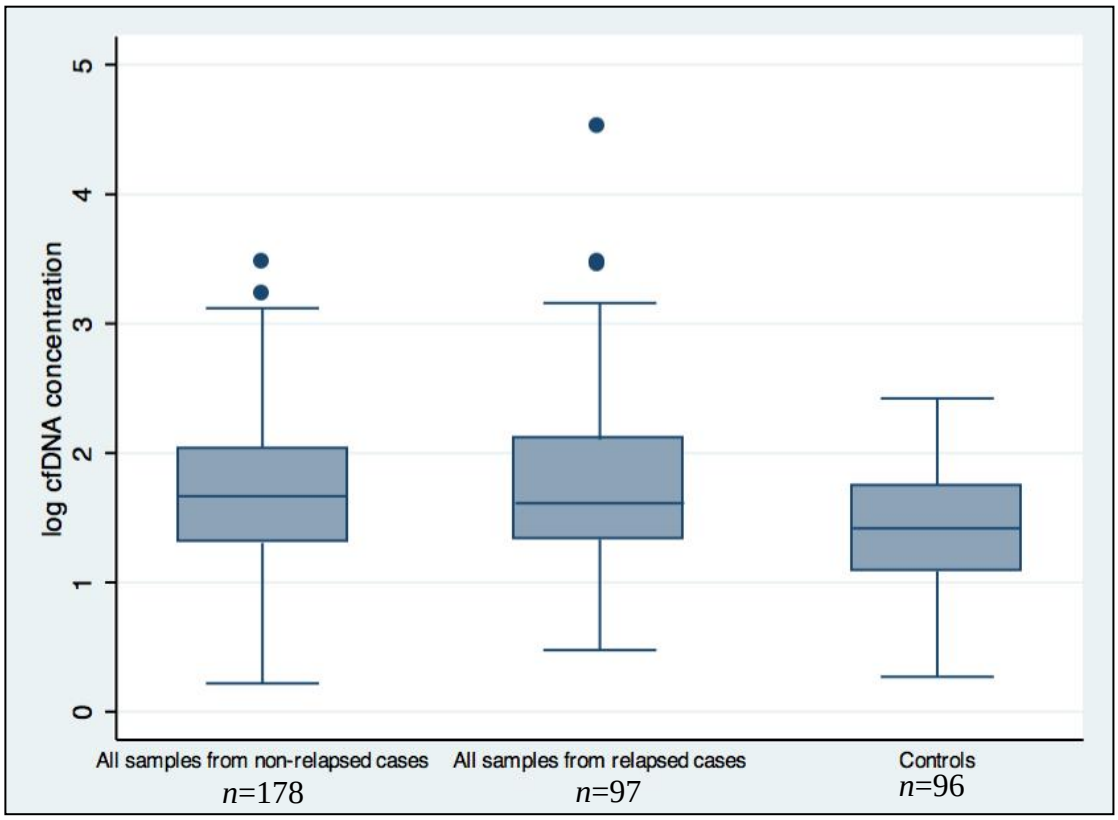


Figure 4.5: Box and whisker plot of the log cfDNA concentration (ng/ml of plasma) in relapsed cases vs. non-relapsed cases vs. controls. No difference in cfDNA concentrations was seen in all samples obtained from cases associated with a relapse compared to all samples from non-relapsed cases (*p*=0.34).

Comparison of baseline cfDNA concentration between relapsed cases, non-relapsed cases and controls

We next examined whether the baseline cfDNA concentration alone was sufficient to distinguish controls, non-relapsed and relapsed cases. (see Figure 4.6 below).

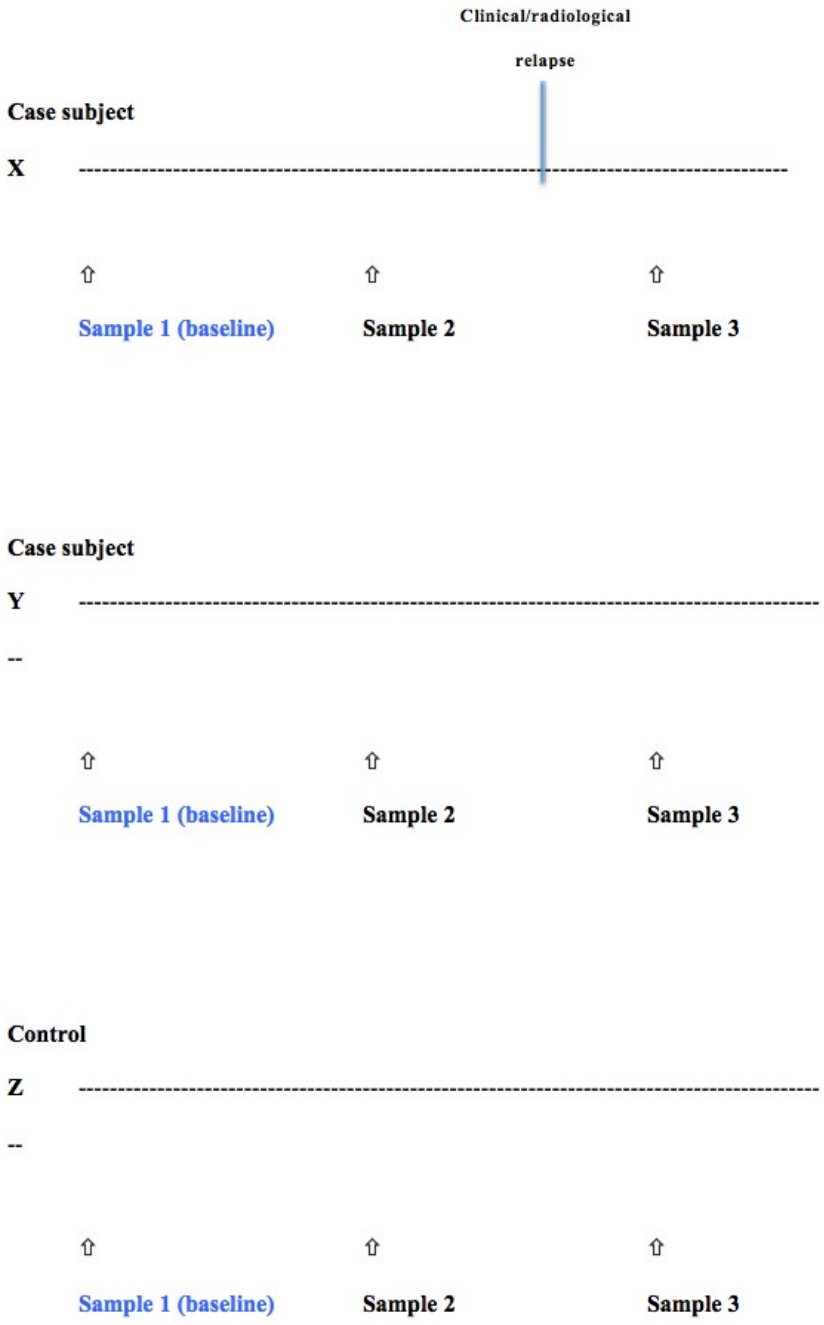


Figure 4.6 – A case was attributed a "relapsed" status if, at any point during the study, a clinical, or radiological, diagnosis of relapse was made. CfDNA concentrations were compared between baseline samples from relapsed cases, non-relapsed samples, and controls**.**

The baseline mean log cfDNA levels for all cases which relapsed on the study were compared with that of non-relapsed cases and controls. The mean baseline log cfDNA concentration in cases which relapsed (*n*=36) was 1.56, SD 0.43; while the mean for cases which did not relapse (*n*=60) was 1.60, SD 0.52, with no significant difference in the mean cfDNA concentration between the two groups (*p*=0.63), see figure 4.7. There was a significant difference in the baseline cfDNA concentration for samples from non-relapsed cases ((*n*=60), mean 1.60, SD 0.52) and controls (n=37, mean 1.38, SD 0.54), *p*=0.043.

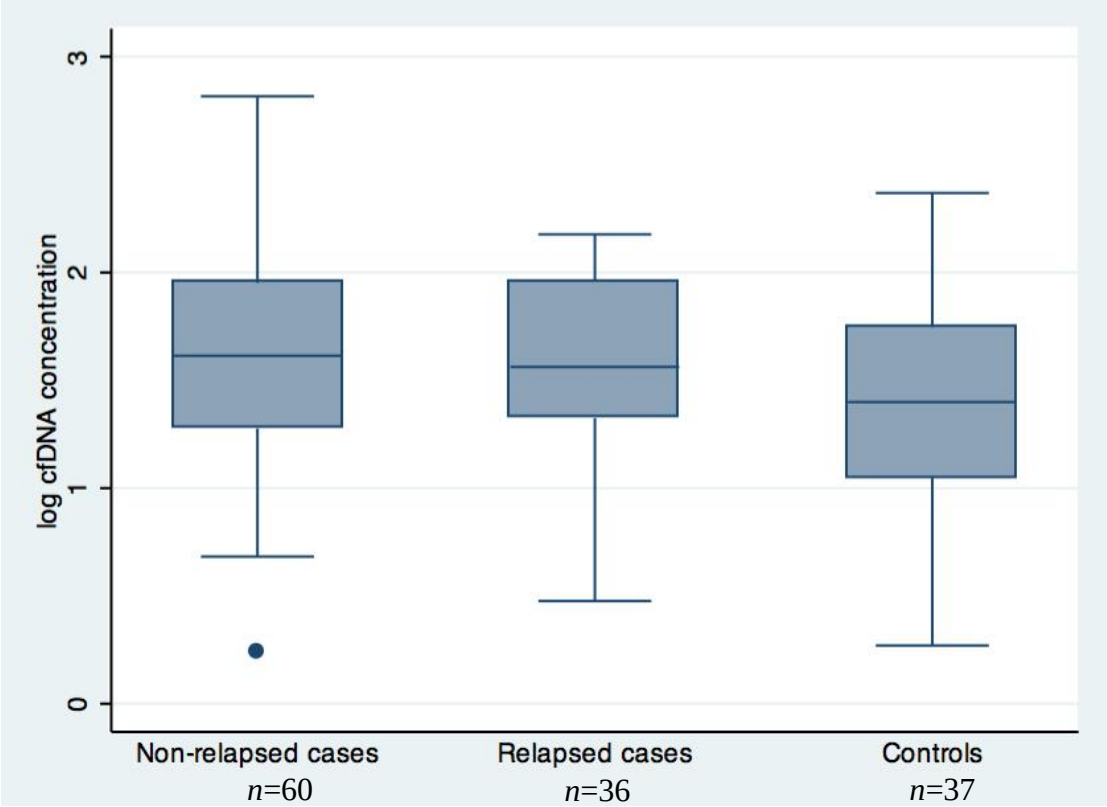


Figure 4.7: Box and whisker plot of the baseline log cfDNA concentration (ng/ml of plasma) in relapsed cases vs. non-relapsed cases vs. controls. No difference in the baseline cfDNA concentrations was seen in cases that relapsed compared to cases which did not relapse.

Comparison of cfDNA concentration in relapsed cases pre- relapse, post relapse and control samples

We finally compared pre- and post-relapse samples. (see Figure 4.8 below)

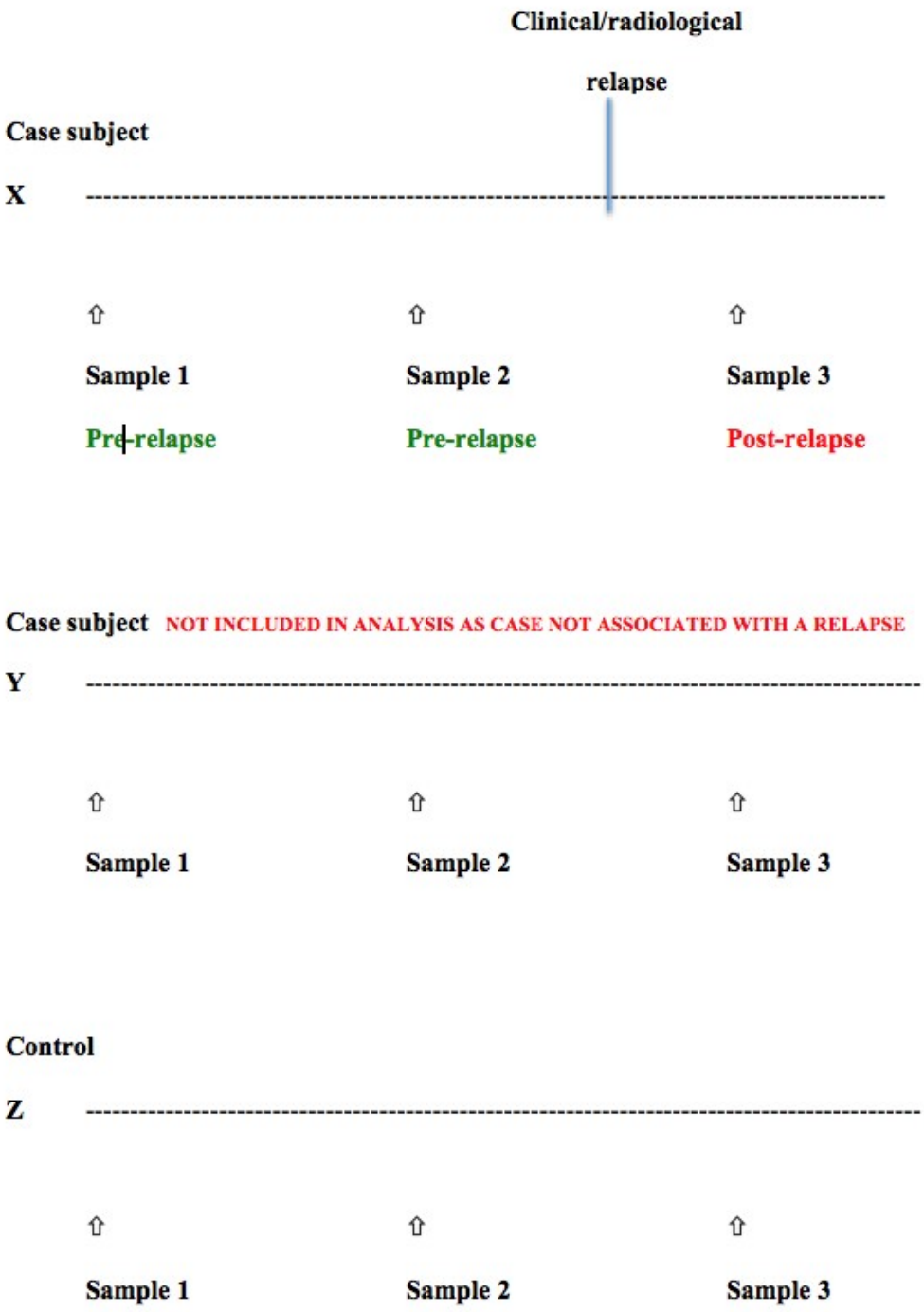


Figure 4.8: For cases associated with relapse (clinical or radiological) during the study, all samples pre-relapse were compared to post-relapse samples. These samples were also compared to control samples.

Among cases that were associated with a relapse event at some time point during the study, we compared cfDNA concentrations across samples prior to the relapse event, with concentrations after the relapse event. Those samples obtained after clinical or radiological evidence of relapse were shown to have higher mean

cfDNA concentration (*n*=38, mean 1.95, SD 0.79), *p*= 0.022. The mean log cfDNA concentration in samples obtained prior to relapse was 1.60 (n= 59, SD 0.54, and was higher than that of controls (n=96, mean 1.41, SD 0.47), *p*= 0.028, see figure 4.9.

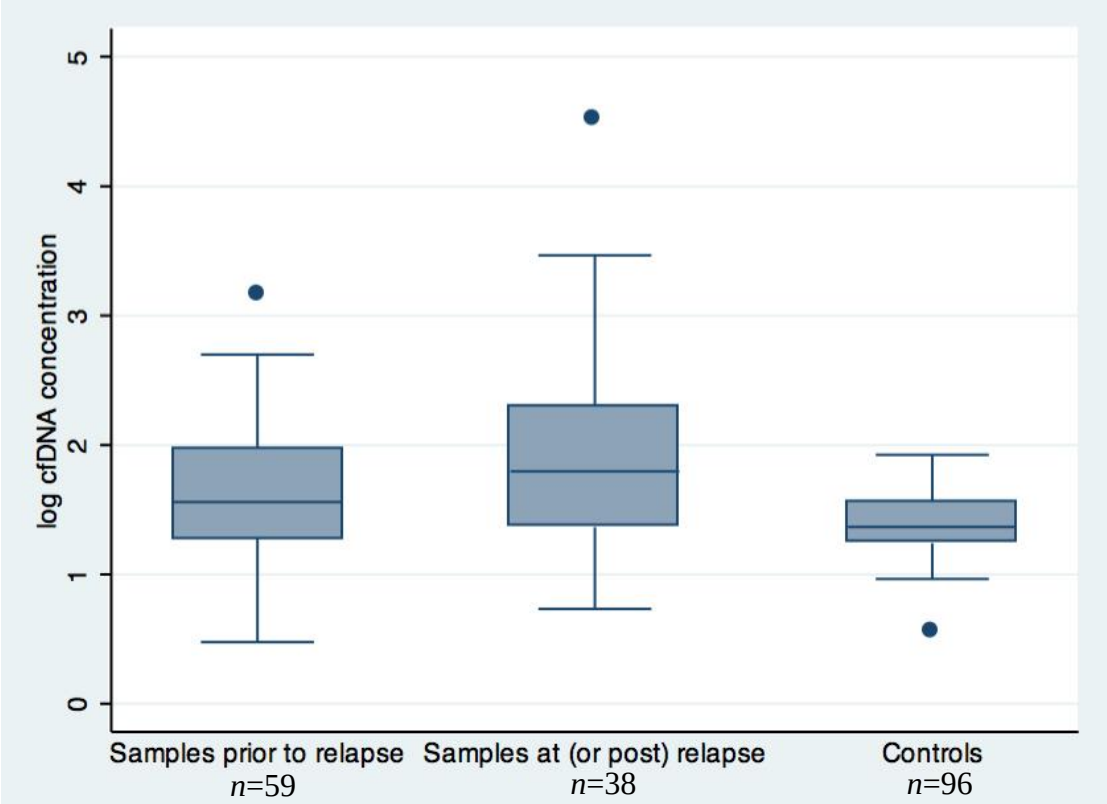


Figure 4.9: Box and whisker plot of the log cfDNA concentration (ng/ml of plasma) from cases who have relapsed, in samples prior to relapse vs. post-relapse vs. controls. In cases that relapsed in the study, a higher mean log cfDNA concentration was seen in samples at (or after) relapse compared to samples prior to relapse

4.3.4 cfDNA concentration over time

To explore the differences in longitudinal trends of cfDNA concentrations in patients who experienced a relapse event during the study, with those who did not, the log cfDNA concentration for each case was plotted longitudinally over time.

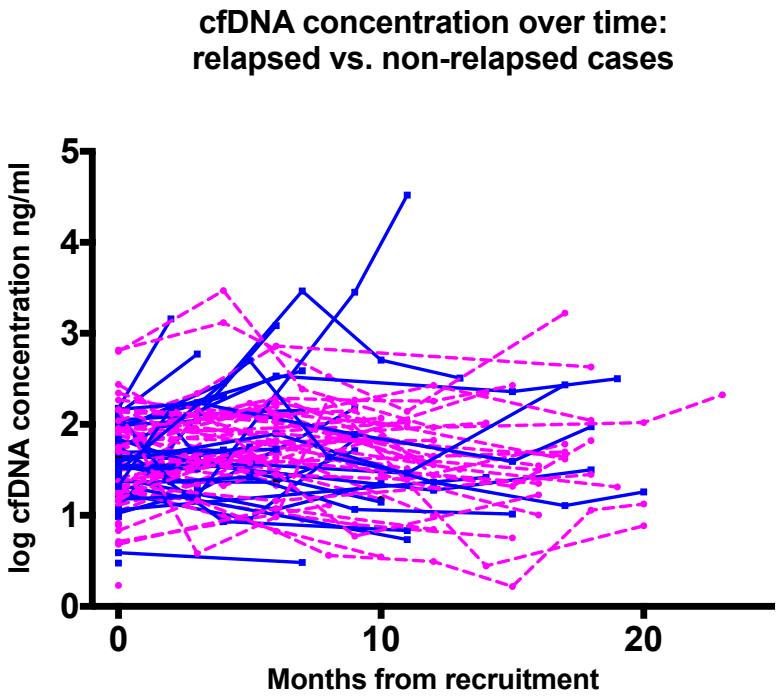


Figure 4.10: Spider plot of log cfDNA concentration over time in cases which relapsed (in blue) and in cases which did not relapse in the study (shown in pink). The x-axis represents time (in months) from recruitment (represented as time 0), and y-axis the log cfDNA concentration (in ng/ml)

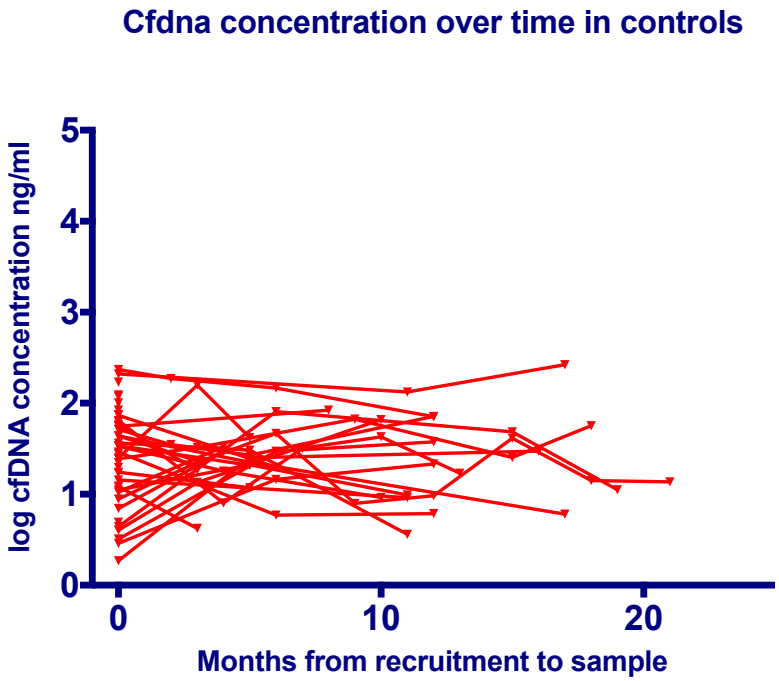


Figure 4.11: Spider plot of log cfDNA concentration over time in control subjects. The x-axis represents time (in months) from recruitment (represented as time 0), and y-axis the log cfDNA concentration (in ng/ml).

As seen from the plots above, the cfDNA concentration does not appear to show any particular trends over time among relapsed cases, compared to non-relapsed cases.

In summary, baseline cfDNA concentration did not differ between relapsed cases and non-relapsed cases. A significant difference was seen when all samples associated with a relapse event were compared with all samples not associated with a relapse event. Also, when all samples prior to a relapse event were compared with all samples following a relapse event, there was again a significant difference, with samples after a relapse having higher cfDNA concentrations.

The log of cfDNA concentration was included as a co-factor in the longitudinal analysis of CNAS described in section 4.8.

4.3.5 Comparison of CNAS in MRM samples

Having demonstrated good reproducibility of the CNAS in Chapter 3, we explored its performance as a biomarker of relapse in our cohort of melanoma patients and controls, by making the following comparisons similar to those carried out for cfDNA concentration:

Comparison of CNAS in relapsed samples, non-relapsed samples and controls

In a comparison of the CNAS in samples associated with a relapse vs. samples not associated with a relapse event (see figure 4.2), using a 2-sided t-test, there was a significant difference between the two groups (p=0.0063), with the samples associated with a relapse having higher log scores (n=38, mean 11.44, SD 1.55), compared to those not associated with a relapse event (n=237, mean 10.70, SD 0.51), see figure 4.12. The non-relapsed samples had a lower mean log CNAS score compared to healthy controls (n=96, mean 10.95, SD 0.66, p=0.0011).

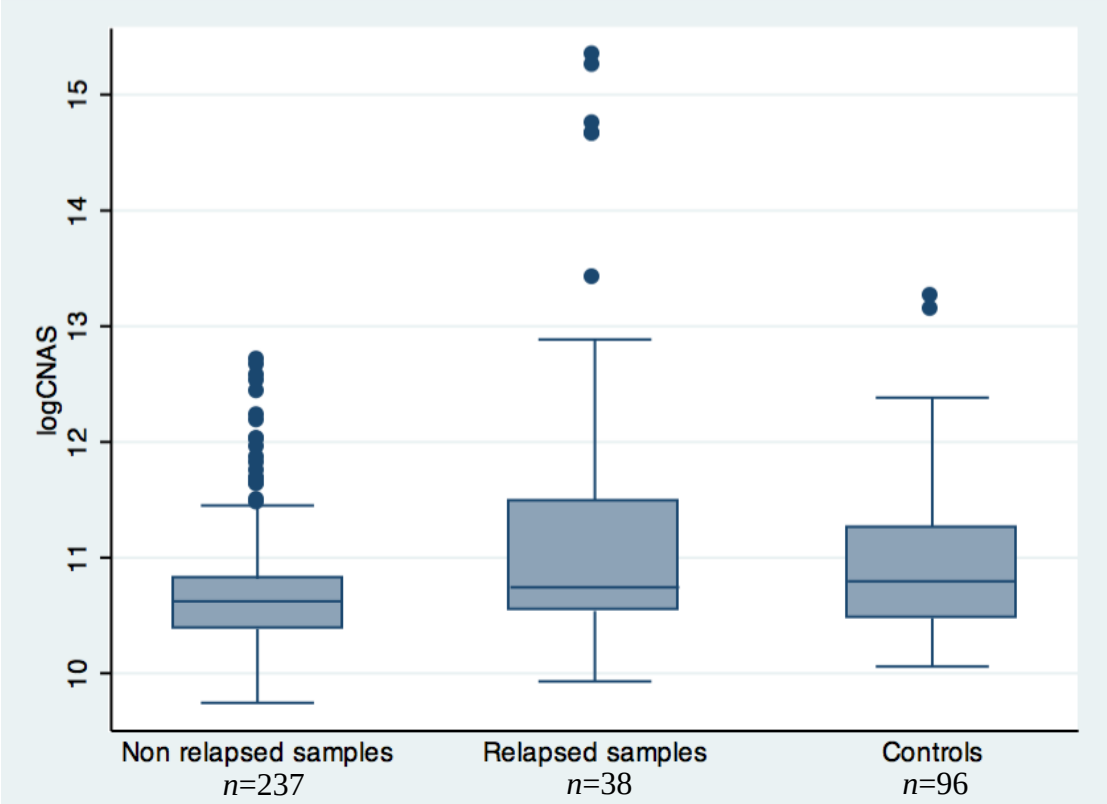


Figure 4.12: Box and whisker plot of the log of CNAS in relapsed samples vs. non-relapsed samples vs. controls. logCNAS in samples associated with a relapse were higher than non-relapsed samples (p=0.0063), while non-relapsed samples had lower mean logCNAS than control samples (p=0.0011)

Comparison of CNAS in relapsed cases, non-relapsed cases and controls

In a separate analysis, each case was attributed either a "relapsed" or "not-relapsed" status, based on whether the case was associated with a relapse event at any point during the study (see figure 4.4). Samples from relapsed cases were associated with higher log CNAS overall (n= 97, mean 11.06, SD 1.10), compared to those from non-relapsed cases (n= 178, mean 10.67, SD 0.50), *p*=0.0012. The samples from non-relapsed cases had a lower mean log CNAS score compared to healthy controls (n=96, mean 10.95, SD 0.66, p=0.0003).

