This is a repository copy of *Genome-wide analysis of circulating cell-free DNA copy number detects active melanoma and predicts survival* .

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/131712/

Version: Accepted Version

Article:

Silva, S., Danson, S., Teare, M.D. et al. (12 more authors) (2018) Genome-wide analysis of circulating cell-free DNA copy number detects active melanoma and predicts survival. Clinical Chemistry, 64 (9). pp. 1338-1346. ISSN 0009-9147

https://doi.org/10.1373/clinchem.2018.290023

© 2018 American Association for Clinical Chemistry. This is an author produced version of a paper subsequently published in Clinical Chemistry. Uploaded in accordance with the publisher's self-archiving policy.

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk/

1 **Title: Genome-wide analysis of circulating cell-free DNA copy number detects active**
2 **melanoma and predicts survival.**
3
4 **Running title**: CfDNA detects melanoma and predicts survival
5
6 **Authors:** S Silva*[1], S Danson*[1], MD Teare[2], F Taylor[1], J Bradford[3], AJG McDonagh[4], A Salawu[1],
7 G Wells[5], G J Burghel[6], I Brock[7], D Connley[7], H Cramp[7], D Hughes[8], N Tiffin[8], A Cox*[7].
8 ***these authors contributed equally to this work**
9
10 **Affiliations list:**
11 [1] Academic Unit of Clinical Oncology and Sheffield Experimental Cancer Medicine Centre,
12 University of Sheffield, Sheffield, S10 2SJ, UK; [2] School of Health and Related Research,
13 University of Sheffield, Sheffield, S1 4DA, UK; [3] Academic Unit of Molecular Oncology Sheffield
14 Medical School, University of Sheffield, Sheffield, S10 2RX, UK; current addres: Almac
15 Diagnostics, 19 Seagoe Industrial Estate, Craigavon, BT63 5QD, UK; [4] Department of
16 Dermatology, Royal Hallamshire Hospital, Sheffield, S10 2JF, UK; [5] Sheffield Genetic Diagnostics
17 Group, Sheffield Children's NHS Foundation Trust, Western Bank Sheffield, S10 2TH, UK; [6] The
18 Manchester Centre for Genomic Medicine, Manchester University NHS Foundation Trust,
19 Manchester, M13 9WL, UK**;** [7] Academic Unit of Molecular Oncology, Sheffield Medical School,
20 University of Sheffield, Sheffield, S10 2RX, UK**;** [8] Department of Histopathology, Royal Hallamshire
21 Hospital, Sheffield, S10 2JF, UK
22
23 **Corresponding author**:
24 Dr Shobha Silva
25 Weston Park Hospital,
26 Whitham Road,
27 Sheffield, S10 2SJ,
28 UK
29 Phone: + 44 114 226 5000
30 Fax: + 44 114 226 5364
31 email: s.silva@sheffield.ac.uk
32
33 **Key words:** circulating cell-free DNA, copy-number, melanoma, highly parallel sequencing

.

List of abbreviations:

BRAF	B-Raf proto-oncogene, serine/threonine kinase
MEK	mitogen-activated protein kinase kinase 1
cfDNA	circulating cell-free DNA
CTLA4	cytotoxic T-lymphocyte associated protein 4
PD1	programmed cell death 1
LDH	lactate dehydrogenase
ctDNA	tumour-derived DNA
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Mb	mega-base
FFPE	formalin-fixed paraffin-embedded

Abstract

Background:

A significant number of melanoma patients develop local or metastic 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% confidence interval 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 Receiver-Operator Characteristic curve of 0.90). Additionally, mortality was higher in those with CNAS above the 75th percentile, compared to those with lower scores (HR 3.4, 95%CI 1.5 – 7.9, p=0.005), adjusting for stage of disease, disease status (active/resected), BRAF status and cfDNA level.

Conclusion:

This study demonstrates the potential of a de novo approach utilising 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.

