

Circulating cell-free DNA-based biomarkers for prognostication and disease monitoring in adrenocortical carcinoma

Lippert, Juliane; Smith, Gabrielle; Appenzeller, Silke; Landwehr, Laura-Sophie; Prete, Alessandro; Steinhauer, Sonja; Asia, Miriam; Urlaub, Hanna; Elhassan, Yasir; Kircher, Stefan; Arlt, Wiebke; Fassnacht, Martin; Altieri, Barbara; Ronchi, Cristina

DOI:

[10.1093/ejendo/lvae022](https://doi.org/10.1093/ejendo/lvae022)

License:

Creative Commons: Attribution-NonCommercial (CC BY-NC)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Lippert, J, Smith, G, Appenzeller, S, Landwehr, L-S, Prete, A, Steinhauer, S, Asia, M, Urlaub, H, Elhassan, Y, Kircher, S, Arlt, W, Fassnacht, M, Altieri, B & Ronchi, C 2024, 'Circulating cell-free DNA-based biomarkers for prognostication and disease monitoring in adrenocortical carcinoma', *European Journal of Endocrinology*. <https://doi.org/10.1093/ejendo/lvae022>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

1 **Circulating cell-free DNA-based biomarkers for prognostication and disease monitoring in**
2 **adrenocortical carcinoma**

3 ^{1,2}Juliane Lippert, ³Gabrielle Smith, ⁴Silke Appenzeller, ¹Laura-Sophie Landwehr, ^{3,5,6}Alessandro Prete,
4 ¹Sonja Steinhauer, ⁷Miriam Asia, ¹Hanna Urlaub, ^{3,7}Yasir S. Elhassan, ⁸Stefan Kircher, ^{3,9}Wiebke Arlt,
5 ¹Martin Fassnacht, ¹Barbara Altieri, ^{3,5}Cristina L. Ronchi

6 *¹Division of Endocrinology and Diabetes, Department of Medicine, University Hospital, University of*
7 *Wuerzburg, Wuerzburg (Germany); ²Institute of Human Genetics, University of Wuerzburg, Wuerzburg*
8 *(Germany); ³Institute of Metabolism and System Research, University of Birmingham, Birmingham (UK);*
9 *⁴Core Unit Bioinformatics, Comprehensive Cancer Center Mainfranken, University of Wuerzburg,*
10 *Wuerzburg (Germany); ⁵Centre for Endocrinology, Diabetes and Metabolism (CEDAM), Birmingham*
11 *Health Partners, Birmingham (UK); ⁶NIHR Birmingham Biomedical Research Centre, University of*
12 *Birmingham and University Hospitals Birmingham NHS Foundation Trust, Birmingham (UK);*
13 *⁷Endocrine Department, Queen Elizabeth Hospital Birmingham NHS Trust, Birmingham (UK);*
14 *⁸Department of Pathology, University of Wuerzburg, Wuerzburg (Germany); ⁹MRC Laboratory of*
15 *Medical Sciences, London (UK).*

16

17 **Short Title:** Role of ccfDNA analysis in management of ACC

18 **Key words:** ccfDNA, adrenal cancer, personalised medicine, liquid biopsy

19 **Word count:** 5238

20

21 **Correspondence and request for reprints to:**

22 Cristina L. Ronchi, MD, PhD

23 Institute of Metabolism and System Research

24 College of Medical and Dental Sciences

25 University of Birmingham

1 Edgbaston B15 2TT, United Kingdom
2 Tel. +44-(0)121-4146857; FAX +44-(0)121-4158712
3 Email: C.L.Ronchi@bham.ac.uk

4 **Abstract**

5 Adrenocortical carcinoma (ACC) is a rare aggressive cancer with heterogeneous behaviour. Disease
6 surveillance relies on frequent imaging, which comes with significant radiation exposure. The aim of the
7 study was to investigate the role of circulating cell-free DNA (ccfDNA)-related biomarkers (BM) for
8 prognostication and monitoring of ACC.

9 We investigated 34 patients with ACC and 23 healthy subjects (HS) as controls. ccfDNA was extracted
10 by commercial kits and ccfDNA concentrations quantified by fluorimeter (BM1). Targeted sequencing
11 was performed using a customised panel of 27 ACC-specific genes. Leucocyte DNA was used to
12 discriminate somatic variants (BM2), while tumour DNA was sequenced in 22/34 cases for comparison.
13 Serial ccfDNA samples were collected during follow-up in 19 ACC patients (median period 9 months)
14 and analysed in relationship with standard radiological imaging.

15 ccfDNA concentrations were higher in ACC than HS (mean±SD, 1.15±1.56 vs 0.05±0.05 ng/μl,
16 $P<0.0001$), 96% of them being above the cut-off of 0.146 ng/μl (mean HS+2SD, positive BM1). At
17 ccfDNA sequencing, 47% of ACC showed at least one somatic mutation (positive BM2). A combined
18 ccfDNA-BM score was strongly associated with both progression-free and overall survival (HR=2.63,
19 95%CI=1.13-6.13, $P=0.010$, and HR=5.98, 95%CI=2.29-15.6, $P=0.0001$, respectively). During disease
20 monitoring, positive BM2 showed the best specificity (100%) and sensitivity (67%) to detect ACC
21 recurrence or progress compared to BM1.

22 In conclusion, ccfDNA-related BMs are frequently detected in ACC patients and represent a promising,
23 minimally invasive tool to predict clinical outcome and complement surveillance imaging. Our findings
24 will be validated in a larger cohort of ACCs with long-term follow-up.

1 **Significance statement**

2 Adrenocortical carcinoma (ACC) is a rare and generally highly aggressive cancer. Despite recent
3 developments, there are still critical unmet clinical needs for patients with ACC. In fact, no markers are
4 available that can predict clinical outcomes at the time of the diagnosis. Moreover, follow-up requires
5 frequent imaging that results in increased radiation exposure and cannot always answer diagnostic
6 questions. In this study, we have developed a method for the evaluation of specific alterations in small
7 fragments of genetic information (=DNA) released from tumour cells into the blood. By correlating these
8 alterations with clinical data and standard radiological imaging, we have demonstrated that this approach
9 can identify markers that could help to better predict the clinical course of ACC patients, and recognize
10 disease relapses and/or progression.

11 **Introduction**

12 Adrenocortical carcinoma (ACC) is a rare malignancy with a generally poor but heterogeneous
13 prognosis¹. Five-year survival rates range from 13% to 80% depending on the European Network for the
14 Study of Adrenocortical Tumors (ENSAT) tumour staging, resection status, and Ki67 index^{1,2}. However,
15 currently available clinical and histopathological factors cannot always reliably distinguish patients with
16 favorable from those with worse prognosis³. Moreover, disease recurrences after resection of the primary
17 tumour are frequent even in lower-risk ENSAT stages, and effective pharmacological therapies for
18 advanced stages are lacking. Therefore, close disease monitoring is essential to allow timely management
19 but relies on frequent radiological imaging², which not only causes relevant radiation exposure for
20 patients but also significant costs for the health systems.

21 Liquid biopsy, i.e. the analysis of tumour material obtained in a minimally invasive manner by sampling
22 of blood or other body fluids, is being increasingly proposed in oncology for molecular profiling,
23 detection of residual disease and monitoring of disease evolution^{4,5}. Decades ago, it was demonstrated
24 that plasma from cancer patients contains higher concentrations of circulating cell-free DNA (ccfDNA)
25 than those from healthy individuals⁶ assuming that at least a part of ccfDNA originates from cancer cells⁷⁻

1 9. As a consequence, even if the origin of ccfDNA cannot be definitively determined, elevated levels of
2 short DNA fragments may be a good marker for the detection of tumour DNA in blood^{10,11} and used to
3 monitor tumour evolution and response to therapy^{12, 13}. Furthermore, tumour-associated genetic
4 alterations, such as single nucleotide variants (SNVs), can be detected in ccfDNA^{14,15}. Sequencing of
5 ccfDNA presents important advantages compared to sequencing of tumour-derived DNA. Firstly, it holds
6 the potential of detecting all the alterations contained in the tumour, while single tissue samples provide
7 only a limited characterisation of the molecular signature¹⁶⁻¹⁹. This is particularly relevant for
8 heterogeneous cancer types, such as ACC. Secondly, serial blood samples are compatible with dynamic
9 and minimally invasive cancer surveillance¹⁷. ccfDNA analysis has been also proposed as a potential
10 prognostic tool. The presence of genetic variants in tumour-specific genes at the ccfDNA level has been
11 associated with worse clinical outcomes and suggested as a predictive marker of response to therapy in
12 multiple cancer types²⁰⁻²². More recently, elevated total ccfDNA concentrations have also been reported
13 as a simple and cheap marker of shorter survival in patients with different cancers^{23, 24}. Finally,
14 sequencing of ccfDNA can be used to identify key treatment targets, both at the time of diagnosis and in
15 case of tumour progression or recurrence²².

16 Only two previous studies performed ccfDNA analysis in patients with ACC^{25,26}. However, these
17 included small case cohorts (only 17 patients in these two studies combined) and used heterogeneous
18 techniques for both ccfDNA isolation and sequencing, and their findings cannot be considered conclusive.
19 Moreover, there is only one case report providing serial targeted ccfDNA analysis for tumour monitoring
20 to date²⁷.

21 The aim of the present pilot study was to investigate ccfDNA-based biomarkers (BM) in a larger well-
22 characterised cohort of patients with ACC and their potential role both as prognostic factors (AIM 1) and
23 as tools for the detection of tumour recurrence or progression (AIM 2).

24

25

1 **Material and methods**

2 *Patient cohort and study design*

3 In the present study, we investigated consecutive patients older than 18 years examined in two tertiary
4 referral centres between 2019 and 2021 (**Figure 1**). Inclusion criteria were: i) patients with adrenal
5 masses suspicious for ACC according to current guidelines^{3,28}, ii) fully available clinical, biochemical
6 and radiological data at the time of diagnosis, and iii) final diagnosis of ACC based on current guidelines<sup>3,
7 28</sup>. These included histopathological confirmation of ACC for patients that underwent adrenal surgery or
8 biopsies, or large, radiologically suspicious adrenal masses associated with severe biochemical and/or
9 clinical steroid excess. Patients who were diagnosed with benign or malignant adrenocortical lesions other
10 than ACC after workup were excluded from the study. Other exclusion criteria included a diagnosis of
11 other active concomitant cancers and severe alterations in liver or kidney functions. After consideration of
12 inclusion and exclusion criteria, the final cohort comprised a total of 34 patients with primary ACC that
13 served for the evaluation of ccfDNA-BMs for prognostic classification of ACC (AIM 1).

14 Peripheral blood samples were collected before surgery (baseline) in all participants with ACC. Blood
15 samples were also collected from 23, as far as known, healthy subjects (HS) recruited among university
16 staff that served as controls for the baseline ccfDNA concentration analysis.

17 For a subgroup of patients with ACC, blood samples were additionally collected during standard follow-
18 up visits after primary surgery (see details below and **Figure 1**) for the evaluation of the role of ccfDNA-
19 based BMs as monitoring tool (AIM 2).

20 The study is compliant with the Declaration of Helsinki. The study protocol was approved by both local
21 ethics committees (#88/11 at the University Hospital of Wuerzburg; HBRC 11/606 and PrimeAct study
22 REC 20/NW/0207 at the University of Birmingham). Written informed consent was obtained from all
23 subjects.