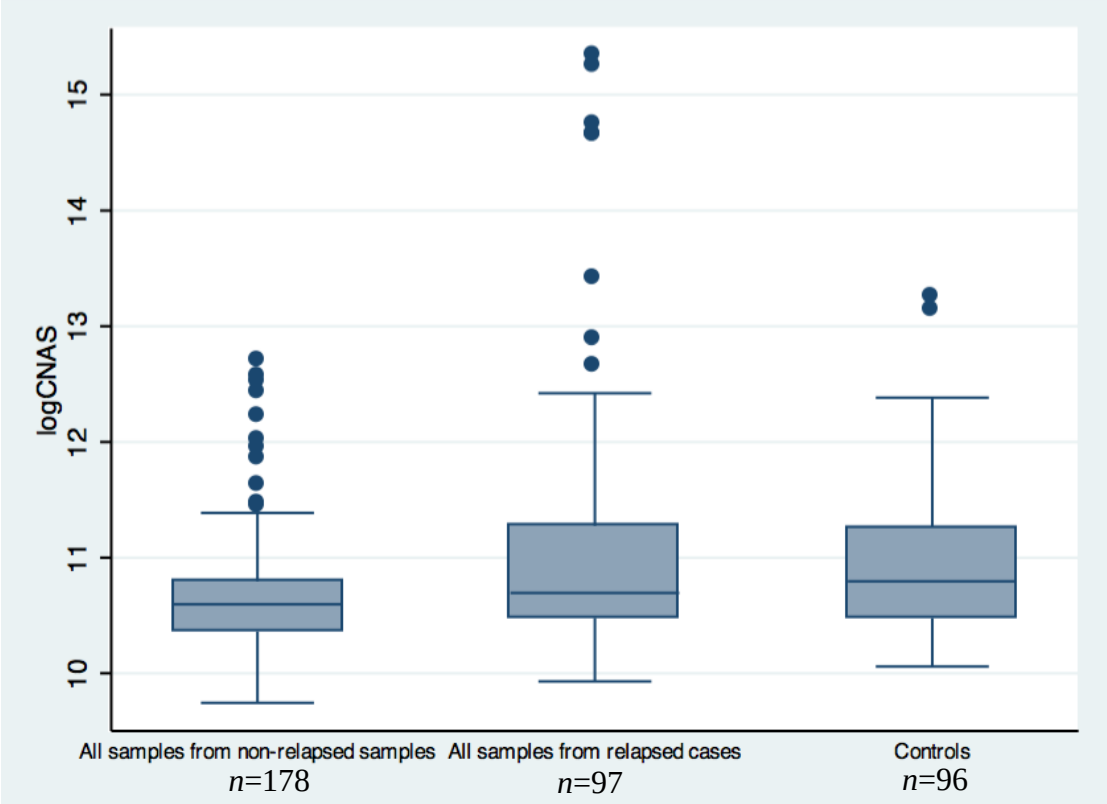


Figure 4.13: Box and whisker plot of the CNAS in relapsed cases vs. non-relapsed cases vs. controls. Higher logCNAS scores overall were seen in all samples obtained from cases associated with a relapse compared to all samples from non-relapsed cases (p=0.0012).
*includes repeated measures

Comparison of baseline CNAS between relapsed cases, non-relapsed cases and controls

A comparison was made between the baseline CNAS for all cases which relapsed on the study, and the baseline CNAS for all non-relapsed cases (see figure 4.6). The mean baseline log CNAS in cases that relapsed (mean 10.97, SD 0.66, n= 36) was not statistically different compared to the baseline mean for cases that did not relapse (mean 10.77, SD 0.54, n=60), p=0.13). The baseline CNAS for non-relapsed cases did not differ significantly from that of the controls (n=37, mean 10.84, SD 0.75), p=0.13.

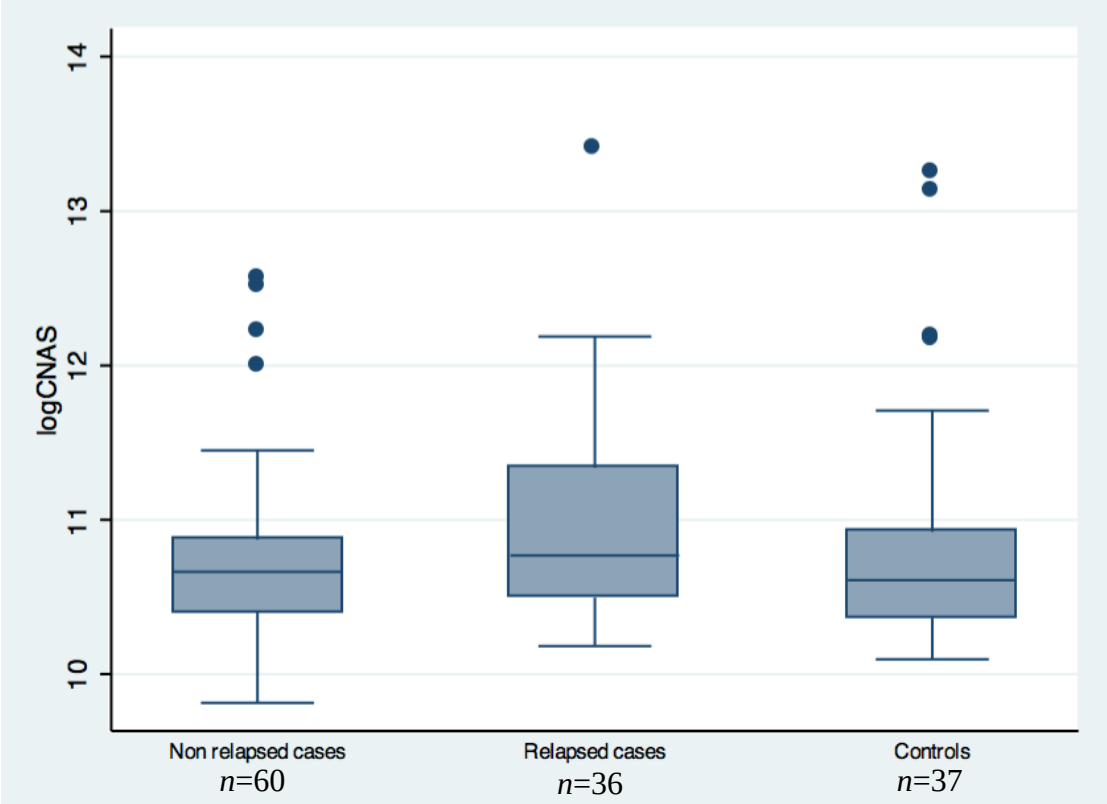


Figure 4.14: Box and whisker plot of the baseline logCNAS in relapsed cases vs. non-relapsed cases vs. controls. No difference in the baseline CNAS was seen in cases that relapsed compared to cases which did not relapse.

Comparison of CNAS in relapsed cases pre- relapse, post relapse and controls

In an analysis of all cases associated with a relapse, the mean of the logCNAS scores in the samples taken at (or after) relapse (n = 38, mean 11.44, SD 1.55, 95% CI 10.93 – 11.95), was significantly higher than the samples taken prior to relapse (n = 59, mean 10.8, 95% CI 10.67 – 10.96), p=0.0057). Samples taken prior to relapse did not differ significantly in log CNAS scores compared to controls (n=96, mean 10.95, SD 0.66, 95% CI 10.82 – 11.09, p= 0.18).

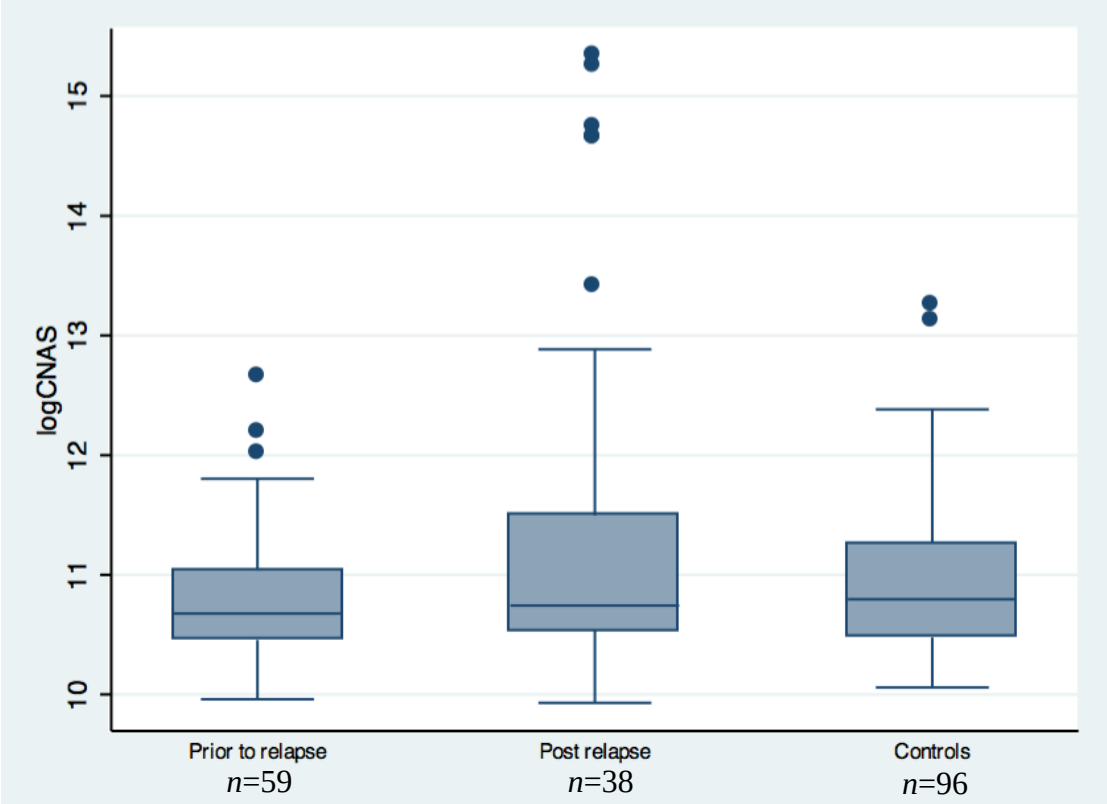


Figure 4.15: Box and whisker plot of the logCNAS from cases who have relapsed, in samples prior to relapse vs. post-relapse vs. controls. In cases that relapsed in the study, a higher mean logCNAS was seen in samples at (or after) relapse compared to samples prior to relapse (p=0.0057) *includes repeated measures.

4.3.6 CNAS as a predictor of the presence of relapsed disease

In order to evaluate the performance of the CNAS as a diagnostic tool in discriminating relapsed disease in the cohort of patients in the Markers of Relapse in Melanoma study, we used logistic regression to explore variables affecting risk of relapse.

Previous CNAS as a discriminator of relapse status

With the purpose of exploring whether CNAS, as calculated at previous sampling, could predict for relapse status at the time of the following sample, a logistic regression analysis was carried out (see table 4.3). Previous CNAS was shown to be a significant predictor of the presence of relapsed disease at the time of

the following sampling (odds ratio for relapse 2.74, 95% confidence interval 1.47-5.11, p=0.001). Stage at recruitment and age were also found to be significant predictors, while previous cfDNA concentration was not a significant predictor. Previous CNAS remained a significant discriminator in multivariable analyses for relapse status (p= 0.002, with area under the Receiver-Operator Characteristic curve of 0.72), see figure 4.12.

Univariable analysis				
	OR	***p*-value**	**95% CI**	**AUC**
Previous log CNAS	**2.74**	**0.001**	**1.47-5.11**	**0.65**
Previous log cfDNA level	0.78	0.54	0.35-1.73	0.57
Stage at recruitment	**3.62**	**0.013**	**1.31-9.98**	**0.64**
Age at recruitment	**0.97**	**0.003**	**0.95-0.99**	**0.66**
Gender	0.42	0.072	0.16-1.08	0.59
BRAF status	0.82	0.67	0.34-1.99	0.52
Multivariable analysis				
	OR	***p*-value**	**95% CI**	-
Previous log CNAS	**2.83**	**0.002**	**1.44-5.56**	-
Stage	2.60	0.092	0.85-7.91	-
Age	0.98	0.066	0.96-1.00	-

Table 4.3: Logistic regression analysis for previous CNAS as a predictor of relapsed disease

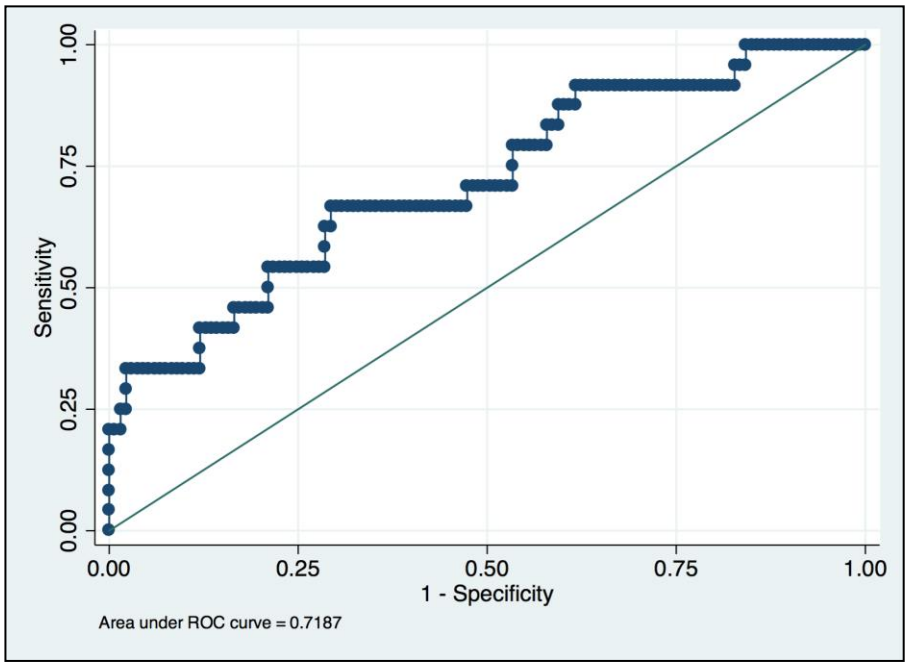


Figure 4.16: ROC multivariable analysis of previous CNAS showed it to be a significant predictor of the presence of relapsed disease at the time of the following sampling (odds ratio for relapse 2.74, 95% confidence interval 1.47-5.11, p=0.001).

Current CNAS as a discriminator of relapse status:

In a separate analysis, CNAS was explored as a discriminator of relapse status at the time of sampling, and was found to be a significant predictor (odds ratio for relapse 2.66, 95% confidence interval 1.71-4.14, p<0.0001). CfDNA level was also found to be a significant predictor of relapse status at the time of sampling, and with multivariable analysis including these variables (as well as stage and age which have previously been shown to be significant predictors), current CNAS remained a significant predictor of relapse status (p=0.001, area under the Receiver-Operator Characteristic curve 0.74).

Univariable analysis				
	OR	***p*-value**	**95% CI**	**AUC**
Current log CNAS	**2.66**	**<0.0001**	**1.71-4.14**	**0.65**
Current log cfDNA level	**2.31**	**0.012**	**1.20-4.45**	**0.60**
Stage	**3.62**	**0.013**	**1.31-9.98**	**0.64**
Age	**0.97**	**0.003**	**0.95-0.99**	**0.66**
Gender	0.42	0.072	0.16-1.08	0.59
BRAF status	0.82	0.67	0.34-1.99	0.52
Multivariable analysis				
	OR	***p*-value**	**95% CI**	-
Current log CNAS	**2.51**	**0.001**	**1.43-4.42**	-
Current log cfDNA level	2.07	0.096	0.88-4.85	-
Stage	2.45	0.11	0.83-7.27	-
Age	**0.97**	**0.004**	**0.95-0.99**	-

Table 4.4: Logistic regression analysis for current CNAS as a predictor of relapsed disease

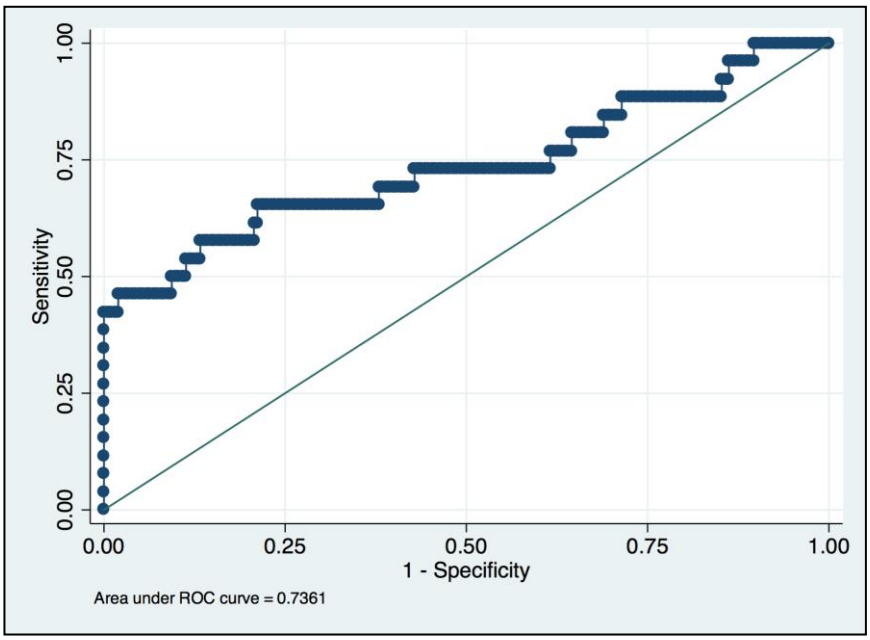


Figure 4.17: ROC multivariable analysis of current CNAS showed it to be a significant predictor of the presence of relapsed disease at the time of sampling (p=0.001).

CNAS above and below the 75th percentile as a predictor of relapse status:

In an exploration of the relationship between CNAS and the presence of active disease in the GEMS study (see Chapter 2), patients were ranked and grouped into four quartiles based on their log CNAS, and those patients with CNAS scores above the 75th percentile were found to have a higher risk of active disease than those with scores below the 75th percentile.

To test the ability of this binary variable (CNAS above and below the 75th percentile) to discriminate relapse status in the MRM cohort, a regression analysis was carried out in two respects. Firstly, a variable was created using CNAS calculated at previous blood sampling (in order to assess if this could predict future relapse status). Secondly, a binary variable using current CNAS was explored to see how this predicted relapse status at the time of blood sampling.

In the first analysis, previous CNAS above and below the 75th percentile was not found to be a significant predictor of future relapse status (odds ratio for relapse 1.05, 95% confidence interval 0.46-2.38, p=0.91).

Current CNAS above and below the 75th percentile, on the other hand, was found to be a significant predictor of relapse status (odds ratio for relapse 2.93, 95% confidence interval 1.27-6.76, p=0.012). However, multivariable regression analysis, including cfDNA level, stage and age as predictors of relapse status, showed current CNAS above and below the 75th percentile no longer to be a significant predictor (p=0.059).

Univariable analysis				
	OR	***p*-value**	**95% CI**	**AUC**
Current log CNAS category	**2.93**	**0.012**	**1.27-6.76**	**0.62**
Multivariable analysis				
	OR	***p*-value**	**95% CI**	-
Current log CNAS category	2.46	0.059	0.97-6.25	-
Current log cfDNA level	**2.99**	**0.004**	**1.43-6.27**	-
Stage	**2.92**	**0.043**	**1.01-8.48**	-
Age	**0.97**	**0.002**	**0.94-0.99**	-

Table 4.5: Logistic regression analysis for current CNAS above and below the 75th percentile as a predictor of relapsed disease

4.3.7 Baseline CNAS as a predictor of future relapse

To mirror the analysis done in the GEMS study, a regression analysis was carried out to investigate the ability of baseline CNAS of cases in the MRM cohort to predict future occurrence of relapse. Baseline CNAS was not found to be a significant predictor of future relapse (odds ratio 1.77, p=0.115, 95% CI 0.87 – 3.62).
In addition, the binary variable of baseline CNAS scores above and below the 75th percentile was also not found to be a significant predictor of future relapse status (odds ration 2.66, p=0.12, 95% CI 0.77 -9.11)

4.3.8 CNAS over time

Having previously demonstrated that the CNAS can predict active disease and survival in melanoma patients (see Chapter 2), we then examined the CNAS in longitudinal samples obtained from all cases recruited in the MRM study (n=96), and

compared the trends seen in patients who experienced a relapse (*n*=36), with those who did not (*n*=60).

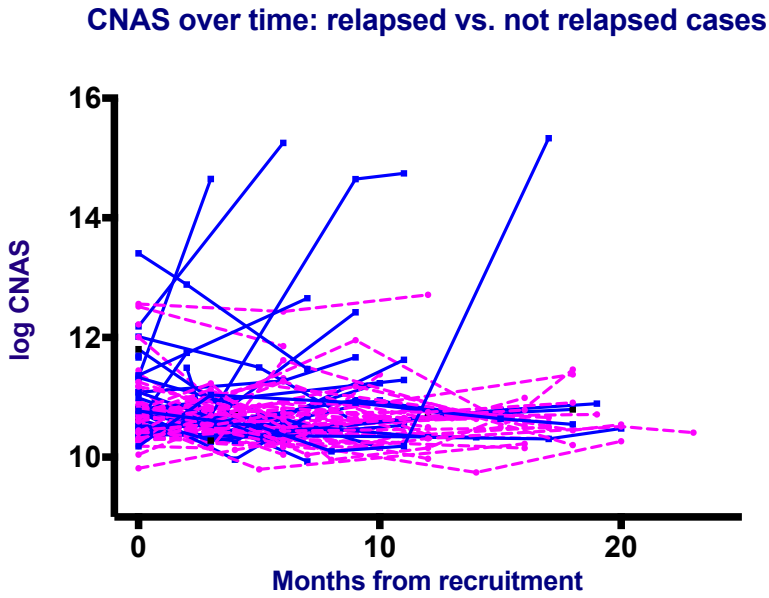


Figure 4.18: Spider plot of log CNAS over time (in months) in cases which relapsed (in blue) and in cases which did not relapse in the study (shown in pink). The x-axis represents time (in months) from recruitment (represented as time 0), and y-axis the log CNAS

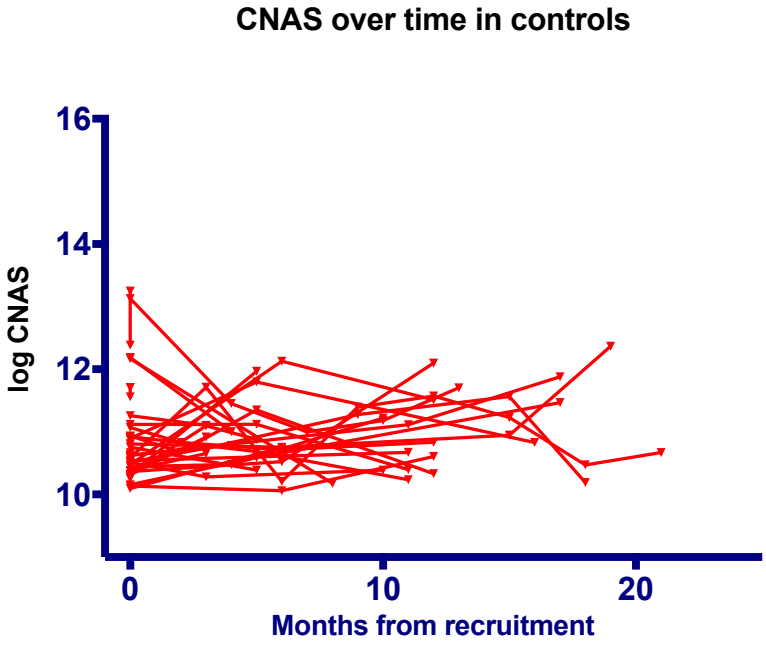


Figure 4.19: Spider plot of log CNAS over time (in months) in controls. Each red line represents a separate control. The x-axis represents time (in months) from recruitment (represented as time 0), and y-axis the log CNAS.

As seen in the plots above in Figures 4.18 and 4.19, there is a clear dramatic rise in the CNAS over time for some relapsed cases. This pattern, however, is evidently not exhibited by all relapsed cases. In contrast, the majority of non-relapsed cases demonstrate relatively stable CNAS scores over time.

4.3.9 Individual CNAS plots:

Longitudinal CNAS plots (with 2 or more time points) were made for 30 of the 36 patients who relapsed on the MRM study (6 patients had only 1 blood sample obtained each, hence it was not possible to chart serial plots for these patients).
Of these 30 patients, 7 of them demonstrated a rise in CNAS (from baseline) ahead of radiological or clinical relapse, and these are shown below (lead time from date of "CNAS relapse" to radiological/clinical relapse given in days) in Figure 4.20. Case 006 had a sharp rise (from baseline) in CNAS nearly a year prior to clinical relapse. The CNAS decreased following surgical excision of the relapsed disease but remained raised above baseline. In case 0026, the CNAS dropped following surgical resection of a relapse, but began to rise again with a lead time of about 5 months (152 days) prior to diagnosis of clinical relapse. The CNAS then fell again after commencing immunotherapy. Case 0029 saw a rise in CNAS from baseline, over a year prior to clinical relapse. Similarly, cases 0035, 0040 and 0050 each had a CNAS several months prior to diagnosis of relapse.

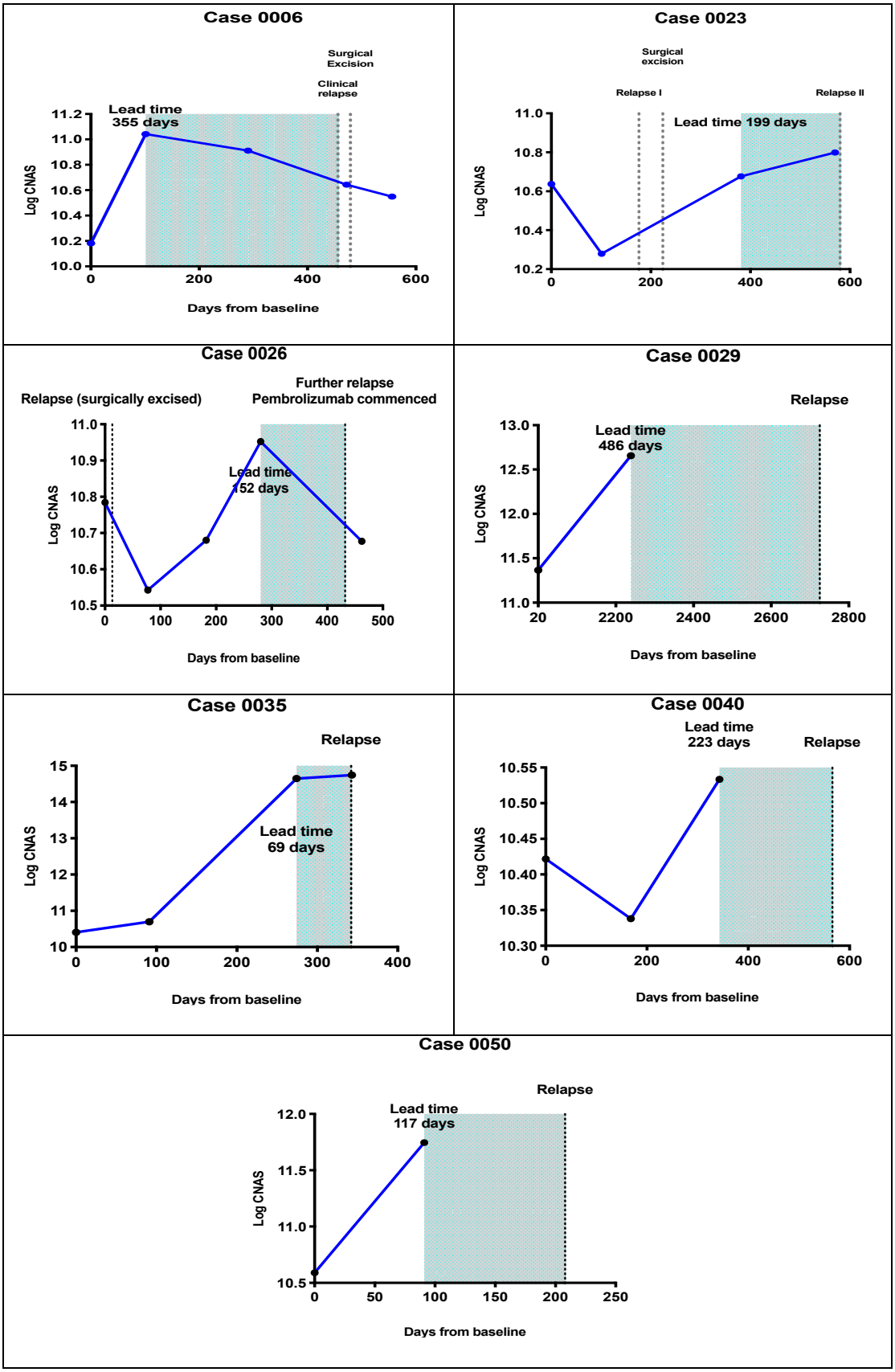


Figure 4.20: Longitudinal logCNAS plotted over time (in days) for 7 separate cases, demonstrating CNAS rise ahead of radiological/clinical relapse. The vertical dotted lines represent significant clinical time-points (labels at the top of the vertical lines specify the nature of the time-point); and the shaded area represents lead time from date of "CNAS relapse" to clinical relapse.

Of the remaining 23 patients, 6 patients had a CNAS rise that coincided with radiological/clinical relapse – this was defined as a CNAS rise within 28 days of radiological/clinical relapse (median interval of 16.86 days, range 0-24 days).