78 **INTRODUCTION**

79 Melanoma is the most aggressive form of skin cancer, and is increasing in incidence throughout
80 the developed world, with around 15,900 new cases and 2,285 deaths from melanoma in the UK in
81 2015 [1]. Prognosis is known to correlate with Breslow thickness, the presence of tumour ulceration
82 and mitotic rate [2]. Patients with thicker melanomas (defined as >2mm with ulceration or >4mm
83 without ulceration) have >50% chance of relapse [3].
84
85 The cornerstone of treatment for loco-regional disease is surgery. However, a significant number of
86 patients later develop local or systemic recurrence. Advanced disease is usually fatal. Until 2011,
87 standard palliative chemotherapy was single agent dacarbazine with a response rate of just 10-
88 15%. Response rates have since improved with the advent of BRAF (B-Raf proto-oncogene,
89 serine/threonine kinase) inhibitors, mitogen-activated protein kinase kinase 1 (MEK) inhibitors [4,5]
90 and immune checkpoint inhibitors that target cytotoxic T-lymphocyte associated protein 4 (CTLA4)
91 and programmed cell death 1 (PD1) [6,7]. Early detection of metastatic relapse (if possible prior to
92 onset of clinical symptoms) is vital to maximise the benefit from the new therapies. At the present
93 time this relies heavily on imaging, but a blood test has the potential to be more sensitive and cost-
94 effective. Although the lactate dehydrogenase (LDH) blood test has been previously used as a
95 prognostic marker, it is not a clinically useful marker of disease status [2] and better biomarkers of
96 relapse need to be identified.
97
98 Plasma cfDNA provides an easily accessible source of tumour-derived DNA (ctDNA). Raised levels
99 of cfDNA are seen in many clinical conditions, when compared with healthy controls [8 -10], and
100 ctDNA has been detected in the plasma of patients with melanoma [11]. An increase in the overall
101 amount of tumour-derived DNA in the plasma is frequently correlated with metastatic disease and
102 relapse, and studies have shown that that tumour specific circulating DNA levels correlate with
103 tumour burden and relapse following treatment [12-14]. Somatic genomic aberrations characteristic
104 of the tumour DNA can frequently be seen in the ctDNA [15]. A number of high-profile publications
105 have demonstrated the potential utility of using genomic profiling techniques to provide biomarkers
106 of metastatic disease and acquired resistance that can capture tumour evolution and heterogeneity
107 [16-18]. Copy number abberations (comprising deletions or duplications of large segments of DNA,
108 ranging in size from a few kilobases to entire chromosomes) are abundant in malignant melanoma
109 [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, United Kingdom over an eighteen month period. Eligible patients had a confirmed diagnosis of cutaneous melanoma of any stage (AJCC melanoma of the skin staging, 7th edition), and no chemotherapy exposure within the previous four weeks. Patients with either active unresected disease, or prior resection of their primary tumour or metastatic disease with clear margins within four weeks of recruitment, were included. Healthy control subjects were co-habiting 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 hours of collection, by centrifugation at 800g for 10 minutes at 4℃, followed by ce ntrifugation at 1600g for 10 minutes at 4℃. Aliquotted plasma was stored at -80℃, and centrifuged at 16 00g prior to extraction of cfDNA.

Circulating DNA was extracted from 1-2ml plasma from 83 melanoma patients on two occasions; in 2009 using an established in-house phenol-chloroform-based method[20] and in 2016 using the QIAamp circulating nucleic acid kit (QIAGEN®) according to the manufacturer protocol, but using a 200ul elution volume. cfDNA levels were quantified by SYBR green quantitative real-time PCR (Life