1 *Clinical, histopathological and radiological data*

2 Patient's age at diagnosis, symptoms at presentation (related to autonomous steroid secretion or mass
3 effect), and initial ENSAT tumour stage were collected for all patients. Ki67 index and resection (R)
4 status were recorded only for the 22 patients who underwent adrenalectomy (**Figure 1**) and used to
5 calculate the S-GRAS score as previously published²⁹. In four additional cases, the Ki67 index was
6 available from adrenal biopsies. A total of 15 patients received adjuvant treatment with mitotane after
7 primary surgery according to current guidelines².

8 Periodical surveillance imaging i.e., by thorax-abdomen-pelvis computed tomography scan with contrast
9 (TAP CT scan), was performed every three months as per current guidelines². The occurrence of disease
10 recurrence or progression as well as the total tumour burden was evaluated at baseline and periodical
11 radiology scans as the sum of all measurable target lesions (in accordance with RECIST v1.1) by expert
12 radiologists. The number and localisation of eventual disease recurrence were also recorded.

13 Overall survival (OS) was defined as the time from primary tumour resection or diagnosis to death.
14 Progression-free survival (PFS) was defined as the time from diagnosis to the first radiological evidence
15 of disease progression. Disease status and survival information were updated up to June 2023.

16 *Sample processing and ccfDNA isolation*

17 We have established a systematic and homogeneous pipeline for sample collection and processing in both
18 our centres aiming to obtain reliable findings using clinically applicable techniques. In brief, 10–20 ml of
19 blood were collected in EDTA tubes and kept on ice until centrifuged (within 2-3 hours of blood
20 collection) for 10 min at room temperature and 800rpm. After centrifugation, plasma was transferred to
21 clean centrifugation tubes without disturbing the buffy coat and centrifuged for another 10 min at 4 °C
22 and 13.000 rpm. Plasma was transferred to a fresh centrifugation tube without disturbing the pellet and
23 stored at -80°C until analysis. ccfDNA was isolated from 2–6 ml of plasma with the QIAamp MinElute
24 ccfDNA Kit (Qiagen, Hilden, Germany) or the Cell3™ Xtract kit (Nonacus, Birmingham, UK) according
25 to manufacturers' instructions. We chose these commercially available kits according to their

1 characteristics of suitability in clinical routine (including costings, time requirements and complexity of
2 protocols, necessity for additional equipment and the amount of usable plasma). To confirm similarities
3 between the two chosen kits, we compared the ccfDNA concentrations obtained in a representative
4 subgroup of samples by isolating the same volume of plasma (1 ml) from same samples. Hereby, we
5 could demonstrate that the ccfDNA concentrations were superimposable the Nonacus and the Qiagen kit
6 (n=6, 0.434 ± 0.203 vs 0.364 ± 0.182 ng/ μ l, P=0.24).

7 ccfDNA was then eluted in 40 μ l of dH₂O and stored at -20 °C until further processing.

10 *ccfDNA analysis*

11 ccfDNA concentration (BM1): ccfDNA concentrations were determined with a Quantus™ Fluorometer
12 (Promega, Fitchburg, United States) according to the manufacturer's instructions. Different volumes of
13 plasma taken for ccfDNA isolation were considered for the designation of the final ccfDNA concentration
14 in a sample. A quality control (QC) for the desired fragment length of the ccfDNA (150-200 bp) was
15 performed on a Bioanalyzer with Agilent High Sensitivity DNA Kit or with the TapeStation High
16 Sensitivity 1000D system (both Agilent, Santa Clara, United States). All ccfDNA samples included in the
17 analysis showed good quality in means of fragment length and no contamination with high molecular
18 weight material. Representative examples of QC by TapeStation in both ACC and HS samples are shown
19 in **Suppl. Fig 1**. According to the QC analysis, we also calculated the calibrated ccfDNA concentrations
20 (based on the percentage of concentrations at 100-250 bps) in a subgroup of 18 samples (including 14
21 patients with ACC and 4 HS). Here, we could observe a very good correlation between total and
22 calibrated concentrations (F=73.3, R=0.906, P<0.0001, **Suppl. Fig 2A-B**). We therefore decided to use
23 the total ccfDNA concentrations for all samples (i.e. baseline and follow ups).

24 ccfDNA sequencing for identification of somatic mutations (BM2): all 34 baseline ccfDNA samples were
25 sequenced. Longitudinal samples collected during follow-up were sequenced for 18 cases of patients that

1 underwent adrenalectomy. In brief, ccfDNA samples were enriched with a customised gene panel, i.e.
2 Cell3™ Target Custom NGS Panel (Nonacus), according to the manufacturer's instructions. The Cell3™
3 Target is a target enrichment system for converting any type of DNA into libraries for next generation
4 sequencing. It uses error suppression technology to ensure confident calling of all mutations down to
5 0.1% variant allele frequency (VAF) and is ideal for rare variant detection in liquid biopsies
6 (www.nonacus.com). The custom panel included 27 genes known to be associated with ACC³⁰⁻³² (**Suppl.**
7 **Table 1**). These included 8 genes that are currently classified as drug targetable at different levels in the
8 OncoKb database (www.oncokb.org) i.e., *TP53*, *KDM6A*, *EGFR*, *FGFR3*, *ATM*, *BRCA2*, *NF1*, and
9 *PTCH1*. The protocol included an end-repair and A-tailing step before adapter ligation at the beginning.
10 After a pre-capture, PCR samples were pooled and target regions were hybridised and therefore enriched
11 with biotin-labelled probes. Unique molecular identifiers (UMIs) were used to reduce the background
12 noise created by PCR and sequencing errors and enable mutation calling of VAF down to 0.1%,
13 especially important when deploying the ultra-deep sequencing necessary for the analysis of cfDNA.
14 After another amplification step via post-capture PCR and quality check, libraries were ready for
15 sequencing. Paired end sequencing was performed on a NextSeq500 with NextSeq 500/550 Mid Output
16 Kit v2.5 (150 Cycles) or on a NextSeq2000 with NextSeq 1000/2000 P2 Reagents (200 Cycles) v3 for
17 estimated 13 Million reads per sample (Illumina, San Diego CA, US).

18 To reliably classify ccfDNA variants as somatic or germline, reference germline DNA was isolated from
19 matched peripheral blood samples using the NucleoSpin Blood L Kit (Macherey-Nagel, Bethlehem, PA,
20 USA) according to the manufacturer's instructions. Library preparation of germline DNA was also
21 conducted with the Cell3™ Target Custom NGS Panel (Nonacus) following the same protocol as for
22 ccfDNA enrichment except of an initial fragmentation step. For those 12 patients where no tumour
23 material was available for sequencing, we analysed genomic DNA from blood for the variants found in
24 ccfDNA via Sanger sequencing.

1 ***Tumour tissue DNA isolation and sequencing***

2 Matched formalin-fixed paraffin-embedded (FFPE) tumour tissues were available for sequencing in the
3 22 patients who underwent adrenalectomy. Tumour localization was annotated by an expert pathologist
4 and tumour cell content was assessed in a representative FFPE slide by haematoxylin-eosin staining
5 before DNA isolation. Tumour cell content reached a high fraction (median 90%, range 60-95). DNA was
6 isolated from tumour material using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) according
7 to the manufacturer's instructions and as previously described³⁰. Library preparation of tumour was also
8 conducted with the Cell3™ Target Custom NGS Panel (Nonacus) following the same protocol as for
9 ccfDNA enrichment except for an initial fragmentation step.

10 ***Sequencing data analysis***

11 Bcl2fastq de-multiplexing was performed as described in the Nonacus user manual (Cell3™ Target: Data
12 Analysis Guidelines, Protocol Guide v1.0). Consensus BAM file preparation was also conducted
13 according to the manufacturer's instructions using NonacusTools, v1.0. Additionally, quality control of
14 the sequencing reads was carried out with FastQC, v0.11.3
15 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and read statistics were calculated using in-
16 house scripts. Variant calling was performed with GensearchNGS (Phenosystems SA, Braine le Chateau,
17 Belgium) for sequencing data from DNA isolated from tumour and blood, as well as ccfDNA. Called
18 variants in tumour samples were compared to variants detected in germline DNA and further filtered for
19 variants with minor allele frequency (MAF) <0.02, VAF >0.2 for tumour DNA and >0.01 for ccfDNA,
20 coverage >100, variant balance >0.2 and variant type worse than synonymous. Detected variants were
21 classified with the use of prediction tools^{33, 34} and databases, such as COSMIC³⁵, ClinVar³⁶ and
22 cBioPortal³⁷. For the final analysis, only variants classified as uncertain, likely pathogenic and pathogenic
23 were considered. In cases where variants detected in ccfDNA were not detected in the corresponding
24 tumour DNA or vice versa, we manually searched for potential variants within the genomic positions (i.e.
25 beyond the given threshold of VAF or coverage).

1 ***ccfDNA-based biomarker (BM) definition***

2 BM1 (quantitative analysis) was defined as positive when the total ccfDNA concentrations were above
3 the cut-off derived from HS i.e., 0.146 ng/ μ l (mean HS+2 standard deviation [SD]). BM1 was defined as
4 very high when the total ccfDNA concentrations were above the arbitrary cut-off of 1 ng/ μ l.

5 BM2 (genomic qualitative analysis) was defined as positive when at least one somatic variant was
6 detected at targeted NGS at the ccfDNA levels.

7 ccfDNA-based BM score was calculated as follows: baseline BM1 (negative=0, positive=1, very high=2)
8 + baseline BM2 (no variants=0, one variant=1, more than one variant=2) for a minimum of 0 and a
9 maximum of 4 points.

10 ***Statistical analysis***

11 Data are shown as mean \pm SD or median and range, as appropriate. Non-parametric Mann-Whitney *U* test
12 and Fisher or χ^2 tests were used to compare baseline continuous and dichotomic data, respectively. Non-
13 parametric Kruskal-Wallis test was used to compare multiple variables, followed by Bonferroni *post-hoc*
14 test. Correlations between two continuous variables were investigated by linear regression. Comparison
15 between total and calibrated ccfDNA concentrations was additionally performed by Bland-Altman test.
16 Kaplan-Meier plots were used to investigate the proportional hazards assumption and to display the
17 unadjusted survival curves for survival outcomes. Hazard ratio (HR), 95% CI, and *P* values calculated by
18 log-rank test (Mantel-Cox) were reported for each survival outcome (OS and PFS). Moreover,
19 multivariable Cox survival models were fitted for OS and PFS, including variables significant at
20 univariable analysis and available for all patients. Statistical analysis was performed using SPSS (version
21 9, IBM Deutschland GmbH, Ehningen, Germany) or GraphPad Prism (version 25, GraphPad Software,
22 Boston, US). *P* value < 0.05 was considered statistically significant.

23
24
25

1 **Results**

2 ***Characteristics of the study cohort***

3 We included a total of 34 patients with primary ACC in place (11M/23F, median age 55.5 years, range
4 23-83) and followed up in one of the two participating centres (Wuerzburg, Germany, and Birmingham,
5 UK). The control group included 23 HS (9M/14F, median age 35 years, range 23-62). An overview of the
6 demographic, clinical, and histopathological characteristics at the time of diagnosis as well as the results
7 of the ccfDNA analysis are shown in **Table 1**.