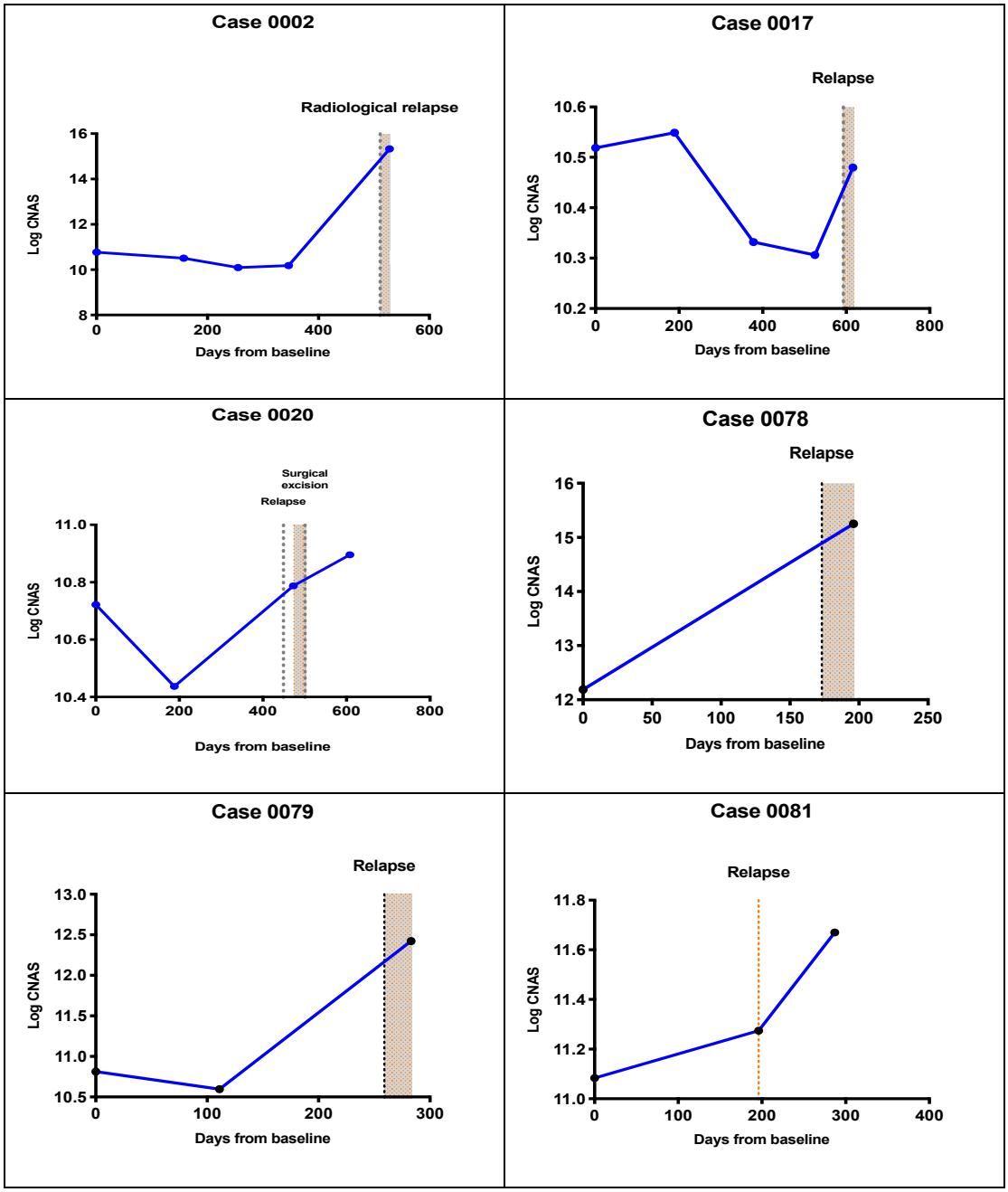


Figure 4.21: Longitudinal logCNAS plotted over time (in days), for 6 separate cases, demonstrating a rise in CNAS which coincided with clinical/radiological relapse.

Thus, 13 out of 30 cases (43.33%) that experienced a relapse event in this cohort demonstrated a rise in their CNAS at, or prior to, radiological/clinical relapse. Of the remaining 17 cases, 7 showed no significant CNAS pattern prior to clinical or radiological relapse, and 9 actually showed a decrease in CNAS prior to their relapse event. One case had relapsed at the time of first blood sampling, and went on to have two lines of systemic treatment, as depicted in the Figure below.

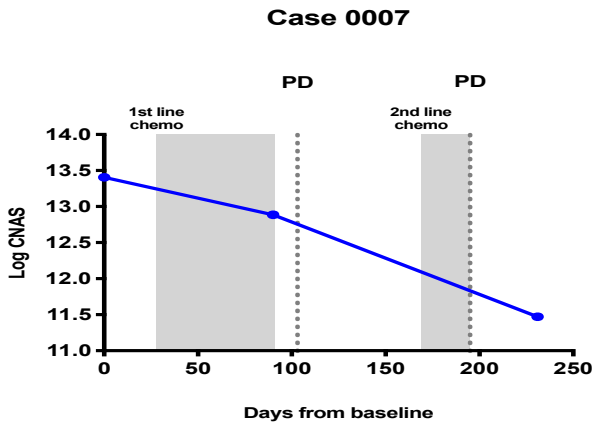


Figure 4.22: Longitudinal logCNAS profile plotted against time (in days, x-axis) showing case 007, who received 2 lines of systemic treatment, with CNAS falling with treatment. Interestingly, CNAS continued to drop despite radiological progression on 2nd line treatment.
PD: disease progression

4.3.10 Random coefficients mixed-model to investigate CNAS as a biomarker of relapse

We established, in the various analyses outlined in section 4.3.5 that CNAS was significantly higher in blood samples taken at/after a relapse event, compared to samples taken prior to relapse. Recognising the importance of taking into consideration the effect of repeated measures in such analyses (as with serial sampling in the MRM study), we investigated further the performance of CNAS as a biomarker. This time, taking into account each patient's trend in CNAS over time and accounting for the differences in CNAS measurements both within and between patients, a mixed effects model regression analysis was carried out:

Current CNAS as a predictor of current relapse status:

To explore the association between current CNAS and relapse status, and to provide an estimate of the effect of patient's trend, a mixed-effects logistic regression was carried out, and current CNAS was shown to be a significant predictor of relapse status (odds ratio 3.41, 95% confidence interval 1.75-6.62, $p<0.0001$). The random-effects estimate was 2.03 (95% confidence interval 1.09-3.79, standard error 0.65). The association remained significant after adjusting for cfDNA level, stage and age ($p=0.009$), with a random-effects estimate of 1.82 (95% confidence interval 0.87-3.77, standard error 0.68)

Univariable analysis				
	OR	***p*-value**	**95% CI**	**Random-effect ± standard error**
log CNAS	**3.41**	**<0.0001**	**1.75-6.62**	**2.03 ± 0.65**
log cfDNA level	**5.33**	**0.011**	**1.46-19.42**	**2.63 ± 0.70**
Stage	**7.08**	**0.038**	**1.11-44.92**	**2.18 ± 0.66**
Age	**0.96**	**0.036**	**0.92-0.99**	**2.12 ± 0.6**
Gender	0.44	0.34	0.08-2.39	2.22 ± 0.65
BRAF status	0.67	0.59	0.15-2.94	1.53 ± 0.55
Multivariable analysis				
	OR	***p*-value**	**95% CI**	**Random-effect ± standard error**
log CNAS	**2.56**	**0.009**	**1.27-5.16**	**1.82 ± 0.68**
log cfDNA level	2.92	0.11	0.79-10.82	-
Stage	3.47	0.15	0.64-18.7	-
Age	**0.96**	**0.034**	**0.92-0.99**	-

Table 4.6: Random-effects regression model of current CNAS as a predictor of relapse status

Previous CNAS as a predictor of current relapse status:

In a univariable mixed regression analysis to explore the ability of previous CNAS to predict future relapse status, previous CNAS (i.e. logCNAS of sample taken at prior sampling) was not shown to be significant predictor of relapse status at the time of next sampling.

Univariable analysis				
	OR	***p*-value**	**95% CI**	**Random-effect ± standard error**
Previous log CNAS	**3.49**	**0.075**	**0.88 – 13.78**	**10.34 ± 5.72**

Table 4.7: Random-effects regression model of previous CNAS as a predictor of relapse status

Previous CNAS as a predictor of current CNAS:

A mixed-effects linear regression was carried out to determine the association between previous CNAS and current CNAS, in order to see if previous CNAS predicts current CNAS. Previous CNAS was found to be a significant predictor of current CNAS (coefficient 0.67, 95% confidence interval 0.47-0.86, $p<0.0001$). This remained significant after adjusting for relapse status (coefficient 0.52, 95% confidence interval 0.33-0.72, $p<0.0001$). Previous cfDNA level, stage, gender, stage, BRAF status and age were not found to be significant predictors of current CNAS.

Univariable analysis				
	Coefficient	***p*-value**	**95% CI**	**Random-effect ± standard error**
Previous log CNAS	**0.67**	**<0.0001**	**0.47-0.86**	**$1.47x10^{-18}$ ± $6.64x10^{18}$**
Previous log cfDNA level	-0.019	0.90	-0.30-0.26	0.34 ± 0.14
Relapse status	**1.00**	**<0.0001**	**0.68-1.32**	**0.18 ± 0.063**
Stage	0.13	0.37	-0.16-0.43	0.24±0.75
Age	-0.0015	0.71	-0.0093-0.0063	0.25±0.076
Gender	-0.040	0.80	-0.34-0.27	0.24±0.076
BRAF status	-0.43	0.063	-0.88-0.024	0.23±0.13
Multivariable analysis				
	Coefficient	***p*-value**	**95% CI**	**Random-effect ± standard error**
Previous log CNAS	**0.52**	**<0.0001**	**0.33-0.72**	**0.028±0.10**
Relapse status	**0.76**	**<0.0001**	**0.41-1.12**	-

Table 4.8: Random-effects regression model of previous CNAS as a predictor of current CNAS

4.3.11 CNAS as a predictor of survival

Univariable Cox regression analysis showed baseline CNAS, as well as baseline CNAS category (baseline log CNAS above and below the 75th percentile), to be significant predictors of overall survival. BRAF status was also identified as a significant predictor of survival. Baseline CNAS concentration, age, gender, and stage at baseline were not found to be significant predictors of overall survival. Mortality was significantly higher (HR (95% CI) 2.63 (1.22-5.69) with increasing logCNAS, p=0.014. After adjusting for the other significant predictors of survival, baseline logCNAS remained a significant predictor of survival (HR (95% CI) 2.76 (1.03 – 7.35), *p*=0.044.

Similarly, baseline CNAS category (baseline log CNAS above and below the 75th percentile) was associated with significantly higher mortality (HR (95% CI) 4.96 (1.36-18.08), p=0.015), and this remained significant after adjusting for BRAF status (HR 7.55 (95% CI 1.59-35.94), p=0.011.

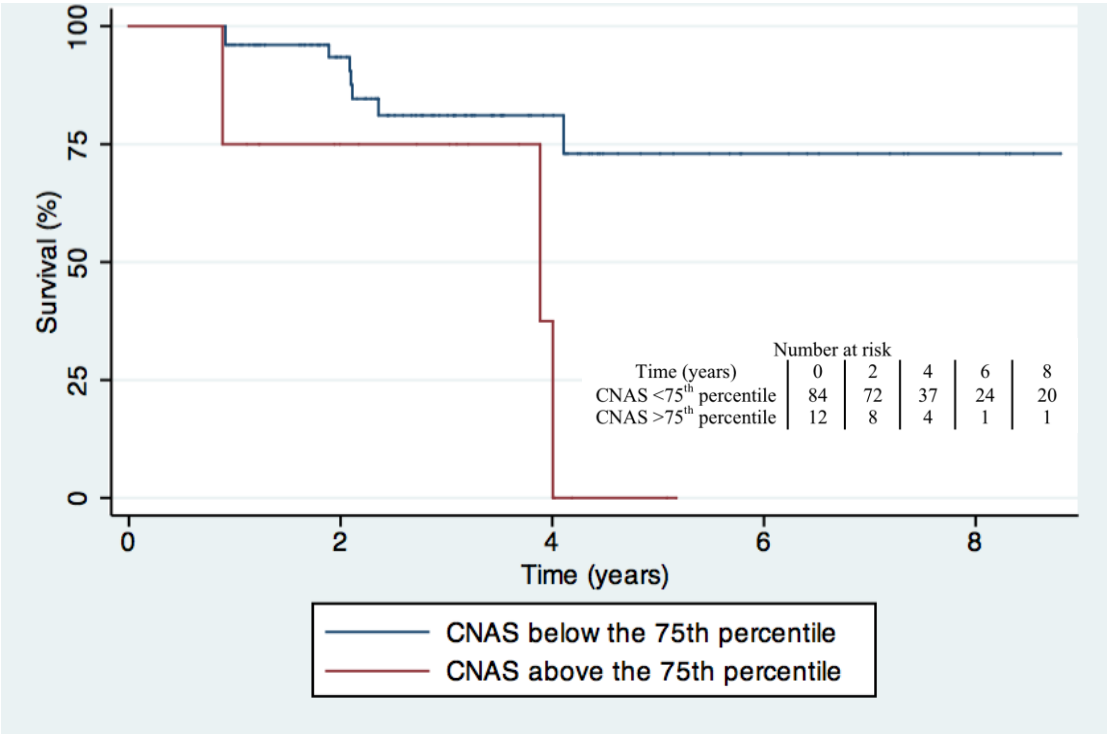


Figure 4.23: Kaplan Meier survival estimates of overall survival, comparing CNAS below (in blue, n=84) and above (in red, n=12) the 75th percentile, showing significantly higher mortality in cases with CNAS above the 75th percentile, after adjusting for BRAF status (HR 7.55 (95% CI 1.59-35.94), p=0.011.

Univariable analysis			
	HR	***p*-value**	**95% CI**
CNAS category	**4.96**	**0.015**	**1.36-18.08**
Stage	1.85	0.36	0.50-6.87
Age	1.003	0.88	0.96-1.05
Gender	2.36	0.18	0.67-8.24
BRAF status	**0.24**	**0.046**	**0.61-0.97**
Multivariable analysis			
CNAS category	**7.55**	**0.011**	**1.59-35.94**
BRAF status	**0.21**	**0.003**	**0.074-0.58**

Table 4.9: Baseline CNAS category as a predictor of survival

4.3.12 Quality control analysis

We had previously demonstrated the reproducibility of the CNAS of DNA libraries prepared from the MDA-MB-435 cell line and sequenced on nine separate runs (section 3.10), with the CNAS showing very little dispersion (coefficient of variation (CV)=0.098), demonstrating high reproducibility of this score. In addition, the CNAS for 10 subjects (8 cases and 2 controls) in duplicate sequencing runs were compared, and the Bland-Altman plot below demonstrates good agreement in the CNAS between the 10 technical repeats.

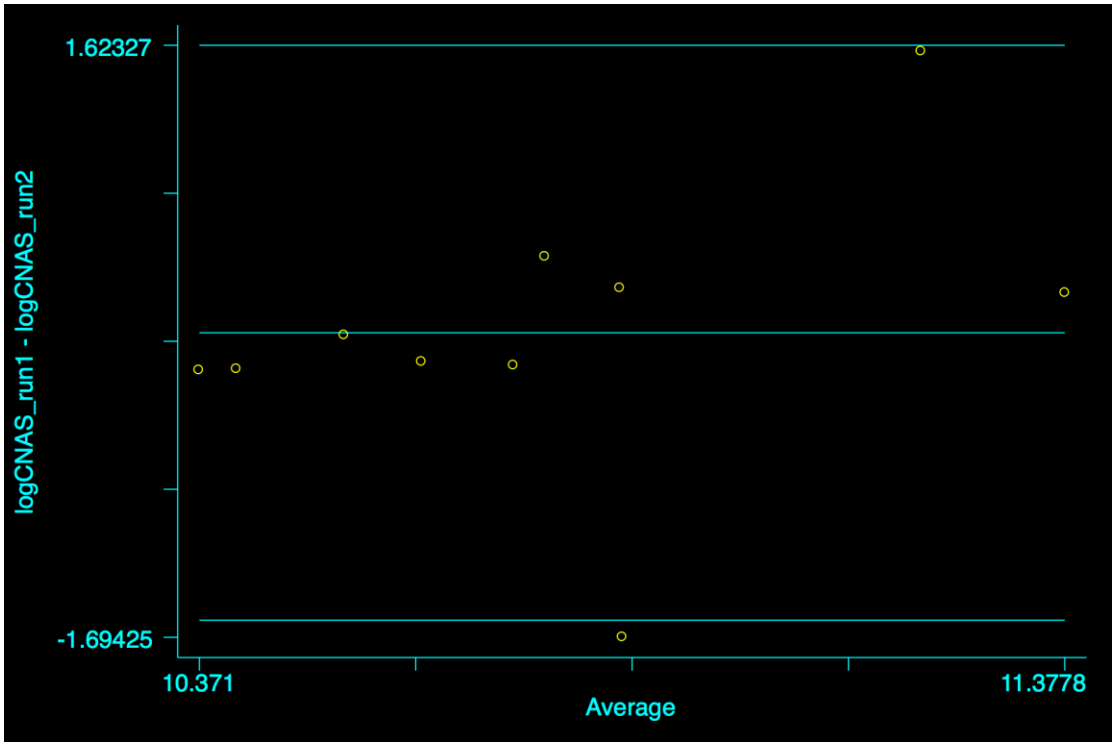


Figure 4.24: Bland-Altman plot of CNAS in duplicate sequencing runs, showing good agreement in CNAS between 10 technical repeats.

To demonstrate that the copy number aberration scores were not related to the amount of input DNA sequenced, a scatter graph and Spearman's correlation analysis was carried out for case and control samples separately. The graphs and Spearman's coefficients are shown in figures 4.25 and 4.26.

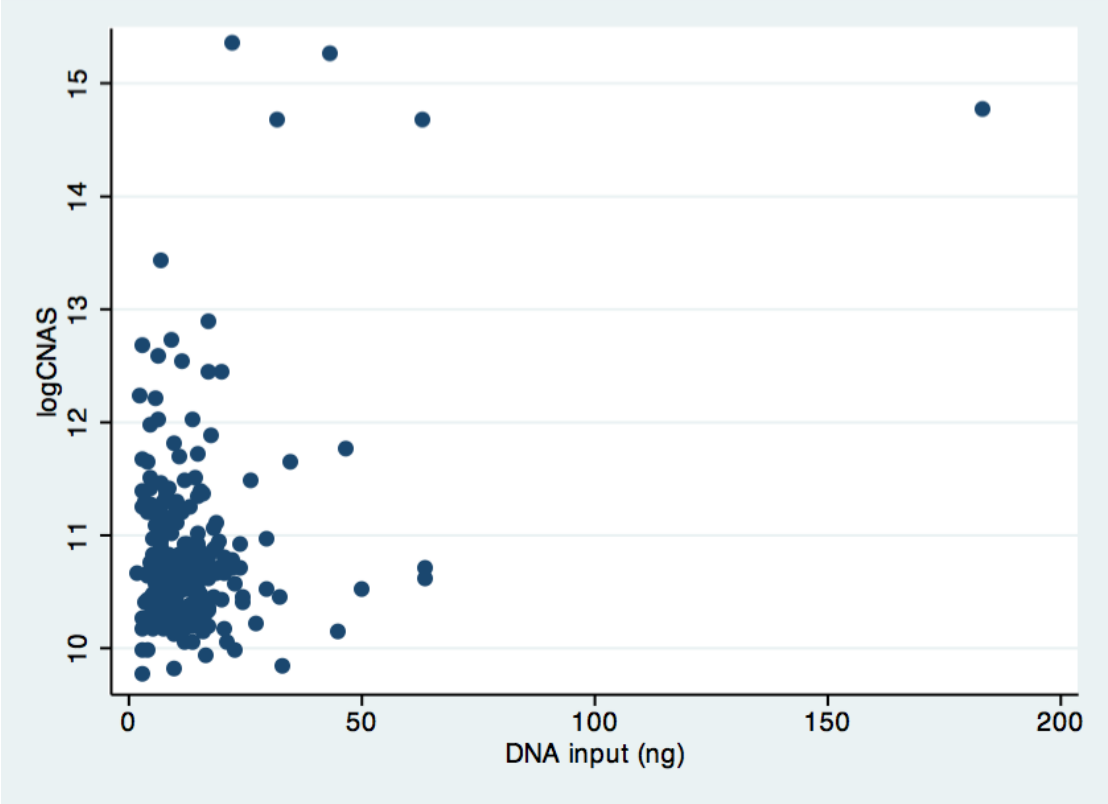


Figure 4.25: Scatter plot of logCNAS for cases vs. quantity of input DNA, showing no association between the two variable (Spearman's coefficient ρ 0.0058, n=275)

Figure 4.25 shows scatter plot of logCNAS scores for all samples obtained from cases versus the quantity of input DNA sequenced for each sample (n=275). The Spearman's coefficient ρ 0.0058 indicates no association between the two variables.

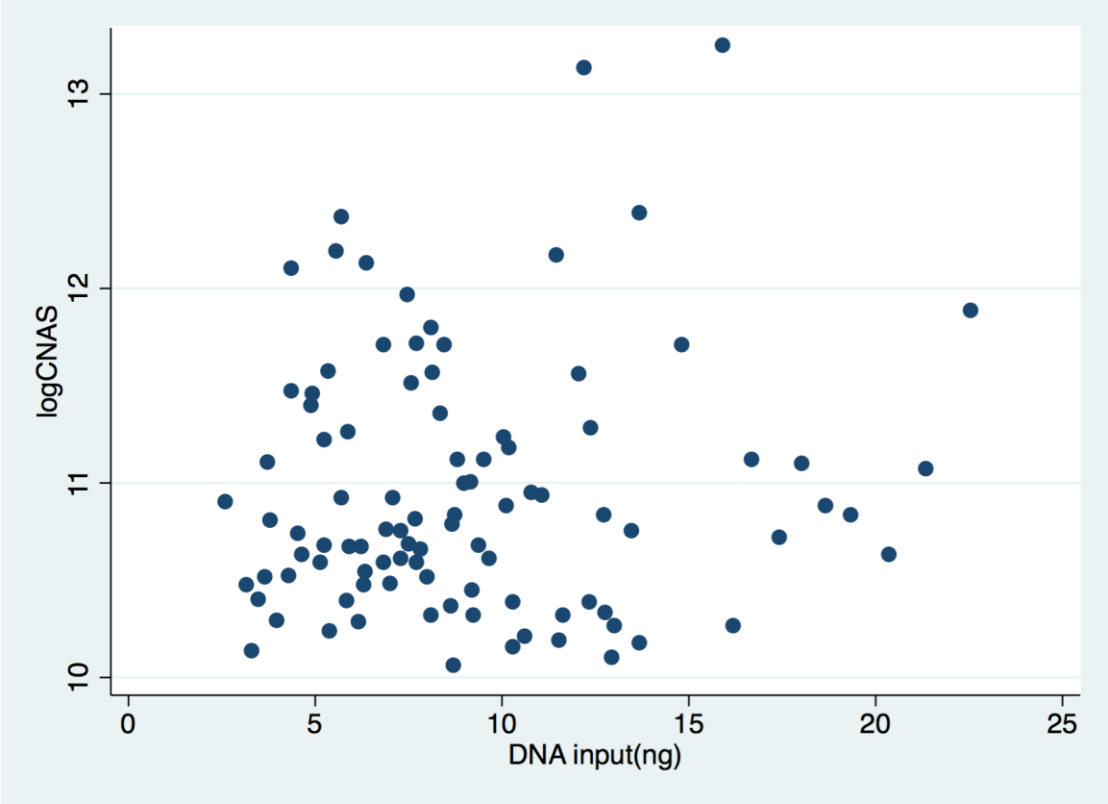


Figure 4.26: Scatter plot of logCNAS for controls vs. quantity of input DNA, showing no association between the two variable (Spearman's coefficient ρ 0.0339, n=96)

Figure 4.26 shows scatter plot of logCNAS scores for all samples obtained from controls versus the quantity of input DNA sequenced for each sample (n=96). The Spearman's coefficient ρ 0.0339 indicates no association between the two variables.

4.3.13 Sensitivity and specificity of logCNAS as a marker of relapse:

The Youden index was calculated to identify the cut-off point of logCNAS which provides the maximum potential effectiveness as a biomarker of relapse status. Adjusting for log cfDNA level, stage and age at diagnosis, the ROC AUC was 0.7068, with a Youden's index of 0.41 at a cut-off logCNAS of 11.28944, providing a sensitivity of 58% and specificity of 83%.

4.3.14 Comparison of MRM and GEMS copy number aberration scores

The baseline copy number aberration scores from cases recruited to the MRM study were compared with those recruited to the GEMS study, to explore how these cohorts differed from each other. The figure below shows the distribution of baseline log CNAS in both cohorts.

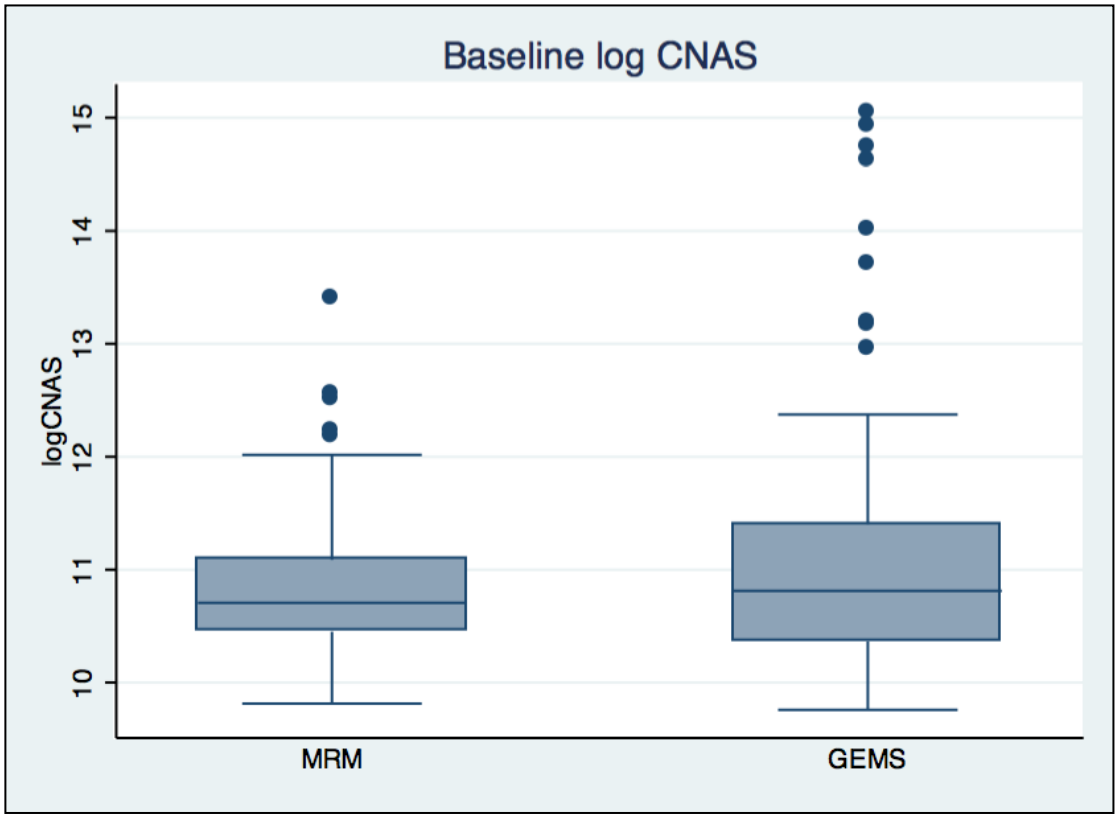


Figure 4.27: Box and whisker plot of the baseline logCNAS in the GEMS cohort (n=83) vs the MRM cohort (n=96), showing a higher mean baseline logCNAS for the GEMS cohort (p=0.03).

The mean baseline log CNAS for the GEMS cohort (*n*=83) was higher than that of the MRM cohort (*n*=96), p = 0.03.

	Mean log CNAS	**95% CI**	***p*-value**
MRM	10.84	10.72 – 10.96	0.03
GEMS	11.14	10.88 – 11.40	-

4.4 Discussion

4.4.1 CNAS vs cfDNA concentration, as a biomarker of relapse

Overall, in our various analyses of predictors of relapsed disease in the MRM cohort, the Copy Number Aberration Score (CNAS) performed better as a marker of relapsed disease, compared to cfDNA concentration, as summarised below:

	CNAS	**cfDNA concentration**
All relapsed samples vs. all non-relapsed samples	Significant difference	Significant difference
All samples from relapsed cases vs. all samples from non-relapsed cases	Significant difference	No significant difference
Baseline samples from cases relapsed or not	No significant difference	No significant difference
Pre-relapse samples vs. post-relapse samples	Significant difference	Significant difference

Table 4.10: Summary of CNAS vs cfDNA concentration as a marker of relapse in the MRM cohort

We found cfDNA concentration showed no differences seen in baseline cfDNA levels between cases that relapsed versus those that did not. This contrasts with our findings in the GEMS study (see section 3.3), where there was a significant difference in cfDNA concentrations between those with active disease and those with surgically excised disease, with patients with active disease having higher concentrations compared to those with excised disease. In the GEMS study, the majority of patients with active disease had advanced stage IV disease (81.8%). In contrast, in the MRM cohort, of those who relapsed, 61.1% had advanced stage IV disease, while 38.9% relapsed with surgically-resectable stage III (i.e. lower volume) disease. So the higher burden of disease in those with active disease in the GEMS

study may account for the significant difference in cfDNA concentrations (and copy-number aberration scores) seen compared to those with surgically excised disease.

Baseline cfDNA levels in samples taken from non-relapsed cases were found to be higher than levels measured in healthy controls. This again contrasts with our findings in the GEMS study, where cfDNA levels amongst those with resected disease were not statistically different from controls. This may be explained by the fact that subjects invited to take part in the MRM study were prevalent melanoma cases undergoing melanoma follow-up at STH, with varying intervals from their surgical excision. This differed from the GEMS study, in which patients were recruited within 4 weeks of their surgical-excision. Therefore, the baseline samples obtained from subjects in the GEMS study would represent 'true" baseline, unlike the baseline samples from the prevalent melanoma cases recruited in the MRM study, some of whom had had their surgical excision several months/years prior to recruitment. This lack of 'true' baseline samples in the MRM cohort could also explain the lack of significant difference seen in the CNAS between cases that went on to relapse in the study, versus those cases that did not relapse.

4.4.2 Baseline CNAS as a predictor of survival

We have shown in this cohort of 96 patients that high baseline CNAS scores (log CNAS above the 75th percentile) were associated with significantly poorer survival. This mirrors the findings in our preliminary analysis in the GEMS study where high CNAS scores were associated with higher mortality (see chapter 3.6). These mirrored findings have potential clinical significance, as they advocate the utility of this minimally invasive baseline blood-test in identifying melanoma patients

with a potentially worse outlook, who are more likely to benefit from adjuvant systemic treatment to improve their outcomes.