142 Technologies), based on an 87bp amplicon in the GAPDH gene. DNA was extracted from patient
143 tumour and blood lymphocyte samples using Qiagen FFPE and Blood DNA kits respectively, and
144 was quantified using QUBIT dsDNA BR fluorometric quantitation. Tumour FFPE material was
145 available for 47 cases, and 21 of these (45%) were previously found to be BRAF V600E mutation
146 positive by Sanger sequencing (Supplemental Methods).
147
148 Whole-genome library preparation and highly-parallel sequencing
149 Tumour and lymphocyte DNA samples were sheared to achieve a target size of 200bp. DNA
150 libraries were prepared using the Ultra II library-prep kit (New England Biolabs®), following the
151 manufacturer's protocol. Libraries were multiplexed in equimolar amounts at 48 samples per flow
152 cell of the Illumina HiSeq-2500®. Samples of melanoma MDA-MB-435 cell line DNA were included
153 in each sequencing run to assess inter-run variability. The software CNANORM was used to
154 generate copy number ratios for cfDNA and tumour DNA for 1 Mb windows across the genome
155 (see Supplemental Methods).
156
157 Calculation of copy number aberration and extreme copy number aberration scores
158 Z-scores for each 1Mb window were calculated by standardizing the copy number ratio to the mean
159 copy-number ratio from a cohort of 20 healthy controls (mean ratio 1.00059, standard deviation
160 0.0049). These controls were relatives (partners or siblings) of melanoma patients recruited to the
161 Markers of Relapse in Melanoma study (REC14/YH/1275). A CNAS was then calculated for each
162 cfDNA sample by summing the square of Z-scores across the genome, as described by Heitzer et
163 al [21]. In addition, we estimated a score based on high-amplitude aberrations ("eCNAS"), by
164 summing the squares of the 95-99th percentile of the standardized z-scores, an approach similar to
165 that used in the plasma genomic abnormality score [22].
166
167 Statistical analyses
168 Univariable logistic regression analyses comparing active and resected melanoma included log
169 CNAS, log eCNAS, log cfDNA level (ng/ml plasma), stage at recruitment (coded as binary I/II vs.
170 III/IV), age at recruitment, gender and BRAF V600E mutation status. Significant variables ($p<0.05$)
171 were included in a multivariable logistic regression, and predicted probabilities based on the model
172 were used to generate Receiver-Operator Characteristic curves.

173 Patient deaths were determined from hospital records, up to a last date of follow-up of 16.08.17.
174 Survival times were plotted using the Kaplan Meier method, and hazard ratios derived in a Cox
175 regression framework with adjustment for time from diagnosis to recruitment. Tumour-related
176 factors significant at the p<0.05 level were included in a multivariable survival analysis All analyses
177 were implemented in Stata (version 12) and statistical tests were two-sided.
178

179 **RESULTS**

180

181 **Characteristics of melanoma patients with active or recently-excised disease**

182 The demographic and clinical characteristics of the study subjects are shown in Supplemental
183 Table 1. Over an 18 month period, 108 eligible patients were approached and 83 (77%) recruited,
184 the majority (75%) through oncology clinics. Thirty-nine cases had recently excised disease, with
185 median (range) time since excision of 25 (10-71) days. Forty-four cases had active unresected
186 melanoma with 95% of these being stage III or stage IV disease at the time of recruitment
187 (Supplemental Table 1). In total, 28 patients (34%) had stage I/II disease and 55 (66%) had stage
188 III/IV disease. Twenty-eight (34%) of the 83 patients remained alive at the data analysis cut-off.
189

190 **Circulating cfDNA levels were stable after long-term plasma storage and were higher in**
191 **patients with active disease**

192 The yields of cfDNA extracted from stored blood plasma samples at two timepoints 7 years apart
193 were highly comparable (Spearman's correlation for log cfDNA level=0.75, p<0.0001;
194 Supplemental Figure 1), with a median drop in yield of 2.8 (interquartile range 0.6-6.2) ng/ml
195 plasma over that time period. The drop in yield was slightly higher in those with stage III/IV disease
196 compared to those with stage I/II disease (p=0.02), but there was no differential drop between
197 active or resected disease (p>0.05). We carried out a pilot analysis to demonstrate that the BRAF
198 V600E mutation could be detected in the cfDNA, and found that 14 of 76 (18%) cfDNA samples
199 successfully amplified were mutation positive, including 6 with recently resected disease.
200

201 As expected, melanoma patients (n= 83) had higher levels of cfDNA compared to control subjects
202 (n= 75) (p=0.004), and patients with active disease (n=44) had higher levels of cfDNA (median
203 (range) 11.5 (0.9-114.3) ng/ml) compared to those who had previously excised disease (n=39;
204 median (range) 5.8 (1.4-19.6) ng/ml; p=0.004; Supplemental Figure 2, Supplemental Table 2). The