8 A complete flowchart showing the final ACC cohort and available follow-ups is shown in **Figure 1**. In
9 brief, a total of 22 patients underwent adrenalectomy (three of whom with debulking purposes) and had
10 available tumour tissue material. The remaining 12 patients did not undergo surgery due to presence of
11 metastatic disease at time of diagnosis or non-operable primary tumours. Nineteen patients that underwent
12 surgery were also tested during post-surgical follow-up for monitoring purposes: 9 of them for at least 3
13 months (short term) and 10 for at least 9 months (long term).

15 ***Relationship between ccfDNA concentrations (BM1) and clinical parameters***

16 Patients with ACC had higher total ccfDNA concentrations than HS (1.15 ± 1.56 vs 0.050 ± 0.048 ng/ μ l,
17 $P < 0.0001$, **Figure 2A**). Overall, 96% of ACC were positive for BM1, while 32% of ACC cases showed
18 very high ccfDNA concentrations, i.e. >1 ng/ μ l (**Figure 2B**). Higher ccfDNA levels were associated with
19 larger tumour burden and more aggressive disease. In fact, patients with advanced stage ACC – i.e. non
20 amenable for complete surgical resection – presented a higher frequency of very high ccfDNA
21 concentrations (11% vs 60%, respectively, $P < 0.001$, **Suppl. Fig 3**). Moreover, patients with ENSAT
22 stage 4 had higher ccfDNA concentrations (2.41 ± 2.06 ng/ μ l) compared to patients with stage 3
23 (0.41 ± 0.31 ng/ μ l, $P = 0.0117$) or 1-2 (0.33 ± 0.43 ng/ μ l, $P = 0.0004$) (**Figure 2C**). Finally, ccfDNA levels
24 correlated positively with both the Ki67 index ($n=22$, $P = 0.0034$, $R = 0.57$, **Figure 2D**) and the number of

1 distant metastases ($n=30$, $P=0.021$, $R=0.42$). There was no significant correlation between the ccfDNA
2 concentrations and age or presence of symptoms at diagnosis.

3 4 ***Relationship between somatic variants at ccfDNA (BM2) and clinical parameters***

5 At ccfDNA sequencing, 47% of ACC showed at least one somatic mutation (positive BM2) and 15% at
6 least two mutations (**Figure 2E-F** and **Table 1**). The most frequently altered genes included: *CTNNB1*
7 (12%), *ZNRF3* (9%), *MEN1* (9%), *TP53* (6%), *ATM* (6%), and *KMT2D* (6%). As expected, the majority
8 of the altered genes belonged to the Wnt/ β -catenin pathway followed by those linked to chromatin
9 remodelling (**Figure 2F** and **Suppl. Table 1**). The VAF were overall comprised between 1.0 and 30.5%.
10 The type of detected genetic variants and the corresponding VAFs are reported in **Table 1** and **Suppl.**
11 **Table 2**.

12 Interestingly, patients with advanced stage ACC – i.e. non amenable for complete surgical resection
13 ($n=15$) – presented a higher frequency of one or more than one somatic variant at baseline ccfDNA
14 compared to those with early stages, i.e. 40% and 20% vs 26% and 11%, respectively ($P=0.0052$, **Table 1**
15 and **Suppl. Fig 3**). In this subgroup of more aggressive cases, the most frequent alterations were observed
16 in *CTNNB1* ($n=3$), *TP53* ($n=2$) and *KMT2D* ($n=2$). Of note, three of these variants affected known drug
17 targetable genes (9% of total): two patients presented missense mutations in *ATM* and one presented a
18 missense mutation in *NF1*.

19 20 ***Comparison between somatic variants in ccfDNA and T-DNA***

21 We compared the the ccfDNA mutational status with available corresponding primary tumour DNA (T-
22 DNA) in 22 patients. These perfectly matched in 68% of cases (**Table 2** and **Suppl. Table 2**).

23 In particular, in 12 out of 14 patients with no somatic variants at baseline ccfDNA that underwent
24 adrenalectomy, T-DNA also showed also no detectable somatic variants. In three cases (13.6%), variants
25 were only found in T-DNA (but not in ccfDNA), including one with *APC* variant (VAF 66.0%), one with

1 *MEN1* variant (VAF 20.9%) and one with variants in *NF1* (VAF 26.4%), *NOTCH1* (VAF 24.6%) and
2 *TP53* (VAF 20.6%)(**Table 2**).

3 Conversely, we observed somatic variants at the ccfDNA level that were not detected at T-DNA in four
4 cases (18.2%) i.e., affecting *APC*, *GNAS*, *NF1* and *ZNR3* genes. Specifically, variants detected in
5 *ZNR3* (VAF 3.9%) and *NF1* (VAF 7.0%) are classified as pathogenic in the COSMIC database, while
6 variants in *APC* (VAF 3.3%) and *GNAS* (VAF 30.5%) are reported as uncertain or not reported yet.

7

8 ***Relationship between ccfDNA-based BMs and clinical outcome (AIM 1)***

9 We then investigated the role of ccfDNA-based BMs for prognostic classification (AIM 1). We first
10 performed univariate survival analysis testing the prognostic role of ccfDNA concentrations (negative
11 BM1, positive BM1, and very high BM1). BM1 was clearly associated with both PFS (HR 3.45, 95% CI
12 1.49-7.96, $P=0.0038$ by log-rank test, median PFS 2 vs 15 months vs undefined, **Figure 3A**) and OS (HR
13 6.28, 95% CI 2.43-17.58, $P=0.0003$, median survival 5 vs 45 months vs undefined, **Figure 3B**). Also the
14 presence of one or more somatic variants in baseline ccfDNA (positive BM2, one or more variants) was
15 able to distinguish patients with unfavourable outcomes, i.e. with a shorter PFS (2 vs 18 months vs
16 undefined; HR 1.89, 95% CI 1.05-3.40, $P=0.0256$ by log-rank test, **Figure 3C**) and OS (8 vs 9 vs 40
17 months; HR 2.47, 95% CI 1.33-4.56, $P=0.0058$, **Figure 3D**).

18 A ccfDNA-based BM score was then calculated starting from baseline ccfDNA-based BMs as described
19 in the Methods for a minimum of 0 and a maximum of 4 points. This score was strongly associated with
20 both PFS and OS at univariate analysis (HR 2.63, 95% CI 1.13-6.13, $P=0.010$, and HR 5.98, 95% CI
21 2.29-15.6, $P=0.0001$, respectively) (**Figure 3E-F**). Importantly, this prognostic role was confirmed at
22 multivariable analysis including the ENSAT tumour stage (model 1 – dichotomic variable: HR=2.86,
23 $P=0.061$ for PFS and HR=8.80, $P=0.004$ for OS, respectively) (model 2 – non-dichotomic variable:
24 HR=1.81, $P=0.009$ for PFS and HR=3.39, $P<0.001$ for OS, respectively) (**Table 3**).

25

1 ***Role of ccfDNA-based BMs in disease monitoring (AIM 2)***

2 Finally, we tested the potential role of ccfDNA-based BMs for longitudinal disease monitoring (AIM 2).
3 Serial blood samples collected during standard follow-up visits after primary surgery were available for
4 19 patients (median duration 9 months, range: 3-12, **Figure 1**).

5 In two of the patients who underwent a debulking surgery due to severe steroid excess (ACC-P5 and
6 ACC-P28), ccfDNA concentrations persisted at very high levels after surgery, which coincided with rapid
7 disease progression. Importantly, in one case (ACC-P28), two somatic variants were detected at baseline
8 and remained detectable at the three-month follow-up analysis (*PRKARIA* VAF from 10.9 to 4.9%, *TERT*
9 VAF from 26.5 to 5.0%) when the radiological imaging showed enlarging liver metastases (**Suppl. Table**
10 **2** and **Suppl. Fig 4**).

11 In six cases, patients presented with disease recurrences during surveillance i.e., at short-term follow-ups
12 (n=2 at 3 months and n=1 at 6 months) or long-term follow-ups (n=1 at 9 months and n=1 at 12 months).

13 In some cases, even if BM2 could not be used during monitoring, ccfDNA concentrations alone could
14 mirror the trend of the radiological imaging, greatly increasing at the time of disease recurrence
15 (representative examples shown in **Figure 4A**). Two cases presented with somatic variants in *MEN1* and
16 *ZNRF3* at baseline ccfDNA (ACC-P3 and ACC-P8, **Suppl. Fig 4**). In one case, these persisted in the
17 three-month sample when the patient showed an early disease recurrence (i.e. liver metastases). At the
18 six-month follow-up, the patient showed a mixed response to treatment with mitotane, but variants could
19 not be detected due to low coverage. The same patient presented a rapid progression 9 months after
20 surgery with a significant increase in the size of the liver lesions and multiple lung metastases (**Figure**
21 **4B**). Simultaneously, both the ccfDNA concentrations and the VAF % of both variants sharply increased
22 (to 46.4 and 45.8%, respectively). In the other case, no variants were detected at the three-month follow-
23 up due to low coverage.

24 Finally, eleven cases showed no evidence of disease recurrence at the last available CT TAP scan. The
25 trend observed in mean total ccfDNA concentrations over time in this group is shown in **Figure 5A**. Of
26 note, ccfDNA levels progressively decreased during surveillance, even if in very few cases remained

1 slightly above the chosen cut-off (representative examples in **Figure 5B and C**). Among these cases, five
2 presented somatic variants at baseline ccfDNA (**Suppl. Fig 4**). Importantly, these variants were no more
3 detectable neither at first post-surgical follow up nor during further surveillance (representative examples
4 are shown in **Figure 5E-G**).

5 Overall, even in this relatively small cohort of cases with fully available data, ccfDNA-related BM1
6 showed a sensitivity of 100% and a specificity of 36% (n=19), while BM2 showed sensitivity of 100%
7 and a specificity of 67% (n=8).

8 Of note, we also sequenced longitudinal ccfDNA samples from 10 patients with no somatic variants
9 detected in neither baseline ccfDNA nor T-DNA (i.e. 3 short-term and 7 long-term). Among these cases,
10 4 developed a disease recurrence over the time (40% of total). Interestingly, none of them showed any
11 somatic variants at the serial ccfDNA samples (**Suppl Table 3**).

13 **Discussion**

14 In the present study, we performed a comprehensive ccfDNA analysis in prospectively collected samples
15 from a large cohort of 34 patients with primary ACC. We could demonstrate that ccfDNA-based
16 biomarkers can be detected in a noticeable proportion of patients and could be proposed for both
17 prognostic classification and disease monitoring.

18 We first investigated the relationship between total ccfDNA concentrations (namely BM1) and clinical
19 parameters at time of diagnosis. To this aim, we used a robust pipeline for sample collection and
20 processing, as well as commercially available, ready-to-use, highly sensitive ccfDNA isolation kits.

21 ccfDNA levels were significantly higher in patients with ACC compared to healthy subjects, and
22 correlated with the tumour burden and aggressiveness, as previously reported for other cancer types³⁸⁻⁴⁰.