Tan et al (Tan et al., 2019) and Lee at al (J. H. Lee et al., 2019) demonstrated, similarly, that baseline ctDNA analysis predicts survival in stage II/III melanoma patients. Further validation of our CNAS in an independent prospective cohort is required to establish its potential to aid the adjuvant-decision making process in melanoma patients, as well as in other tumour-sites.

4.4.3 Sensitivity/specificity and utility of CNAS as a marker of melanoma relapse

In the sensitivity/specificity analysis of logCNAS as a biomarker of relapse, the Youden's index provided the cut-off point of logCNAS which optimises its ability to discriminate relapsed disease from non-relapsed status at the time of blood-sampling. The low sensitivity of 58%, demonstrates the limited ability of logCNAS to correctly identify relapsed cases. The high specificity of 83% demonstrates that a low logCNAS is likely to correctly identify non-relapsed status. Further exploratory analyses are required to explore scoring algorithms which combine the copy-number score with other potential biomarkers (e.g. ctDNA mutation burden) to maximise sensitivity, while preserving the high specificity provided by the logCNAS score.

In choosing the mixed-effects random coefficients model for the longitudinal analysis of CNAS, the unstructured method was utilised as the least restrictive covariance assumption, in order to be as inclusive as possible, utilising all available data points in the longitudinal sampling. As the unstructured matrix imposes no constraints on the values within the model, with each variance and covariance uniquely estimated from the data, allowing for the best possible model fit, this matrix

was justified, because there was no reason to expect the variances in this dataset to be equal, or that covariances would display a specific pattern.

This model showed that logCNAS a significant predictor of relapse status at the time of blood sampling. In addition, baseline logCNAS was shown, by logistic regression analysis, to be a significant predictor of survival in this cohort. While this needs prospective validation in a separate cohort, this could potentially have utility in estimating individual risk of relapse, therefore tailoring the need for adjuvant therapies. While other studies have shown similar association of baseline ctDNA with survival in melanoma patients (J. H. Lee et al., 2019; Tan et al., 2019), these studies have relied on the presence of mutations (BRAF/NRAS/KIT), which could be measured in ctDNA. While this approach can improve the sensitivity of the test, it does restrict the scope of these biomarkers. Our analysis has demonstrated proof of principle that low-coverage copy-number analysis can be utilised as a marker of relapse in melanoma, and warrants further exploration and validation.

Chapter 5: Common Copy number aberrations in melanoma

5.1 Introduction

Genomic instability is one of the hallmarks of cancer, characterised by the accumulation of genetic abnormalities, which facilitate the acquisition of additional mechanisms that promote tumour growth and proliferation (Hanahan & Weinberg, 2011). These genetic abnormalities include gene mutations, chromothripsis, and copy number aberrations. Various copy number aberrations have been studied in melanoma, mainly in FFPE samples, but also in frozen tumour samples.

In chapters 3 & 4, using whole genome scores, we described the genome-wide copy number changes detected in cfDNA extracted from 2 cohorts of patients (GEMS and MRM). In this chapter, we have attempted to explore individual copy number aberrations, to see if those described in published literature, including The Cancer Genome Atlas (TCGA) are identifiable in our cfDNA samples from MRM.

The aim of the work described in this chapter was to identify, from published literature, chromosomes which were associated with copy number aberrations in cutaneous melanoma, and to then see if these chromosomes of interest were similarly aberrant in the circulating cell free DNA extracted from our patients' samples. In the first instance, we analysed baseline samples from all patients recruited in the MRM study, and we then went on to study the CNAs in the 36 samples associated with a relapse event in the MRM study.

The objectives of the chapter were:

1. Compare each chromosome of interest identified in the literature with baseline cfDNA samples from the Markers of Relapse in Melanoma (MRM) study.
2. Compare chromosome aberrations in cfDNA from relapsed samples from the MRM study with the chromosomes of interest from the literature.

3. Explore how copy number aberration data from The Cancer Genome Atlas (TGCA) melanoma database compared to those from the relapsed samples from the MRM study.

5.2 Analysis of chromosomes of interest in the baseline samples from the Markers of Relapse in Melanoma (MRM) study

Having conducted a search in PubMed of relevant studies which reported the common copy number aberrations in melanoma tumour (FFPE) samples, we compiled a list of chromosomes of interest, where amplifications, deletions, or duplications had been identified. Relevant studies were identified using the keywords “copy number” and “melanoma”, and the search was limited to articles published in the last 40 years. In identifying chromosomes of interest, where frequency of aberrations were provided in the literature, we included those with a frequency of 10% or more in our comparisons. The table below summarises the common gains and losses reported in melanoma tumour DNA. We set out to see how these compared with the cfDNA samples extracted from baseline blood samples obtained from patients recruited to the MRM study.

	Bastion 2003 (Bastian et al., 2003) n=132	Bastion 1998 (Bastian et al., 1998) n=32	Curtin 2005 (Curtin et al., 2005) n=70	Jonsson 2007 (Jönsson et al., 2007) n=47	Gandolfi 2016 (Gandolfi et al., 2016) n=41	Hoefsmit 2020 (Hoefsmit et al., 2020) n=38	TCGA 2015 n=333
Gain	1q (33%)	1q (25%)		1q	1p 5% 1q (17%)		1p13
Gain		2 (13%)					
Gain					3p (5%)		3p13
Gain							4q12
Gain							5p15
Gain	6p (37%)	6p (28%)	6p		6p (19%)	6p	
Gain	7p (32%) 7q (32%)	7 (50%)	7	7	7p (17%) 7q (22%)	7	7q34
Gain	8q (25%)	8 (commonly q) (34%)	8q	8q		8	
Gain							9p24
Gain							11q13
Gain							17q11
Gain	17q (24%)	17(commonly q) (13%)	17q	17q			
Gain	20q (22%)	20 (commonly q) (13%)	20q	20q		20	
Loss					1p (7%)		
Loss					3p (7%)		
Loss				4			
Loss	6q (26%)	6q (28%)	6q	6q		6q	

Loss					7p (2%)	
Loss		8p (22%)	8p			8p
Loss	9p (64%) 9q (36%)	9p (81%)	9p	9p	9p (39%)	9
Loss	10q (36%) 10p (30%)	10 (whole or q) (63%)	10	10	10q (56%)	10
Loss	11q (21%)		11q	11q	11q (7%)	
Loss			13			
Loss			21q			

Table 5.1: Summary of key copy number aberrations identified in melanoma tumour FFPE samples in the literature. Where provided, the frequency of aberration (expressed as a percentage of samples affected) is shown in brackets.

5.3 Creation of summary plots of copy number ratios across individual chromosomes of interest

As outlined in Chapter 2, the cfDNA extractions from the baseline samples from the MRM study and subsequent quantification, DNA library preparation, pooling for sequencing, were carried out myself. Copy number ratios were generated using the pipeline outlined in Chapter 2. Dr Mark Dunning at Sheffield University of Sheffield provided bioinformatics support to generate summary plots. Violin plots of the copy number ratio of each 1 Mb window for the ninety-six baseline samples were plotted against chromosomal position for the identified chromosomes of interest from Table 5.1. We defined a copy number gain as a copy number ratio of greater than 1.1, and a copy number loss as a copy number ratio of less than 0.9. This was used as a guide, accounting for the fact that tumour-derived cfDNA is diluted within wild-type cfDNA in blood. A summary of all amplifications and deletions, identified using these criteria in the chromosomes of interest, in the baseline samples is presented in Appendix E.

The three most commonly affected aberrations of interest in the published literature have been selected (chromosomes 7, 9 and 10), and their copy number profiles in the MRM baseline samples are summarized below:

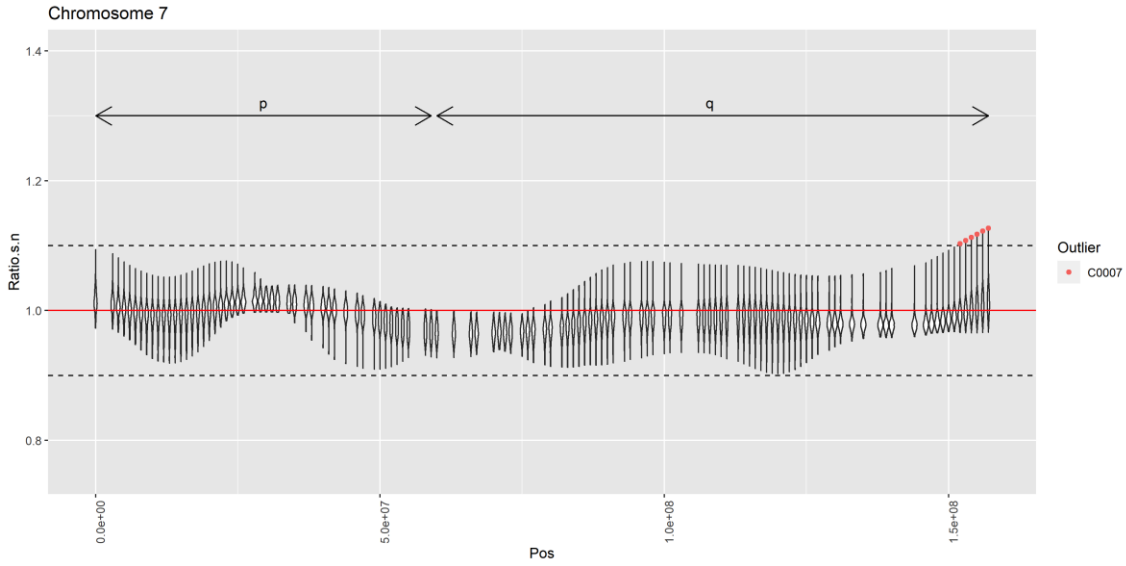


Figure 5.1: Copy number ratios of each 1Mb window of chromosome 7 in the MRM baseline samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

The published literature shows quite high levels of gain of chromosome 7 (both p and q arms), This is not strongly evident in our MRM baseline samples, as seen in Figure 5.1. Only one sample showed clear evidence of gain at the end of the q arm, and there is a trend for gains in the p arm, as well as a trend for losses in the beginning of the q arm.

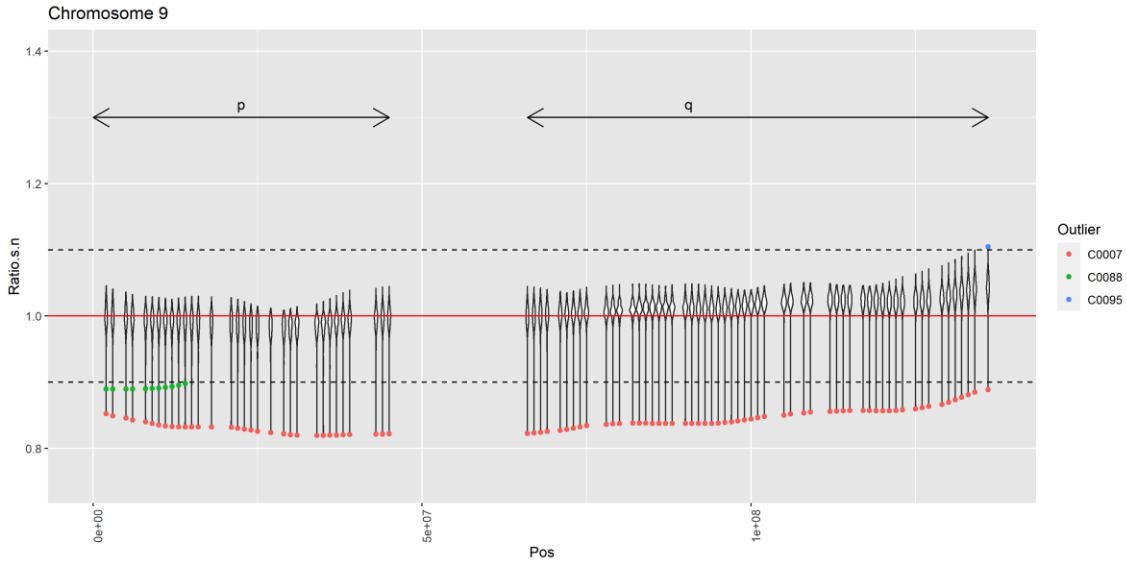


Figure 5.2: Copy number ratios of each 1Mb window of chromosome 9 in the MRM baseline samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

Melanoma is associated with deletions on chromosome 9p21, a region that contains the CDKN2A tumour suppressor gene ((Quelle et al., 1995). Two samples in our cohort showed evidence of loss in 9p, and one of these samples also showed a loss in the q arm (see Figure 5.2 above). Overall, there was a trend for losses in the p arm, and gains in q.

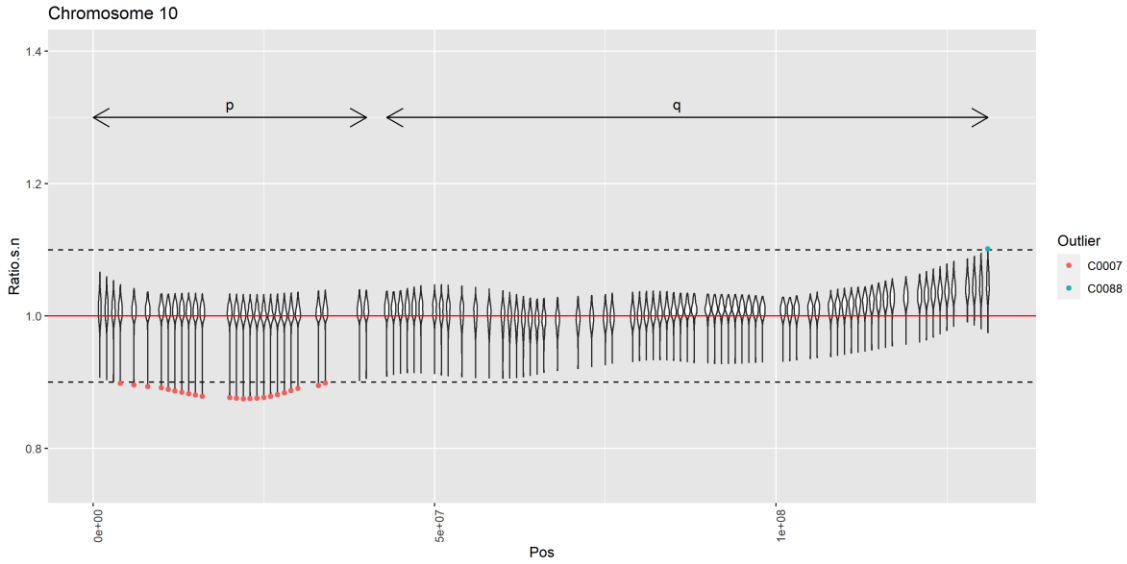


Figure 5.3: Copy number ratios of each 1Mb window of chromosome 10 in the MRM baseline samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

Another frequently deleted chromosome cited in the literature is chromosome 10 (frequently 10q, but whole chromosome deletions as well as deletions affecting the p arm have also been described). One sample in the MRM cohort demonstrated loss in 10p, and another sample showed a 10q gain, as seen in Figure 5.3 above. Overall, however, there was a trend for gains at the end of the q arm.

In addition to three most commonly affected chromosomes in the literature highlighted above, the profile below represents the chromosome with the highest number of defined aberrations from our baseline samples.

One paper showed loss in 21q (Curtin et al., 2005). In Figure 5.4, there is evidence of losses in thirty samples in the MRM baseline samples (affecting p and/or q arms). However, four samples showed gains (1 in 21q, and 3 in 21p).

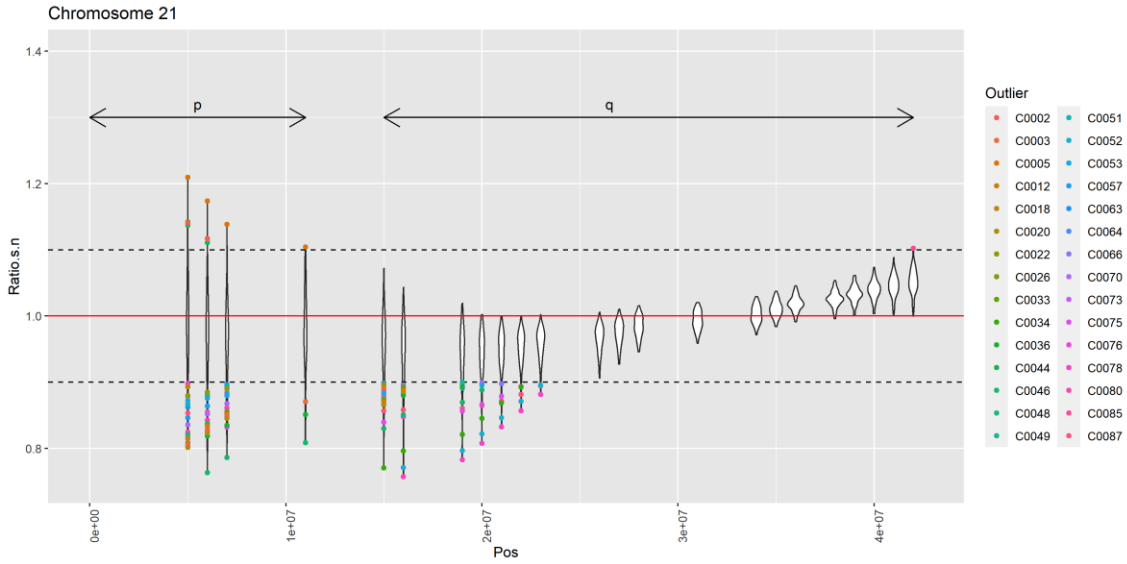


Figure 5.4: Copy number ratios of each 1Mb window of chromosome 21 in the MRM baseline samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

Chromosome	Defined gain	Weaker evidence for gain	Defined loss	Weaker evidence for loss
1		1p	1q (4 outliers)	1q
2		2p	2p (1 outlier)	
4		4p	4p/4q (7 outliers)	4p/4q
6	6p (1 outlier)		6q (same outlier)	6q
7	7q (1 outlier)			7q
8	8q (1 outlier)	8q	8q (1 outlier)	8p, 8q
9	9q (1 outlier)	9q	9p/q (2 outliers)	9p
10	10q (1 outlier)	10p/q	10p (1 outlier)	
11	11 q (1 outlier)	11p/q	11p (same outlier)	
13			13q (16 samples)	13p
17		17q	17p (1 outlier)	17q
20		20p/q		
21	21 p (3 samples) 21q (1 sample)	21q	21p/q (30 samples)	

Table 5.2: Summary of gains and losses seen in the baseline samples from the Markers of Relapse in Melanoma study. The defined gains and losses refer to those that met the criteria set for copy number gains and losses (copy number ratio >1.1 and less than 0.9 respectively). The columns for weaker evidence of gains/losses represent those aberrations that did not strictly meet the set criteria, but showed a trend for these aberrations.

5.4 Analysis of chromosomes of interest in the relapsed samples from the Markers of Relapse in Melanoma (MRM) study

Having explored the copy number aberrations in the baseline samples, we proceeded to analyse the aberrations in the cfDNA samples associated with a clinical and/or radiological relapse. Again, we focused on aberrations seen within the three most commonly affected aberrations of interest in the published literature (chromosomes 7, 9 and 10).

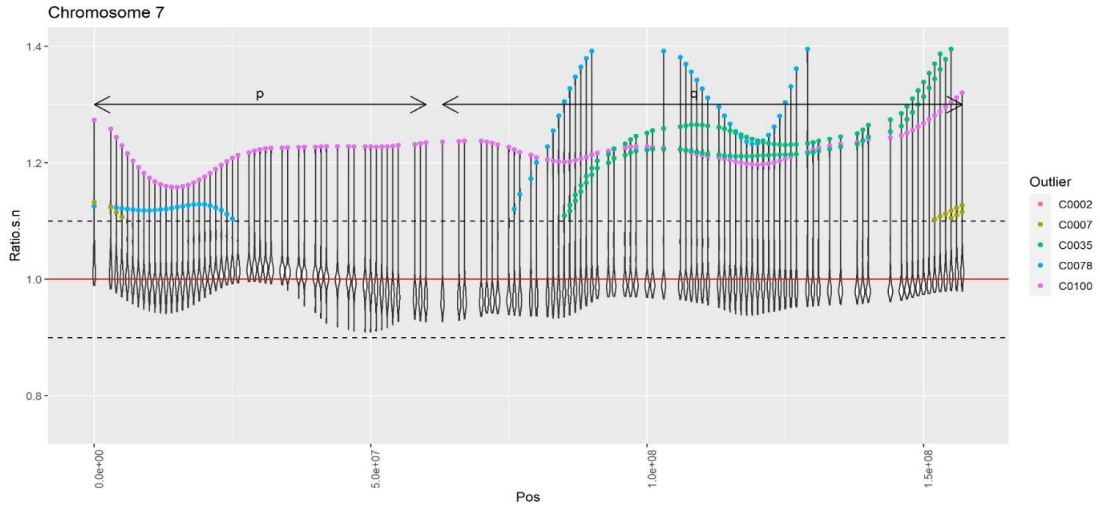


Figure 5.5: Copy number ratios of each 1Mb window of chromosome 7 in the MRM relapsed samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

As seen in Figure 5.5 above, we have seen more copy number changes associated with chromosome 7 in the relapsed samples compared to the baseline samples. One sample had copy number gains across the entire chromosome. Two samples had gains predominantly in the q arm, but also in the early part of the p arm. Two further samples (both from the same patient) showed gains across the q arm. Overall, there appeared to be a trend for losses in p and q arms across the cohort.

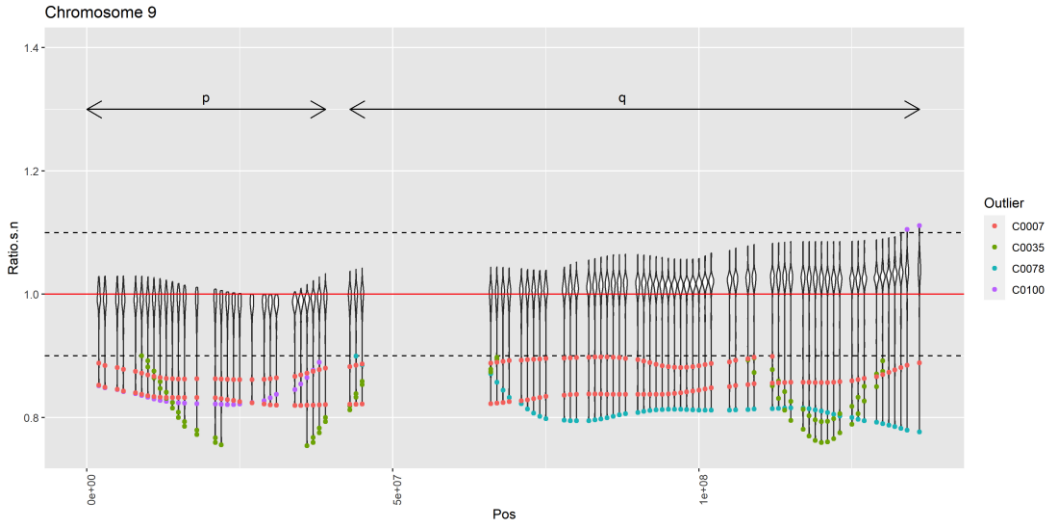


Figure 5.6: Copy number ratios of each 1Mb window of chromosome 9 in the MRM relapsed samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

Three samples (two of which were taken from the same patient at different time points of relapse) showed losses of chromosome 9 across nearly the entire chromosome, as seen in Figure 5.6 above. Another sample showed a loss on the q arm only. A further sample showed losses in the p arm, as well as a gain in the terminal aspect of the q arm. Overall, however, the samples in this relapsed cohort showed a trend towards gains in the q arm, with some suggestion of p arm loss.

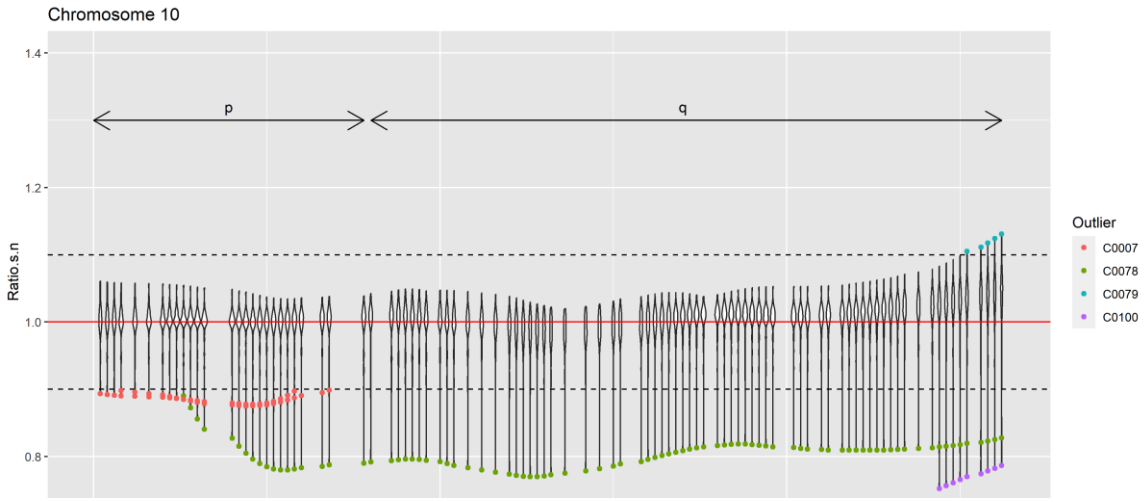


Figure 5.7: Copy number ratios of each 1Mb window of chromosome 10 in the MRM relapsed samples. The black arrows highlight p and q arms (with the centromere between). The red line highlights the copy number ratio of 1.0.

In the figure above, one sample in this cohort demonstrated losses across the entire chromosome 10, while two other samples showed losses in the p and q arm respectively. A further sample showed a gain at the terminal part of the q arm. There appeared to be a trend in the samples overall towards gains in the q arm.

Chromosome	Defined gain	Weaker evidence for gain	Defined loss	Weaker evidence for loss
1	1p (3 samples) 1q (4 samples)	1p	1q (2samples 1q (1 sample))	1q
2	2p (1 sample) 2q (3 samples)	2q	2p (1 outlier)	
4	4p (1 sample) 4q (1 sample)		4p (4 samples) 4q (2 samples)	4p/4q
6	6p (3 samples) 6q (2 samples)		6q (3 samples)	6q
7	7p (3 samples) 7q (4 samples)	7p		
8	8p (3 samples) 8q (4 samples)	8q	8q (1 samples)	8q
9	9q (1 sample)	9q	9p (4 samples) 9q (3 samples)	9p
10	10q (1 outlier)	10q	10p (2 samples) 10q (2 samples)	
11	11p (1 sample) 11q (1 sample)	11p/q	11p (2 samples) 11q (4 samples)	
13	13q (2 samples)		13q (6 samples)	13q
17	17p (2 outliers) 17q (3 outliers)	17q	17p (3 samples) 17q (2 samples)	17p
20	20p (3 samples)	20p/q		
21	21 p (2 samples) 21q (3 samples)	21q	21p/q (11 samples)	21q

Table 5.3: Summary of gains and losses seen in the relapsed samples from the Markers of Relapse in Melanoma study. The defined gains and losses refer to those that met the criteria set for copy number gains and losses (copy number ratio >1.1 and less than 0.9 respectively). The columns for weaker evidence of gains/losses represent those aberrations that did not strictly meet the set criteria, but showed a trend for these aberrations.