205 cfDNA levels in those with recently excised disease were similar to those in healthy controls (n=75;
206 median (range) 5.3 (0.7-33.7) ng/ml; p=0.45; Supplemental Figure 2).
207
208 **Low coverage copy number analysis of cfDNA**
209 All 83 cfDNA samples were successfully sequenced, with a median of 15.3 million reads per
210 sample (range 6.6–36.1 million reads). On average, 89% of reads aligned to the human reference-
211 genome GRCh38, with a range of 0.1-0.9X coverage of the genome (median 0.4X coverage).
212 Genomic DNA extracted from the melanoma cell line MDA-MB-435 was analysed on 10
213 sequencing runs, and the eCNAS and CNAS were calculated to quantify the gains and losses
214 genome-wide (Supplemental Figure 3). The CNAS yielded highly reproducible results (coefficient of
215 variation (CV)=0.098), although the eCNAS score was more variable (CV=0.60). To estimate assay
216 sensitivity, 12ng each of serial dilutions of MDA-MB-435 DNA in lymphocyte DNA were analysed.
217 Copy-number aberrations could be detected as a raised CNAS above baseline at dilutions as low
218 as 6.25% cell-line DNA (Supplemental Figure 4).
219
220 Figure 1 shows representative examples of CNA plots for cfDNA compared to matched tumour
221 FFPE DNA. The blood sample for patient 60 was taken 1 year after the date of the tumour FFPE
222 sample, and, consistent with active disease and an an evolving tumour, some CNA are present in
223 both sample types, but in addition CNA are lost and gained in the cfDNA sample compared to the
224 earlier tumour FFPE sample. The blood sample for patient 5 was taken only 28 days after resection
225 of the primary tumour, and the cfDNA sample shows some remaining CNA despite the recent
226 resection of the tumour. This observation is consistent with the presence of residual disease, and
227 this patient's subsequent relapse. The CNAS for all patients are listed in Supplemental Table 2.
228
229 **CfDNA CNAS can distinguish active melanoma from recently-excised disease**
230 CNAS for cfDNA were higher for cases with active disease compared to those with recently-
231 excised disease (p=0.0011). However there was no significant difference in the eCNAS scores
232 between the two groups (p=0.14; Figure 2).
233
234 Univariable logistic regression analysis comparing patients with active melanoma to those with
235 resected disease identified log CNAS, log cfDNA level and disease stage as significant predictors
236 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 (95% CI) 46.7 (5.0, 431.6), p=0.001) compared to those in the lower three quartiles. A multivariable logistic regression analysis, adjusting for cfDNA level 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 (95% CI) 17.4 (1.6, 190.9), p=0.019, area under ROC=0.90, Table 1).

The cfDNA CNAS is associated with patient survival

Univariable Cox regression analysis identified log CNAS, log cfDNA level, 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 (95% CI) of 7.7 (4.2,14.2); p<0.0001, Figure 3). Median overall survival for those with CNAS 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 (95%CI) 3.4, (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 tumour, unlike methods which track tumour-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 sub-types. While we have presented representative examples of CNA plots in cfDNA compared with matched FFPE tumour DNA in Figure 1, we did find that for some cases with active disease, the cfDNA CNA plots showed a paucity of copy-number aberrations, despite the corresponding tumour DNA harbouring multiple aberrations. While some of this may be explained by the 'dilution effect' (tumour-derived aberrant DNA occurring in the background of predominantly wild-type circulating DNA makes smaller aberrations more dificult 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 (where the fraction of tumour-derived DNA is generally