23 Importantly, using an arbitrary cut-off based on levels observed in healthy subjects, BM1 was considered
24 positive in 96% of ACC. The proportion of circulating tumoural DNA (ctDNA) in the background of
25 overall ccfDNA has been historically reported as highly variable, ranging from 0.01% to 90%^{9,16,41}. In
26 fact, many factors may influence the concentration of ctDNA, including tumour volume, localisation and

1 vascularisation, hepatic and renal clearance, as well as anti-cancer treatments⁴². In our patient cohort, all
2 baseline samples were collected at time of diagnosis, when patients were treatment-naïve, and had normal
3 liver and kidney function, therefore excluding these potential interferences. We could also demonstrate
4 that positive BMI was strongly associated with worst clinical outcome, both in terms of PFS and OS, in
5 agreement with recent reports showing the potential prognostic role of ccfDNA concentrations alone²⁰.
6 These findings are of particular interest considering that measuring ccfDNA levels is a minimally
7 invasive, cheap, and straightforward technique that could easily be implemented in clinical practice to
8 further improve prognostic classification of ACC.

9 The information about somatic genetic events detected at the ccfDNA level showed to be of additional
10 importance. Here, we used a library preparation procedure incorporating an error suppression technology
11 to ensure confident calling of mutations down to 0.1% VAF. In fact, almost 50% of primary ACC
12 presented at least one somatic variant at ccfDNA. This proportion of positive cases is higher than those
13 described in previous studies on smaller cohorts of patients i.e., 20-30%^{25,26}. This could be at least in part
14 due to 1) the use of a more homogeneous series of patients with primary ACC in place, 2) a robust
15 pipeline for collection, processing, isolation and measurement of ccfDNA, and 3) the utilization of a
16 highly sensitive customized ACC-specific gene panel. Another recent study, using a different approach,
17 i.e. Guardant360 (Guardant Health, Inc., Redwood City, CA, USA) that allows to analyse not only
18 SNV/indels but also gene fusions and copy number amplifications, identified alterations in ccfDNA in up
19 to 80% of patients with ACC⁴³.

20 In our cohort, somatic alterations detected in ccfDNA samples matched with available T-DNA in almost
21 70% of cases. Moreover, in about 20% of cases, variants in ACC-specific genes (*ZNRF3*, *APC*, *GNAS*
22 and *NFI*) were detected only in ccfDNA – but not in T-DNA. These findings further confirm the
23 additional value and the potential clinical utility of ccfDNA sequencing in molecular profiling –
24 compared to tissue sequencing^{19,44-46}. This is particularly relevant for highly heterogeneous cancers such
25 as ACC. Of note, at ccfDNA sequencing, the most frequently affected pathway confirmed to be Wnt/beta
26 catenin and chromatin remodeling – as previously reported in multiple pan-genomic or targeted studies on

1 both snap-frozen and FFPE tumour samples³⁰⁻³². On the contrary, only a few variants were detected at the
2 ccfDNA level affecting genes in the p53/Rb pathway.

3 The presence of somatic variants at ccfDNA showed to have prognostic value (AIM 1), being linked to a
4 shorter progression-free and overall survival, in agreement with previous studies on other solid tumours
5 ⁴⁷⁻⁴⁹. In fact, the ccfDNA-based BM score calculated by merging BM1 and BM2, was found to be strongly
6 associated with clinical outcomes, remaining a significant, independent prognostic factor at multivariable
7 analysis including the ENSAT tumour stage. This is of particular interest considering that ACC is a
8 generally aggressive cancer with heterogeneous and difficult-to-predict clinical outcomes. Therefore, we
9 could suggest that ccfDNA-based BM evaluated at the time of diagnosis could be used for improving the
10 prognostic classification of patients with ACC.

11 It is important to mention that at least eight genes contained in our customized panel of ACC-specific
12 genes are classified as drug-targetable. At ccfDNA sequencing, somatic variants were detected in one of
13 these genes in three patients (9% of total), i.e. two presented missense mutations in *ATM* (targetable by
14 PARP inhibitors) and one a missense mutation in *NFI* (targetable by MEK inhibitors). Interestingly, in
15 two cases, information about druggable genetic events could only be gained by analysing ccfDNA. In
16 fact, in one case the patient did not undergo surgery due to the presence of disseminated disease and in the
17 other case, T-DNA sequencing did not detect the presence of any variants. These findings further
18 corroborate the potential clinical utility of ccfDNA analysis for molecular profiling and identification of
19 targetable events in ACC⁴³, similar to what has been proposed for other cancer types^{18,22}.

20 We also evaluated the potential role of ccfDNA analysis for disease monitoring (AIM 2) in serial samples
21 of 19 patients who underwent standard follow-up visits². We observed a relatively good correspondence
22 between the ccfDNA-based BMs and the radiological evidence of tumour manifestations. For instance,
23 patients with advanced ACC who underwent debulking surgery presented very high levels of ccfDNA
24 after surgery in agreement with rapid disease progression. When present at baseline, somatic variants
25 remained detectable in most cases during surveillance and matched with the radiological disease
26 progression. Moreover, among patients with disease recurrences, one presented with baseline ccfDNA

1 somatic variants that persisted at the first post-surgical follow-up when the patient showed an early
2 disease recurrence, while both the ccfDNA concentrations and VAF% sharply increased at time of rapid
3 disease progression. Finally, in patients with no evidence of recurrent disease, somatic variants were not
4 detectable in ccfDNA neither at first post-surgical follow-up nor during further surveillance.

5 In the relatively small cohort of cases with fully available data, both BM1 and BM2 showed a very high
6 sensitivity (both 100%), while BM2 showed a better specificity than BM1 (67% vs. 36%). Therefore, we
7 hypothesise that ccfDNA analysis could be useful to complement radiological surveillance for both the
8 detection of early recurrences in patients with successfully resected ACC and monitoring of disease
9 evolution and/or response to treatment in patients with advanced ACC, similar to what has been proposed
10 for other solid tumours⁵⁰. However, further studies on larger cohorts of patients with longer follow-up
11 periods are required to validate our findings.

12 Overall, ccfDNA analysis has evolved since its inception with improvements in the technologies and
13 detection limits and represents a set of research tools that appear poised to enter routine clinical care^{51,52}.

14 As a matter of fact, an FDA-approved ctDNA assay, the Cobas epidermal growth factor receptor (EGFR)
15 Mutation Test (Roche, Basel, Switzerland), is available to detect *EGFR* mutations and drive the use of
16 EGFR tyrosine kinase inhibitor therapy in non-small cell lung cancer. Moreover, there are two CLIA-
17 certified commercially available ctDNA platforms: the mentioned Guardant360 panel for the assessment
18 of 73 cancer genes and the PlasmaSelect (Personal Genome Diagnostics, Inc., Baltimore, MD, USA) with
19 a 64-gene panel. However, this approach is still quite expensive and not readily available, especially for
20 rare cancer types.

21 Limitations of the study are represented by the relatively short period of follow up (up to max. 12 months)
22 and the lack of an evaluation of ccfDNA-related biomarkers in the early post-operative time-point (i.e. 6-
23 8 weeks). These aspects were beyond the scope of this pilot study and will be the aim of a future long-
24 term project. Moreover, in few cases some follow-up visits (and therefore blood collections) have been
25 missing due to the restrictions related to the COVID-19 pandemic that significantly reduced the face-to-
26 face access to the health systems.

1 In conclusion, ccfDNA-related BMs are frequently detected in patients with ACC and may represent a
2 promising, minimally invasive tool to predict early disease progression and complement imaging in
3 disease surveillance. Further studies are however required before these BMs could be proposed for
4 implementation in clinical practice.

5 **Acknowledgements:** The authors are grateful to Ms. Michaela Haaf for coordinating the clinical data
6 record in Wuerzburg and to Antonia Lorey for their skillful technical assistance. This work has been
7 carried out with the help of the Interdisciplinary Bank of Biomaterials and Data of the University Hospital
8 of Wuerzburg and the Julius Maximilian University of Wuerzburg (IBDW). The implementation of the
9 IBDW has been supported by the Federal Ministry for Education and Research (grant number FKZ:
10 01EY1102). We also thank the EU COST Action CA20122 Harmonisation for supportive networking
11 (www.goharmonisation.com).

12 **Author disclosure summary:** The Authors declare no potential conflict of interest in relation to this
13 work. Co-author Wiebke Arlt are on the editorial board of EJE. They were not involved in the review or
14 editorial process for this paper, on which they are listed as authors.

15 **Financial support:** This work has been supported by the Deutsche Krebshilfe (70113526 M.F., C.L.R,
16 and S.A.), the AMEND (ACC Research Award 2019 and 2021 to C.L.R.), and the European Reference
17 Network on Rare Endocrine Conditions (Endo-ERN). A.P. receives support from the National Institute
18 for Health and Care Research (NIHR) Birmingham Biomedical Research Centre at the University
19 Hospitals Birmingham NHS Foundation Trust and the University of Birmingham (grant reference number
20 NIHR203326), while C.L.R receives support from HRA Pharma Rare Disease (research grant). The
21 funders of the study had no role in the study design, data collection, data analysis, data interpretation, or
22 writing of the report. The views expressed are those of the author(s) and not necessarily those of the
23 NIHR or the Department of Health and Social Care UK.

24

1 **Legend to the Figures**

2 **Figure 1. Flowchart of the patient cohort with primary adrenocortical carcinoma (ACC)**

3 Legend: BM1=ccfDNA-based biomarker 1 (ccfDNA concentrations), BM2=ccfDNA-based biomarker 2
4 (somatic variants detected at ccfDNA level). Long-term follow up = at least 9 months from surgery,
5 short-term follow up: at least 3 months after surgery

6
7 **Figure 2. Baseline total ccfDNA concentrations and somatic variants detected in ccfDNA samples**
8 **by targeted Next-Generation Sequencing from 34 patients with primary adrenocortical carcinoma**
9 **(ACC-P).**

- 10 A) Comparison of ccfDNA concentrations between ACC and 23 healthy subjects (HS);
11 B) Comparison of ccfDNA concentrations between ACC and HS as follows: ccfDNA positive if levels
12 above the cut-off of 0.146 ng/μl, ccfDNA very high levels if above 1 ng/μl;
13 C) Relationship between ccfDNA concentrations and ENSAT tumour stage in ACC. Statistics by
14 Kruskal-Wallis test followed by Bonferroni *post-hoc* test;
15 D) Correlation between ccfDNA concentrations and Ki67 proliferation index in ACC. Statistics by linear
16 regression;
17 E) Pie chart showing the proportion of cases with one or more than one somatic variant in ACC-specific
18 genes;
19 F) Pie chart showing the proportion of samples with individual somatic variants (i.e. gene names).
20 Highlighted in blue are the genes members of the Wnt/β-catenin pathway.

21
22 **Figure 3. Prognostic role of ccfDNA-related biomarkers (AIM 1) - Relationship between ccfDNA-**
23 **related biomarkers (i.e. BM1, BM2 and ccfDNA-based BM score) and clinical outcomes in 34**
24 **patients with adrenocortical carcinoma.**

1 A-C-E) Kaplan-Meier curves for progression-free survival (PFS), B-D-F) Kaplan-Meier curves for
2 overall survival (OS). Statistical analysis by log-rank test.