5.5 Analysis of aberrations of interest from the TCGA copy number dataset

The Cancer Genome Atlas (TCGA) programme carried out multi-platform analyses to characterize the somatic alterations in frozen tumour samples from 333 melanomas (Network, 2015). These samples comprised 20% thick primary cutaneous melanomas, and 80% metastases (regional lymph nodes/skin/soft tissue and distant metastases). Based on the most prevalent significantly mutated genes, they defined four sub-types within the melanoma samples as follows: mutant BRAF (45% of samples), mutant RAS (28%), mutant NF1 (8%), and triple- wild type (14%). DNA copy-number profiling by Affymetrix SNP 6.0 arrays was carried out across the samples using an established protocol (McCarroll et al., 2008). They reported similar arm-level copy-number aberrations across all four sub-types. However, they noted some differences between the subtypes – specifically in focal amplifications targeting known oncogenes, as summarised below:

Triple-wild type subtype

4q12 amplification (KIT, PDGFRA, KDR)

12q 14 (MDM2, CDK4)

CCND1 (11q13)

TERT (5p15)

Mutant BRAF subtype

BRAF (7q34)

MITF (3p13)

PD-L1 CD274 (9p24)

Mutant RAS subtype

NRAS (1p13)

NF1 mutant subtype

NF1 (17q11.2)

Using the TCGA copy-number data available for melanoma, we produced plots on the proportion of amplifications / deletions across the chromosomes of interest (7,9,10). These are shown below:

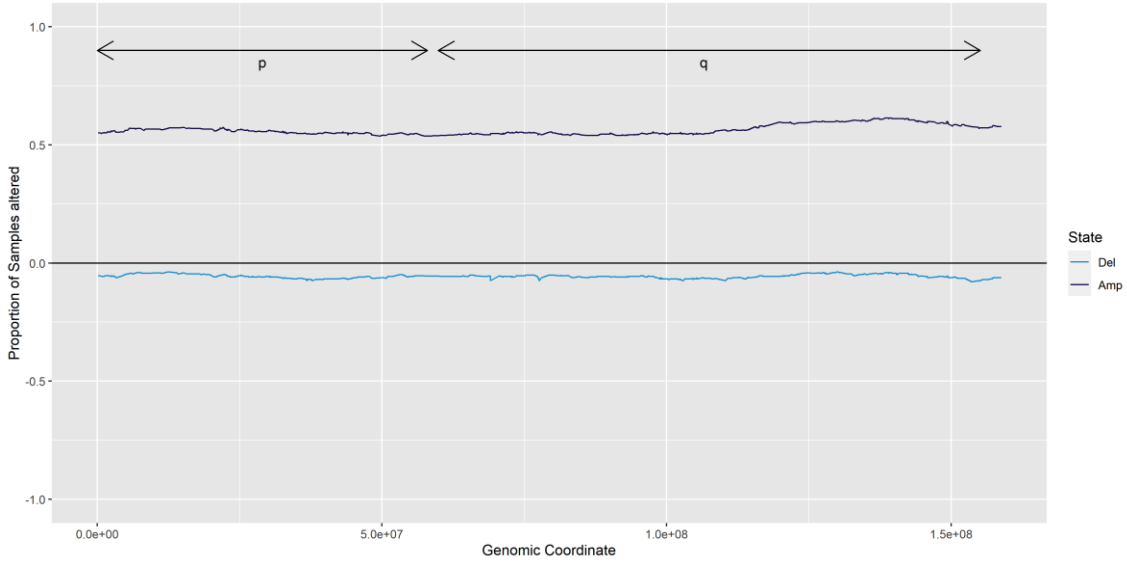


Figure 5.8: Proportion of samples demonstrating amplifications/deletions at chromosome 7. The proportion of samples in the TCGA melanoma samples (n=333) demonstrating amplifications (dark blue) in chromosome 7 exceeded 50%. There was a much smaller proportion (<10%) of samples which demonstrated deletions (light blue). The x-axis represents genomic coordinates across chromosome 7; while the y-axis represents proportion of deletions (expressed as a proportion from 0 to -1) and amplifications (0 to +1)

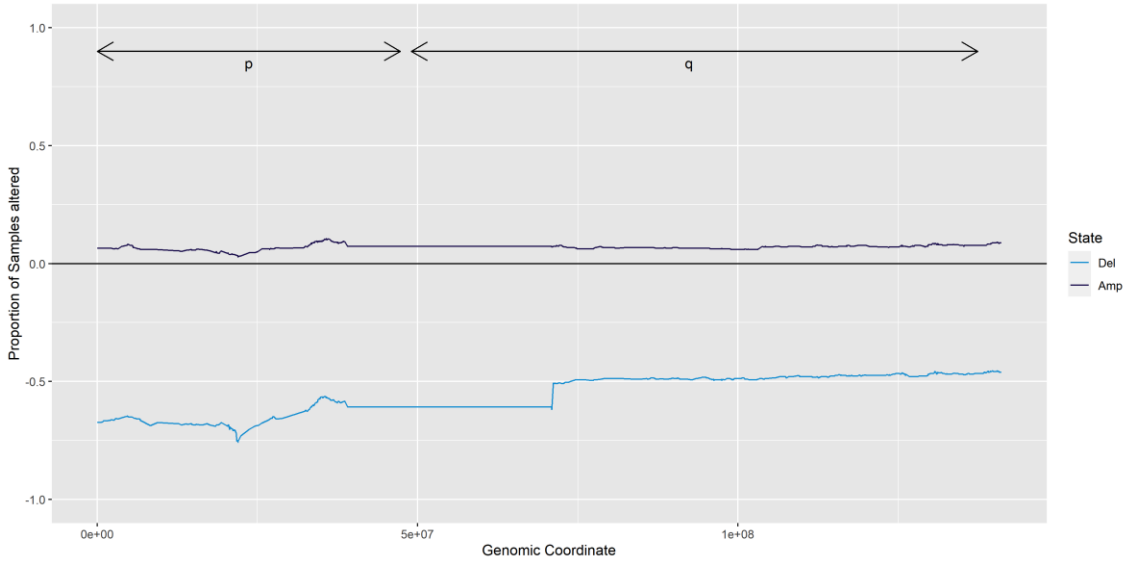


Figure 5.9: Proportion of samples demonstrating amplifications/deletions at chromosome 9. Amplifications and deletions detected across chromosome 9 in the TCGA melanoma dataset (n= 333) are here expressed as a proportion on the y-axis, with proportion of samples demonstrating deletions expressed 0 to -1.0, and proportion of samples showing amplifications expressed from 0 to +1.0) Amplifications (dark blue) across this chromosome were minimal (<10%); while deletions (light blue) were seen in at least 50% of samples (predominantly in the p arm). The x-axis represents genomic coordinates across chromosome 9.

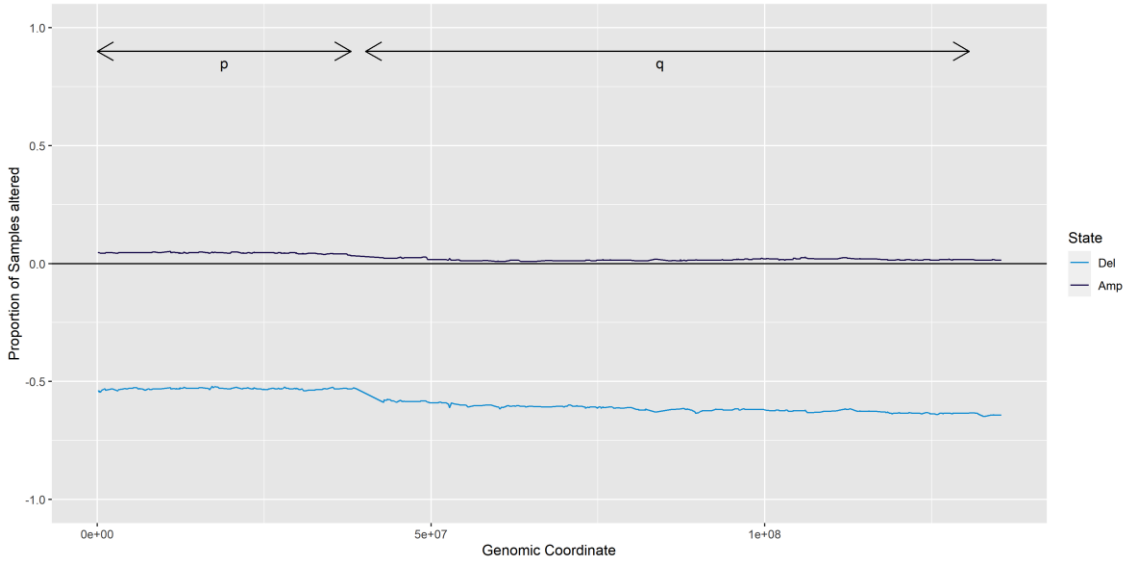


Figure 5.10: Proportion of samples demonstrating amplifications/deletions at chromosome 10. Amplifications and deletions detected across chromosome 10 in the TCGA melanoma dataset (n= 333) are here expressed as a proportion on the y-axis, with proportion of samples demonstrating deletions expressed 0 to -1.0, and proportion of samples showing amplifications expressed from 0 to +1.0) There were minimal amplifications (dark blue, <5%) noted across the p arm of chromosome 10 (while deletions (light blue) were seen in at least 50% of samples (more so in the q arm). The x-axis represents genomic coordinates across chromosome 10.

As seen in figures 5.7-5.9 above, over half of the samples in the TGCA melanoma dataset demonstrated amplifications in chromosome 7, and deletions in chromosomes 9 and 10. Comparatively, much smaller proportions of gains/losses were seen in the corresponding chromosomes in the relapsed samples from our MRM cohort. In the MRM cohort, 7 out of 36 samples had definite gains (3 in 7p, and 4 in 7q); while in chromosome 9, losses in 9p were noted in 4/36 samples, and 3/36 in 9q. 1 out of the 36 samples showed a gain in 9p. A similar proportion was seen in chromosome 10, with 2 out 36 samples showing loss in 10p, 2/36 in 10q, and 1/36 showing a 10p gain.

5.6 Discussion

Somatic copy number aberrations are very common in cancers, and are detectable in about 90% of solid tumours (Pleasance et al., 2010). Having detected copy number aberrations (CNAs) in DNA extracted from serial samples of blood taken from patients recruited in the Markers of Relapse in Melanoma study, we have explored how these CNAs compare with the commonest aberrations listed in published literature. We have also compared the rates of aberrations found in the melanoma cohort in The Cancer Genome Atlas.

While there were very few samples in the baseline cohort that met the threshold set for defined copy number gains or losses, the majority of the samples had sections of chromosomes with copy number ratios greater than, or less than, 1.0, reflecting the common CNAs summarised in Tables 5.1 and 5.2. It is well established that tumour-derived cfDNA is diluted within wild-type DNA in circulation(Diehl et al., 2005), which explains the lack of more extreme copy number ratios in cfDNA. In addition, the detection of copy-number aberrations would be influenced by the

fraction of cfDNA present in circulation, the magnitude of the aberrations within the cfDNA, as well as the coverage utilised in identifying them (Chan et al., 2013; Heitzer et al., 2013). We had demonstrated our limit of detection of detection of copy number aberrations to be 6.25%, with low-coverage (0.1x) sequencing. Recently, Nguyen et al demonstrated their ability to detect copy number aberrations in plasma samples with tumour-derived DNA content as low as 1%, using IIlumina HiSeq at a coverage of 1000x. This increased sensitivity of detection comes at a price in terms of cost of sequencing, which would need to be taken into consideration when developing a biomarker of relapse for use within a resource-constrained health service(Nguyen et al., 2023). A proof-of-concept study in colorectal patients using low-coverage WGS sequencing (6x coverage) copy number alterations, in combination with epigenetic signatures and cfDNA fragmentation patterns, demonstrated the ability of this approach as a biomarker for colorectal cancer detection and treatment monitoring (Hallermayr et al., 2022). Similarly, a post-hoc analysis of the PEARLY study utilised low-coverage WGS to analyse copy number aberrations in triple-negative breast cancer. This showed that copy-number aberration burden at baseline was found to accurately predict for recurrence risk (Kim et al., 2022).

Interestingly, there were more copy-number aberrations identified in the chromosomes of interest in the MRM cohort of relapsed samples, compared with the baseline samples. This is expected, as it is established that ctDNA correlates with tumour burden (Dawson et al., 2013; Schreuer et al., 2017), such that relapsed disease is more likely to be associated with detectable copy-number aberrations.

A significant limitation in these analyses of copy-number aberrations was that by fixing the copy number windows at 1Mb, smaller aberrations would have been missed as a result of lower resolution.

In summary, some of the copy number aberrations seen in the published literature are found in this dataset, although the signals are much weaker in cfDNA. This limits the potential utility of our copy number aberration approach as a biomarker of relapse in melanoma, unless the limit of detection (and therefore, the sensitivity and sensitivity of the biomarker) can be improved. As discussed, this could potentially be achieved by increasing coverage of WGS, and/or combining with other markers, including ctDNA mutational analysis (e.g. BRAF).

Chapter 6: Conclusion and Future Work

Outcomes for patients with advanced (metastatic or inoperable) melanoma have improved dramatically with the introduction of systemic therapies, including BRAF (B-Raf proto-oncogene, serine/threonine kinase) inhibitors, mitogen-activated protein kinase 1 (MEK) inhibitors (J. Larkin et al., 2014; C. Robert et al., 2019) and immune checkpoint inhibitors that target cytotoxic T-lymphocyte associated protein 4 (CTLA4) and programmed cell death 1 (PD1) (Hodi et al., 2010; C. Robert, Schachter, et al., 2015; Wolchok et al., 2013). These therapies have been shown to significantly improve overall survival in patients, with durable responses (measurable in years) seen in a proportion of patients. Therefore, early detection of metastatic relapse is crucial to maximise potential benefit for patients. The need for a clinically useful biomarker of melanoma relapse formed the premise for this piece of research, which investigated copy-number analysis in circulating cell-free DNA, as a marker of relapse.

To begin with, pre-analytical testing was carried out on cfDNA samples obtained from a feasibility study (conducted for patient and healthy control recruitment, and for cfDNA sample collection), to optimise DNA yield for downstream analysis, and to investigate stability of cfDNA over time.

Focus was then made on exploring the application of published genomic copy-number scoring algorithms in this pilot cohort of melanoma patients and healthy controls, and the CNAS (an algorithm which captures the whole spectrum of copy-number aberrations across the genome regardless of amplitude or length of aberration) proved more robust. It was shown to be a good discriminator of active disease, and a good predictor of survival (with higher scores associated with poorer survival) in the pilot cohort of melanoma cases.

Following this proof-of–concept in the pilot study, patients and healthy controls were recruited into a separate longitudinal study, to test the hypothesis that individual changes in CNAS over time could be utilised as a biomarker of melanoma relapse. A random coefficients mixed-effects statistical model was utilized, taking into consideration inter- and intra-patient variations in CNAS, to investigate the performance of this scoring algorithm as a biomarker of relapse. This model showed that the CNAS was a significant predictor of relapse status at the time of blood-sampling. Nearly half (46.67%) of the cases that had a relapse event in this cohort, demonstrated a rise in CNAS rise at, or prior to, clinical/radiological relapse. Baseline CNAS, while not shown to be a significant predictor of future relapse status, was found to be a good predictor of survival. The low sensitivity (55%) of the CNAS in this cohort, indicates its limited ability to correctly identify relapsed cases.

It is crucial that precise biomarkers are developed to guide management decisions in all aspects of melanoma management – from deciding on the extent of surgical resection of the primary and/or nodal disease, to guiding decision-making on the need for adjuvant therapies, to tailoring systemic treatment in metastatic disease. There is, as yet, no validated predictive/prognostic blood-based biomarker that can reliably guide these decisions. Blood-based cfDNA analysis (the liquid biopsy), as a minimally invasive, cost-effective, reproducible test, has potential for application in these aspects of melanoma management, provided further development of the technology can improve their sensitivity and specificity.

The main limitation in both the pilot study and the separate longitudinal study conducted as part of this research was their retrospective nature, which limits the

interpretation of the analyses. In addition, the small size of both studies, and relatively small number of serial samples obtained in the longitudinal study, as well as the varying intervals between those samples, limited the conclusions that could be reliably drawn from the longitudinal analyses. Furthermore, in our copy-number analysis, we utilized the CNAnorm bioinformatics pipeline in calculating copy-number ratios, a method which was developed in-house. This pipeline was utilised in our pilot study, and for consistency, and to enable comparison across both studies, we maintained this as the pipeline of choice in data analysis in the longitudinal study. There are other potentially more sensitive and accurate copy-number analysis tools, notably ichorCNA, which has become accepted as the gold-standard for low-coverage copy-number analysis(Adalsteinsson et al., 2017; Allsopp et al., 2023; Hallermayr et al., 2022; Sato et al., 2024; Wallander et al., 2021).

Future work will focus on validating the CNAS in a separate larger cohort prospectively, with emphasis on regular interval sampling to maximise serial collection for genomic analysis. To improve the sensitivity of copy-number analysis as a marker of melanoma relapse, it would be interesting to combine this approach with other biomarkers (e.g. ctDNA BRAF/NRAS mutational burden). There has been considerable research of recent that has shown dPCR as a tool for identifying rare alleles at low frequency, with high sensitivity and accuracy (Marczynski, Laus, dos Reis, Reis, & Vazquez, 2020; Postel, Roosen, Laurent-Puig, Taly, & Wang-Renault, 2018). Therefore, combining copy-number number low-coverage whole-genome sequencing analysis with BRAF/NRAS dPCR would present a potentially highly sensitive marker of relapse, while maintaining the cost-effectiveness of such a test.

The copy-number approach also has potential applications beyond being utilised as a marker of relapse. Significant advances have been made recently, in the adjuvant melanoma setting, with melanoma recurrence rates reduced, and progression-free survival improved through the use of immunotherapy and BRAF-targeted therapy in patients with resected nodal and visceral disease. Therefore, copy-number analysis can potentially have a role in informing decisions in adjuvant therapies in melanoma patients. Furthermore, as copy-number aberrations are not limited to melanoma, the investigation of CNAS analysis for detection and/or prediction of relapse in other tumour sites is warranted.

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Chapter 8: Appendices

Appendix A: A selection of technologies used in detecting genetic/epigenetic changes in DNA

Denaturing capillary electrophoresis (Fiala et al., 2013; Levy et al., 2012)	Denatured PCR fragments are separated (at an optimum temperature, or during a temperature gradient) based on the electrophoretic properties of mutated vs. non-mutated fragments. Detectable fraction of mutant alleles 0.3% Pros: Simple principle Applicable on a variety of commercial sequencers Cons: Requires a panel of mutations to be pre-selected
Sanger Sequencing (Kheterpal & Mathies, 1999; Sanger, Nicklen, & Coulson, 1992)	Fragments of DNA are created by incorporating dideoxynucleotide analogues (terminators). Each terminator can have a fluorescent molecule which can be uniquely identified. Terminator products are purified, then separated by size using the fluorescence generated by the terminators. Amplification and sequencing typically occur separately (but can be done in a single reaction) **Pros:** Can detect known as well as new mutations simultaneously **Cons:** Low specificity Detectable fraction of mutant alleles 15-20% Can miss rare mutations
Coamplification at lower denaturation temperature PCR **COLD PCR** (J. Li et al., 2008) (Mancini et al., 2010)	By lowering the temperature of the PCR annealing step, mutated fragments are preferentially amplified, then partly melted, allowing primer annealing. At this lower temperature, non-mutated fragments remain double-stranded, so primer annealing is prevented. **Pros:** Able to detect low-prevalence mutations (0.1-0.5%) **Cons:** Simultaneous enrichment of multiple amplicons (with different melting temperatures) requires additional techniques e.g. compartmentalization of reactions and gradual increase of temperature

BEAMing beads, emulsion, amplification and magnetics (Diehl et al., 2008; M. Li, Diehl, Dressman, Vogelstein, & Kinzler, 2006)	Tumour mutations are identified by direct sequencing. Total number of DNA fragments in plasma is estimated by real time-PCR. PCR products are bound to beads, which are analysed by flow-cytometry or optical scanning to determine the fraction of DNA fragments that contains these mutations. This fraction is multiplied by the total number of fragments to get the number of mutated fragments. Detectable fraction of mutant alleles 0.001 - 1.7% **Pros:** Counts pre-specified mutations Can identify rare variants High sensitivity & specificity **Cons:** Requires tumour sample for personalised mutation assay
Tagged-amplicon deep sequencing (TAM-Seq) (Forshew et al., 2012)	Short DNA amplicons (150-200bp) are amplified in a 2-step process. The resulting libraries are tagged by unique "barcodes" prior to sequencing. Rare mutations that are present above the background rate across multiple samples can be detected. **Pros:** High specificity and sensitivity Can identify mutations which lack hotspots **Cons:** Detectable fraction of mutant alleles limited to 2% Requires personalized assays
Pyrosequencing (Ogino et al., 2005)	"Sequencing by synthesis": a complementary DNA strand is synthesized for the DNA strand whose sequence is being determined. Each time a nucleotide is incorporated into the complementary strand, a pyrophosphate group is released, producing detectable light, the intensity of which is specific for each nucleotide. Thus, the sequence of DNA template is determined. **Pros:** Detectable fraction of mutant alleles 5% No requirement for a purification step Suitable for detecting methylation changes **Cons:** Low sensitivity and specificity

Whole genome/exome sequencing by massive parallel/next generation sequencing (Chan et al., 2013; Dawson et al., 2013; Heitzer et al., 2013; Leary et al., 2010; Leary et al., 2012; Murtaza et al., 2013; Shaw et al., 2012)	DNA is purified, sheared, assembled into "libraries" which are amplified by PCR. The PCR products are then sequenced at the same time i.e. "in parallel". Illumina sequencing: fluorescently-labelled nucleotides are incorporated by the PCR products or "clusters". A high-resolution digital camera registers the resulting colour changes, and the sequence is thus determined. Ion torrent sequencing: beads coated with amplified DNA fragments are "washed" sequentially with nucleotides. A semi-conductor registers the resulting pH changes as specific nucleotides are incorporated, and the sequence is determined based on the pattern of pH changes. **Pros:** Can detect various DNA alterations (including copy number changes and chromosomal rearrangements) in a multiplexed manner High specificity **Cons:** Requires considerable expertise Personalised assay required False–negatives where burden of disease is low
Droplet digital PCR (Kinugasa et al., 2015; Reinert, 2015; Taly et al., 2013)	DNA sample is separated into partitions ("droplets"), and then amplified. Each partition is labelled as positive or negative for the target sequence, then quantification is done by counting the positive partitions. **Pros:** High specificity and sensitivity Multiplex methodology enables highly efficient analysis **Cons:** Requires personalized assays Expensive
Nanopore (single molecule) sequencing http://www.nanoporetech.com	Based on principle of the Coulter counter. An insulating membrane containing an aperture (nanopore) separates two chambers containing conductive electrolyte. When DNA molecules are driven through the nanopore across an electrical gradient, changes in ionic current across the nanopore can be measured in time, to give information on the nucleotide sequence of the DNA molecule. Detectable fraction of mutant alleles 0.0015 – 3% **Pros:** Eliminates the need for amplification Long read lengths Potentially low cost

	Cons: Technology still under development Instability of biologic nanopores Signal noise may limit single-nucleotide resolution

Appendix B: Summary of biomarkers in melanoma

BIOMARKER	TYPE	MEASUREMENT	USES	LIMITATIONS
Lactate dehydrogenase (Balch et al., 2009; Karagiannis, Fittall, & Karagiannis, 2014; Kelderman et al., 2014; Palmer, Erickson, Ichetovkin, Knauer, & Markovic, 2011; Solassol, Du-Thanh, Maudelonde, & Guillot, 2011; Tandler, Mosch, & Pietzsch, 2012)	Enzyme, catalyses pyruvate conversion to lactate in the presence of hypoxia	Serum assay (colorimetric, ELISA assays)	Strongest prognostic indicator in clinical use currently Correlates with tumour burden As a prognostic indicator in patients receiving Ipilimumab treatment Increasing level correlates negatively with survival	Non-specific Some studies failed to show its prognostic/predictive value
S100β (Egberts et al., 2012; Guo, Stoffel-Wagner, Bierwirth, Mezger, & Klingmuller, 1995; Henze, Dummer, Joller-Jemelka, Boni, & Burg, 1997; Kruijff et al., 2009)	Protein, downregulates p53	ELISA estimation in serum	Correlates with tumour burden Negative correlation between levels and survival	Non-specific
Tyrosinase (Garbe et al., 2003; Quaglino et al., 2007; H. Schmidt, Sorensen, Fode, Nexo, & von der Maase, 2005; Visus et al., 2007)	Enzyme, involved in melanin synthesis	RT-PCR in blood	Prognostic indicator of tumour progression Correlates with survival	Some studies failed to show prognostic value
Melanoma-inhibitory activity MIA (Diaz-Lagares et al., 2011; Juergensen et al., 2001; Meral et al., 2001; Perrotta et al., 2010)	Amino-acid, acts as an autocrine growth factor, promotes tumour invasion and metastasis		Increased levels associated with advanced disease and poorer prognosis Correlates with treatment response and relapse	Less specific than LDH or S100B
Vascular endothelial growth factor (Ugurel, Rappl, Tilgen, & Reinhold, 2001) (Pelletier et al., 2005)	Glycoprotein, stimulates tumour angiogenesis	ELISA estimation of serum level	As a prognostic and predictive marker Correlates with tumour stage/disease burden Increasing level associated with poor survival	Non-specific Several studies failed to reproduce correlation with survival
Cyclooxygenase-2	Enzyme involved in prostaglandin synthesis	IHC staining of tissue specimen	As a prognostic and predictive marker	Invasive process, as requires tissue specimen

(Becker, Siegelin, Rompel, Enk, & Gaiser, 2009) (Kuzbicki, Lange, Straczynska-Niemiec, & Chwirot, 2012)	Expressed in tumours Induced by stimuli e.g. inflammation		Correlates with Breslow thickness, disease-specific survival Differentiates between early melanoma lesions and benign naevi	Non-specific
Osteopontin (Rangel et al., 2008) (Alonso et al., 2007)	Glyco-phosphoprotein, initiates various signal transduction pathways, enhancing tumour growth and micro-environment	IHC of tissue specimen ELISA estimation of serum level	An independent predictor of survival Increased expression in primary tumour is associated with increased incidence of metastases	Non-specific (elevated in auto-immune diseases, infections)
C-reactive protein (Fang et al., 2015) (Deichmann, Kahle, Moser, Wacker, & Wust, 2004)	Acute phase reactant produced in the liver in response to pro-inflammatory cytokines	ELISA estimation of serum level	An independent prognostic marker: elevated levels associated with poorer survival High levels associated with disease progression and distant metastases	Non-specific
Matrix metalloproteinase (Nikkola et al., 2005) (Nikkola et al., 2002) (Rotte, Martinka, & Li, 2012)	Endopeptidases involved in remodelling tumour microenvironment	ELISA estimation of serum level IHC of tissue sample	As a prognostic marker MMP-1 and -3 correlate with disease-free survival MMP-9 correlates with overall survival Increased MMP-2 expression differentiates melanoma lesions from normal/dysplastic naevi	Not tumour-specific (elevated in other malignancies)
YKL-40 (H. Schmidt et al., 2006) (H. Schmidt et al., 2005) (Egberts et al., 2012)	Glycoprotein with uncertain cellular function.	ELISA estimation of serum level	As a prognostic marker Correlates with tumour stage and disease progression Independently correlates with survival (conflicting evidence for this)	Limited sensitivity Non-specific Level affected by treatment with immunotherapy
Galectin-3 (Brown et al., 2012) (Vereecken et al., 2009) (Buljan, Situm, Tomas, Milosevic, & Kruslin, 2011)	Protein secreted by melanoma cells and inflammatory cells Promotes tumourigenesis and metastasis	IHC of tumour specimen	As a prognostic marker Independently correlates with survival Inversely relates with tumour thickness Increased expression associated with more aggressive phenotype	Not tumour-specific
Carcinoembryonic antigen-related cell adhesion molecule (Thies et al., 2002) (Sivan et al., 2012)	Membrane protein, stimulates angiogenesis, confers protection to tumour cell from immune attack		As a prognostic marker An independent factor for risk of metastasis Correlates with tumour stage and survival	Not tumour-specific

Appendix C: Research Ethics Committee approval

Revised 10 February 2015

NHS
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National Research Ethics Service

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23 January 2015

Dr Shobha Silva
Academic Unit of Clinical Oncology
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S10 2SJ

Dear Dr Silva

Study title: **Markers of relapse in melanoma**
REC reference: **14/YH/1275**
IRAS project ID: **167721**

Thank you for your email of 20 January 2014. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 11 December 2014

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Letter from Dr Shobha Silva]		16 December 2014
Letters of invitation to participant	1.1	20 January 2015
Other [Questionnaire]	1.1	15 December 2014
Other [Email from Dr Shobha Silva]		20 January 2015
Participant information sheet (PIS) [Information Leaflet Controls]	1.2	20 January 2015
Participant information sheet (PIS) [Information Leaflet Patients]	1.2	20 January 2015

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Cover letter]	1.0	17 November

Clinical Trials Executive approval

The University Of Sheffield.

Department Of Oncology.

PJW/MAR

16 December 2014

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Dear Shobha

Re: Markers of Relapse in Melanoma

Thank you for introducing this trial to the Clinical Trials Executive on 8 December 2014. Reproduced below is the relevant extract from the minutes of that meeting:

"Dr Silva introduced this population-based case-control longitudinal study, which aims to collect detailed epidemiological data on cutaneous melanoma over time. The project is being undertaken as part of her PhD and is funded by Weston Park Cancer Charity. Plasma circulating cell-free DNA (ccfDNA) provides an easily accessible source of tumour derived DNA and the aim of the project is to identify whether cancer-specific somatic mutations detected in ccfDNA and/or circulating levels of ccfDNA, could act as early markers of relapse in melanoma. This will be tested by assessing the relationship between levels of ccfDNA, ccfDNA molecular markers and metastatic relapse over time. It is hoped that being able to detect relapses before the manifestation of clinical symptoms would enable earlier initiation of treatment, and therefore improve the effectiveness of these treatments.

It is proposed to recruit 120 patients with melanoma and 120 healthy controls (co-habiting partners or siblings) over 2 years, and obtain blood samples every 3 months from recruitment for up to 5 years, which will involve up to 20 extra blood tests. There will be no extra visits for patients but up to 20 visits for controls over 5 years. to coincide with patient visits. It is also aimed to assess the relationship between inherited variation in key genes and gene expression in healthy skin, and this will involve obtaining a single skin biopsy from some patients. Patients will be recruited at WPH and RHH, and will all continue with their current treatment and/or follow-up. Study visits will take place alongside each patient's routine NHS 3-monthly follow-up. These will be conducted by a member of the research team and involve questionnaire completion (initial visit only) and obtaining a blood sample.