269 below 1%). However, the promise of this approach in detecting relapsed metastatic disease (which
270 is often of moderate-high volume in terms of disease burden) should be highlighted. The sensitivity
271 of this approach can be improved by combining it with targeted point-mutation detection assays,
272 given the high prevalence of BRAF/NRAS mutations in melanoma. The detection rate of the BRAF
273 V600E mutation in our cfDNA samples (18%) is much lower than the expected prevalence of
274 BRAF-mutated melanoma, reflecting the limited sensitivity of the technique used (Sanger
275 sequencing). The use of more sensitive techniques, such as droplet digital PCR, can significantly
276 increase the detection rate.
277
278 We demonstrated that cfDNA is relatively stable in plasma that is appropriately processed and
279 stored at -80℃, for up to 7 years, with a low median decrease in yield over that ti me period. We
280 have previously shown that the phenol-chloroform based extraction method gave higher cfDNA
281 yields compared to kit-based extraction, (although kit-based approaches are more amenable to
282 high-throughput) [20]. It is likely that the different extraction methods used on the two occasions
283 could also be contributing to the difference in yields, suggesting that the median drop of 2.8 ng/ml
284 could be an over-estimate of the effects of storage time. As others have shown [11-13,23], we
285 found higher levels of cfDNA in melanoma patients, in particular in those with active disease,
286 compared to controls. It is well established that cfDNA levels can be elevated in other pathological
287 processes, other than cancer, and following recent surgery, therefore cfDNA level analysis alone is
288 not a sufficient indicator of the presence of active melanoma. In our cohort, the median duration
289 from surgery to sampling was 25 days, so, given the short half-life of cfDNA, any temporary rise in
290 cfDNA levels due to surgery would have recovered by the time of blood-sampling.
291
292 Comparison of the CNAS and eCNAS methods of scoring demonstrated the CNAS to be more
293 reproducible. The CNAS is a genome-wide score (Heitzer et al [21]) which captures the entire
294 spectrum of copy-number aberrations regardless of amplitude or length of aberration. In contrast,
295 the eCNAS score (similar to the PGA score [22,24]) focuses on the 95-99th percentile of copy-
296 number ratios, ignoring the potentially significant contribution made by smaller aberrations, which
297 may be quite numerous, and it may also exclude some large aberrations.
298
299 The representative cfDNA CNA plots in Figure 1 illustrate that there may be potential to use this
300 approach to track disease evolution and progression. However the CNA plots for some cases with

301 active disease showed few copy-number aberrations compared to the corresponding primary
302 tumour DNA. This can arise due to reduction in tumour burden following treatment, and the dilution
303 of the circulating tumour DNA in the plasma, making detection of CNA more challenging. Despite
304 this issue, the CNAS for all cases in the present study were above the limit of detection shown in
305 Supplemental Figure 4, allowing meaningful interpretation of the results.
306
307 Our results showed that the CNAS for cases with active disease were significantly higher than
308 those with resected disease (p=0.0011). We note however that there is a wide variation in the
309 CNAS, with some overlap between the scores for active and resected cases. It is possible that the
310 presence of microscopic residual disease in some patients with resected disease may be
311 contributing to higher scores in those patients, as suggested by the CNA profile for patient 5 shown
312 in Figure 1.
313
314 We have also established a multivariable model (based on the CNAS, cfDNA level and disease
315 stage) which is a good discriminator of active disease, with area under ROC of 0.90. There have
316 been a number of recent publications which have utilised cfDNA to differentiate melanoma cases
317 from controls [9] or to assess treatment response in melanoma [25,26]. These studies utilised
318 either cfDNA levels, mutation analysis (predominantly BRAF) or cfDNA integrity indices (or a
319 combination of these approaches) in their analyses. We further demonstrated, using a multivariable
320 Cox regression analysis correcting for other factors known to affect survival, that high CNAS scores
321 (>75th percentile) were associated with significantly poorer survival. Although our study was not
322 powered to determine differences by disease stage,, these results are comparable to those of
323 others [27, 28] who observed that cfDNA predicts relapse and survival in stage II/III melanoma
324 patients. It would be of value to explore the effects of stage in a larger cohort.
325
326 The study has some limitations. The modest limit of detection (6.25%) suggests that the CNAS is
327 unlikely to be sensitive enough to detect very early-stage disease (where the fraction of tumour-
328 derived DNA is generally below 1%). In order to improve sensitivity, the CNA approach could be
329 combined with other genomic markers such as mutation burden in cfDNA. The low detection rate of
330 the BRAF V600E mutation in our cfDNA (18% compared to 45% in tumour material) reflects the
331 limited sensitivity of the technique used for this pilot (Sanger sequencing). A further limitation of this
332 study is the relatively small sample size of the study cohort. While the study had over 90% power to