3 BM1=total ccfDNA concentrations, BM2=somatic variants at ccfDNA. HR=hazard ratio, 95% CI=95%
4 confidence interval.

5

6 **Figure 4. Monitoring role of ccfDNA-related biomarkers (AIM 2) - Two representative examples of**
7 **longitudinal ccfDNA analysis from serial samples collected in patients with primary adrenocortical**
8 **carcinoma (ACC-P) that developed disease recurrence during follow-up after successful surgery.**

9 A) Patient with ACC with no detected somatic variants at both baseline ccfDNA and tumour-DNA (T-
10 DNA). Disease recurrence was observed at surveillance imaging 12 months after adrenalectomy during
11 adjuvant treatment with mitotane;

12 B) Patient with ACC with two somatic variants at baseline ccfDNA. First surveillance imaging 3 months
13 after adrenalectomy showed an early disease recurrence with evidence of liver metastases. An initial
14 mixed response at mitotane was observed at 6-month and 9-month follow-up followed by progressive
15 disease with increase in size of liver metastases and new multiple lung metastases. This was followed by
16 start of systemic chemotherapy with etoposide-doxorubicin-cisplatin (EDP-M).

17 VAF=variant allele frequency, Sx=surgery, TF=tumour free, Rec=disease recurrence, PD=progressive
18 disease, mixed=mixed response to treatment.

19

20 **Figure 5. Monitoring role of ccfDNA-related biomarkers (AIM 2) - Overview and five**
21 **representative examples of longitudinal ccfDNA analysis from serial samples collected in patients**
22 **with primary adrenocortical carcinoma (ACC-P) that remained tumour-free at radiological**
23 **imaging during follow-up after successful surgery. A) Total ccfDNA concentrations prior to surgery**

24 **and during follow-up (mean±standard deviation); B-C) Patients without somatic variants at baseline**
25 **ccfDNA; D-F) Patients with detected somatic variants at baseline ccfDNA.**

26 VAF=variant allele frequency.

1 Bibliography

- 2 1. Else T, Kim AC, Sabolch A, Raymond VM, Kandathil A, Caoili EM, Jolly S, Miller BS,
3 Giordano TJ & Hammer GD. Adrenocortical carcinoma. *Endocr Rev* 2014 **35** 282-326.
- 4 2. Fassnacht M, Dekkers OM, Else T, Baudin E, Berruti A, de Krijger R, Haak HR, Mihai R, Assie
5 G & Terzolo M. European Society of Endocrinology Clinical Practice Guidelines on the
6 management of adrenocortical carcinoma in adults, in collaboration with the European Network
7 for the Study of Adrenal Tumors. *Eur J Endocrinol* 2018 **179** G1-G46.
- 8 3. Fassnacht M, Tsagarakis S, Terzolo M, Tabarin A, Sahdev A, Newell-Price J, Pelsma I, Marina
9 L, Lorenz K, Bancos I, Arlt W & Dekkers OM. European Society of Endocrinology clinical
10 practice guidelines on the management of adrenal incidentalomas, in collaboration with the
11 European Network for the Study of Adrenal Tumors. *Eur J Endocrinol* 2023 **189** G1-G42.
- 12 4. Poulet G, Massias J & Taly V. Liquid Biopsy: General Concepts. *Acta Cytol* 2019 **63** 449-455.
- 13 5. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R &
14 Rosenfeld N. Liquid biopsies come of age: towards implementation of circulating tumour DNA.
15 *Nat Rev Cancer* 2017 **17** 223-238.
- 16 6. Leon SA, Shapiro B, Sklaroff DM & Yaros MJ. Free DNA in the serum of cancer patients and
17 the effect of therapy. *Cancer Res* 1977 **37** 646-650.
- 18 7. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C & Beljanski M. Neoplastic characteristics
19 of the DNA found in the plasma of cancer patients. *Oncology* 1989 **46** 318-322.
- 20 8. Giacona MB, Ruben GC, Iczkowski KA, Roos TB, Porter DM & Sorenson GD. Cell-free DNA
21 in human blood plasma: length measurements in patients with pancreatic cancer and healthy
22 controls. *Pancreas* 1998 **17** 89-97.
- 23 9. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD & Knippers R. DNA
24 fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from
25 apoptotic and necrotic cells. *Cancer Res* 2001 **61** 1659-1665.
- 26 10. Umetani N, Giuliano AE, Hiramatsu SH, Amersi F, Nakagawa T, Martino S & Hoon DS.
27 Prediction of breast tumor progression by integrity of free circulating DNA in serum. *J Clin*
28 *Oncol* 2006 **24** 4270-4276.
- 29 11. Yan YY, Guo QR, Wang FH, Adhikari R, Zhu ZY, Zhang HY, Zhou WM, Yu H, Li JQ & Zhang
30 JY. Cell-Free DNA: Hope and Potential Application in Cancer. *Front Cell Dev Biol* 2021 **9**
31 639233.
- 32 12. Huang ZH, Li LH & Hua D. Quantitative analysis of plasma circulating DNA at diagnosis and
33 during follow-up of breast cancer patients. *Cancer Lett* 2006 **243** 64-70.
- 34 13. Shaw JA, Guttery DS, Hills A, Fernandez-Garcia D, Page K, Rosales BM, Goddard KS, Hastings
35 RK, Luo J, Ogle O, Woodley L, Ali S, Stebbing J & Coombes RC. Mutation Analysis of Cell-
36 Free DNA and Single Circulating Tumor Cells in Metastatic Breast Cancer Patients with High
37 Circulating Tumor Cell Counts. *Clin Cancer Res* 2017 **23** 88-96.
- 38 14. De Mattos-Arruda L & Caldas C. Cell-free circulating tumour DNA as a liquid biopsy in breast
39 cancer. *Mol Oncol* 2016 **10** 464-474.
- 40 15. Adamo P, Cowley CM, Neal CP, Mistry V, Page K, Dennison AR, Isherwood J, Hastings R, Luo
41 J, Moore DA, Howard PJ, Miguel ML, Pritchard C, Manson M & Shaw JA. Profiling tumour
42 heterogeneity through circulating tumour DNA in patients with pancreatic cancer. *Oncotarget*
43 2017 **8** 87221-87233.
- 44 16. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, Diaz LA, Jr., Goodman SN, David KA, Juhl
45 H, Kinzler KW & Vogelstein B. Detection and quantification of mutations in the plasma of
46 patients with colorectal tumors. *Proc Natl Acad Sci U S A* 2005 **102** 16368-16373.
- 47 17. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll
48 L, Szabo SA, Kinzler KW, Vogelstein B & Diaz LA, Jr. Circulating mutant DNA to assess tumor
49 dynamics. *Nat Med* 2008 **14** 985-990.

- 1 18. Joesse SA & Pantel K. Circulating DNA and Liquid Biopsies in the Management of Patients with
2 Cancer. *Cancer Res* 2022 **82** 2213-2215.
- 3 19. Vietsch EE, Graham GT, McCutcheon JN, Javaid A, Giaccone G, Marshall JL & Wellstein A.
4 Circulating cell-free DNA mutation patterns in early and late stage colon and pancreatic cancer.
5 *Cancer Genet* 2017 **218-219** 39-50.
- 6 20. Tan G, Chu C, Gui X, Li J & Chen Q. The prognostic value of circulating cell-free DNA in breast
7 cancer: A meta-analysis. *Medicine (Baltimore)* 2018 **97** e0197.
- 8 21. Bachet JB, Laurent-Puig P, Meurisse A, Bouche O, Mas L, Taly V, Cohen R, Gornet JM, Artru
9 P, Louafi S, Thirot-Bidault A, Baumgaertner I, Coriat R, Tougeron D, Lecomte T, Mary F,
10 Aparicio T, Marthey L, Blons H, Vernerey D & Taieb J. Circulating tumour DNA at baseline for
11 individualised prognostication in patients with chemotherapy-naïve metastatic colorectal cancer.
12 An AGEO prospective study. *Eur J Cancer* 2023 **189** 112934.
- 13 22. Cohen SA, Liu MC & Aleshin A. Practical recommendations for using ctDNA in clinical
14 decision making. *Nature* 2023 **619** 259-268.
- 15 23. Fernandez-Garcia D, Hills A, Page K, Hastings RK, Toghil B, Goddard KS, Ion C, Ogle O,
16 Boydell AR, Gleason K, Rutherford M, Lim A, Guttery DS, Coombes RC & Shaw JA. Plasma
17 cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast
18 cancer. *Breast Cancer Res* 2019 **21** 149.
- 19 24. Gristina V, Barraco N, La Mantia M, Castellana L, Insalaco L, Bono M, Perez A, Sardo D,
20 Inguglia S, Iacono F, Cutaia S, Bazan Russo TD, Francini E, Incorvaia L, Badalamenti G, Russo
21 A, Galvano A & Bazan V. Clinical Potential of Circulating Cell-Free DNA (cfDNA) for
22 Longitudinally Monitoring Clinical Outcomes in the First-Line Setting of Non-Small-Cell Lung
23 Cancer (NSCLC): A Real-World Prospective Study. *Cancers (Basel)* 2022 **14**.
- 24 25. Creemers SG, Korpershoek E, Atmodimedjo PN, Dinjens WNM, van Koetsveld PM, Feelders
25 RA & Hofland LJ. Identification of Mutations in Cell-Free Circulating Tumor DNA in
26 Adrenocortical Carcinoma: A Case Series. *J Clin Endocrinol Metab* 2017 **102** 3611-3615.
- 27 26. Garinet S, Nectoux J, Neou M, Pasmant E, Jouinot A, Sibony M, Orhant L, Pipoli da Fonseca J,
28 Perlemoine K, Bricaire L, Groussin L, Soubrane O, Dousset B, Libe R, Letourneur F, Bertherat J
29 & Assie G. Detection and monitoring of circulating tumor DNA in adrenocortical carcinoma.
30 *Endocr Relat Cancer* 2018 **25** L13-L17.
- 31 27. McCabe MJ, Pinese M, Chan CL, Sheriff N, Thompson TJ, Grady J, Wong M, Gauthier MA,
32 Puttick C, Gayevskiy V, Hajdu E, Wong SQ, Barrett W, Earls P, Lukeis R, Cheng YY, Lin RCY,
33 Thomas DM, Watkins DN, Dinger ME, McCormack AI & Cowley MJ. Genomic stratification
34 and liquid biopsy in a rare adrenocortical carcinoma (ACC) case, with dual lung metastases. *Cold
35 Spring Harb Mol Case Stud* 2019 **5**.
- 36 28. Fassnacht M DO, Else T, Baudin E, Berruti A, de Krijger RR, Haak HR, Mihai R, Assie G,
37 Terzolo M. European Society of Endocrinology Clinical Practice Guidelines on the Management
38 of Adrenocortical Carcinoma in Adults, in collaboration with the European Network for the Study
39 of Adrenal Tumors. *Eur J Endocrinol* 2018.
- 40 29. Elhassan YS, Altieri B, Berhane S, Cosentini D, Calabrese A, Haissaguerre M, Kastelan D,
41 Fragoso M, Bertherat J, Al Ghuzlan A, Haak H, Boudina M, Canu L, Loli P, Sherlock M, Kimpel
42 O, Lagana M, Sitch AJ, Kroiss M, Arlt W, Terzolo M, Berruti A, Deeks JJ, Libe R, Fassnacht M
43 & Ronchi CL. S-GRAS score for prognostic classification of adrenocortical carcinoma: an
44 international, multicenter ENSAT study. *Eur J Endocrinol* 2021 **186** 25-36.
- 45 30. Lippert J, Appenzeller S, Liang R, Sbiera S, Kircher S, Altieri B, Nanda I, Weigand I, Gehrig A,
46 Steinhauer S, Riemens RJM, Rosenwald A, Muller CR, Kroiss M, Rost S, Fassnacht M & Ronchi
47 CL. Targeted Molecular Analysis in Adrenocortical Carcinomas: A Strategy Toward Improved
48 Personalized Prognostication. *J Clin Endocrinol Metab* 2018 **103** 4511-4523.
- 49 31. Assie G, Letouze E, Fassnacht M, Jouinot A, Luscap W, Barreau O, Omeiri H, Rodriguez S,
50 Perlemoine K, Rene-Corail F, Elarouci N, Sbiera S, Kroiss M, Allolio B, Waldmann J, Quinkler
51 M, Mannelli M, Mantero F, Papatomas T, De Krijger R, Tabarin A, Kerlan V, Baudin E, Tissier