Dr Wadsley and Lesley Bruce provided supportive peer review. Dr Wadsley thought the study scientifically interesting. However, more detail was required regarding patient eligibility as it was not clear from the protocol about what would be expected of the controls and if they would be required to have 3 monthly blood tests for 5 years. He asked if there was a record of controls being willing to commit to this and Dr Silva acknowledged that it was a lot to ask. Dr Wadsley thought it an ambitious recruitment target and wondered if it was realistic in the timeframe but Dr Silva was confident that it could be achieved and cited the track record with the GEMS study. It was not clear whether patients are to be in remission at recruitment or could already have relapsed. Dr Wadsley noted that the follow up is up to 5 years and wondered if there would be enough data at the end of year 3 when Dr Silva finishes, but she confirmed that

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Participant information leaflets/consent forms/invitation letter

Letter of invitation

Sheffield Teaching Hospitals NHS
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Whitham Road, Sheffield S10 2SJ
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Fax: +44 (0) 114 226 536

Dear ,

We are working with researchers at the University of Sheffield, who are looking for people with melanoma, as well as healthy volunteers, to participate in a study looking at ways to detect melanoma early. We would like to invite you to participate, and we hope you are willing to help.

The study is looking at the genes (DNA) that may be responsible for Melanoma, to find out how they work, in order to help develop better tests and treatments for the future.

In addition to patients with melanoma, the study needs healthy volunteers who may be partners or siblings of patients. If a relative (partner/sibling) will be attending your appointment with you, you may want to discuss this with them.
Please find enclosed an information leaflet about the study. A member of the research team will talk to you about the study when you attend your appointment, if you are interested, and will answer any questions you may have regarding the study.
Thank you very much for reading this letter.
Yours sincerely,

Dr Sarah Danson
Mr AJ Stephenson
Dr Helen Ramsay
Dr Andrew McDonagh
Dr James Lester
Mr M Brotherston

Patient information leaflet

Academic Unit of Clinical Oncology
Head: Professor Penella Woll
Cancer Clinical Trials Centre
Broomcross Building
Weston Park Hospital
Whitham Road, Sheffield S10 2SJ
Telephone: +44 (0) 114 226 5235
Fax: +44 (0) 114 226 5364

INFORMATION SHEET FOR PATIENTS

Markers of relapse in melanoma

Thank you for reading this.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives, your GP and others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?

This study is designed to collect together important clinical information, detailed questionnaires, blood samples and any surplus tissue samples from patients with a diagnosis of melanoma (skin cancer). We will be asking for permission to approach the partners and siblings (brothers or sisters) of these individuals.

Many factors contribute to whether or not an individual develops melanoma, at what age, how aggressive their cancer is, and how well it responds to treatment. This study will be used to identify possible factors, which may improve the diagnosis and treatment of melanoma in the future.

2. Why have I been chosen?

We are inviting people with a diagnosis of melanoma to take part in this study. We will also be asking for permission to approach the partners and siblings of these individuals.

3. Do I have to take part?
It is up to you to decide whether or not to take part. A member of the research team will talk to you about this study and give you time to think about whether or not you would like to take part. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Whether you take part or not will not affect your treatment or the standard of care you receive.

4. What will happen to me if I take part in the study?
If you agree to take part, we will meet with you when you attend your regular 3 monthly appointments for up to 5 years. Also, in between these regular appointments, if you attend clinic for any reason related to your melanoma diagnosis, we would like to meet with you. There will be no extra hospital visits for the purposes of this study. At the first visit, you will have the opportunity to discuss the study, and be asked to sign the consent form if you want to take part.

You will be given a questionnaire at the first clinic visit and a member of the research team will help you complete this if you wish. This will include questions on:

- Your age, place of birth and ethnic group.
- Your health and previous illnesses.
- The history of cancer in your family, for example who has or had cancer and at what age it was diagnosed.
- Your exposure to sun.

The questionnaire will take approximately 30 minutes to complete.

At the first, and each of the subsequent clinic visits, you will be asked to give a single 50ml blood sample. This should take about 5 minutes. Neither you nor your doctors will receive a result from this blood sample because it is purely for research. This sample will be carefully processed to allow us to study the DNA (substance that makes up genes) and proteins in it. We will analyse any differences over time, in the DNA from you and your partner/sibling to try and identify genes that may (along with many other factors) affect how a tumour develops.
At each clinic visit you will be asked about any new medications you are taking and will be examined.

We will also analyse stored spare biopsy material and pathology specimens. Such specimens will have been taken when you had your biopsy or surgery, for examination by the hospital pathologist, to determine your diagnosis. We would like to use any tissue that is left over from this procedure to extract DNA to look for any genetic changes within the tissue itself.

We will also ask for permission to perform an additional skin biopsy. This will take about 15 minutes, and is a smaller biopsy than the initial biopsy you would have had for your diagnosis. This is additional to the blood tests. However, if you wish not to have an additional skin biopsy this does not affect you participating in the study or any of your other medical care.

We will obtain information on your disease and treatment from the medical records, to link in with the work described above. This information will include information about any cancer and the treatment received.

All this information will be stored anonymously (i.e. labelled with a code number and not your name) on a secure computer database.

5. What are the possible disadvantages and risks of taking part?
You will have the discomfort of a blood test (and skin biopsy if this is performed) and the inconvenience of taking part in a questionnaire interview. You would be asked to attend the hospital routinely for check-ups on a 3-monthly basis, even if not on the study, so participation in the study should not incur extra clinic visits. You would be able to continue with any other treatments you require (such as surgery, chemotherapy or radiotherapy) whilst on this study.
Since all of the data will be anonymous, individual results from this research study will not be fed back to you, so there are no implications for your treatment.

6. What are the possible benefits of taking part?
There is no immediate clinical benefit to you in taking part. This research may lead to the development of a new genetic test or treatment that may benefit those with melanoma at some point in the future.

7. What if new information becomes available?
If any important new information becomes available that may affect your health during the period of the study, the investigators will contact you to tell you about it.

8. What happens when the research study stops?
The blood and biopsy samples that you give us will be kept for up to 20 years. The samples will only be used for research purposes and not for any financial gain. The samples will be treated as a gift from you to us. Additional studies will be planned in the future and may use your samples. Any such studies would have to be reviewed and gain approval from a research ethics committee.

9. Will my taking part in the study be confidential?
Yes. Anything you say will be treated in confidence, no names will be mentioned in any reports of the study and care will be taken so that individuals cannot be identified from details in reports of the results of the study. Everyone who takes part in the study will be assigned a code number, and all of the data relating to each person will be held on a computer database and will only be linked to that code number, and not to people's names or addresses. We would like to inform your GP of your participation.

10. What will happen to the results of the study?
The results will be presented at scientific conferences and published in scientific journals. You will not be identified in any reports or publications. If you would like a summary of the study results, please contact a member of the study team (see contact details below).

11. Who is organising and funding this study?
The study is being organised by Dr Sarah Danson, and Dr Shobha Silva at Weston Park Hospital, Sheffield, in collaboration with researchers in the Royal Hallamshire

Hospital and the University of Sheffield. The study is funded by Weston Park Hospital Cancer Charity.

12. Who has reviewed the study?
This research protocol has been reviewed in the hospitals were patients will be seen and by the University of Sheffield. It has also been scientifically reviewed by external reviewers, and by Sheffield Research Ethics Committee.

13. What if something goes wrong?
If you are harmed by your participation in this study, there are no *special* compensation arrangements. We will only be taking a blood sample, which carries negligible risk. If you are harmed due to someone's negligence, then you may have grounds for a legal action. If you have *any* cause to complain about *any* aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study.

If you have any complaints or concerns please contact in the first instance the project co-ordinator, Dr Shobha Silva at Weston Park Hospital, Whitham Road, Sheffield, S10 2SJ. Tel: 0114 226 5000.

Or contact Dr David Throssell, Medical Director, Sheffield Teaching Hospitals NHS Foundation Trust, 8 Beech Hill Road, Sheffield, S10 2SB. Tel: 0114 271 2178.

14. Contact for further information.
If you need more information or have any questions concerning this study, please contact the study team:

Dr Shobha Silva, Weston Park Hospital, Sheffield, S10 2SJ. Tel: 0114 226 5000. Email: shobha.silva@sth.nhs.uk or Dr Sarah Danson, Weston Park Hospital, Sheffield, S10 2SJ. Tel: 0114 226 5000. Email: s.danson@sheffield.ac.uk

Research Nurse: Sister Helen Cramp, Institute for Cancer Studies, University of Sheffield Medical School, Sheffield S10 2TH. Tel: 0114 271 2354. Email: h.e.cramp@sheffield.ac.uk.

Thank you for reading this and for taking an interest in this research study.

Control information leaflet

Academic Unit of Clinical Oncology
Head: Professor Penella Woll
Cancer Clinical Trials Centre
Broomcross Building
Weston Park Hospital
Whitham Road, Sheffield
S10 2SJ
Telephone: +44 (0) 114 226 5235
Fax: +44 (0) 114 226 5364

INFORMATION SHEET FOR CONTROLS

Markers of relapse in melanoma

Thank you for reading this.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives, your GP and others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?
This study is designed to collect together important clinical information, detailed questionnaires, blood samples and any surplus tissue samples from patients with a diagnosis of melanoma (skin cancer), their partners and their siblings (brothers or sisters).

Many factors contribute to whether or not an individual develops melanoma, at what age, how aggressive their cancer is, and how well it responds to treatment. This study will be used to identify whether blood tests can help to identify the development, or recurrence, of a melanoma in its early stages.

2. Why have I been chosen?
You have been asked because either your sibling or partner has a diagnosis of melanoma.

3. Do I have to take part?
It is up to you to decide whether or not to take part. A member of the research team will talk to you about this study and give you time to think about whether or not you would like to take part. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

4. What will happen to me if I take part in the study?
If you agree to take part, we will ask you to attend the clinic every 3 months for up to 5 years, to coincide with clinic visits for your partner/sibling. At the first visit, have the opportunity to discuss the study, and be asked to sign the consent form if you want to take part.
You will then be given a questionnaire and the research team will help you complete this if you wish. This will include questions on:

- Your age, place of birth and ethnic group.
- Your health and previous illnesses.
- The history of cancer in your family, for example who has or had cancer and at what age it was diagnosed.
- Your exposure to sun.

The questionnaire will take approximately 30 minutes to complete.

At the first, and each of the subsequent clinic visits, you will be asked to give a 50ml blood sample. This will take about 5 minutes. Neither you nor your doctors will receive a result from this blood sample because it is purely for research. This sample will be carefully processed to allow us to study the DNA (substance that makes up genes) and proteins in it. We will analyse any differences over time, in the DNA between you and your partner/sibling who is a patient to try and identify genes that may (along with many other factors) affect how a tumour develops. Individual abnormalities detected in the DNA will not be communicated to you, as the relevance of these abnormalities is not known.

All information will be stored anonymously (i.e. labelled with a code number and not your name) on a secure computer database.

5. What are the possible disadvantages and risks of taking part?
You will have the discomfort of a blood test and the inconvenience of attending the hospital for repeated visits, as well as the inconvenience of taking part in a questionnaire interview. Since all of the data will be anonymous, individual results from this research study will not be fed back to you, so there are no implications for your health.

6. What are the possible benefits of taking part?
There is no immediate clinical benefit to you in taking part. This research may lead to the development of a new genetic test or treatment that may benefit those with melanoma at some point in the future.

7. What if new information becomes available?
If any important new information becomes available that may affect your health during the period of the study, the investigators will contact you to tell you about it.

8. What happens when the research study stops?
The blood samples that you give us will be kept for up to 20 years. The samples will only be used for research purposes and not for any financial gain. The samples will be treated as a gift from you to us. Additional studies will be planned in the future and may use your samples. Any such studies would have to be reviewed and gain approval from a research ethics committee.

9. Will my taking part in the study be confidential?
Yes. Anything you say will be treated in confidence, no names will be mentioned in any reports of the study and care will be taken so that individuals cannot be identified from details in reports of the results of the study. Everyone who takes part in the study will be assigned a code number, and all of the data relating to each person will be held on a computer database and will only be linked to that code number, and not to people's names or addresses. We would like to inform your GP of your participation.

10. What will happen to the results of the study?
The results will be presented at scientific conferences and published in scientific journals. You will not be identified in any reports or publications. If you would like a summary of the study results, please contact a member of the study team (see contact details below).

11. Who is organising and funding this study?
The study is being organised by Dr Sarah Danson and Dr Shobha Silva at Weston Park Hospital, in collaboration with researchers in the Royal Hallamshire Hospital and the University of Sheffield. The study is funded by Weston Park Hospital Cancer Charity.

12. Who has reviewed the study?
This research protocol has been reviewed in the hospitals were patients will be seen and by the University of Sheffield. It has also been scientifically reviewed by external reviewers, and by Sheffield Research Ethics Committee.

13. What if something goes wrong?
If you are harmed by your participation in this study, there are no *special* compensation arrangements. We will only be taking a blood sample, which carries negligible risk. If you are harmed due to someone's negligence, then you may have grounds for a legal action. If you have *any* cause to complain about *any* aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study.

If you have any complaints or concerns please contact in the first instance the project co-ordinators, Dr Shobha Silva at Weston Park Hospital, Whitham Road, Sheffield, S10 2SJ. Tel: 0114 226 5000.

Or contact Dr David Throssell, Medical Director, Sheffield Teaching Hospitals NHS Foundation Trust, 8 Beech Hill Road, Sheffield, S10 2SB.

14. Contact for further information.

If you need more information or have any questions concerning this study, please contact the study team:

Dr Shobha Silva, Weston Park Hospital, Sheffield, S10 2SJ. Tel: 0114 226 5000. Email: shobha.silva@sth.nhs.uk or Dr Sarah Danson, Weston Park Hospital, Sheffield, S10 2SJ. Tel: 0114 226 5000. Email: s.danson@sheffield.ac.uk

Research Nurse: Sister Helen Cramp, Institute for Cancer Studies, University of Sheffield Medical School, Sheffield S10 2TH. Tel: 0114 271 2354. Email: h.e.cramp@sheffield.ac.uk.

Thank you for reading this and for taking an interest in this research study.

Patient consent form

Academic Unit of Clinical Oncology
Head: Professor Penella Woll
Cancer Clinical Trials Centre
Broomcross Building
Weston Park Hospital
Whitham Road, Sheffield S10 2SJ
Telephone: +44 (0) 114 226 5235
Fax: +44 (0) 114 226 5364

PATIENT CONSENT FORM

Markers of relapse in melanoma

Name of Researcher: Identification Number for this study:…….

Please initial box

1. I confirm that I have read and understand the information sheet dated 28th October 2015 (Version 1.3) for the above study and have had the opportunity to ask questions.	
2.I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.I give permission for samples of blood to be obtained from me, at intervals over the next 5 years. I understand that DNA (the substance that genes are made of) will be extracted from the blood sample and analysed to identify genes related to the development of melanoma.	
4.I give consent for the storage of clinical information about me on a computer database; I understand that the laboratory and clinical information will be labelled with a code number and that no other personal information will be held with the DNA or clinical information (i.e. it will not have my name or address on it).	
5.I agree to the use of data about my health provided by the Office of National Statistics and Trent Cancer Registry.	
6.I understand that sections of any of my medical notes may be looked at by responsible individuals from Sheffield Teaching Hospitals or the University of Sheffield or from regulatory authorities where it is relevant to my taking part in research. I give permission for such individuals directly involved in this study to have access to my records.	

7.I give permission for surplus biopsy material and pathology specimens to be used in this Study.	
8.I understand that all data (personal and clinical) collected will be treated in accordance with European and national laws for the protection of data.	
9.I agree to the use of my samples and data for future research.	
10.I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test, but that a proportion of any profits may go towards further research in this field.	
11. OPTIONAL: I agree for an additional skin biopsy to be taken for research purposes.	
12. I agree for my GP to be informed of my participation in this study.	
11.I agree to take part in the above study.	

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Control consent form

Academic Unit of Clinical Oncology
Head: Prof Penella Woll
Cancer Clinical Trials Centre
Broomcross Building
Weston Park Hospital
Whitham Road, Sheffield S10 2SJ
Telephone: +44 (0) 114 226 5235
Fax: +44 (0) 114 226 5364

CONTROL CONSENT FORM

Markers of Relapse in Melanoma

Name of Researcher: Identification Number for this study:……..

Please initial box

1.I confirm that I have read and understand the information sheet dated 20th January 2015 (Version 1.2) for the above study and have had the opportunity to ask questions.	
2.I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.I give permission for samples of blood to be obtained from me at intervals over the next 5 years. I understand that DNA (the substance that genes are made of) will be extracted from the blood sample and analysed and compared with that from melanoma patients.	
4.I give consent for the storage of clinical information about me on a computer database; I understand that the laboratory and clinical information will be labelled with a code number and that no other personal information will be held with the DNA or clinical information (i.e. it will not have my name or address on it).	
5.I agree to the use of data about my health provided by the Office of National Statistics.	
6.I understand that all data (personal and clinical) collected will be treated in accordance with European and national laws for the protection of data.	

7.I agree to the use of my samples and data for future research.	
8.I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test, but that a proportion of any profits may go towards further research in this field.	
9. I agree for my GP to be informed of my participation in this study.	
10.I agree to take part in the above study.	

_____	_____	_____
Name of Control	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix D: Patient and control characteristics from the Genetics and Epidemiology of Melanoma in Sheffield (GEMS) study.

	Patients	Controls	
Male	47 (56.6%)	26 (34.7%)	p=0.006
Female	36 (43.4%)	49 (65.3%)	
Median age in years at diagnosis (range)	57 (20-87)	60 (18-83)	p=0.76
Other cancers	10/83 (12.0%)	3/75 (4.0%)	p=0.066
Family history of melanoma	3/83 (3.6%)	0/55 (0%)*	p=0.154
Family history of cancer	42/83 (50.6%)	22/55 (40.0%)*	p=0.221

Stage at sampling	**Recently excised**	**Active disease**
I	15	1
II	11	1
III	11	6
IV	2	36
Total	39	44

Appendix E: Research Ethics application form.

NHS REC Form | Reference: 14/YH/1275 | IRAS Version 3.5

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Markers of relapse in melanoma

1. Is your project research?

(●) Yes ( ) No

2. Select one category from the list below:

( ) Clinical trial of an investigational medicinal product
( ) Clinical investigation or other study of a medical device
( ) Combined trial of an investigational medicinal product and an investigational medical device
( ) Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
( ) Basic science study involving procedures with human participants
( ) Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
( ) Study involving qualitative methods only
(●) Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
( ) Study limited to working with data (specific project only)
( ) Research tissue bank
( ) Research database

If your work does not fit any of these categories, select the option below:

( ) Other study

2a. Please answer the following question(s):

a) Will you be taking new samples primarily for research purposes (i.e. not surplus or existing stored samples), including any removal of organs or tissue from the deceased?	(●) Yes	( ) No
b) Will you be using surplus tissue or existing stored samples identifiable to the researcher?	(●) Yes	( ) No
c) Will you be using only surplus tissue or existing stored samples not identifiable to the researcher?	( ) Yes	(●) No
d) Will you be processing identifiable data at any stage of the research (including in the identification of participants)?	(●) Yes	( ) No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

[x] England
[ ] Scotland

Date: 17/11/2014 | 1 | 167721/696966/1/108

☐ Wales
☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

◉ England
○ Scotland
○ Wales
○ Northern Ireland
○ This study does not involve the NHS

4. Which review bodies are you applying to?

☑ NHS/HSC Research and Development offices
☐ Social Care Research Ethics Committee
☑ Research Ethics Committee
☐ National Information Governance Board for Health and Social Care (NIGB)
☐ National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information Forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

◉ Yes ○ No

5a. Are all the research costs and infrastructure costs for this study provided by an NIHR Biomedical Research Centre, NIHR Biomedical Research Unit, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC) or NIHR Research Centre for Patient Safety & Service Quality in all study sites?

○ Yes ◉ No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP).

5b. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN) support and inclusion in the NIHR Clinical Research Network (CRN) Portfolio? Please see information button for further details.

○ Yes ◉ No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP) and you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form immediately after completing this project filter and before completing and submitting other applications.

6. Do you plan to include any participants who are children?

○ Yes ◉ No

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

○ Yes ◉ No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the

guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?

○ Yes ◉ No

9. Is the study or any part of it being undertaken as an educational project?

◉ Yes ○ No

Please describe briefly the involvement of the student(s):
Part of a clinical PhD fellowship

9a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

◉ Yes ○ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?

○ Yes ◉ No

11. Will identifiable patient data be accessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?

○ Yes ◉ No

Integrated Research Application System
Application Form for Research limited to working with human tissue samples and/or data

NHS
Health Research Authority

Application to NHS/HSC Research Ethics Committee

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Markers of relapse in melanoma

Please complete these details after you have booked the REC application for review.

REC Name:
NRES Committee Yorkshire & The Humber Sheffield

REC Reference Number:
14/YH/1275

Submission date:
17/11/2014

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:

Markers of relapse in melanoma

A2-1. Educational projects

Name and contact details of student(s):

Student 1

Title	Forename/Initials	Surname
Dr	Shobha	Silva

Address	Academic Unit of Clinical Oncology Weston Park Hospital, Whitham Road Sheffield
Post Code	S10 2SJ
E-mail	shobha.silva@sth.nhs.uk
Telephone	01142265000
Fax	01142265364

Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/ degree:
PhD

Name of educational establishment:
University of Sheffield

Name and contact details of academic supervisor(s):

Academic supervisor 1

	Title	Forename/Initials	Surname
	Professor	Angela	Cox
Address	YCR Institute for Cancer Studies		
	Department of Oncology, University of Sheffield		
	Sheffield		
Post Code	S10 2RX		
E-mail	a.cox@sheffield.ac.uk		
Telephone	01142712373		
Fax	01142711602		

Academic supervisor 2

	Title	Forename/Initials	Surname
	Dr	Sarah	Danson
Address	Academic Unit of Clinical Oncology		
	Weston Park Hospital, Whitham Road		
	Sheffield		
Post Code	S10 2SJ		
E-mail	s.danson@sheffield.ac.uk		
Telephone	01142265000		
Fax	01142265364		

Academic supervisor 3

	Title	Forename/Initials	Surname
	Dr	Dawn	Teare
Address	School of Health and Related Research		
	University of Sheffield,		
	Regent Court, 30 Regent Street, Sheffield		
Post Code	S1 4DA		
E-mail	m.d.teare@sheffield.ac.uk		
Telephone	01142226398		
Fax	01142220749		

Please state which academic supervisor(s) has responsibility for which student(s):
Please click "Save now" before completing this table. This will ensure that all of the student and academic supervisor details are shown correctly.

Student(s)	Academic supervisor(s)
Student 1 Dr Shobha Silva	☑ Professor Angela Cox ☑ Dr Sarah Danson

☑ Dr Dawn Teare

A copy of a current CV for the student and the academic supervisor (maximum 2 pages of A4) must be submitted with the application.

A2-2. Who will act as Chief Investigator for this study?

- (•) Student
- ( ) Academic supervisor
- ( ) Other

A3-1. Chief Investigator:

	Title	Forename/Initials	Surname
	Dr	Shobha	Silva
Post	Clinical Research Fellow		
Qualifications	MBBS, MRCP(UK), MRCP (Medical Oncology)		
Employer	University of Sheffield		
Work Address	Academic Unit of Clinical Oncology Weston Park Hospital, Whitham Road Sheffiled		
Post Code	S10 2SJ		
Work E-mail	shobha.silva@sth.nhs.uk		
* Personal E-mail			
Work Telephone	01142265000		
* Personal Telephone/Mobile			
Fax	01142265364		

** This information is optional. It will not be placed in the public domain or disclosed to any other third party without prior consent.*
A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application.

A4. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project?
This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

	Title	Forename/Initials	Surname
	Ms	Rachel	Simpson
Address	Clinical Research Office D floor, Royal Hallamshire Hospital Glossop Road, Sheffield		
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A5-1. Research reference numbers. *Please give any relevant references for your study:*

Applicant's/organisation's own reference number, e.g. R & D (if available):

Sponsor's/protocol number:
Protocol Version: 1.0
Protocol Date:
Funder's reference number: N/A
Project website: N/A

Additional reference number(s):

Ref.Number Description	Reference Number
Sheffield Teaching Hospital Research Department	STH18695

Registration of research studies is encouraged wherever possible. You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you have registered your study please give details in the "Additional reference number(s)" section.

A5-2. Is this application linked to a previous study or another current application?

(●) Yes ( ) No

Please give brief details and reference numbers.
STH15777, Research Committee reference number 07/H1308/119 and 10/H1003/72
Translational study on samples collected in the GEMS (Genetics and Epidemiology of Melanoma in Sheffield)study

2. OVERVIEW OF THE RESEARCH

To provide all the information required by review bodies and research information systems, we ask a number of specific questions. This section invites you to give an overview using language comprehensible to lay reviewers and members of the public. Please read the guidance notes for advice on this section.

A6-1. Summary of the study. *Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, this summary will be published on the website of the National Research Ethics Service following the ethical review.*

Melanoma is the most serious form of skin cancer. The 5-year survival (for melanomas measuring more than 3.5mm thick) is only 50%, due to the high relapse rate. Following metastatic relapse, average prognosis is around 6-9 months. With conventional chemotherapy options, response rates are low; while with newer targeted agents, response rates are higher and outlook is better, but only in a proportion of patients. An on-going issue is how best to monitor patients. Once clinical symptoms of relapse have manifested, often it is too late for available therapies to be of significant benefit. Therefore, it would be immensely beneficial if reliable markers of relapse or progression can be developed, to enable early initiation of treatment. Plasma circulating cell-free DNA (ccfDNA) provides an easily accessible source of tumour-derived DNA which has potential for the development of markers of relapse. An increase in the amount of tumour-derived DNA has been correlated with tumour burden and relapse. Also, somatic genetic mutations (eg BRAF in melanoma), can be identified in ccfDNA. However, the clinical utility of ccfDNA has not been proven, partly due to the lack of longitudinal data from both melanoma patients and healthy controls. This study aims to address this by performing a longitudinal-study monitoring ccfDNA levels in melanoma patients and health controls. Patients with a diagnosis of melanoma would be eligible, and asked to complete a questionnaire and provide a blood sample 3-monthly for upto 5 years at their routine hospital appointments. Concurrently, their partners/family(as healthy controls) would be asked to provide the same. The recent feasibility study (GEMS) confirmed that patients and healthy controls can be recruited, and that ccfDNA can be extracted from blood samples obtained. The study showed a statistically significant difference in the level of ccfDNA in patients with active disease compared to those without.

A6-2. Summary of main issues. *Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.*

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex

organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

The study involves genetic research, and this will be made clear to all participants at the beginning.

Potential issues include:

1. Confidentiality - samples/questionnaires will be anonymised and protected.
2. Medical insurance - individual results will not be available, so there will not be these implications.
3. Use of diagnostic material - any archived material sampled will be surplus to requirements.
4. Controls will be asked for 3-monthly blood samples when their partner/family attends hospital for routine review - the inconvenience of these visits will be kept to a minimum.

A6-3. Proportionate review of REC application *The initial project filter has identified that your study may be suitable for proportionate review by a REC sub-committee. Please consult the current guidance notes from NRES and indicate whether you wish to apply through the proportionate review service or, taking into account your answer to A6-2, you consider there are ethical issues that require consideration at a full REC meeting.*

○ Yes - proportionate review ◉ No - review by full REC meeting

Further comments (optional):

Note: This question only applies to the REC application.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. *Please tick all that apply:*

- ☐ Case series/ case note review
- ☐ Case control
- ☑ Cohort observation
- ☐ Controlled trial without randomisation
- ☐ Cross-sectional study
- ☐ Database analysis
- ☑ Epidemiology
- ☐ Feasibility/ pilot study
- ☑ Laboratory study
- ☐ Metanalysis
- ☐ Qualitative research
- ☑ Questionnaire, interview or observation study
- ☐ Randomised controlled trial
- ☐ Other (please specify)

A10. What is the principal research question/objective? *Please put this in language comprehensible to a lay person.*

The aim of the study is to identify whether cancer-specific somatic (acquired) mutations (genetic changes) detected in ccfDNA, and/or circulating levels of ccfDNA, could act as early markers of relapse or progression in melanoma. We will test this by assessing the relationship between levels of ccfDNA, ccfDNA molecular markers, and metastatic relapse over time.
The objectives are:
1. To recruit 120 patients with melanoma and 120 healthy controls (co-habiting partners or siblings) over 2 years, and obtain blood samples/complete questionnaires at recruitment, then 3-monthly for up to 5 years.
2. To determine plasma ccfDNA levels, and identify if mutations are present.
3. To assess if there is a temporal relationship between these and the onset of symptoms of relapse
4. To confirm the stability of ccfDNA levels in controls over time

A11. What are the secondary research questions/objectives if applicable? *Please put this in language comprehensible to a lay person.*

This study will also assess the relationship between inherited variation in key genes and gene expression in healthy skin. This will involve obtaining a single skin biopsy from patients at recruitment (optional).

A12. What is the scientific justification for the research? *Please put this in language comprehensible to a lay person.*

Melanoma is the most aggressive form of skin cancer. The incidence has been rising since the mid-1970s, mostly due to changes in sun-related behaviour. There were 13,348 new cases diagnosed in the UK in 2011, and 2,148 deaths from melanoma in 2012 (Cancer Research UK statistics).
The 5-year survival (for melanomas measuring 3.5mm or more) is only 50%, and the average lifespan once metastatic relapse has occurred is around 12 months. Conventional chemotherapy has poor response rates, while newer targeted treatments offer better response rates and improved outlook in a proportion of patients. It is postulated that being able to detect relapses before the manifestation of clinical symptoms will enable earlier initiation of treatment, and hence may improve the effectiveness of these treatments.
This study hopes to develop a robust way of detecting relapses earlier, and this would be of significant benefit in clinical management of melanomas.