333 detect the observed difference in mean log CNAS between active and resected disease, a larger
334 replication cohort is required to validate the results and fully assess the performance of the CNAS
335 controlling for other relevant factors including disease stage and treatment.
336
337 To our knowledge, this is the first application of a cfDNA copy-number approach to predict active
338 disease and survival in melanoma . Furthermore, our observed associations of CNAS with these
339 traits suggest that longitudinal analysis of copy-number profiles in melanoma patients is warranted.
340 The CNAS may act as an early marker of relapsed disease, that could be applied in both research
341 and clinical settings.
342
343 **ACKNOWLEDGEMENTS:** We are grateful to all the patients and their relatives who took part in
344 this study. We would like to thank Emillie Jarratt for her help with highly parallel sequencing.
345

346 **REFERENCES**

347 1. http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-
348 type/skin-cancer (Accessed May 2018).
349 2. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR et al. Final
350 version of 2009 AJCC melanoma staging and classification. J Clin Oncol 2009;27(36):6199-206.
351 3. Salti GI, Kansagra A, Warso MA, Ronan SG, Das Gupta TK. Clinical node-negative thick
352 melanoma. Archives of surgery (Chicago, Ill : 1960). 2002;137(3):291-5.
353 4. Larkin J, Ascierto PA, Dreno B, Atkinson V, Liszkay G, Maio M et al. Combined vemurafenib
354 and cobimetinib in BRAF-mutated melanoma. N Engl J Med 2014; 371:1867-1876.
355 5. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A. Stroiakovski D et al.
356 Improved overall survival in melanoma with combined dabrafenib and trametinib. N Engl J Med
357 2015; 372:30-39.
358 6. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB et al. Improved
359 survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363(8):711-23.
360 7. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM et al. Nivolumab
361 plus ipilimumab in advanced melanoma. N Engl J Med 2013;369(2):122-33.
362 8. Teare MD, Woll PJ. Genomic tests: unreliable for cancer? A focus on circulating DNA and
363 lung cancer. Expert Rev Mol Diagn 2007;7(6):699-702.
364 9. van der Vaart M, Pretorius PJ. Is the role of circulating DNA as a biomarker of cancer being
365 prematurely overrated? Clin Biochem 2010;43(1-2):26-36
366 10. Mori T, O'Day SJ, Umetani N, Martinez SR, Kitago M, Koyanagi K, et al. Predictive Utility of
367 Circulating Methylated DNA in Serum of Melanoma Patients Receiving Biochemotherapy. J Clin
368 Oncol. 2005;23(36):9351-8.
369 11. Bettegowda C, Sausen M, Leary RJ et al. Detection of circulating tumor DNA in early- and
370 late-stage human malignancies. Sci Transl Med 2014;6(224):224ra24.
371 12. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to
372 monitor treatment response and detect acquired resistance in patients with metastatic melanoma.
373 Oncotarget. 2015;6(39):42008-18.
374 13. Girotti MR, Gremel G, Lee R, Galvani E, Rothwell D, Viros A, et al. Application of
375 Sequencing, Liquid Biopsies, and Patient-Derived Xenografts for Personalized Medicine in
376 Melanoma. Cancer discovery. 2016;6(3):286-99.
377 14. Wong SQ, Raleigh JM, Callahan J, Vergara IA, Ftouni S, Hatzimihalis A, et al. Circulating
378 Tumor DNA Analysis and Functional Imaging Provide Complementary Approaches for
379 Comprehensive Disease Monitoring in Metastatic Melanoma. JCO Precision Oncology. 2017(1):1-
380 14.
381 15. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol
382 2014;32(6):579-86.
383 16. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM et al. Non-invasive
384 analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature
385 2013;497(7447):108-12.
386 17. Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical
387 validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nature Med
388 2014;20(4):430-5.
389 18. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking
390 in circulating tumor DNA predicts relapse in early breast cancer. Science Transl Med
391 2015;7(302):302ra133.
392 19. Network CGA. Genomic classification of cutaneous melanoma. Cell 2015;161(7):1681-96.