- 1 F, Dousset B, Groussin L, Amar L, Clauser E, Bertagna X, Ragazzon B, Beuschlein F, Libe R, de
2 Reynies A & Bertherat J. Integrated genomic characterization of adrenocortical carcinoma. *Nat*
3 *Genet* 2014 **46** 607-612.
- 4 32. Zheng S, Cherniack AD, Dewal N, Moffitt RA, Danilova L, Murray BA, Lerario AM, Else T,
5 Knijnenburg TA, Ciriello G, Kim S, Assie G, Morozova O, Akbani R, Shih J, Hoadley KA,
6 Choueiri TK, Waldmann J, Mete O, Robertson AG, Wu HT, Raphael BJ, Shao L, Meyerson M,
7 Demeure MJ, Beuschlein F, Gill AJ, Sidhu SB, Almeida MQ, Fragoso M, Cope LM, Kebebew E,
8 Habra MA, Whitsett TG, Bussey KJ, Rainey WE, Asa SL, Bertherat J, Fassnacht M, Wheeler
9 DA, Cancer Genome Atlas Research N, Hammer GD, Giordano TJ & Verhaak RGW.
10 Comprehensive Pan-Genomic Characterization of Adrenocortical Carcinoma. *Cancer Cell* 2016
11 **30** 363.
- 12 33. Schwarz JM, Cooper DN, Schuelke M & Seelow D. MutationTaster2: mutation prediction for the
13 deep-sequencing age. *Nat Methods* 2014 **11** 361-362.
- 14 34. Pertea M, Lin X & Salzberg SL. GeneSplicer: a new computational method for splice site
15 prediction. *Nucleic Acids Res* 2001 **29** 1185-1190.
- 16 35. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal
17 PA, Stratton MR & Wooster R. The COSMIC (Catalogue of Somatic Mutations in Cancer)
18 database and website. *Br J Cancer* 2004 **91** 355-358.
- 19 36. Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D,
20 Jang W, Karapetyan K, Katz K, Liu C, Maddipatla Z, Malheiro A, McDaniel K, Ovetsky M,
21 Riley G, Zhou G, Holmes JB, Kattman BL & Maglott DR. ClinVar: improving access to variant
22 interpretations and supporting evidence. *Nucleic Acids Res* 2018 **46** D1062-D1067.
- 23 37. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R,
24 Larsson E, Cerami E, Sander C & Schultz N. Integrative analysis of complex cancer genomics
25 and clinical profiles using the cBioPortal. *Sci Signal* 2013 **6** p11.
- 26 38. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber
27 B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA,
28 Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao
29 Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto
30 GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih I M, Oba-
31 Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD,
32 Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA,
33 Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N & Diaz LA, Jr. Detection of
34 circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014 **6**
35 224ra224.
- 36 39. Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, Salari R, Le Quesne J,
37 Moore DA, Veeriah S, Rosenthal R, Marafioti T, Kirkizlar E, Watkins TBK, McGranahan N,
38 Ward S, Martinson L, Riley J, Fraioli F, Al Bakir M, Gronroos E, Zambrana F, Endozo R, Bi
39 WL, Fennessy FM, Sponer N, Johnson D, Laycock J, Shafi S, Czyzewska-Khan J, Rowan A,
40 Chambers T, Matthews N, Turajlic S, Hiley C, Lee SM, Forster MD, Ahmad T, Falzon M, Borg
41 E, Lawrence D, Hayward M, Kolvekar S, Panagiotopoulos N, Janes SM, Thakrar R, Ahmed A,
42 Blackhall F, Summers Y, Hafez D, Naik A, Ganguly A, Kareht S, Shah R, Joseph L, Marie Quinn
43 A, Crosbie PA, Naidu B, Middleton G, Langman G, Trotter S, Nicolson M, Remmen H, Kerr K,
44 Chetty M, Gomersall L, Fennell DA, Nakas A, Rathinam S, Anand G, Khan S, Russell P, Ezhil
45 V, Ismail B, Irvin-Sellers M, Prakash V, Lester JF, Kornaszewska M, Attanoos R, Adams H,
46 Davies H, Oukrif D, Akarca AU, Hartley JA, Lowe HL, Lock S, Iles N, Bell H, Ngai Y, Elgar G,
47 Szallasi Z, Schwarz RF, Herrero J, Stewart A, Quezada SA, Peggs KS, Van Loo P, Dive C, Lin
48 CJ, Rabinowitz M, Aerts H, Hackshaw A, Shaw JA, Zimmermann BG, consortium TR,
49 consortium P & Swanton C. Phylogenetic ctDNA analysis depicts early-stage lung cancer
50 evolution. *Nature* 2017 **545** 446-451.

- 1 40. Volckmar AL, Sultmann H, Riediger A, Fioretos T, Schirmacher P, Endris V, Stenzinger A &
2 Dietz S. A field guide for cancer diagnostics using cell-free DNA: From principles to practice and
3 clinical applications. *Genes Chromosomes Cancer* 2018 **57** 123-139.
- 4 41. Han X, Wang J & Sun Y. Circulating Tumor DNA as Biomarkers for Cancer Detection.
5 *Genomics Proteomics Bioinformatics* 2017 **15** 59-72.
- 6 42. Fleischhacker M & Schmidt B. Circulating nucleic acids (CNAs) and cancer--a survey. *Biochim*
7 *Biophys Acta* 2007 **1775** 181-232.
- 8 43. Nazha B, Zhuang TZ, Dada HI, Drusbosky LM, Brown JT, Ravindranathan D, Carthon BC,
9 Kucuk O, Goldman J, Master VA & Bilen MA. Blood-Based Next-Generation Sequencing in
10 Adrenocortical Carcinoma. *Oncologist* 2022 **27** 462-468.
- 11 44. Oikkonen J & Hautaniemi S. Circulating tumor DNA (ctDNA) in precision oncology of ovarian
12 cancer. *Pharmacogenomics* 2019 **20** 1251-1253.
- 13 45. Leighl NB, Page RD, Raymond VM, Daniel DB, Divers SG, Reckamp KL, Villalona-Calero MA,
14 Dix D, Odegaard JI, Lanman RB & Papadimitrakopoulou VA. Clinical Utility of Comprehensive
15 Cell-free DNA Analysis to Identify Genomic Biomarkers in Patients with Newly Diagnosed
16 Metastatic Non-small Cell Lung Cancer. *Clin Cancer Res* 2019 **25** 4691-4700.
- 17 46. Aggarwal C, Thompson JC, Black TA, Katz SI, Fan R, Yee SS, Chien AL, Evans TL, Bauml JM,
18 Alley EW, Ciunci CA, Berman AT, Cohen RB, Lieberman DB, Majmundar KS, Savitch SL,
19 Morrisette JJD, Hwang WT, Elenitoba-Johnson KSJ, Langer CJ & Carpenter EL. Clinical
20 Implications of Plasma-Based Genotyping With the Delivery of Personalized Therapy in
21 Metastatic Non-Small Cell Lung Cancer. *JAMA Oncol* 2019 **5** 173-180.
- 22 47. Cullinane C, Fleming C, O'Leary DP, Hassan F, Kelly L, O'Sullivan MJ, Corrigan MA &
23 Redmond HP. Association of Circulating Tumor DNA With Disease-Free Survival in Breast
24 Cancer: A Systematic Review and Meta-analysis. *JAMA Netw Open* 2020 **3** e2026921.
- 25 48. Schraa SJ, van Rooijen KL, Koopman M, Vink GR & Fijneman RJA. Cell-Free Circulating
26 (Tumor) DNA before Surgery as a Prognostic Factor in Non-Metastatic Colorectal Cancer: A
27 Systematic Review. *Cancers (Basel)* 2022 **14**.
- 28 49. Hallermayr A, Steinke-Lange V, Vogelsang H, Rentsch M, de Wit M, Haberl C, Holinski-Feder
29 E & Pickl JMA. Clinical Validity of Circulating Tumor DNA as Prognostic and Predictive
30 Marker for Personalized Colorectal Cancer Patient Management. *Cancers (Basel)* 2022 **14**.
- 31 50. Hallermayr A, Benet-Pages A, Steinke-Lange V, Mansmann U, Rentsch M, Holinski-Feder E &
32 Pickl JMA. Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in
33 Residual Disease Detection and Treatment Monitoring. *Clin Chem* 2021 **67** 1483-1491.
- 34 51. Crowley E, Di Nicolantonio F, Loupakis F & Bardelli A. Liquid biopsy: monitoring cancer-
35 genetics in the blood. *Nat Rev Clin Oncol* 2013 **10** 472-484.
- 36 52. Haber DA & Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and
37 circulating tumor DNA. *Cancer Discov* 2014 **4** 650-661.

38
39
40
41

1 **Table 1. Demographic, clinical, and histopathological characteristics of 34 patients with primary adrenocortical carcinoma (ACC) and**
 2 **results of circulating cell-free DNA (ccfDNA) analysis.**