A13. Please summarise your design and methodology. *It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.*

Overview:
The study aims to collect detailed epidemiological data on cutaneous melanoma over time. It will involve collecting blood samples (and skin biopsies from some patients) for analyses of genes and ccfDNA, and to detect changes in these over time. Cohabiting partners/siblings will be approached as controls to provide blood samples.
Hypothesis:
Cancer-specific somatic mutations detected in ccfDNA, and/or circulating levels of ccfDNA could act as early markers of relapse in melanoma.
Recruitment and consent:
Over a period of 2 years, we aim to recruit 120 cutaneous melanoma patients, and at least one cohabiting partner/sibling control for each (we will not exclude those patients without a control). Patients will be recruited at Weston Park Hospital and Royal Hallamshire Hospital. The Dermatology service at Royal Hallamshire Hospital sees approximately 200 new cases of melanoma per year, and the high risk patients (around 120 per year) are referred to Weston Park Hospital for shared care with the oncology team. Eligible patients will be identified from clinic lists and MDT meetings. We aim to identify and recruit all eligible new patients seen in our clinics, plus as many prevalent cases as possible. A log will be kept of all patients not recruited, with reasons for non-recruitment. Patients will be approached by Dr Shobha Silva or Sister Helen Cramp (research nurse) to discuss the study if they express interest in participating. Information leaflets will be provided, and after time for consideration and discussion, written informed consent will be taken from interested participants. Consent will be obtained for 3-monthly blood samples, questionnaire completion, access to medical records, a skin biopsy in patients (optional) and access to surplus tumour tissue (for patients).
Study visits and blood sample collection:
Study visits will coincide with each patient's 3-monthly NHS follow-up. The intial visit will involve obtaining consent, a blood sample and completing a questionnaire, and will take approximately 30 minutes. The questionnaire has been adapted from a previously validated questionnaire (kindly provided by Professor Tim Bishop, Leeds Institute of Molecular Medicine). An additional proforma will be completed at recruitment detailing tumour pathology, stage, treatment, and outcome. Tumour specimens will be requested in batches from relevant pathology laboratories, coordinated by Dr Nick Tiffin. Patients will be asked for permission for their excised tumour to be accessed and analysed for DNA and methylation studies. Patients will be invited to provide a single skin biopsy sample, either at the initial visit, or at a subsequent visit (optional). All samples and data will be anonymised (labelled with a study identification number), and data stored on a password-protected computer. Subsequent follow-up visits will involve a blood sample, as well as clinical information (to be done by a member of their direct clinical team) regarding their health.
Sample analysis:
Gene analysis- Patients and controls will consent to have blood samples to check for variants in candidate genes involved in the development of melanoma and the likelihood of response to treatment.
ccfDNA: DNA will be extracted from blood samples using QIAamp circulating nucleic acid kit. The study aims to determine if changes in levels of /mutations in ccfDNA correlate with tumour burden, by collecting samples over time, and correlating these with the clinical picture.
Data analysis:
Data will be collected and analysed to determine:

- associations between mutations and melanoma
- differences between levels of ccfDNA in patients and controls over time

Benefits/Risks:
There are no additional benefits to individual patients/controls participating in the study. Patients will not receive individual test results. Study visits will coincide with regular NHS follow-up visits, so should not be a significant burden on participants' time. In our experience, patients and relatives are generally happy to participate in such studies as the results may benefit future patients.

Time-line:
Participants will be recruited over a 2 year period, and will be followed up for up to 5 years. Sample analysis and data entry will occur alongside patient recruitment, and extend into the 3rd year of the study. The data will be finalised and presented as a PhD thesis at the end of the 3rd year. Further follow-up information and analysis will continue for up to 5 years post recruitment.

A14-1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

- [x] Design of the research
- [ ] Management of the research
- [x] Undertaking the research
- [ ] Analysis of results
- [x] Dissemination of findings
- [ ] None of the above

Give details of involvement, or if none please justify the absence of involvement.
A patient representative will be approached regarding involvement in reviewing the patient information leaflets.

4. RISKS AND ETHICAL ISSUES

RESEARCH PARTICIPANTS

A17-1. Please list the principal inclusion criteria (list the most important, max 5000 characters).

Patients:
- diagnosis of cutaneous/conjunctival melanoma of any stage
- no chemotherapy within the previous four (4) weeks
- capable and willing to complete study procedures

Controls:
- co-habiting partners or siblings of study patients
- capable and willing to complete study procedures

A17-2. Please list the principal exclusion criteria (list the most important, max 5000 characters).

- non-cutaneous melanoma, other than conjunctival melanoma (as this is probably a different disease entity)
- those felt to be too unwell to be approached
- anyone unable to give informed consent

RESEARCH PROCEDURES, RISKS AND BENEFITS

A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. *These include seeking consent, interviews, non-clinical observations and use of questionnaires.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?

3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Consent to study	1	0	30 minutes	Dr Shobha Silva (chief investigator) or Sister Helen Cramp (research nurse), in hospital outpatient clinic
Clinical interview	20	20	15 minutes	Trained member of medical team, in hospital outpatient clinic
Questionnaire	1	0	30 minutes	Dr Shobha Silva or Sister Helen Cramp

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. *These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.*

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

Intervention or procedure	1	2	3	4
Blood sample	20	0	2 minutes	Dr Shobha Silva or Sister Helen Cramp, in hospital outpatient clinic
Skin biopsy (optional)	1	0	15 minutes	Dr Shobha Silva

A21. How long do you expect each participant to be in the study in total?

Up to 5 years

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

Venepuncture and skin biopsy may cause transient discomfort and/or bruising, but will be conducted by trained staff. Inconvenience of completing the detailed questionnaire, this will be kept to a minimum

A24. What is the potential for benefit to research participants?

No direct benefit for individual patients, but will potentially lead to a better understanding of the patterns of relapse, and help guide prompt treatment.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27-1. How will potential participants, records or samples be identified? Who will carry this out and what resources will

be used?*For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under arrangements with the responsible care organisation(s).*

We aim to recruit 120 patients and 120 controls. We will approach all patients diagnosed with cutaneous melanoma of any stage. Patients will be identified and approached through NHS clinics at Weston Park Hospital (Dr Sarah Danson & James Lester) and Royal Hallamshire Hospital (Dr Andrew McDonagh and Dr Helen Ramsay). We aim to identify all eligible patients through review of clinical notes and MDT lists. An invitation letter will be posted to patients (from the clinicians involved in their direct clinical care) prior to their planned clinic appointment. When they attend their appointment, patients will be approached by Dr Shobha Silva or Sister Helen Cramp (research nurse) to discuss the study if they express interest in participating. Information leaflets will be provided, and after time for consideration and discussion, written informed consent will be taken from interested participants.Controls will be recruited through the patients. We will anonymously log the reasons for non-recruitment. The North Trent Cancer Network has an annual incidence of melanoma of between 400-500 cases. The low and intermediate-risk patients are seen by the dermatologists (The Royal Hallamshire Hospital sees about 200 cases per year), while the high risk patients are referred to Weston Park Hospital (about 120 cases per year) for joint follow-up between the oncologists and dermatologists.

A27-2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

(●) Yes ( ) No

Please give details below:
We aim to identify all eligible patients through review of clinical notes, as well as MDT lists. Eligible patients will be identified by members of the clinical team directly involved in the patients' care (ie Dr Sarah Danson/Dr James Lester at Weston Park Hospital and Dr Andrew McDonagh/Dr Helen Ramsay at Royal Hallamshire Hospital)

A27-4. Will researchers or individuals other than the direct care team have access to identifiable personal information of any potential participants?

( ) Yes (●) No

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

( ) Yes (●) No

A29. How and by whom will potential participants first be approached?

Patients will be approached by a member of their direct care team (Dr Sarah Danson/Dr James Lester's outpatient clinic at Weston Park Hospital, and Dr Andrew McDonagh's outpatient clinic at Royal Hallamshire Hospital). An invitation letter will be sent (by a member of their direct care team) prior to their clinic appointment. Newly diagnosed patients will be approached at a clinic visit following their diagnosis; while for existing patients, they will be approached at a time felt to be appropriate by the direct care team.

A30-1. Will you obtain informed consent from or on behalf of research participants?

(●) Yes ( ) No

If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Written informed consent will be obtained by Dr Shobha Silva, Sister Helen Cramp, Dr Sarah Danson, Dr James Lester, Dr Andrew McDonagh, Dr Helen Ramsay or an appropriately trained and supervised sub-investigator. A copy of this written consent form will be provided to participants.

If you are not obtaining consent, please explain why not.

Please enclose a copy of the information sheet(s) and consent form(s).

A30-2. Will you record informed consent (or advice from consultees) in writing?

(●) Yes ( ) No

A31. How long will you allow potential participants to decide whether or not to take part?

As long as the patient needs to make an informed decision (a minimum of 24 hours). Patients will have received an invitation letter and information leaflet at least 24 hours prior to their initial visit, and following discussion with the research team, would still have the opportunity to take the information leaflet and consent form away with them prior to making a decision.

A33-1. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?*(e.g. translation, use of interpreters)*

Due to the complexity of the questionnaires, entry will be restricted to those who can assimilate the information, with the help of the research team. Translators (face-to-face or via telephone) will be provided if required.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

(●) The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.

( ) The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.

( ) The participant would continue to be included in the study.

( ) Not applicable – informed consent will not be sought from any participants in this research.

( ) Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)?*(Tick as appropriate)*

- [x] Access to medical records by those outside the direct healthcare team
- [x] Electronic transfer by magnetic or optical media, email or computer networks
- [ ] Sharing of personal data with other organisations
- [ ] Export of personal data outside the EEA
- [ ] Use of personal addresses, postcodes, faxes, emails or telephone numbers
- [ ] Publication of direct quotations from respondents

- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [ ] Manual files including X-rays
  - [ ] NHS computers
  - [ ] Home or other personal computers
  - [x] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details:
The examination of clinical records will be undertaken by Dr Shobha Silva and Sister Helen Cramp after written informed consent has been obtained. Dr Silva and Sister Cramp will keep a separate database containing personal identifier and contact information for the purposes of obtaining follow-up data on the participants. The data is to be stored on a single university computer, will not be shared, and will be password-protected. The computer will be stored in a secure room when not in use

A38. How will you ensure the confidentiality of personal data? *Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.*

Aside from the database described in A36, the rest of the data will be anonymised using a study allocation number.

A40. Who will have access to participants' personal data during the study? *Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.*

All investigators of the study. Consent for access will be sought during the consenting process.

Storage and use of data after the end of the study

A43. How long will personal data be stored or accessed after the study has ended?

- ○ Less than 3 months
- ○ 3 – 6 months
- ◉ 6 – 12 months
- ○ 12 months – 3 years
- ○ Over 3 years

INCENTIVES AND PAYMENTS

A46. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A47. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?

○ Yes ◉ No

A48. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

NOTIFICATION OF OTHER PROFESSIONALS

A49-1. Will you inform the participants' General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?

◉ Yes ○ No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A49-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?

◉ Yes ○ No

It should be made clear in the participant's information sheet if the GP/health professional will be informed.

PUBLICATION AND DISSEMINATION

A50. Will the research be registered on a public database?

○ Yes ◉ No

Please give details, or justify if not registering the research.
No suitable register exists.

Registration of research studies is encouraged wherever possible.
You may be able to register your study through your NHS organisation or a register run by a medical research charity, or publish your protocol through an open access publisher. If you are aware of a suitable register or other method of publication, please give details. If not, you may indicate that no suitable register exists. Please ensure that you have entered registry reference number(s) in question A5-1.

A51. How do you intend to report and disseminate the results of the study? *Tick as appropriate:*

- ☑ Peer reviewed scientific journals
- ☑ Internal report
- ☑ Conference presentation
- ☐ Publication on website
- ☐ Other publication
- ☐ Submission to regulatory authorities
- ☐ Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- ☐ No plans to report or disseminate the results
- ☐ Other (please specify)

A53. Will you inform participants of the results?

○ Yes ◉ No

Please give details of how you will inform participants or justify if not doing so.
Patients can have copies of published results if they wish to. However, the results of the study will not have a direct impact on individual patient's treatment.

5. Scientific and Statistical Review

A54. How has the scientific quality of the research been assessed? *Tick as appropriate:*

- [x] Independent external review
- [ ] Review within a company
- [ ] Review within a multi-centre research group
- [x] Review within the Chief Investigator's institution or host organisation
- [ ] Review within the research team
- [ ] Review by educational supervisor
- [ ] Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
The project was previously reviewed as a potential Yorkshire Cancer Research/Cancer Research UK PhD project in 2012, and deemed to be suitable. However, the candidate dropped out just before the project was due to start. The project was reviewed externally for Weston Park Cancer Charity funding in 2013 (for a research nurse/data manager) but did not go ahead as a research fellow had not been appointed, and it was suggested that we reapply this year if that had occurred.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/ institution.

A56. How have the statistical aspects of the research been reviewed? *Tick as appropriate:*

- [ ] Review by independent statistician commissioned by funder or sponsor
- [ ] Other review by independent statistician
- [ ] Review by company statistician
- [ ] Review by a statistician within the Chief Investigator's institution
- [x] Review by a statistician within the research team or multi-centre group
- [ ] Review by educational supervisor
- [ ] Other review by individual with relevant statistical expertise
- [ ] No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Dr	Dawn	Teare
Department	School of Health and Related Research		
Institution	University of Sheffield		
Work Address	Regent Court, 30 Regent Street		
	Sheffield		
Post Code	S1 4DA		
Telephone	01142226398		

Fax	01142220749
Mobile	
E-mail	m.d.teare@sheffield.ac.uk

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Changes in ccfDNA levels and mutations over time in patients with melanoma compared with healthy controls.

A58. What are the secondary outcome measures? *(if any)*

Feasibility of recruitment to the study

A59. What is the sample size for the research? *How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.*

Total UK sample size:	240
Total international sample size (including UK):	240
Total in European Economic Area:	240

Further details:
All to be recruited from a single centre.

A60. How was the sample size decided upon? *If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.*

Based on the GEMS study, we anticipate a recruitment of 5 patients per month. Assuming a study duration of 2 years, and 50% marker prevalence (typical for BRAF V600E), 120 patients will provide at least 80% power to detect a 2-fold difference, at the 5% level in typical median relapse-free survival times (10 months vs 5 months, log rank test).

A61. Will participants be allocated to groups at random?

○ Yes ◉ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Statistical analysis will focus on the effects of the categorical and quantitative markers on relapse-free survival time, taking account of the repeated measurements of these variables

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. *Please include all grant co-applicants, protocol co-authors and other key members of the Chief Investigator's team, including non-doctoral student researchers.*

	Title	Forename/Initials	Surname
	Dr	Andrew	McDonagh
Post	Consultant Dermatologist		
Qualifications			
Employer	Sheffield Teaching Hospitals NHS Trust		

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Post Code	
Telephone	01142711900
Fax	
Mobile	
Work Email	a.j.mcdonagh@sheffield.ac.uk

Title	Forename/Initials	Surname
Dr	Nick	Tiffin

Post	Consultant Pathologist
Qualifications	
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Post Code	
Telephone	01142711900
Fax	
Mobile	
Work Email	nick.tiffin@sth.nhs.uk

Title	Forename/Initials	Surname
Dr	Helen	Ramsay

Post	Consultant Dermatologist
Qualifications	
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Title	Forename/Initials	Surname
Dr	James	Lester

Post	Clinical Oncology Consultant
Qualifications	
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Telephone	01142265000
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	Title	Forename/Initials	Surname
	Dr	Sarah	Danson
Post	Consultant Medical Oncologist		
Qualifications			
Employer			
Work Address	Weston Park Hospital Whitham Road Sheffield		
Post Code			
Telephone	01142265000		
Fax			
Mobile			
Work Email	s.danson@sheffield.ac.uk		

A64. Details of research sponsor(s)

A64-1. Sponsor

Lead Sponsor

Status:
- (●) NHS or HSC care organisation
- ( ) Academic
- ( ) Pharmaceutical industry
- ( ) Medical device industry
- ( ) Local Authority
- ( ) Other social care provider (including voluntary sector or private organisation)
- ( ) Other

Commercial status:

If Other, please specify:

Contact person

Name of organisation	Sheffield Teaching Hospitals NHS Trust
Given name	Rachel
Family name	Simpson
Address	Royal Hallamshire Hospital
Town/city	Sheffield
Post code	
Country	
Telephone	01142265940
Fax	
E-mail	rachel.simpson@sth.nhs.uk

Is the sponsor based outside the UK?

( ) Yes (●) No

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

A65. Has external funding for the research been secured?

- [x] Funding secured from one or more funders
- [ ] External funding application to one or more funders in progress
- [ ] No application for external funding will be made

What type of research project is this?

- ○ Standalone project
- ○ Project that is part of a programme grant
- ○ Project that is part of a Centre grant
- ◉ Project that is part of a fellowship/ personal award/ research training award
- ○ Other

Other – please state:

Please give details of funding applications.

Organisation	Weston Park Hospital Cancer Charity
Address	Weston Park Cancer Charity Whitham Road
Post Code	S10 2SJ
Telephone	
Fax	
Mobile	
Email	samantha.kennedy@sth.nhs.uk

Funding Application Status: ◉ Secured ○ In progress

Amount:

Duration
Years: 3
Months:

If applicable, please specify the programme/ funding stream:

What is the funding stream/ programme for this research project?
Not applicable.

A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

○ Yes ◉ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A6-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68-1. Give details of the lead NHS R&D contact for this research:

	Title	Forename/Initials	Surname
	Miss	Rachel	Simpson
Organisation	Sheffield Teaching Hospitals NHS Trust		
Address	Clinical Research Office		
	D Floor, Royal Hallamshire Hospital		
	Glossop Road, Sheffield		
Post Code	S10 2JF		
Work Email	rachel.simpson@sth.nhs.uk		
Telephone	01142265940		
Fax	01142265937		
Mobile			

Details can be obtained from the NHS R&D Forum website: http://www.rdforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 02/01/2015
Planned end date: 01/10/2035
Total duration:
Years: 20 Months: 9 Days: 0

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)

On publication of results if analysis of data.

A71-2. Where will the research take place? *(Tick as appropriate)*

- [x] England
- [ ] Scotland
- [ ] Wales
- [ ] Northern Ireland
- [ ] Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
( ) Yes (•) No

A72. What host organisations (NHS or other) in the UK will be responsible for the research sites? *Please indicate the type of organisation by ticking the box and give approximate numbers of planned research sites:*

[x] NHS organisations in England	1
[ ] NHS organisations in Wales	
[ ] NHS organisations in Scotland	
[ ] HSC organisations in Northern Ireland	
[ ] GP practices in England	
[ ] GP practices in Wales	
[ ] GP practices in Scotland	
[ ] GP practices in Northern Ireland	

- ☐ Social care organisations
- ☐ Phase 1 trial units
- ☐ Prison establishments
- ☐ Probation areas
- ☐ Independent hospitals
- ☐ Educational establishments
- ☐ Independent research units
- ☐ Other (give details)

Total UK sites in study: 1

A76. Insurance/ indemnity to meet potential legal liabilities

Note: in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland

A76-1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? *Please tick box(es) as applicable.*

Note: Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

- ☑ NHS indemnity scheme will apply (NHS sponsors only)
- ☐ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-2. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? *Please tick box(es) as applicable.*

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

- ☑ NHS indemnity scheme will apply (protocol authors with NHS contracts only)
- ☐ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A76-3. What arrangements will be made for insurance and/ or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

Note: Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

- ☑ NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)
- ☐ Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

Part B: Section 4 – Use of residual or existing stored human tissue(or other human biological materials)

1. What types of human tissue or other biological material will be included in the study?

Archived tumour samples, surplus to requirements, will be collected for for assessment of DNa and methylation studies.

2. Will the samples be released to the researcher:

In fully anonymised form? *(link to stored tissue and data is broken)*
○ Yes ◉ No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
○ Yes ◉ No

In a form in which the donor could be identifiable to researchers?
◉ Yes ○ No

If Yes, please justify.

Tissue samples released from MHS pathology laboratories will be labelled with a pathology database number. Further samples prepared from this will be labelled with the participant's study identifcation number, but no personal identifiers.

3. Has consent been obtained previously to use the samples for research

○ Consent has been given for all samples
○ Consent has been given for some of the samples
◉ No consent has been given

5. Is it proposed to seek further consent to use the samples in this research?

◉ Yes ○ No

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

○ Yes ◉ No

8. What types of test or analysis will be carried out on the samples?

Comparing gene expression in biopsy specimens from healthy tissue with gene variations in tumour samples

9. Will the research involve the analysis or use of human DNA in the samples?

◉ Yes ○ No

10. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

11. If so, will arrangements be made to notify the individuals concerned?

○ Yes
○ No
◉ Not applicable

12. Who is the holder of the samples?

Please tick either/both boxes as applicable.

☑ NHS pathology department(s) / diagnostic archive(s)
Specific details of each department/archive are not required

☐ Other research tissue bank(s) or sample collection(s)
Please provide further details of each bank/collection below

13. Will any of the samples be imported from outside the UK?

○ Yes ◉ No

14. Please give details of where the samples will be stored, who will have access and the custodial arrangements.

The samples will be stored in the Sheffield Teaching Hospitals (STH) Clinical Histological Archive. the responsible organisation of this bank is STH Directorate of Laboratory Medicine, and their contact is Dr Nick Tiffin, Consultant Histopathologist.

15. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☐ Return to current holder of the samples
☑ Transfer to another tissue bank

(If the bank is in England, Wales or Northern Ireland a licence from the Human Tissue Authority will be required to store relevant material for possible further research.)

☐ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a storage licence for research from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act
☐ Disposal in accordance with the Human Tissue Authority Code of Practice
☐ Other
☐ Not yet known

Please give further details of the proposed arrangements:
Once the ethics approval of this study expires, then the collection will be transferred to the Sheffield Teaching Hospitals tissue bank under the HTA biorepository licence held by Simon Heller

Part B: Section 5 – Use of newly obtained human tissue(or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?

Blood samples via venepuncture
Skin biopsy (in a subgroup of patients)

2. Who will collect the samples?

Investigators of the study, or trained phlebotomy staff within the hospital

3. Who will the samples be removed from?

- [x] Living donors
- [ ] The deceased

4. Will informed consent be obtained from living donors for use of the samples? *Please tick as appropriate*

In this research?
(•) Yes ( ) No

In future research?
(•) Yes ( ) No ( ) Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?

( ) Yes (•) No

8. Will the samples be stored: *[Tick as appropriate]*

In fully anonymised form? *(link to donor broken)*
( ) Yes (•) No

In linked anonymised form? *(linked to stored tissue but donor not identifiable to researchers)*
(•) Yes ( ) No

If Yes, say who will have access to the code and personal information about the donor.
Chief and co-investigators

In a form in which the donor could be identifiable to researchers?
( ) Yes (•) No

9. What types of test or analysis will be carried out on the samples?

DNA will be extracted using validated techniques, and levels of ccfDNA will be quantified using real-time PCR. Also a validated assay to detect BRAF V600E is in place which can be used to detect this mutation.

10. Will the research involve the analysis or use of human DNA in the samples?

(•) Yes ( ) No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?

○ Yes ◉ No

12. If so, will arrangements be made to notify the individuals concerned?

○ Yes ○ No ◉ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Blood and biopsy samples will be stored in freezers in the university's medical school. The samples will only be accessed by investigators.
Prof Angela Cox will act as custodian of the blood samples for the duration of the study.

14. What will happen to the samples at the end of the research? *Please tick all that apply and give further details.*

☑ Transfer to research tissue bank

(If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

☐ Storage by research team pending ethical approval for use in another project

(Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

☐ Storage by research team as part of a new research tissue bank

(The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act

☐ Disposal in accordance with the Human Tissue Authority's Code of Practice

☐ Other

☐ Not yet known

Please give further details of the proposed arrangements:

Once the ethics approval of this study expires, then the collection will be transferred to the Sheffield Teaching Hospital tissue bank under the HTA biorepository licence held by Simon Heller

PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. *For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.*

Research site		Investigator/ Collaborator/ Contact	
Institution name	Sheffield Teaching Hospital NHS Trust	Title	Dr
Department name	Weston Park Hospital	First name/ Initials	Shobha
Street address	Whitham Road	Surname	Silva
Town/city	Sheffield		
Post Code	S10 2SJ		

PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.

4. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendment.

5. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.

6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2006.

7. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.

8. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 1998.

9. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:

    - Will be held by the REC (where applicable) until at least 3 years after the end of the study; and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
    - May be disclosed to the operational managers of review bodies, or the appointing authority for the REC (where applicable), in order to check that the application has been processed correctly or to investigate any complaint.
    - May be seen by auditors appointed to undertake accreditation of RECs (where applicable).
    - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.
    - May be sent by email to REC members.

10. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

11. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

Contact point for publication*(Not applicable for R&D Forms)*

NRES would like to include a contact point with the published summary of the study for those wishing to seek further information. We would be grateful if you would indicate one of the contact points below.

(●) Chief Investigator

( ) Sponsor

- ○ Study co-ordinator
- ○ Student
- ○ Other – please give details
- ○ None

Access to application for training purposes *(Not applicable for R&D Forms)*

Optional – please tick as appropriate:

☑ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

This section was signed electronically by Dr Shobha Silva on 15/11/2014 18:10.

Job Title/Post:	Clinical Research Fellow/Medical Oncology Registrar
Organisation:	Weston Park Hospital
Email:	scsilva1@sheffield.ac.uk

D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co-sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.
3. Any necessary indemnity or insurance arrangements, as described in question A76, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.
4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
6. The duties of sponsors set out in the Research Governance Framework for Health and Social Care will be undertaken in relation to this research.
7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.
8. Specifically, for submissions to the Research Ethics Committees (RECs) I declare that any and all clinical trials approved by the HRA since 30th September 2013 (as defined on IRAS categories as clinical trials of medicines, devices, combination of medicines and devices or other clinical trials) have been registered on a publically accessible register in compliance with the HRA registration requirements for the UK, or that any deferral granted by the HRA still applies.

This section was signed electronically by Dr Dipak Patel on 17/11/2014 10:29.

Job Title/Post:	Research Manager
Organisation:	Sheffield Teaching Hospitals NHS Foundation Trust
Email:	dipak.patel@sth.nhs.uk

D3. Declaration for student projects by academic supervisor(s)

1. I have read and approved both the research proposal and this application. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level.

2. I undertake to fulfil the responsibilities of the supervisor for this study as set out in the Research Governance Framework for Health and Social Care.

3. I take responsibility for ensuring that this study is conducted in accordance with the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research, in conjunction with clinical supervisors as appropriate.

4. I take responsibility for ensuring that the applicant is up to date and complies with the requirements of the law and relevant guidelines relating to security and confidentiality of patient and other personal data, in conjunction with clinical supervisors as appropriate.

Academic supervisor 1

This section was signed electronically by M. Dawn Teare on 17/11/2014 12:14.

Job Title/Post:	Senior Lecturer in Genetic Epidemiology
Organisation:	University of Sheffield
Email:	m.d.teare@sheffield.ac.uk

Academic supervisor 2

This section was signed electronically by Dr Sarah Danson on 16/11/2014 12:24.

Job Title/Post:	Reader in Medical Oncology
Organisation:	University of Sheffield/Sheffield Teaching Hospitals NHS Trust
Email:	s.danson@shef.ac.uk

Academic supervisor 3

This section was signed electronically by Angela Cox on 16/11/2014 12:29.

Job Title/Post:	Prof. of Cancer Genetic Epidemiology
Organisation:	University of Sheffield
Email:	a.cox@sheffield.ac.uk

Appendix F: Silva S et al. Genome-Wide Analysis of Circulating Cell-Free DNA Copy Number Detects Active Melanoma and Predicts Survival

Clinical Chemistry 64:9
1338–1346 (2018)

Cancer Diagnostics

Genome-Wide Analysis of Circulating Cell-Free DNA Copy Number Detects Active Melanoma and Predicts Survival

Shobha Silva,[1*†] Sarah Danson,[1†] Dawn Teare,[2] Fiona Taylor,[1] James Bradford,[3‡] Andrew J.G. McDonagh,[4] Abdulazeez Salawu,[1] Greg Wells,[5] George J. Burghel,[6] Ian Brock,[7] Daniel Connley,[7] Helen Cramp,[7] David Hughes,[8] Nick Tiffin,[8] and Angela Cox[7†]

BACKGROUND: A substantial number of melanoma patients develop local or metastatic recurrence, and early detection of these is vital to maximise benefit from new therapies such as inhibitors of BRAF and MEK, or immune checkpoints. This study explored the use of novel DNA copy-number profiles in circulating cell-free DNA (cfDNA) as a potential biomarker of active disease and survival.

PATIENTS AND METHODS: Melanoma patients were recruited from oncology and dermatology clinics in Sheffield, UK, and cfDNA was isolated from stored blood plasma. Using low-coverage whole-genome sequencing, we created copy-number profiles from cfDNA from 83 melanoma patients, 44 of whom had active disease. We used scoring algorithms to summarize copy-number aberrations and investigated their utility in multivariable logistic and Cox regression analyses.

RESULTS: The copy-number aberration score (CNAS) was a good discriminator of active disease (odds ratio, 3.1; 95% CI, 1.5–6.2; $P = 0.002$), and CNAS above or below the 75th percentile remained a significant discriminator in multivariable analysis for active disease ($P = 0.019$, with area under ROC curve of 0.90). Additionally, mortality was higher in those with CNASs above the 75th percentile than in those with lower scores (HR, 3.4; 95% CI, 1.5–7.9; $P = 0.005$), adjusting for stage of disease, disease status (active or resected), BRAF status, and cfDNA concentration.