393 20. Xue XY, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating
394 cell-free DNA from plasma and serum. Clin Chim Acta 2009;404(2):100-4.
395 21. Heitzer E, Ulz P, Belic J, Gutschi S, Quehenberger F, Fischereder K, et al. Tumor-
396 associated copy number changes in the circulation of patients with prostate cancer identified
397 through whole-genome sequencing. Genome Med 2013;5(4):30.
398 22. Xia S, Huang CC, Le M, Dittmar R, Du M, Yuan T, et al. Genomic variations in plasma cell
399 free DNA differentiate early stage lung cancers from normal controls. Lung Cancer 2015;90(1):78-
400 84.
401 23. Daniotti M, Vallacchi V, Rivoltini L, Patuzzo R, Santinami M, Arienti F, et al. Detection of
402 mutated BRAFV600E variant in circulating DNA of stage III-IV melanoma patients. Int J Cancer
403 2007;120(11):2439-44.
404 24. Xia S, Kohli M, Du M, Dittmar RL, Lee A, Nandy D, et al. Plasma genetic and genomic
405 abnormalities predict treatment response and clinical outcome in advanced prostate cancer.
406 Oncotarget 2015;6(18):16411-21.
407 25. Tsao SC, Weiss J, Hudson C, Christophi C, Cebon J, Behren A, Dobrovic A. Monitoring
408 response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR
409 for BRAF and NRAS mutations. Sci Rep 2015;5:11198.
410 26. Lipson EJ, Velculescu VE, Pritchard TS, Sausen M, Pardol DM, Topalian SL, Diaz LA, Jr.
411 Circulating tumor DNA analysis as a real-time method for monitoring tumor burden in melanoma
412 patients undergoing treatment with immune checkpoint blockade. J Immunother Cancer
413 2014;2(1):42.
414 27. Lee RJ, Gremel G, Marshall A, Myers KA, Fisher N, Dunn JA, et al. Circulating tumor DNA
415 predicts survival in patients with resected high risk stage II/III melanoma. Ann Oncol 2017 [Epub
416 ahead of print].
417 28. Valpione S, Gremel G, Mundra P, Middlehurst P, Galvani E, Girotti MR, et al. Plasma total
418 cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for
419 survival in metastatic melanoma patients. Eur J Cancer 2018 Jan;88:1-9.
420

421

Table 1: Logistic regression analysis

Univariable analysis				
	OR	**p-value**	**95% CI**	**AUC**
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[a]	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
Gender	1.2	0.69	0.5 - 2.9	0.52
BRAF status	1.3	0.59	0.5 - 3.2	0.53
Multivariable analysis				
	OR	**p-value**	**95% CI**	-
CNAS category[b]	17.4	0.019	1.6 - 190.90	-
cfDNA category[a]	2.7	0.26	0.5 - 15.1	-
Stage	24.6	<0.0001	4.4 - 136.9	-

[a] log cfDNA level above and below the 75th percentile, [b] log CNAS above and below the 75th percentile

422

423 **Table 2: Cox regression analysis**
424

	HR	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
Gender	0.7	0.23	0.4 - 1.3
Active/Resected	9.3	<0.0001	4.9 - 17.3
Multivariable Cox regression			
CNAS category[a]	3.4	0.005	1.5 - 7.9
cfDNA category[b]	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

425 [a] log CNAS above and below the 75th percentile, [b] log cfDNA level above and below the 75th percentile
426

427 **Figure 1: Copy-number aberration plot derived from cfDNA extracted from a patient with**
428 **active melanoma reflects some aberrations seen in the original tumour biopsy specimen.**
429 Examples of copy-number aberration plots for cfDNA compared to matched tumour FFPE DNA, in
430 a case with active disease (case 60; top figure) and resected disease (case 5; bottom figure).
431
432 **Figure 2: The copy-number aberration scores (CNAS) for cfDNA were higher for cases with**
433 **active disease compared to those with recently-excised disease; while the eCNAS showed**
434 **no significant difference between the two groups.**
435 Box and whisker plot of the log scores (log eCNAS on the right, log CNAS on the left) in cases with
436 active melanoma compared to those with recently-excised disease.
437
438 **Figure 3: Mortality was higher in patients with log CNAS above the 75th percentile**
439 Kaplan-Meier survival graph comparing survival for patients with log CNAS above (red) and below
440 (blue) the 75th percentile. Inset shows the numbers of patients at risk at each time point.