Patient-ID	Sex/age	Symptoms	ENSAT tumour stage	Metastasis at diagnosis	ccfDNA levels (ng/μL of plasma)	ccfDNA sequencing Gene name	Surgery	R status	Ki67 index	S-GRAS score	Available Follow-up
ACC-P1	F/55	Yes	4	Liver, lung, LN	0.195	<i>CTNNB1</i> (NM_001098209.1)	No	NA	NA	NA	No
ACC-P2	M/83	Yes	3	None	0.301	No variants identified	Yes	0	9	3	Long
ACC-P3	M/43	Yes	3	None	Yes	<i>MEN1</i> (NM_130799.2) <i>ZNRF3</i> (NM_001206998.1)	Yes	0	30	4	Long
ACC-P4	M/71	No	3	None	0.173	No variants identified	Yes	0	40	4	Short
ACC-P5	F/23	Yes	4	Lung	1.935	No variants identified	Yes	2	40	8	Short
ACC-P6	M/36	Yes	2	None	0.151	No variants identified	Yes	0	3	1	Long
ACC-P7	F/68	Yes	4	Liver	2.325	No variants identified	Yes	2	30	9	No
ACC-P8	F/74	No	3	None	1.120	<i>MEN1</i> (NM_130799.2) <i>ZNRF3</i> (NM_001206998.1)	Yes	1	40	6	Short
ACC-P9	M/37	Yes	4	Liver, lung	0.735	<i>TP53</i> (NM_000546.5)	No	NA	NA	NA	No
ACC-P10	F/54	Yes	4	Lung	2.700	<i>ZNRF3</i> (NM_001206998.1)	No	NA	NA	NA	No
ACC-P11	M/64	Yes	4	Liver	4.350	<i>CTNNB1</i> (NM_001098209.1) <i>DAXX</i> (NM_001141970.1) <i>RBI</i> (NM_000321.2) <i>TP53</i> (NM_000546.5)	No	NA	NA	NA	No
ACC-P12	F/73	Yes	4	Liver	6.000	<i>KMT2D</i> (NM_003482.3)	No	NA	NA	NA	No
ACC-P13	M/57	Yes	4	Liver	0.401	<i>KMT2D</i> (NM_003482.3)	No	NA	NA	NA	No
ACC-P14	F/36	Yes	3	None	0.193	No variants identified	Yes	1	30	6	Short
ACC-P15	F/57	Yes	3	None	0.890	No variants identified	Yes	1	10	6	Long
ACC-P16	M/50	No	2	None	0.062	<i>NFI</i> (NM_000267.3)*	Yes	0	17	1	Short
ACC-P17	F/65	Yes	3	None	0.385	<i>GNAS</i> (NM_000516.5)	Yes	1	25	7	Short
ACC-P18	F/60	Yes	4	Lung, LN	5.500	<i>CTNNB1</i> (NM_001098209.1) <i>ATM</i> (NM_000051.3)*	No	NA	NA	NA	No
ACC-P21	F/56	No	2	None	0.251	No variants identified	Yes	0	30	3	Long
ACC-P22	F/50	No	3	None	0.247	No variants identified	Yes	0	18	3	Long
ACC-P23	F/27	Yes	3	None	0.189	No variants identified	Yes	0	40	4	No
ACC-P24	M/31	Yes	2	None	0.163	No variants identified	Yes	0	12	2	Long
ACC-P25	F/56	No	3	None	0.454	No variants identified	Yes	0	5	2	Long
ACC-P26	F/39	No	2	None	0.185	<i>CTNNB1</i> (NM_001098209.1)	Yes	0	40	2	Short
ACC-P27	M/64	No	1	None	0.114	No variants identified	Yes	0	23	3	No

ACC-P28	F/35	Yes	4	Lung	1.473	<i>TERT</i> (NM_198253.2) (NM_002734.4)	Yes	2	80	8	Short
ACC-P29	F/56	Yes	3	None	0.302	No variants identified	Yes	0	12	4	Short
ACC-P30	F/53	Yes	2	None	0.368	<i>APC</i> (NM_000038.5)	Yes	0	30	4	Long
ACC-P31	F/51	Yes	2	None	1.380	<i>ATM</i> (NM_000051.3)*	Yes	0	22	4	Long
ACC-P32	F/26	Yes	4	Lung	0.414	No variants identified	No	NA	19°	NA	No
ACC-P33	M/48	Yes	4	Liver, lung	0.846	No variants identified	No	NA	NA	NA	No
ACC-P34	F/62	Yes	4	Liver	0.781	No variants identified	No	NA	20°	NA	No
ACC-P35	F/58	Yes	4	Liver	5.758	No variants identified	No	NA	75°	NA	No
ACC-P36	F/59	Yes	4	Liver, lung and LN	2.800	<i>MEN1</i> (NM_130799.2)	No	NA	20°	NA	No

1 ¹ki67 index available from adrenal tumour biopsy. *known drug targetable genes according to OncoKb database.

2 Legend: R status=resection status of primary tumour, S-GRAS score calculated as previously published (Elhassan YS et al., *Eur J Endocrinol* 2021, DOI:
3 10.1530/EJE-21-0510), F=female, M=male, LN=lymph node, NA=not applicable, short follow-up=at least 3 months, long follow-up=at least 9 months.

4

5 **Table 2. Comparison between targeted next-generation sequencing in matched tumour DNA and circulating cell-free DNA in 22 primary**
6 **adrenocortical carcinoma (ACC-P).**

Sample-ID	ENSAT tumour stage	T-DNA sequencing			ccfDNA sequencing			Correspondence between T-DNA and ccfDNA
		Gene name	Variant	VAF (%)	Gene name	Variant	VAF (%)	
ACC-P2	3	No variants identified			No variants identified			YES
ACC-P3	3	<i>MEN1</i> (NM_130799.2)	p.Tyr351*	74.80	<i>MEN1</i> (NM_130799.2)	p.Tyr351*	8.2	YES Only in ccfDNA
					<i>ZNFR3</i> (NM_001206998.1)	p.Cys333*	3.9	
ACC-P4	3	No variants identified			No variants identified			YES
ACC-P5	4	No variants identified			No variants identified			YES
ACC-P6	2	No variants identified			No variants identified			YES
ACC-P7	4	No variants identified			No variants identified			YES
ACC-P8	3	<i>MEN1</i> (NM_130799.2)	p.Arg460*	84.4	<i>MEN1</i> (NM_130799.2)	p.Arg460*	2.6	YES
		<i>ZNFR3</i> (NM_001206998.1)	p.Phe474Argfs*95	74.8	<i>ZNFR3</i> (NM_001206998.1)	p.Phe474Argfs*95	1.9	YES
ACC-P14	3	No variants identified			No variants identified			YES
ACC-P15	3	No variants identified			No variants identified			YES
ACC-P16	2	No variants identified			<i>NF1</i> (NM_000267.3)*	p.Leu2735Met	7.0	Only in ccfDNA
ACC-P17	3	No variants identified			<i>GNAS</i> (NM_000516.5)	p.Arg265=	3.3	Only in ccfDNA

ACC-P21	2	No variants identified			No variants identified			YES
ACC-P22	3	No variants identified			No variants identified			YES
ACC-P23	3	<i>APC</i> (NM_000038.5)	p.Arg876*	66.0	No variants identified			Only in T-DNA
ACC-P24	2	No variants identified			No variants identified			YES
ACC-P25	3	No variants identified			No variants identified			YES
ACC-P26	2	<i>CTNNB1</i> (NM_001098209.1)	p.Ser45Ala	74.0	<i>CTNNB1</i> (NM_001098209.1)	p.Ser45Ala	4.5	YES
		<i>MEN1</i> (NM_130799.2)	p.Asp180_Trp183del	20.9				Only in T-DNA
ACC-P27	1	<i>NF1</i> (NM_000267.3)	p.Arg2343Gln	26.4	No variants identified			Only in T-DNA
		<i>TP53</i> (NM_000546.59)	p.Ala159Val	24.6				Only in T-DNA
		<i>NOTCH1</i> (NM_017617.5)	p.Arg2104His	20.6				Only in T-DNA
ACC-P28	4	<i>PRKARIA</i> (NM_002734.49)	c.Glu55*	76.6	<i>PRKARIA</i> (NM_002734.49)	p.Glu55*	10.9	YES
		<i>TERT</i> (NM_198253.2)	p.Leu234Phe	28.0	<i>TERT</i> (NM_198253.2)	p.Leu234Phe	26.5	
ACC-P29	3	No variants identified			No variants identified			YES
ACC-P30	2	No variants identified			<i>APC</i> (NM_000038.5)	p.Asn32Ile	30.5	Only in ccfDNA
ACC-P31	2	<i>ATM</i> (NM_000051.3)*	p.Arg337Cys	88.6	<i>ATM</i> (NM_000051.3)*	p.Arg337Cys	9.9	YES

1 Legend: T-DNA=tumour DNA, ccfDNA=circulating cell-free DNA, VAF=variant allele frequency. Highlighted in grey = somatic variants detected both in
 2 ccfDNA and T-DNA. Highlighted in light yellow = somatic variants detected only in ccfDNA. Highlighted in light blue = somatic variants detected only in T-
 3 DNA. *known drug targetable genes according to OncoKb database.

4

5 **Table 3. Uni- and multivariable survival analysis for progression-free survival (PFS) and overall survival (OS) in 34 patients with**

6 **adrenocortical carcinomas.**

Parameter (n of patients if different from 34)	Univariable analysis				Multivariable analysis			
	PFS		OS		PFS		OS	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Age (≤ 50 vs > 50 years)	0.88 (0.33-2.40)	0.814	0.65 (0.28-1.51)	0.314				
Symptoms at diagnosis (yes vs no)	2.44 (0.72-8.30)	0.154		0.146				
ENSAT tumour stage (1-2 vs 3 vs 4)	6.40 (2.34-17.5)	<0.001	6.60 (2.51-17.3)	<0.001	6.02 (2.14-16.9)	<0.001	9.49 (2.89-31.2)	<0.001
Resection status (n=22) (R0 vs R1 vs R2)	1.77 (1.06-2.95)	0.030	2.59 (1.25-5.37)	0.011				
Ki67 index (n=26) (< 10 vs $10-19$ vs ≥ 20)	2.43 (0.92-6.42)	0.074	5.54 (0.84-36.6)	0.075				

S-GRAS score (n=22) (group 1 vs 2 vs 3 vs 4)	2.14 (1.10-4.16)	0.025	7.52 (1.83-30.9)	0.005				
ccfDNA-based BM1 (0 vs 1 vs 2)	3.45 (1.49-7.96)	0.004	6.28 (2.24-17.6)	<0.001	2.01 (0.67-6.02)	0.213	2.25 (0.68-7.39)	0.183
ccfDNA-based BM2 (0 vs 1 vs 2)	1.89 (1.05-3.40)	0.033	2.47 (1.33-4.56)	0.004	1.71 (0.86-3.38)	0.125	4.16 (1.86-9.31)	<0.001
ccfDNA-based scores - Model 1								
ENSAT tumour stage (1-2 vs 3 vs 4)	6.40 (2.34-17.5)	<0.001	6.60 (2.51-17.3)	<0.001	5.51 (2.08-14.6)	<0.001	5.43 (2.12-13.9)	<0.001
ccfDNA-based BM score (positive vs negative)	2.87 (1.20-6.90)	0.018	8.24 (2.29-29.7)	0.001	2.86 (0.95-7.54)	0.061	8.80 (2.01-38.4)	0.004
ccfDNA-based scores - Model 2								
ENSAT tumour stage (1-2 vs 3 vs 4)	6.40 (2.34-17.5)	<0.001	6.60 (2.51-17.3)	<0.001	6.10 (2.19-16.9)	<0.001	7.71 (2.82-21.1)	<0.001
ccfDNA-based BM score (0 vs 1 vs 2 vs 3 vs 4)	1.80 (1.22-2.66)	0.003	2.37 (1.51-3.71)	<0.001	1.81 (1.15-2.81)	0.009	3.39 (1.83-6.29)	<0.001

- 1 Legend: S-GRAS score calculated as previously published (Elhassan YS et al., *Eur J Endocrinol* 2021, DOI: 10.1530/EJE-21-0510), ccfDNA=circulating cell-free DNA, BM1=total ccfDNA concentrations, BM2=somatic variants detected in ccfDNA. In bold significant variable available in all patients.
- 2