CONCLUSIONS: This study demonstrates the potential of a de novo approach utilizing copy-number profiling of cfDNA as a biomarker of active disease and survival in melanoma. Longitudinal analysis of copy-number profiles as an early marker of relapsed disease is warranted.

Melanoma is the most aggressive form of skin cancer and is increasing in incidence throughout the developed world, with around 15 900 new cases and 2285 deaths from melanoma in the UK in 2015 *(1)*. Prognosis is known to correlate with Breslow thickness, the presence of tumor ulceration and mitotic rate *(2)*. Patients with thicker melanomas (defined as >2 mm with ulceration or >4 mm without ulceration) have >50% chance of relapse *(3)*.

The cornerstone of treatment for locoregional disease is surgery. However, a substantial number of patients later develop local or systemic recurrence. Advanced disease is usually fatal. Until 2011, standard palliative chemotherapy was single-agent dacarbazine, with a response rate of just 10%–15%. Response rates have since improved with the advent of B-Raf protooncogene, serine/threonine kinase (BRAF)[9] inhibitors, mitogen-activated protein kinase kinase 1 (MEK) inhibitors *(4, 5)*, and immune checkpoint inhibitors that target cytotoxic T-lymphocyte associated protein 4 (CTLA4) and programmed cell death 1 (PD1) *(6, 7)*. Early detection of metastatic relapse (if possible before onset of clinical symptoms) is vital to maximize the benefit from the new therapies. At the present time, this relies heavily on imaging, but a blood test has the potential to be more sensitive and cost-effective. Although the lactate dehydrogenase blood test has been previously used as a prognostic

[1] Academic Unit of Clinical Oncology and Sheffield Experimental Cancer Medicine Centre, University of Sheffield, Sheffield, UK; [2] School of Health and Related Research, University of Sheffield, Sheffield, UK; [3] Academic Unit of Molecular Oncology, Sheffield Medical School, University of Sheffield, Sheffield, UK; [4] Department of Dermatology, Royal Hallamshire Hospital, UK; [5] Sheffield Genetic Diagnostics Group, Sheffield Children's NHS Foundation Trust, Western Bank Sheffield, UK; [6] The Manchester Centre for Genomic Medicine, Manchester University NHS Foundation Trust, Manchester, UK; [7] Academic Unit of Molecular Oncology, Sheffield Medical School, University of Sheffield, Sheffield, S10 2RX, UK; [8] Department of Histopathology, Royal Hallamshire Hospital, Sheffield, UK.

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† S. Silva, S. Danson, and A. Cox contributed equally to this work.

‡ Current affiliation: Almac Diagnostics, 19 Seagoe Industrial Estate, Craigavon, BT63 5QD, UK.

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[9] Nonstandard abbreviations: BRAF, B-Raf protooncogene, serine/threonine kinase; cfDNA, circulating cell-free DNA; ctDNA, tumor-derived DNA; FFPE, formalin-fixed paraffin-embedded.

marker, it is not a clinically useful marker of disease status *(2)*, and better biomarkers of relapse need to be identified.

Plasma circulating cell-free DNA (cfDNA) provides an easily accessible source of tumor-derived DNA (ctDNA). Raised concentrations of cfDNA are seen in many clinical conditions, when compared with healthy controls *(8–10)*, and ctDNA has been detected in the plasma of patients with melanoma *(11)*. An increase in the overall amount of tumor-derived DNA in the plasma is frequently correlated with metastatic disease and relapse, and studies have shown that tumor-specific circulating DNA concentrations correlate with tumor burden and relapse following treatment *(12–14)*. Somatic genomic aberrations characteristic of the tumor DNA can frequently be seen in the ctDNA *(15)*. A number of high-profile publications have demonstrated the potential utility of using genomic profiling techniques to provide biomarkers of metastatic disease and acquired resistance that can capture tumor evolution and heterogeneity *(16–18)*. Copy-number aberrations (comprising deletions or duplications of large segments of DNA, ranging in size from a few kilobases to entire chromosomes) are abundant in malignant melanoma *(19)*. Such aberrations occur throughout the genome and can be detected by whole-genome, highly parallel sequencing at low coverage, in contrast to single-nucleotide mutations, the detection of which requires high-coverage targeted sequencing. Copy-number analysis in cfDNA, therefore, has great potential as a source of biomarkers in melanoma.

We carried out a feasibility study for patient recruitment, cfDNA sample collection, yield, and stability. To investigate the potential usefulness of low-coverage cfDNA sequencing for relapse detection in melanoma, we investigated whether cfDNA copy-number profiles could be used to differentiate melanoma patients with active disease from those with recently resected disease.

Materials and Methods

PATIENTS

Patients were recruited from dermatology and oncology outpatient clinics in Sheffield and Barnsley Hospitals, UK, over an 18-month period. Eligible patients had a confirmed diagnosis of cutaneous melanoma of any stage (American Joint Committee on Cancer, Melanoma of the Skin Staging, 7th edition) and no chemotherapy exposure within the previous 4 weeks. Patients with either active unresected disease or prior resection of their primary tumor or metastatic disease with clear margins within 4 weeks of recruitment were included. Healthy control participants were cohabiting partners of study patients or siblings of study patients with no previous cancer diagnosis. All participants gave informed consent, and the study was approved by the local research ethics committee (REC10/H1003/72).

CIRCULATING DNA EXTRACTION AND QUANTIFICATION

Plasma was prepared from blood collected in EDTA vacutainers and processed within 2 h of collection, by centrifugation at 800*g* for 10 min at 4 °C, followed by centrifugation at 1600*g* for 10 min at 4 °C. Aliquoted plasma was stored at −80 °C, and centrifuged at 1600*g* before extraction of cfDNA.

Circulating DNA was extracted from 1–2 mL of plasma from 83 melanoma patients on 2 occasions: in 2009 with an established in-house phenol-chloroform-based method *(20)*, and in 2016 with the QIAamp circulating nucleic acid kit (QIAGEN®), according to the manufacturer protocol but with a 200 μL elution volume. cfDNA concentrations were quantified by SYBR green quantitative real-time PCR (Life Technologies), based on an 87-bp amplicon in the glyceraldehyde-3-phosphate dehydrogenase gene. DNA was extracted from patient tumor and blood lymphocyte samples with use of Qiagen formalin-fixed paraffin-embedded (FFPE) and Blood DNA kits, respectively, and was quantified with QUBIT double-stranded DNA BR fluorometric quantification. Tumor FFPE material was available for 47 cases, and 21 of these (45%) were previously found to be *BRAF*[10] *V600E* mutation positive by Sanger sequencing (see Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue9).

WHOLE-GENOME LIBRARY PREPARATION AND HIGHLY PARALLEL SEQUENCING

Tumor and lymphocyte DNA samples were sheared to achieve a target size of 200 bp. DNA libraries were prepared with the Ultra II library-prep kit (New England Biolabs®), in accordance with the manufacturer's protocol. Libraries were multiplexed in equimolar amounts at 48 samples per flow cell of the Illumina HiSeq-2500®. Samples of melanoma MDA-MB-435 cell line DNA were included in each sequencing run to assess interrun variability. The software CNANORM was used to generate copy-number ratios for cfDNA and tumor DNA for 1-Mb windows across the genome (see Methods in the online Data Supplement).

CALCULATION OF COPY-NUMBER ABERRATION AND EXTREME CNAs

Z scores for each 1-Mb window were calculated by standardizing the copy-number ratio to the mean copy-number ratio from a cohort of 20 healthy controls (mean

[10] Human Gene: *BRAF*, B-Raf protooncogene, serine/threonine kinase.

ratio, 1.00059; SD, 0.0049). These controls were relatives (partners or siblings) of melanoma patients recruited to the Markers of Relapse in Melanoma study (REC14/YH/1275). A copy-number aberration score (CNAS) was then calculated for each cfDNA sample by summing the square of Z scores across the genome, as described by Heitzer et al. *(21)*. In addition, we estimated a score based on high-amplitude aberrations ("eCNAS"), by summing the squares of the 95th to 99th percentile of the standardized Z scores, an approach similar to that used in the plasma genomic abnormality score *(22)*.

STATISTICAL ANALYSES

Univariable logistic regression analyses comparing active and resected melanoma included log CNAS, log eCNAS, log cfDNA concentration (ng/mL plasma), stage at recruitment (coded as binary I and II vs III and IV), age at recruitment, sex, and *BRAF V600E* mutation status. Significant variables ($P < 0.05$) were included in a multivariable logistic regression, and predicted probabilities based on the model were used to generate ROC curves.

Patient deaths were determined from hospital records, up to a last date of follow-up of August 16, 2017. Survival times were plotted with the Kaplan–Meier method, and hazard ratios derived in a Cox regression framework with adjustment for time from diagnosis to recruitment. Tumor-related factors significant at the $P < 0.05$ level were included in a multivariable survival analysis. All analyses were implemented in Stata (version 12), and statistical tests were 2-sided.

Results

CHARACTERISTICS OF MELANOMA PATIENTS WITH ACTIVE OR RECENTLY EXCISED DISEASE

The demographic and clinical characteristics of the study participants are shown in Table 1 in the online Data Supplement. Over an 18-month period, 108 eligible patients were approached and 83 (77%) recruited, the majority (75%) through oncology clinics. Thirty-nine cases had recently excised disease, with median (range) time since excision of 25 (10–71) days. Forty-four cases had active unresected melanoma, with 95% of these being stage III or stage IV disease at the time of recruitment (see Table 1 in the online Data Supplement). In total, 28 patients (34%) had stage I or II disease and 55 (66%) had stage III or IV disease. Twenty-eight (34%) of the 83 patients remained alive at the data analysis cutoff.

CIRCULATING cfDNA CONCENTRATIONS WERE STABLE AFTER LONG-TERM PLASMA STORAGE AND WERE HIGHER IN PATIENTS WITH ACTIVE DISEASE

The yields of cfDNA extracted from stored blood plasma samples at 2 time points 7 years apart were highly comparable (Spearman correlation for log cfDNA concentration = 0.75, $P < 0.0001$; see Fig. 1 in the online Data Supplement), with a median drop in yield of 2.8 (interquartile range, 0.6–6.2) ng/mL plasma over that period. The drop in yield was slightly higher in those with stage III or IV disease than in those with stage I or II disease ($P = 0.02$), but there was no differential drop between active or resected disease ($P > 0.05$). We carried out a pilot analysis to demonstrate that the *BRAF V600E* mutation could be detected in the cfDNA and found that 14 of 76 (18%) cfDNA samples successfully amplified were mutation positive, including 6 with recently resected disease.

As expected, melanoma patients (n = 83) had higher concentrations of cfDNA than healthy controls (n = 75) ($P = 0.004$), and patients with active disease (n = 44) had higher concentrations of cfDNA (median, 11.5; range, 0.9–114.3 ng/mL) than those who had previously excised disease (n = 39; median, 5.8; range, 1.4–19.6 ng/mL; $P = 0.004$; see Fig. 2 and Table 2 in the online Data Supplement). The cfDNA concentrations in those with recently excised disease were similar to those in healthy controls (n = 75; median, 5.3; range, 0.7–33.7 ng/mL; $P = 0.45$; see Fig. 2 in the online Data Supplement).

LOW-COVERAGE COPY-NUMBER ANALYSIS OF cfDNA

All 83 cfDNA samples were successfully sequenced, with a median of 15.3 million reads per sample (range, 6.6–36.1 million reads). On average, 89% of reads aligned to the human reference genome GRCh38, with a range of 0.1 to 0.9× coverage of the genome (median, 0.4× coverage). Genomic DNA extracted from the melanoma cell line MDA-MB-435 was analyzed on 10 sequencing runs, and the eCNAS and CNAS were calculated to quantify the gains and losses genome wide (see Fig. 3 in the online Data Supplement). The CNAS yielded highly reproducible results (CV = 0.098), although the eCNAS was more variable (CV = 0.60). To estimate assay sensitivity, serial dilutions of 12 ng each of MDA-MB-435 DNA in lymphocyte DNA were analyzed. Copy-number aberrations could be detected as a raised CNAS above baseline at dilutions as low as 6.25% cell line DNA (see Fig. 4 in the online Data Supplement).

Fig. 1 shows representative examples of CNA plots for cfDNA compared to matched tumor FFPE DNA. The blood sample for patient 60 was taken 1 year after the date of the tumor FFPE sample, and, consistent with active disease and an evolving tumor, some CNAs are present in both sample types, but in addition CNAs are lost and gained in the cfDNA sample compared to the earlier tumor FFPE sample. The blood sample for patient 5 was taken only 28 days after resection of the primary tumor, and the cfDNA sample shows some remaining CNA despite the recent resection of the tumor. This

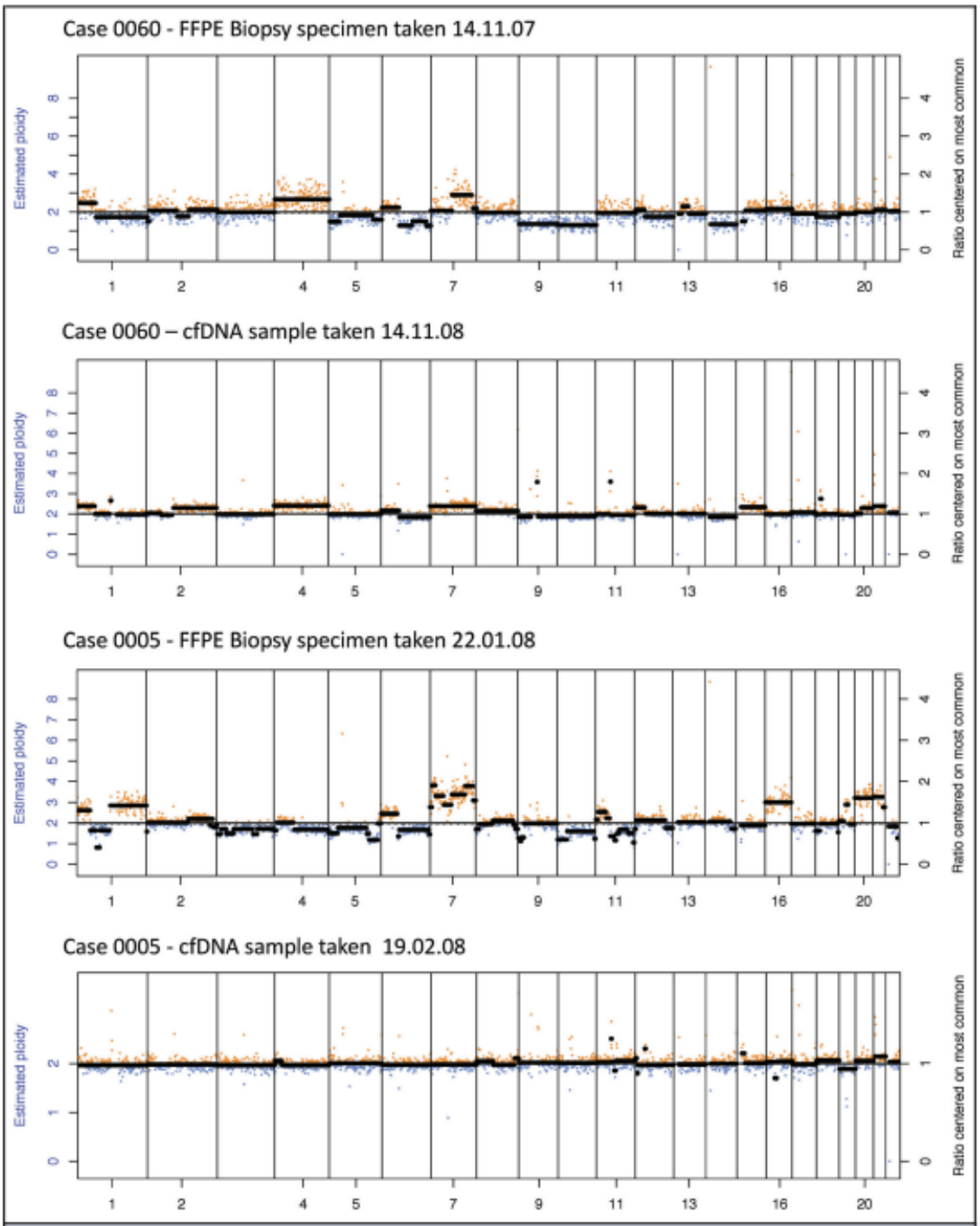

Fig. 1. Copy-number aberration plot derived from cfDNA extracted from a patient with active melanoma reflects some aberrations seen in the original tumor biopsy specimen.

Examples of copy-number aberration plots for cfDNA compared to matched tumor FFPE DNA, in a case with active disease (case 60; top Fig.) and resected disease (case 5; bottom Fig.).

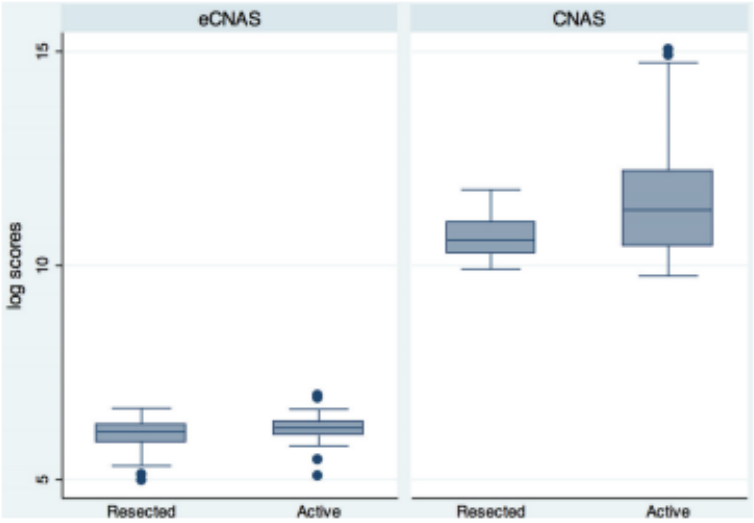

Fig. 2. The CNASs for cfDNA were higher for cases with active disease than those with recently excised disease, but the eCNAS showed no significant difference between the 2 groups.

Box and whisker plot of the log scores (log eCNAS on the left, log CNAS on the right) in cases with active melanoma compared to those with recently excised disease.

observation is consistent with the presence of residual disease and this patient's subsequent relapse. The CNASs for all patients are listed in Table 2 in the online Data Supplement.

cfDNA CNASs CAN DISTINGUISH ACTIVE MELANOMA FROM RECENTLY EXCISED DISEASE

CNASs for cfDNA were higher for cases with active disease than for those with recently excised disease ($P = 0.0011$). However, there was no significant difference in the eCNASs between the 2 groups ($P = 0.14$; Fig. 2).

Univariable logistic regression analysis comparing patients with active melanoma to those with resected disease identified log CNAS, log cfDNA concentration, and disease stage as significant predictors for the presence of active disease (Table 1). To explore the relationship between CNAS and the presence of active disease, patients were grouped according to their CNAS quartile. Those in the highest quartile had a significantly increased risk of active disease (OR, 46.7; 95% CI, 5.0–431.6; $P = 0.001$) compared to those in the lower 3 quartiles. A multivariable logistic regression analysis, adjusting for cfDNA concentration and stage and including a binary coding of logCNAS (above and below the 75th percentile), showed that CNAS was a significant predictor of active disease (OR, 17.4; 95% CI, 1.6–190.9; $P = 0.019$; area under ROC curve = 0.90; Table 1).

THE cfDNA CNAS IS ASSOCIATED WITH PATIENT SURVIVAL

Univariable Cox regression analysis identified log CNAS, log cfDNA concentration, disease stage, *BRAF V600E* status, and the presence of active disease as significant predictors of overall survival in melanoma patients (Table 2). Mortality was higher among those with log CNAS above the 75th percentile, with HR of 7.7 (95% CI, 4.2–14.2; $P < 0.0001$; Fig. 3). Median overall survival for those with CNASs above the 75th percentile was 13.5 months, compared to 73.8 months for those with scores <75th percentile. This association remained significant after adjusting for the other variables (HR, 3.4; 95% CI, 1.5–7.9; $P = 0.005$) (Table 2).

Discussion

This study aimed to use low-coverage copy-number analysis in cfDNA to detect active disease in melanoma. This assay does not require any prior knowledge about which mutations are present in the tumor, unlike methods that track tumor-specific mutations. We have successfully generated copy-number profiles, using low-coverage whole-genome sequencing with very low-input DNA, thus demonstrating the utility of this de novo approach, which could have clinical applicability in a variety of other cancer subtypes. While we have presented representative examples of CNA plots in cfDNA compared with matched FFPE tumor DNA in Fig. 1, we did find that for

Table 1. Logistic regression analysis of predictors of active disease.

	OR[a]	*P* value	95% CI	AUC
Univariable analysis				
log CNAS	3.1	0.002	1.5–6.2	0.71
log eCNAS	3.1	0.099	0.8–12.2	0.59
log cfDNA level	4.2	<0.0001	1.9–9.2	0.75
cfDNA category[b]	12.8	0.001	2.7–60.0	0.68
Stage	42	<0.0001	8.8–201.3	0.81
Age	1.0	0.79	0.96–1.02	0.53
Sex	1.2	0.69	0.5–2.9	0.52
BRAF status	1.3	0.59	0.5–3.2	0.53
	OR	***P* value**	**95% CI**	–
Multivariable analysis				
CNAS category[c]	17.4	0.019	1.6–190.90	–
cfDNA category[b]	2.7	0.26	0.5–15.1	–
Stage	24.6	<0.0001	4.4–136.9	–

[a] AUC, area under the ROC curve; OR, odds ratio.
[b] log cfDNA level above and below the 75th percentile.
[c] log CNAS above and below the 75th percentile.

some cases with active disease, the cfDNA CNA plots showed a paucity of copy-number aberrations, despite the corresponding tumor DNA harboring multiple aberrations. While some of this may be explained by the "dilution effect" (tumor-derived aberrant DNA occurring in the background of predominantly wild-type circulating DNA makes smaller aberrations more difficult to detect in cfDNA), this represents a limitation of this approach. The modest limit of detection (6.25%) of our low-coverage approach suggests it is unlikely to be sensitive enough to detect the presence of active disease in very early-stage disease (in which the fraction of tumor-derived DNA is generally below 1%). However, the promise of this approach in detecting relapsed metastatic disease (which is often of moderately high volume in terms of disease burden) should be highlighted. The sensitivity of this approach can be improved by combining it with targeted point-mutation detection assays, given the high prevalence of BRAF and NRAS mutations in melanoma. The detection rate of the BRAF V600E mutation in our cfDNA samples (18%) is much lower than the expected prevalence of BRAF-mutated melanoma, reflecting the limited sensitivity of the technique used (Sanger sequencing). The use of more sensitive techniques, such as droplet digital PCR, can significantly increase the detection rate.

Table 2. Cox regression analysis of predictors of survival.

	HR[a]	*P* value	95% CI
Univariable Cox regression			
log CNAS	2.1	<0.0001	1.6–2.7
log eCNAS	2.1	0.12	0.8–5.4
log cfDNA level	5.5	<0.0001	3.4–8.9
Stage	9.4	<0.0001	4.2–21.0
BRAF status	2.6	0.001	1.4–4.5
Sex	0.7	0.23	0.4–1.3
Active/resected	9.3	<0.0001	4.9–17.3
Multivariable Cox regression			
CNAS category[b]	3.4	0.005	1.5–7.9
cfDNA category[c]	2.3	0.032	1.1–4.8
Stage	3.2	0.016	1.2–8.2
BRAF status	3.2	<0.0001	1.7–6.1
Active/resected	4.7	<0.0001	2.1–10.6

[a] HR, hazard ratio.
[b] log CNAS above and below the 75th percentile.
[c] log cfDNA level above and below the 75th percentile.

We demonstrated that cfDNA is relatively stable in plasma that is appropriately processed and stored at −80 °C, for up to 7 years, with a low median decrease in yield over that period. We have previously shown that the phenol-chloroform-based extraction method gave higher cfDNA yields than kit-based extraction, (although kit-based approaches are more amenable to high throughput) *(20)*. It is likely that the different extraction meth-

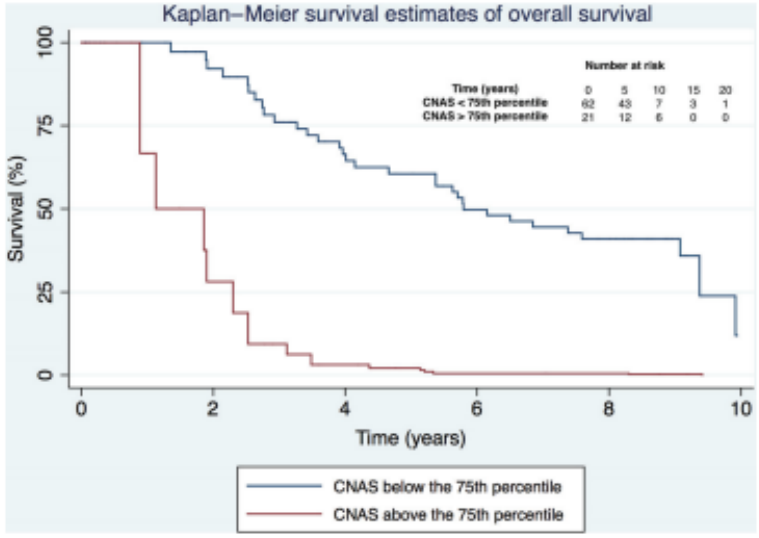

Fig. 3. Mortality was higher in patients with log CNAS above the 75th percentile.
Kaplan–Meier survival graph comparing survival for patients with log CNAS above (red) and below (blue) the 75th percentile. Inset shows the number of patients at risk at each time point.

ods used on the 2 occasions could also be contributing to the difference in yields, suggesting that the median drop of 2.8 ng/mL could be an overestimate of the effects of storage time. As others have shown *(11–13, 23)*, we found higher concentrations of cfDNA in melanoma patients, in particular in those with active disease, than in healthy controls. It is well established that cfDNA concentrations can be increased in pathological processes other than cancer and after recent surgery; therefore, cfDNA concentration analysis alone is not a sufficient indicator of the presence of active melanoma. In our cohort, the median duration from surgery to sampling was 25 days; therefore, given the short half-life of cfDNA, any temporary rise in cfDNA concentrations due to surgery would have recovered by the time of blood sampling.

Comparison of the CNAS and eCNAS methods of scoring demonstrated the CNAS to be more reproducible. The CNAS is a genome-wide score (Heitzer et al. *(21)*), which captures the entire spectrum of copy-number aberrations regardless of amplitude or length of aberration. In contrast, the eCNAS (similar to the PGA score *(22, 24)*) focuses on the 95th–99th percentile of copy-number ratios, ignoring the potentially significant contribution made by smaller aberrations, which may be quite numerous, and it may also exclude some large aberrations.

The representative cfDNA CNA plots in Fig. 1 illustrate that there may be potential to use this approach to track disease evolution and progression. However, the CNA plots for some cases with active disease showed few copy-number aberrations compared to the corresponding primary tumor DNA. This can arise because of reduction in tumor burden after treatment and the dilution of the circulating tumor DNA in the plasma, making detection of CNA more challenging. Despite this issue, the CNASs for all cases in the present study were above the limit of detection shown in Fig. 4 in the online Data Supplement, allowing meaningful interpretation of the results.

Our results showed that the CNASs for cases with active disease were significantly higher than those with resected disease ($P = 0.0011$). We note, however, that there is a wide variation in the CNAS, with some overlap between the scores for active and resected cases. It is possible that the presence of microscopic residual disease in some patients with resected disease may be contributing to higher scores in those patients, as suggested by the CNA profile for patient 5, shown in Fig. 1.

We have also established a multivariable model (based on the CNAS, cfDNA concentration, and disease stage) that is a good discriminator of active disease, with area under ROC curve of 0.90. There have been a number of recent publications that have used cfDNA to differentiate melanoma cases from controls *(9)* or to assess treatment response in melanoma *(25, 26)*. These studies used either cfDNA concentrations, mutation analysis (predominantly BRAF), or cfDNA integrity indices (or a combination of these approaches) in their analyses. We

further demonstrated, using a multivariable Cox regression analysis correcting for other factors known to affect survival, that high CNAS scores (>75th percentile) were associated with significantly poorer survival. Although our study was not powered to determine differences by disease stage, these results are comparable to those of others *(27, 28)* who observed that cfDNA predicts relapse and survival in stage II and III melanoma patients. It would be of value to explore the effects of stage in a larger cohort.

The study has some limitations. The modest limit of detection (6.25%) suggests that the CNAS is unlikely to be sensitive enough to detect very early-stage disease (in which the fraction of tumor-derived DNA is generally below 1%). To improve sensitivity, the CNA approach could be combined with other genomic markers such as mutation burden in cfDNA. The low detection rate of the BRAF V600E mutation in our cfDNA (18% compared to 45% in tumor material) reflects the limited sensitivity of the technique used for this pilot (Sanger sequencing). A further limitation of this study is the relatively small sample size of the study cohort. While the study had over 90% power to detect the observed difference in mean log CNASs between active and resected disease, a larger replication cohort is required to validate the results and fully assess the performance of the CNAS while controlling for other relevant factors including disease stage and treatment.

To our knowledge, this is the first application of a cfDNA copy-number approach to predict active disease and survival in melanoma. Furthermore, our observed associations of CNAS with these traits suggest that longitudinal analysis of copy-number profiles in melanoma patients is warranted. The CNAS may act as an early marker of relapsed disease, which could be applied in both research and clinical settings.

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