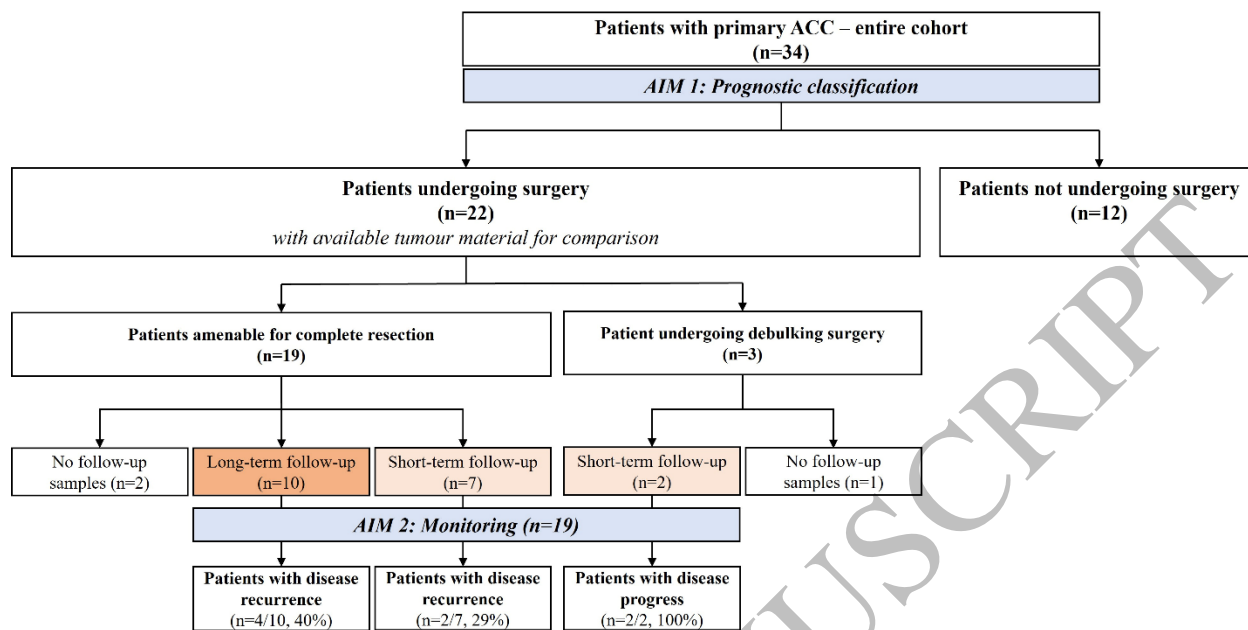


Figure 1
284x144 mm (DPI)

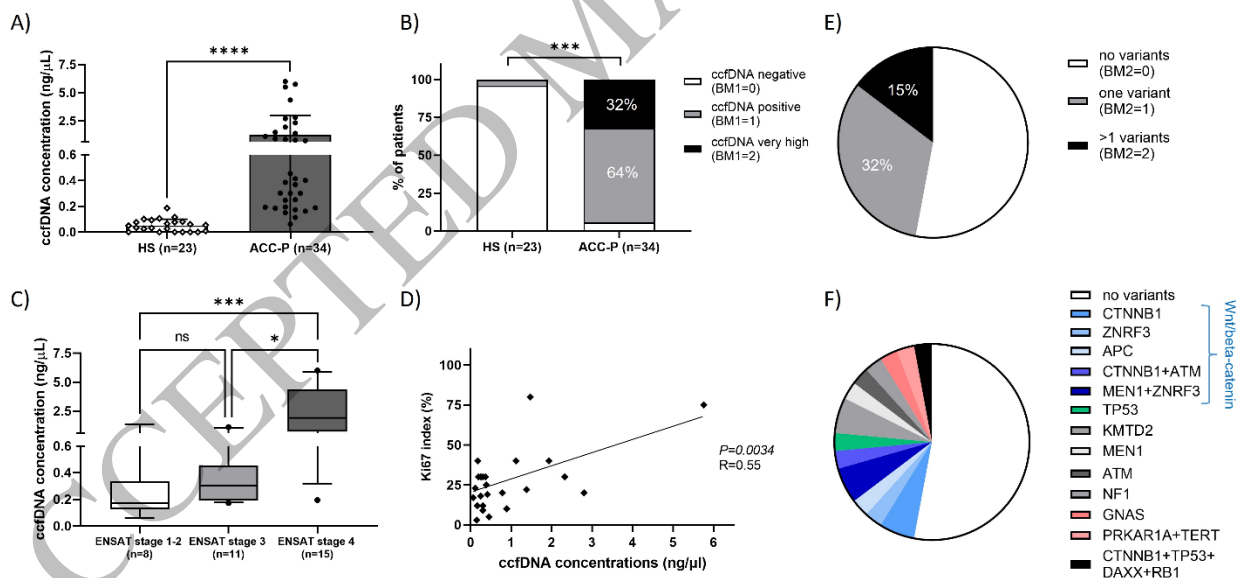


Figure 2
315x150 mm (DPI)

1
2
3
4

5
6
7
8

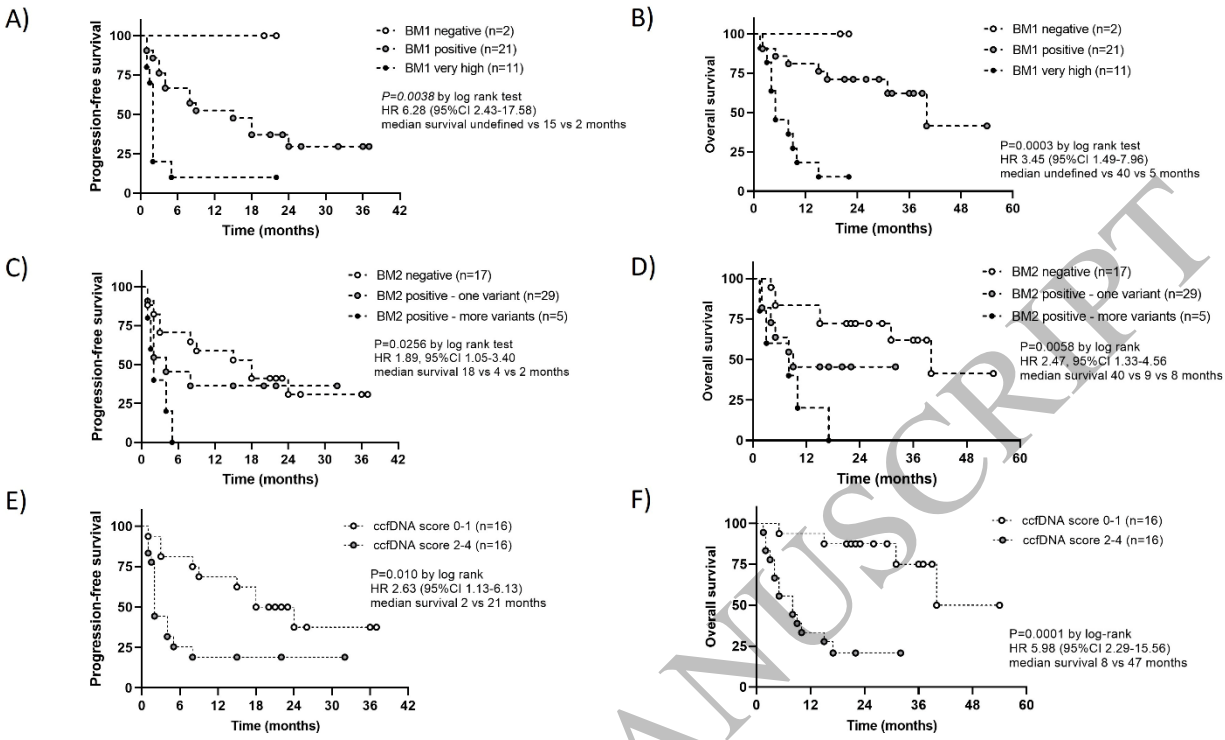


Figure 3
264x160 mm (DPI)

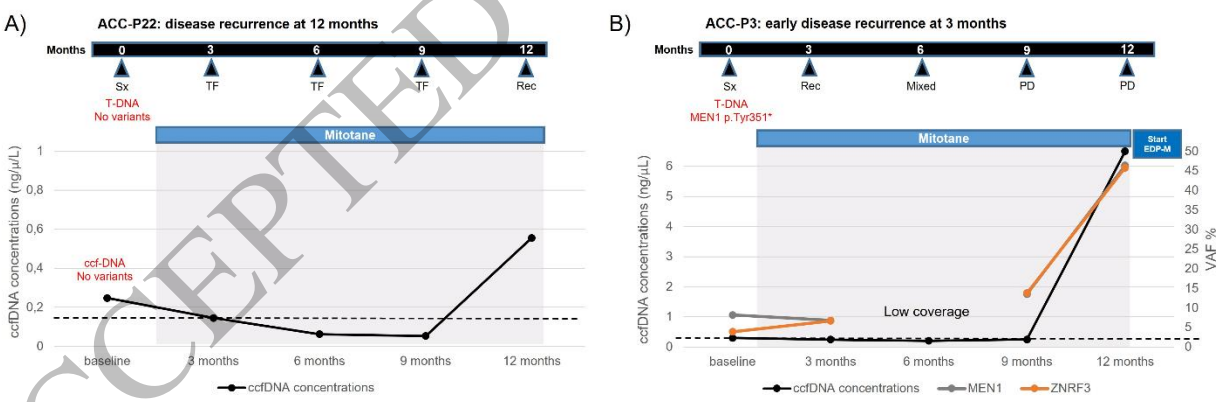


Figure 4
301x97 mm (DPI)

1
2
3
4
5
6
7
8

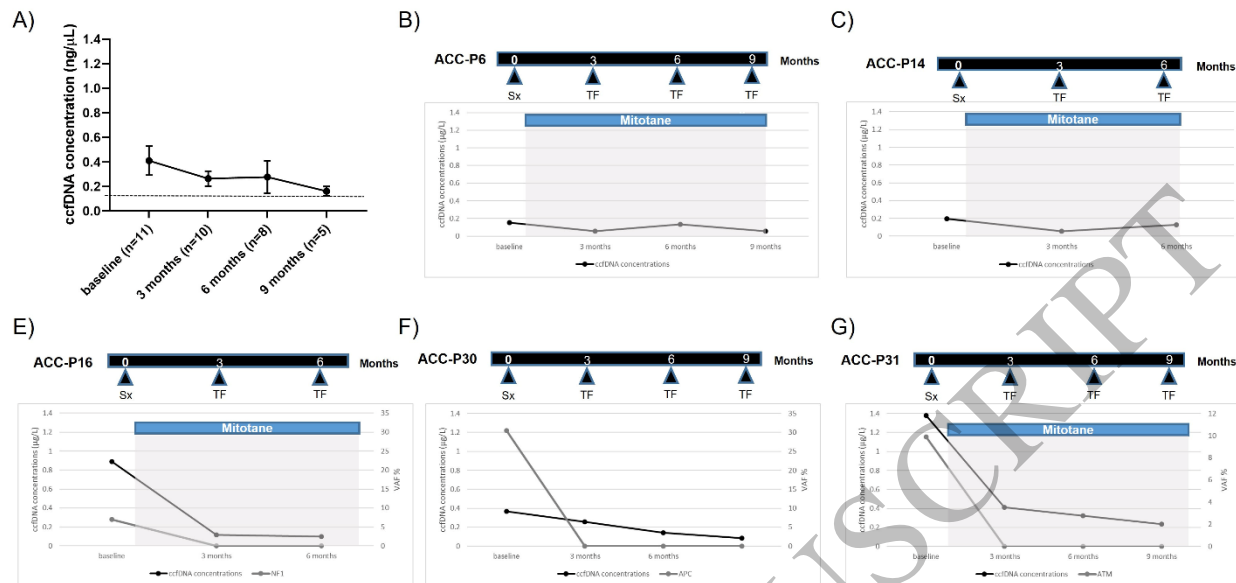


Figure 5
300x141 mm (DPI)

1
2
3